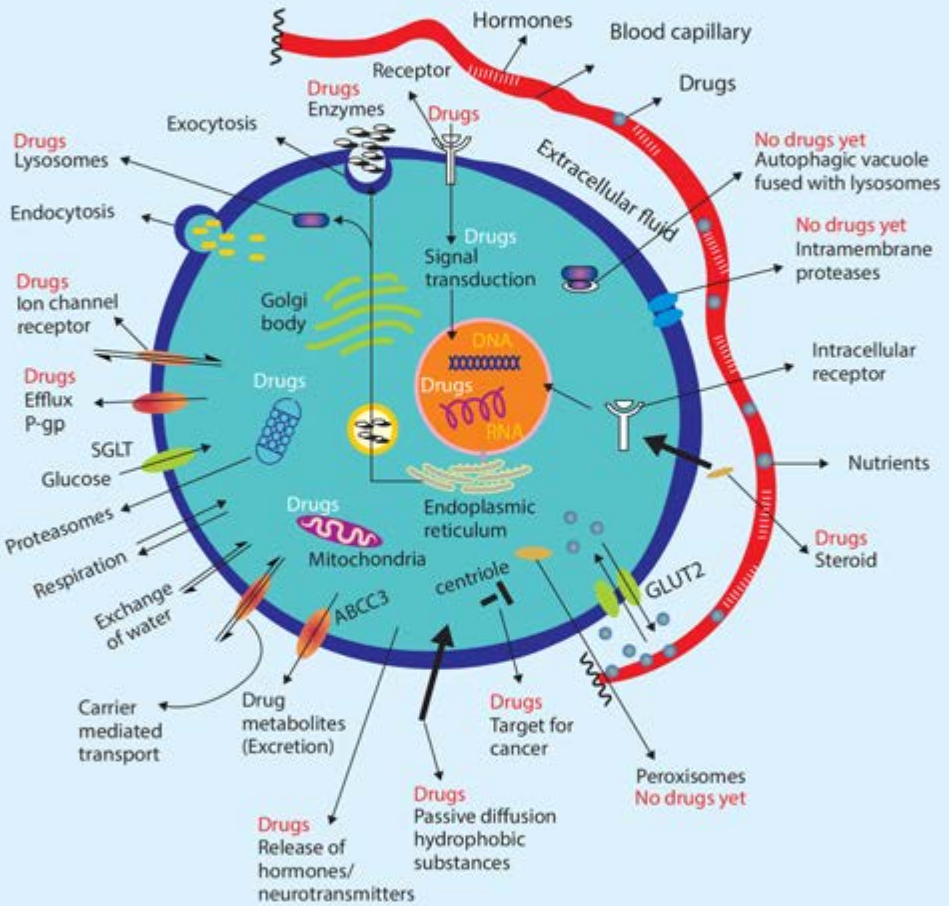
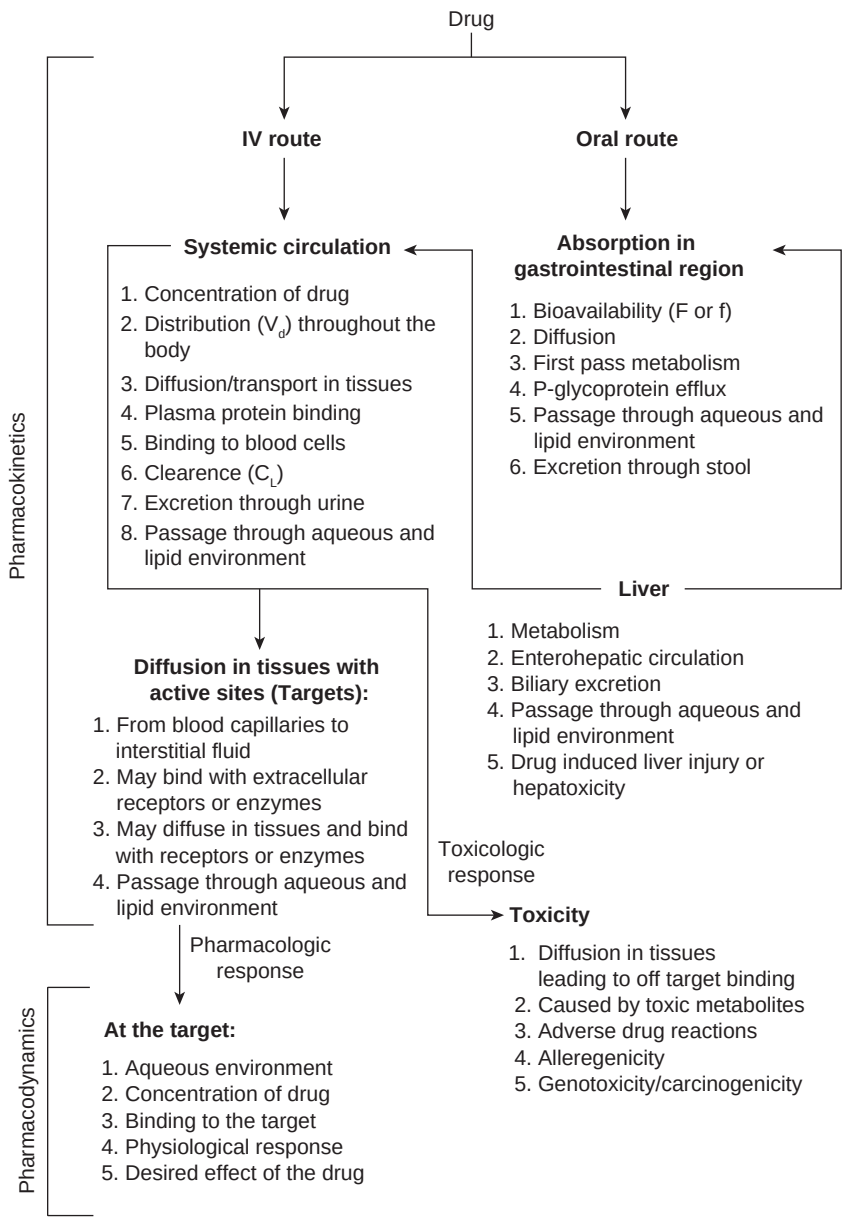
An Introduction to Medicinal Chemistry



Dr. Bijoy Kundu

WILEY

The Journey of drugs through the Body



An Introduction to **Medicinal Chemistry**

An Introduction to
Medicinal Chemistry

Dr. Bijoy Kundu

Ex-Chief Scientist & Former Head Medicinal & Process Chemistry Division
Professor at AcSIR, New Delhi
Former Head: Sophisticated & Analytical Instrument Facility
Ex-Sc-In-Charge: Academy Affairs and Electron Microscopy Units
Former Dean: NIPER, Raebarelly
CSIR-Central Drug Research Institute Lucknow, U.P.

НАРОДНЫЙ ФРОНТ
https://pobeda.onf.ru/
LET'S HELP THE RUSSIA

WILEY

НАРОДНЫЙ ФРОНТ
https://pobeda.onf.ru/
LET'S HELP THE RUSSIA

An Introduction to
Medicinal Chemistry

Copyright © 2020 by Wiley India Pvt. Ltd., 4436/7, Ansari Road, Daryaganj, New Delhi-110002.

All rights reserved. No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or scanning without the written permission of the publisher.

Limits of Liability: While the publisher and the author have used their best efforts in preparing this book, Wiley and the author make no representation or warranties with respect to the accuracy or completeness of the contents of this book, and specifically disclaim any implied warranties of merchantability or fitness for any particular purpose. There are no warranties which extend beyond the descriptions contained in this paragraph. No warranty may be created or extended by sales representatives or written sales materials. The accuracy and completeness of the information provided herein and the opinions stated herein are not guaranteed or warranted to produce any particular results, and the advice and strategies contained herein may not be suitable for every individual. Neither Wiley India nor the author shall be liable for any loss of profit or any other commercial damages, including but not limited to special, incidental, consequential, or other damages.

Disclaimer: The contents of this book have been checked for accuracy. Since deviations cannot be precluded entirely, Wiley or its author cannot guarantee full agreement. As the book is intended for educational purpose, Wiley or its author shall not be responsible for any errors, omissions or damages arising out of the use of the information contained in the book. This publication is designed to provide accurate and authoritative information with regard to the subject matter covered. It is sold on the understanding that the Publisher is not engaged in rendering professional services.

Trademarks: All brand names and product names used in this book are trademarks, registered trademarks, or trade names of their respective holders. Wiley is not associated with any product or vendor mentioned in this book.

Other Wiley Editorial Offices:

John Wiley & Sons, Inc. 111 River Street, Hoboken, NJ 07030, USA

Wiley-VCH Verlag GmbH, Pappellaee 3, D-69469 Weinheim, Germany

John Wiley & Sons Australia Ltd, 42 McDougall Street, Milton, Queensland 4064, Australia

John Wiley & Sons (Asia) Pte Ltd, 1 Fusionopolis Walk #07-01 Solaris, South Tower, Singapore 138628

John Wiley & Sons Canada Ltd, 22 Worcester Road, Etobicoke, Ontario, Canada, M9W 1L1

First Edition: 2020

ISBN: 978-81-265-9988-2

ISBN: 978-81-265-9068-1 (ebk)

www.wileyindia.com

Printed at:

Dedicated to my parents:

Late (Mrs) Kamla Kundu

(Former District Basic Education Officer, U.P. India)

&

Late Dr. B. N. Kundu

Preface

Drugs generally have their origin in organic compounds derived either from nature or from synthetic sources. However, in the last two decades biologics, involving proteins, hormones and monoclonal antibodies are also gaining significance and more and more of these macromolecules have started reaching clinics as therapeutic agents. This has particularly gained importance in view of the emerging fact that communication among cells through intercellular and intracellular cell signaling is crucial for the survival of the multicellular organism.

In general, a drug may activate or inhibit intracellular or intercellular signals that are responsible for numerous cellular/physiological functions. The cell signals are produced following binding of external and endogenous messenger molecules to extracellular and intracellular therapeutic macromolecular targets (receptor and enzymes). Thus, primary objective of drug design is to mimic the messenger molecules (ligands/substrates) with the view to modulate the function(s) of macromolecular targets. Indeed, drug design still remains a challenging task, due to continuous unraveling of new insights in the mechanism of physiological functioning.

The present book "An Introduction to Medicinal Chemistry" is an introduction to the chemical and biological parameters, challenges and underlying principles involved in the early stages of drug discovery from bench to preclinical stage. My main objective has been to compile various aspects of drug discovery principles in the form of a handbook instead a textbook. The book may provide one-stop information dealing with mechanistic, biological and chemical aspects of drug design and improvisation of their actions. This has been exemplified by providing relevant examples of drugs for a better understanding. The entire book revolves around five basic components of drug discovery: cellular signals, therapeutic targets, physico-chemical properties of test compounds, physiological barriers and life span of drugs in the body. The book will be more suitable for beginners including undergraduates/postgraduates/Ph.D scholars studying medicinal chemistry and pharmaceutical chemistry.

The book has been structured into fourteen chapters of a sensible size. Chapter 1 deal with cell signaling and diseases. In this chapter etiology of most diseases has been discussed in the light of a common cause involving intracellular and intercellular signaling generated in response to external and endogenous cues (messengers). Next, different classification methods to group diseases that inflict mankind have been discussed. Chapter 2 deals definition and types of drug followed by an overview of drug action that covers the journey of drug through the human physiological system from the site of administration to tissues with site of action. Next, the modern drug development strategy comprising target-based approaches has been discussed in Chapter 3. This has been followed by discussion on strategies for identifying hit molecules and drug design/lead optimization strategies in Chapters 4 and 5 respectively.

In Chapter 6, the significance of physico-chemical properties of chemical structures, as one of the most critical parameters in drug design strategies, have been discussed. It regulates both PK and PD parameters of drugs in the physiological environment.

In Chapters 7 to 10, role of various receptors as therapeutic targets in drug discovery and design, drug receptor binding theories, significance of receptor agonists and antagonists and contribution of drug-receptor interactions in drug development have been discussed.

In Chapter 11, role of pharmacokinetic (PK) parameters absorption, distribution, metabolism, excretion and toxicity (ADMET), in countering resistance imposed by physiological barriers following administration of drugs has been discussed.

In Chapter 12, pharmacodynamics (PD) of drugs involving manifestation of biological activity at the designated site in terms of binding affinity, efficacy, potency, and PD drug-drug interaction has been discussed. This has been followed by discussion on factors affecting enyzme activity, classification of enzymes and therapeutic role of enzymes in drug discovery.

In Chapters 13 and 14, role of enzymes in drug metabolism and digestion of food followed by their therapeutic implications has been discussed respectively.

In summary, the book covers basic components involved in the discovery of preclinical candidates, starting from hit molecules. Further many components and their effect on drug discovery have been explained with hand-drawn figures for better clarity and understanding.

About the Author

Dr. Bijoy Kundu after completing his Ph.D in Chemistry in 1981 from CSIR-Central Drug Research Institute (CSIR-CDRI), Lucknow, India worked as post-doctoral fellow at City University of New York, USA with Prof. Fred Naider for two years (1981–82), and at University of Illinois, Urbana-Champaign, Illinois, USA from 1989–91with Prof. K.L. Rinehart. After his return from USA in 1982, he joined as Scientist B at CSIR-CDRI in the division of Biopolymers and within two years of his joining, he was selected as Scientist C in the same division at CSIR-CDRI. Finally, after putting in 30 years of service he superannuated as Chief scientist and Head of the Department of Medicinal Chemistry of CSIR-CDRI, Lucknow, India.

During his tenure at CSIR-CDRI, he worked on solid and solution phase synthesis of peptides and heterocycles of biological interest, dealing with diseases such as diabetes, malaria, allergy/asthma, cardiovascular and those of the immune system. He has filed one international patent on a compound with anti-allergic activity along with several Indian patents. Besides, he has published several scientific papers in international journals of high impact factor such as Organic Letters, Journal of Organic Chemistry, Chem. Eur. J. and Angew Chem. In 1997, he was deputed by the institute to work for one year on combinatorial chemistry at University of Tubingen, in Germany under a WHO fellowship. He was also involved in setting up the state-of-the-art facilities in the institute on Combinatorial Chemistry and on National Repository for Organic Compounds.

His work on immunomodulators led Editors of Burger's Medicinal Chemistry, USA to invite him to write a chapter on "Organ Transplant Drugs" in their sixth series (Sixth series, Volume V, 485, 2003). He is the second Indian to have been invited to write an article for this internationally established series on Medicinal Chemistry.

While carrying out R&D work on drug development at CDRI, Dr. Kundu developed tremendous interest in teaching and taught medicinal Chemistry to the pre-Ph.D students at CSIR-CDRI and at the Indian Institute of Public Health-*Delhi* (*IIPH-Delhi*) New Delhi, India. In 2010, he was made Scientist-in-charge of Academy/Academic affairs unit in CSIR-CDRI where he restructured the pre-Ph.D course work for chemical and biological sciences in accordance with Jawahar Lal Nehru University (JNU) and Academy of Scientific and Innovative Research (AcSIR), New Delhi guidelines. After his superannuation in July 2015, he has been teaching medicinal, pharmaceutical and organic chemistry to undergraduate and postgraduate students of the department of Chemistry, University of Lucknow, India as guest faculty.

Acknowledgement

At the outset, I thank almighty God for giving me the strength to write this book. I also wish to place on record my gratitude to all my teachers who laid the foundation of Chemistry during the tenure of my graduation and post-graduation at Gorakhpur.

I shall always remain indebted to my alma mater – CSIR (Central Drug Research Institute), Lucknow, from where I did my Ph.D. and thereafter, worked as a scientist for thirty years in the departments of Biopolymers and Medicinal Chemistry. I am grateful to the former directors, my seniors and colleagues at the CDRI who not only provided the needed exposure to acquaint with various components of Medicinal Chemistry, but also for honing my administrative skills.

I extend my sincere thanks to the faculty, Department of Chemistry, Lucknow University, Lucknow, who gave me an opportunity to teach Organic, Medicinal and Pharmaceutical Chemistry at the graduate and postgraduate levels.

I express my special thanks to Dr. Ashim Ghatak, M.D. who has been my pillar of strength and with whom I held hours of discussions while I was writing this book.

Finally, a token of thanks to my Ph.D. students Dr. S. K. Khare, M.S., Dr. S. Shukla, M.S., Dr. M. Shukla, M.S., G. Singh, S.K. Rastogi, T. Srinivasan, G. Srivastava, S. K. Singh, Ms. P. Gupta, S. Duggineni, D. Sawant, A. Kesarwani, S. Sharma, P. K. Agarwal, S. Sharma, S. Gupta, M. Saifuddin, R. Arigela, A. Kumar, S. Samla and R. Kumar who, besides working diligently in incorporating my ideas always confronted me with new research challenges.

Last but not the least, I thank all the reviewers who took utmost pain and reviewed the chapters within the given time frame:

- **Dr. Amit Shard**, Assistant Professor, Department of Medicinal Chemistry, NIPER, Ahmedabad
- **Dr. Neelima D Passi**, Assistant Professor, Pharmaceutical Chemistry, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh
- **Dr. Poonam Dhiman**, Associate Professor, Chitkara College of Pharmacy, Chitkara University, Rajpura, Panjab
- **Prof. (Dr.) R. Mahesh**, Pharmacy Department, Birla Institute of Technology & Science, Pilani
- **Dr. Sandeep Sundriyal**, Assistant Professor, Department of Pharmacy, Birla Institute of Technology and Science, Pilani
- **Prof. (Dr.) Prashant S. Kharkar**, Department of Pharmaceutical Science and Technology, Institute of Chemical Technology, Mumbai.

Dr. Bijoy Kundu

Abbreviations

AC	Adenylyl cyclase
ALERT	Undesirable functional groups
ANP	Atrialnatriuretic peptide
AROM	Number of aromatic rings
ARB	Angiotensin II receptor blocker
ATP	Adenosinetriphosphate
ADP	Adenosinediphosphate
BBB	Blood brain barrier
Caco-2	A human colon adeno-carcinoma cell line
CADD	Computer aided drug design
cAMP	Cyclic adenosine monophosphate
CAMs	Cell adhesion molecules
cGMP	Cyclic guanosine monophosphate
CREB	cAMP-responsive element-binding protein
CRF	Corticotropin-releasing factor
CYP	Cytochrome P450
ECF	Extracellular fluid
ECM	Extracellular matrix
ER	Endoplasmic reticulum
GABA	γ-aminobutyric acid
$GABA_A$ receptor	γ-aminobutyric acid receptor (ion channel)
$GABA_B$ receptor	γ-aminobutyric acid receptor (GPCR derived)
GIT	gastro-intestinal tract
GnRH	Gonadotropin releasing hormone
GREBP	cGMP-response element-binding protein
GPCR	G-protein coupled receptor
DAG	1,2-diacylglycerol
DDI	Drug drug interaction
IC	Inhibitory concentration
IP3	Inositol 1,4,5-trisphosphate
EC	Effective concentration
ECF	Extracellular fluid
ED	Effective dose
FSH	Follicle stimulating hormone
GLUT	Glucose transporters
GMP	Guanine monophosphate
GLP-1	Glucagon-like peptide-1

GRF	Growth hormone releasing factor
HBD	Hydrogen bond donor
HBA	Hydrogen bond acceptor
LH	Lutienising hormone
MDR1	Multidrug resistance protein 1
mAb	Monoclonal antibody
NSAID	Nonsteroidal anti-inflammatory drugs
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLC	Phosphiolipase
PK	Pharmacokinetics
PD	Pharmacodynamics
PPB	Plasma protein binding
PSA	Polar surface area
PTH	Parathyroid hormone
sGC	Soluble guanylyl cyclase
SST	Somatostatin
RAS	Renin angiotensin system
rGC	Guanylyl cyclase linked receptor
ROTB	Number of rotatable bonds
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone

Contents

Preface vii
About the Author ix
Acknowledgement xi
Abbreviations xiii

Chapter 1 Cell Signaling and Diseases 1

1.1 Introduction 1
1.1.1 Components of Cell-Signaling Pathways 1
1.2 Chemical Messengers 2
1.2.1 First Messengers (Signaling Molecules) 3
1.2.2 Second Messengers 5
1.3 Signal Transduction 11
1.3.1 Signal Transduction Enzyme 11
1.4 Effectors 13
1.5 Signal Transduction Modulators as Drugs 15
1.6 Intercellular (Between-Cells) Signaling 18
1.6.1 Fluid Compartment 19
1.6.2 Extracellular Matrix 20
1.6.3 Types of Intercellular Signaling (Communication) between Cells 21
1.7 Homeostasis Regulated by Signaling Pathways 27
1.8 Signaling in Cellular Metabolism 30
1.8.1 Metabolic Pathway 30
1.8.2 Metabolites 31
1.8.3 Metabolomics 32
1.9 Diseases 38
1.9.1 Main Causes of Diseases 38
1.9.2 Classification of Diseases 40
1.9.3 Additional Disease Concepts 41
1.10 Understanding Signaling Pathways in the Pathogenesis of Diseases 43
1.11 Unexplored and Emerging Intracellular Signaling Components as Therapeutic Targets 44
1.11.1 Peroxisomes 44
1.11.2 Lysosomes 44
1.11.3 Autophagy 44
1.11.4 Proteasomes 45

1.12 History of Drug Research 45
1.12.1 Classification of Drugs Based on their Action on Signaling Pathways 45
Exercises 46
Competitive Exam Questions 47
References 47

Chapter 2 An Overview of Drug Action 49

2.1 Definition of Drugs 49
2.2 Types of Pharmacological Action of the Drugs (Theories of Drug Activity) 50
2.2.1 Structurally Nonspecific Drugs 50
2.2.2 Structurally Specific Drugs 50
2.2.3 Drugs that Don't Act on Receptors 51
2.3 Classification of Drugs Based on Their Origin 51
2.3.1 Naturally Occurring Compounds as Drugs 52
2.3.2 Synthetic Compounds 53
2.4 Drug Action 53
2.4.1 Absorption (A) 54
2.4.2 Distribution (D) 55
2.4.3 Metabolism 61
2.4.4 Excretion 61
2.4.5 Toxicity 62
2.5 Factors Affecting ADMET 63
2.5.1 Physicochemical Parameters of Drugs 63
2.5.2 Physiological Factors 64
Exercises 91
Competitive Exam Questions 91
References 92

Chapter 3 The Modern Drug Development Process 93

3.1 Introduction 93
3.2 The Modern Drug Development Process Comprises Five Steps 94
3.2.1 Drug Discovery and Development 94
3.2.2 Preclinical Research 97
3.2.3 Clinical Trials 99
3.2.4 FDA Review 99
3.2.5 FDA Monitoring 99
3.3 Initial Steps in Drug Discovery Process (Medicinal Chemistry) 99
3.3.1 Generation of Library of Chemical Compounds 100
3.3.2 Screening of Chemical Libraries 101
3.4 *In Vivo* Screening Assays 115

3.5 Screening in Model Organisms (Our Distant Fast Breeding Cousins) 116
3.5.1 Danio Rerio (Zebrafish) 117
3.5.2 Caenorhabditis Elegans 117
3.5.3 Drosophila Melanogaster 117
Exercises 118
Competitive Exam Questions 118
References 118

Chapter 4 Strategies for Identifying Hit Molecules 121

4.1 Hit Molecules 121
4.1.1 Random in vitro High-Throughput Screening of Chemical Libraries against Biological Targets/Whole Cells 121
4.1.2 In Vitro High Throughput Screening of Focused Chemical Libraries against a Validated Biological Targets or Whole Cells 122
4.1.3 Characteristics of Hits 122
4.1.4 Strategies for Identifying Hits Leading to Drug Candidates 123
4.1.5 Sources for Hits 123
4.2 Serendipity in Drug Discovery 125
4.2.1 Chlorpromazine 125
4.2.2 Minoxidil 125
4.2.3 Aspartame 125
4.2.4 Viagra (Sildenafil citrate) 125
4.2.5 Warfarin 125
4.2.6 CNS Drugs 125
4.3 Endogenous Sources (Derived from Humans or Animals) for the Identification of Drug Candidate(s) 126
4.3.1 Endocrine Hormones and their Therapeutic Applications 126
4.3.2 Steroid Hormones as Endogenous Source for Drugs 127
4.3.3 Peptide/Protein Endocrine Hormones as Endogenous Source for Drugs 131
4.4 Monoclonal Antibody Therapy 134
4.4.1 Monoclonal Antibodies in Diagnostics 135
4.4.2 Treating Disease at the RNA Level with Oligonucleotides 135
4.5 Drugs of Microbial Origin 135
4.5.1 Drugs Isolated from Actinomycetes 135
4.5.2 Drugs Isolated from Fungi 135
4.5.3 Drugs Isolated from Bacteria 136
4.6 Traditional Methods as a Source for Hit Identification (Exogenous Sources) 136
4.6.1 Paclitaxel (Taxol) 136
4.7 Target-Based Screening as a Rationalized Approach for the Identification of Hit/Lead Molecules 139

4.7.1 Quality of Chemical Libraries 139
4.7.2 Fragment-Based Drug Discovery for Identifying Hits 143
4.7.3 Virtual Screening for Identifying Hits 145
4.7.4 Phenotypic Screening for Identifying Hits 147
4.7.5 Repositioning of Drugs for Identifying Hits 147
4.8 Difference Between Fragments, Hits, and Leads 147
Exercises 149
Competitive Exam Questions 149
References 149

Chapter 5 Drug Design Strategies 151

5.1 Introduction 151
5.2 Hit to Lead 152
5.2.1 Characteristics of Lead Molecules 152
5.3 Characteristics of Preclinical Candidates 152
5.4 Lead Optimization/Drug Design Strategies 153
5.4.1 Pharmacophore Identification 154
5.4.2 Extensive Structure Activity Relationship to Improve Potency and Selectivity 155
5.4.3 Functional Group Modification 164
5.4.4 Structural Modifications to Increase the Activity 165
5.4.5 Structure-Based Drug Design 171
5.4.6 Bioisosterism and Drug Design 174
5.4.7 Stereoisomerism in Drug Design 181
5.4.8 Drug Design Strategies to Improve Absorption via Modulating Polarity 184
5.4.9 Preventing Degradation of Drugs (Drug Stability) 186
5.4.10 Drug Design Strategies Used for Targeting Drugs 190
5.4.11 Prodrug Strategy 192
5.4.12 Structure–Property Relationship 201
5.4.13 Allosteric Modulators as an Emerging Concept in Drug Design 204
5.4.14 'Me Too' Strategies for Drug Design 205
5.4.15 Antimetabolite Strategy for Drug Design 207
5.4.16 Role of DMPK for the Selection of Preclinical Candidate 211
5.5 Timeline of a Pharmaceutical Drug Development from Laboratory to Clinics 212
Exercises 214
Competitive Exam Questions 214
References 214

Chapter 6 Role of Physicochemical Properties of Chemical Structures in Drug Design 217

6.1 Introduction 217
6.2 Lipinski's Rule of Five 220

	6.2.1 The Rule of Three (RO3)	221
6.3	Ionization Constant (pK_a)	221
	6.3.1 Ionization of Base	223
	6.3.2 Ionization of Acid	223
	6.3.3 Role of pK_a in ADMET and Target-Binding	224
	6.3.4 Strength of Acidic/Basic Compounds in Periodic Table	225
6.4	Solubility of Drugs	226
6.5	Lipophilicity	228
	6.5.1 Effect of pK_a on Lipophilicity	229
	6.5.2 Effect of Lipophilicity on Some of the PK/PD Parameters in vitro	230
	6.5.3 Apparent Permeation Coefficient (P_{app})	230
	6.5.4 Distribution Coefficient (logD)	230
	6.5.5 Lipophilic Efficiency	231
	6.5.6 Effect of Lipophilicity on Local Anesthetics	232
	6.5.7 Effect of pK_a on Local Anesthetics	233
6.6	Hydrogen Bond Acceptor (HBA)/Hydrogen Bond Donor (HBD)	234
6.7	Surface Activity	235
	6.7.1 Applications	236
6.8	Number of Rotatable Bonds	236
6.9	Number of Aromatic Rings	236
6.10	Undesirable Functional Groups (ALERT)	237
6.11	Steric Factor	237
6.12	Molecular Size	238
	6.12.1 Molecular Weight	238
	6.12.2 Electron Density	238
	6.12.3 Polar Surface Activity	239
6.13	Physicochemical Properties of a Successful CNS Drug	242
6.14	Physicochemical Parameters Required for a Preclinical Candidate Drug	243
	Exercises	244
	Competitive Exam Questions	244
	References	245

Chapter 7 Receptorology 247

7.1	Introduction	247
7.2	Receptors as Drug Target	248
	7.2.1 Comparison between Receptors and Enzymes	249
	7.2.2 Intravital Ligands (Endogenous)	250
	7.2.3 Ligands that Enter Cells	250
	7.2.4 Ligands that do not Enter Cells	250
	7.2.5 Signal Transduction	252
7.3	Distribution of Receptors in the Cell	253
	7.3.1 Cell-Surface Receptors	253
	7.3.2 Enzyme-Linked Receptors	267

7.3.3 Ionotropic Receptors 274
7.3.4 Intracellular Receptors (Nuclear Receptors) 281
Exercises 282
Competitive Exam Questions 283
References 283

Chapter 8 Drug Receptor Theories 285

8.1 Drug–Receptor Occupation Theories 285
8.1.1 Hypothesis of Clark 286
8.1.2 Hypothesis of Ariens and Stephenson 286
8.1.3 Simple Occupancy Theory 286
8.1.4 Rate Theory 286
8.1.5 Macromolecular Perturbation Theory 287
8.1.6 Activation Aggregation Theory 287
8.1.7 The Receptor Cooperativity Model 287
8.1.8 The Mobile Receptor Hypothesis 287
8.1.9 Two-State Receptor Theory 287
8.2 General Mechanism of Drug–Receptor Interaction 287
8.2.1 Lock and Key Hypothesis 288
8.2.2 Induced Fit Model 288
8.3 Drug–Receptor Complex Formation and Dissociation 288
8.3.1 The Dose–Response Relationship 289
8.3.2 Binding Affinity and K_D 290
8.3.3 Relationship between Formations of Drug–Receptor Complexes with Occupancy of Receptors 290
8.3.4 Calculation of Receptor Occupancy 291
8.3.5 K_D and EC_{50} 291
8.4 Agonists/Partial Agonists/Inverse Agonists 292
8.4.1 Drug Agonists 293
8.4.2 Partial Agonists 297
8.4.3 Inverse Agonists 298
8.5 Spare Receptors 299
8.5.1 Examples of Spare Receptors 300
Exercises 300
References 301

Chapter 9 Receptor Antagonists 303

9.1 Introduction 303
9.2 Orthosteric Binding Site 303
9.3 Allosteric Site 304
9.3.1 Positive Allosteric Modulators (PAMs) 306

9.3.2 Negative Allosteric Modulation (NAM) 306
9.3.3 Silent Allosteric Modulators 307
9.4 Receptor Antagonists 311
9.4.1 Antagonists for Cell Surface Receptors 311
9.4.2 Enzyme-Linked Receptor Blockers 312
9.4.3 Ion Channel Blockers (Ionotropic Receptor Antagonists) 312
9.4.4 Antagonists for Intracellular Nuclear Receptor 312
9.4.5 Types of Antagonism 313
Exercises 318
References 318

Chapter 10 Drug–Target Interactions 319

10.1 Introduction 319
10.1.1 Structural Features in Compounds Involved in Binding 319
10.1.2 Structure of Protein Targets 320
10.1.3 Role of Water in Drug-Protein Interaction 321
10.2 Reversible Interaction 321
10.2.1 Hydrogen Bonds 322
10.2.2 Ionic Bond (Salt Bridge) 328
10.2.3 Van der Waals Forces 329
10.2.4 Dipole-Dipole/Ion-Dipole/Dipole-Dipole Induced Forces 330
10.2.5 Hydrophobic Interaction 332
10.3 Irreversible Drug-Target Interaction (Covalent Bonds) 334
10.4 Summary of Drug-Target Interactions 334
10.4.1 Weak Interactions during Binding of Drug with GPCR 335
Exercises 336
References 336

Chapter 11 Pharmacokinetics 337

11.1 Introduction 337
11.1.1 Gastrointestinal Region as a Physiological Barrier 338
11.1.2 Liver as a Physiological Barrier 339
11.1.3 Physiological Barriers during Blood Circulation 340
11.1.4 Aqueous/Lipid Environment as a Physiological Barrier 340
11.1.5 pH of Aqueous Environment as a Physiological Barrier 340
11.1.6 Effect of Physicochemical Properties of Drugs/Test Compounds on ADME 340
11.2 Kinetics of ADME 341
11.2.1 Absorption 342
11.2.2 Distribution 346
11.2.3 Metabolism 352
11.2.4 Excretion 356

11.3 Methods for Assessing Major Physicochemical Properties of Compounds that Affect ADME Parameters 362
11.3.1 Measuring Solubility 362
11.3.2 Measuring Lipophilicity 362
11.3.3 Measuring pK_a 363
11.3.4 Measuring Permeability 363
11.4 Summary of Important PK Parameters 363
Exercises 364
Competitive Exam Questions 364
References 365

Chapter 12 Pharmacodynamics 367

12.1 Introduction 367
12.2 Biochemistry of Enzymes 368
12.2.1 Enzymes as Biocatalysts 369
12.2.2 Active Site 369
12.2.3 Cofactors 372
12.2.4 Enzyme-Catalysed Reactions 373
12.2.5 Enzyme–Substrate Complex 373
12.2.6 Enzyme-Substrate Interactions 374
12.2.7 Factors Affecting Enzyme Activity 375
12.3 Allosteric Enzymes 389
12.3.1 Allosteric Activation (PAM) 390
12.3.2 Allosteric Inhibition (NAM) 391
12.3.3 Silent Allosteric Modulators (SAM) 392
12.3.4 Effect of Allosteric Modulation on Reaction Kinetics 393
12.4 Nomenclature of Enzymes 393
12.4.1 The EC Numbers 393
12.5 Role of Enzymes 398
12.5.1 Diagnostic Enzymes 398
12.5.2 Therapeutic Use of Enzymes 398
12.5.3 Enzymes as Therapeutic Targets 399
Exercises 403
Competitive Exam Questions 404
References 405

Chapter 13 Role of Enzymes in Metabolism 407

13.1 Introduction 407
13.1.1 Microsomal Enzymes 407
13.1.2 Non-Microsomal Enzymes 408

13.2 CYP-Mediated Metabolism 408
13.2.1 Types of Metabolic Reactions 408
13.3 Examples of Phase I and II Drug Metabolism 416
13.4 Cytochrome P450 Enzyme Induction and Inhibition 418
13.4.1 Enzyme Induction 418
13.4.2 Enzyme Inhibition 418
13.5 CYP Enzyme Induction by Exogenous Substances (Pharmacokinetic Drug-Drug Interactions) 420
13.5.1 Physiological Consequences of CYP Enzyme Induction 420
13.6 Pharmacodynamic Drug-Drug Interactions 420
13.7 Drug-Design Approach for Making Drugs More Resistant to CYP Metabolism 421
13.8 Examples of Other Non-Microsomal Metabolisms 422
Exercises 422
Competitive Exam Questions 423
References 423

Chapter 14 Digestive Enzymes 425

14.1 Introduction 425
14.1.1 Release of Digestive Enzymes 426
14.2 Digestion of Proteins 427
14.2.1 Proteases 427
14.3 Zymogens (Proenzymes) 435
14.3.1 Pepsinogen 435
14.3.2 Pancreatic Zymogens 436
14.4 Protein Homeostasis 437
14.5 Digestion of Lipids 439
14.5.1 Emulsification and Digestion 439
14.6 Digestion of Carbohydrates 443
Exercises 444
Competitive Exam Questions 444
References 445

Index 447

CHAPTER

1

Cell Signaling and Diseases

KEY THINGS TO LEARN FROM THIS CHAPTER

- Role of cellular communication in the pathogenesis of diseases
- Components of cell signaling pathways:
  - Intracellular signaling: First and second messengers, Signal transduction
  - Intercellular signaling: Endocrine, paracrine, autocrine and direct signaling
- Participation of signaling pathways in homeostasis
- Participation of signaling pathways in cell metabolism
- Classification of diseases
- Emerging intracellular signaling components as therapeutic targets

1.1 Introduction

In a multicellular organism, the cells continuously communicate with each other through endogenous cell-specific signals and carry on with their normal cellular/physiological functions assigned to them. Each cell depending on the assigned task not only communicates intracellularly through signal transduction but also communicates intercellularly with other cells. Besides endogenous signals, cells also respond to exogenous cues and try to keep the organism healthy and free from any disorders by maintaining the cellular functions in an orchestrated manner (through homeostasis/feedback loops). Any defect/impairment in signaling pathways involving intercellular or intracellular communication may disrupt the normal cellular/physiological functions and result in a disease. Numerous phenotypic and genotypic diseases have been attributed to defects/errors in signaling pathways. For example, concentration of glucose level in bloodstream is regulated by two signaling molecules insulin and glucagon (endogenous cues), however, deficiency or excess of either one of the signaling molecules leads to disruption in downstream signaling pathways within cells and cause diabetes.

1.1.1 Components of Cell-Signaling Pathways

Each cell carries cell-surface receptors made up of proteins that can detect structurally diverse chemical signals (ligands/first messenger) in a highly specific manner. The chemical signals that cannot diffuse through plasma membrane bind with cell surface receptors whereas the ones that can diffuse through the membrane

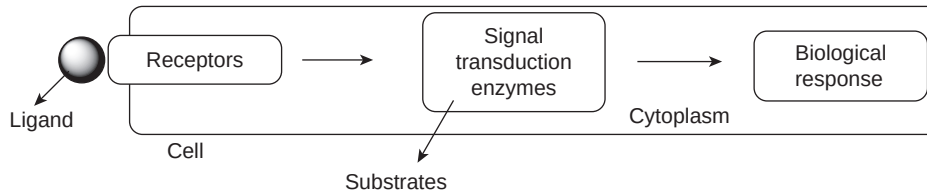


Figure 1.1 The three components of cell signaling are reception, transduction and cellular response.

binds with intracellular receptors. The ligand-receptor interaction is followed by transduction of signal into the cell, called intracellular signaling, which is then responsible for downstream signaling pathway leading to the cellular response. Within cells, the intracellular signaling pathway amplifies its signal through a series of second messengers, effector molecules (primarily proteins), signaling enzymes that together are responsible for cellular/physiological functions. The endogenous first messengers/ligands are generally released by cells/tissues within the organism in the form of neurotransmitter/hormone in the bloodstream. Through circulation, they either travel to a distant part of the body to the cell bearing its receptor or may act on nearby cells for intercellular signaling. Thus, together these intra- and inter-cellular signaling pathways play a crucial role in the overall maintenance of the physiological function in the multicellular organism. The three key components of cell signaling are reception through receptors, signal transduction through effectors/enzymes and cellular response as a result of downstream signaling (Figure 1.1). The molecular components in signaling pathways leading to a response provide options for developing drugs with agonistic or antagonistic properties with the view to modulate the functioning of receptors (using ligands) or enzymes (using substrates). In general, pathogenesis of any disease arises primarily due to the impairment in the functioning of enzymes or receptors involved in the signaling pathways.

There are two scenarios that can lead to defect in any of the signaling pathways thereby causing various diseases.[1] In one scenario, any exogenous signal (cue) that has entered the body in the form of xenobiotics can induce defect in the signaling pathway and in the other scenario, aberration with the endogenous cues (first messenger) may result in the defect in the signaling pathway. This defect in the signaling system ultimately results in the pathogenesis of disease.

1.2 Chemical Messengers

A chemical messenger is a compound that serves to transmit message via signal for the purpose of communication among cells in the organism. This communication among cells via signal regulates basic functions of cells and plays an important role in maintaining homeostasis (maintenance of equilibrium/stability of internal physiological conditions within the organism in response to external stimuli) in the organism.

The mode of action for signaling molecules are governed by their physicochemical properties preferentially hydrophilicity/hydrophobicity, pK_a, solubility, molecular weight/size, etc. The hydrophilic molecules preferentially bind to the receptors present on the cell surface of the target as they are unable to penetrate the lipid bilayer of the cell membrane. On the contrary, hydrophobic molecules such as steroid hormones, thyroid hormone, vitamin D_3, and retinoic acid readily diffuse through lipid bilayer cell membrane and bind to intracellular receptors in the cytoplasm or nucleus for intracellular signaling. Once signaling molecules bind to these receptors, they give rise to intracellular signal transduction through several cytosolic chains of molecules. Thus, there are two kinds of messengers: first and second messengers.

1.2.1 First Messengers (Signaling Molecules)

First messengers also known as ligands are chemicals present in the extracellular domain that elicit cellular response within a cell by binding to a receptor. The chemicals that act as first messenger are structurally diverse in nature and could be intravital or entering the body through environment. The chemical nature of these molecules within an organism may range from neurotransmitters to hormones (peptides) such as epinephrine, acetylcholine, serotonin, growth hormone, and insulin. Since these chemical messengers are hydrophilic in nature, they are unable to diffuse through bilayer cell membrane and therefore bind with receptors (made up of proteins) present on the surface of the cell. Binding of these hydrophilic extracellular first messengers to cell-surface receptors is followed by change in its conformation that triggers transduction of signal through the intracellular component of the cell-surface protein receptors extending within the cell. The signal transduction is propagated intracellularly (signal amplification), which regulates the cellular/physiological functions through a series of events involving second messenger/effectors, etc., (intracellular signaling pathways).

Besides cell-surface receptors, there are intracellular nuclear receptors present in either cytoplasm or nucleus and are involved in genomic signaling, thereby modulating gene expression. These receptors primarily bind to steroid hormones as ligand (first messenger). Ion channel receptors (ligand-gated) present in membranes of cells and cytosolic organelles comprise yet another class of receptors and are involved in the movement of ions across the membranes. (Details of structure and function of receptors have been discussed in Chapter 7)

1.2.1.1 Examples of First Messenger

Glucagon-like peptide (GLP-1) as First Messenger

Glucagon-like peptide (GLP-1), a hormone secreted by intestinal enteroendocrine L cells, acts as first messenger/signaling molecule by binding with receptor present at the cell surface and relay signal within the cell for the release of insulin.

Thus GLP-1, an endogenous ligand, acts as a first messenger and after binding with cell membrane receptor activates the release of insulin through secondary mediators. However, under diabetic conditions, Dipeptidyl peptidase (DPP-4) enzymes present on the cell surface degrade GLP-1, thus blocking the binding of GLP-1 with the receptor and in turn prevent the release of insulin. Interestingly, researchers identified exenatide, as a 39-amino acid peptide that is resistant to DPP-4 degradation, binds with the receptor as an exogenous ligand/first messenger, and stimulates secretion of insulin (Fig. 1.2).



Additional Information

Neurotransmitters

They are signaling molecules involved in the process of communication (a nerve impulse) from neuron to neuron, neuron to muscle (contraction/relaxation) and neuron to endocrine system, to secrete hormones. In the unidirectional neuron to neuron signaling, presynaptic neurons release signaling molecules that bind with receptors present on post-synaptic neurons.

In the gastrointestinal region, the gut neurotransmitters are secreted by network of neurons (100 million neurons – more than the spinal cord) lining the gut, and the network is known as enteric nervous system. Major neurotransmitters in the gut comprise: acetylcholine, serotonin, dopamine, glutamate, norepinephrine (NE), small brain proteins: neuropeptides, and nitric oxide (NO).

Neuropeptides

These substances play an important role in the bidirectional gut–brain communication. For example, luteinizing hormone and follicle-stimulating hormone, gonadotropins, neurokinins, and Corticotropin-releasing factor (CRF).

Both neuropeptides and gut hormones act as first messengers and target the same cell-surface receptors (typically G protein-coupled receptors) and lead to the different biological implications.

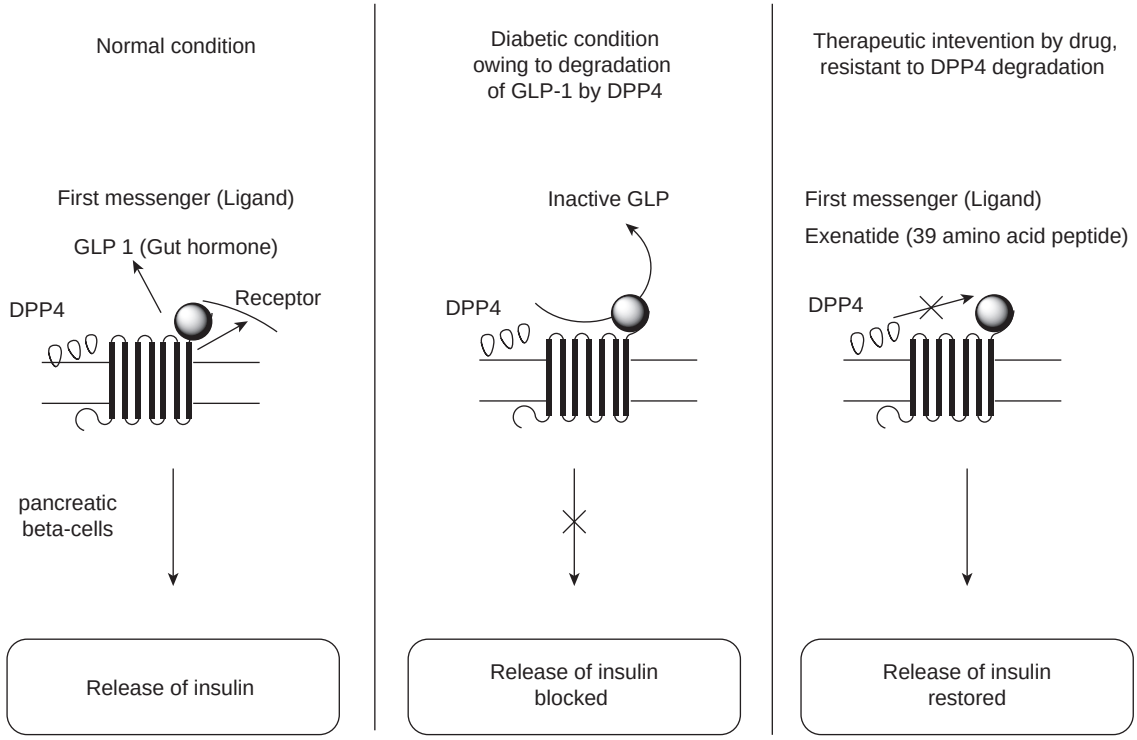


Figure 1.2 Substituting intravital first messenger GLP-1 with exogenous drug exenatide to treat diabetes (DPP4 = dipeptidyl peptidase 4).

Signaling molecules can be categorized under endogenous signal produced by our body cells (intravital) and exogenous signals that occur naturally all around us in the environment and keep entering our body through our gastrointestinal and respiratory tracts. Because cells of all species use many of the same signal molecules, these molecules often induce a response in the body and can affect both our physiological function and our behavior. They can be beneficial or deadly, but they all work by activating or blocking receptors/enzymes in our cells.

Exogenous signal molecules can be divided into the four major sources: bacterial, multicellular tissue, pheromones, and xenobiotics (including drugs). It is interesting to note that several exogenous signal molecules have corresponding endogenous receptors that can readily identify them. For example, plant products such as nicotinic (from tobacco), muscarinic (from mushrooms), and cannabinoid (from cannabis), all have corresponding endogenous receptors in our human body.

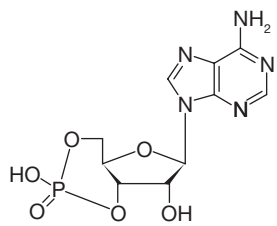
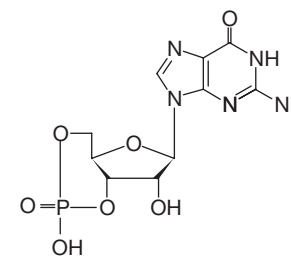
Serine Protease as First Messenger

In the last two decades, serine proteases have been identified as first messenger that activate Protease-activated receptors (PARs)[2] present on cell surface of platelets. However, they bind differently in comparison to conventional first messengers based on neurotransmitters, hormones, and steroids. Thrombin, for example is a serine protease which binds with PARs and plays an important role in coagulation.

1.2.2 Second Messengers

The binding of first messenger/ligand to receptors on the cell surface is followed by change in its conformation that triggers transduction of signal through the intracellular component of the cell-surface protein receptors extending within the cell. The signal transduction within the cell commences with the production of small chemicals called second messengers via effectors. The latter are intracellular signaling molecules which receive extracellular signal from receptor and initiate intracellular signaling or signal transduction pathways. Two of the ubiquitous effectors involved in intracellular signaling pathways are adenylyl cyclases (ACs) and guanylyl cyclases (GCs). In general, the signal transduction pathways initiated by these effectors commence with production of second messengers primarily responsible for downstream signaling pathways. Some of the prominent second messenger molecules include (Table 1.1): nitric oxide (NO), inositol trisphosphate (IP_3), diacylglycerol (DAG), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and calcium. Notably, NO is the only second messenger that is gas and binds to soluble guanylyl cyclase enzyme (effector), which acts as a receptor. The downstream signaling pathways within the cells involve amplification and relay of signals via multiple intracellular signal molecules such as enzymes and effector proteins. The latter, in turn, triggers downstream cascade by phosphorylating other enzymes or transcription factors responsible for cell proliferation, differentiation, migration, survival, and apoptosis.

Table 1.1 Molecular structure and examples of second messengers

 cAMP	 cGMP
Examples of Second Messengers	**Examples of Hormones which Utilize this System**
cGMP	Atrial natriuretic hormone, NO
cAMP	Neurotransmitters epinephrine and NE, LH, FSH, Thyroid-stimulating hormone (TSH), calcitonin, Parathyroid hormone (PTH)
IP_3 and DAG	TSH, vasopressin, neurotransmitter GABA
Calcium	Neurotransmitters epinephrine and NE, Angiotensin II (Ang II), GnRH, Thyrotropin-releasing hormone (TRH)
NO and CO (gases)	Plays a role in the activation of an intracellular enzyme, soluble guanyl cyclase that catalyzes formation of the second messenger cGMP.

1.2.2.1 Examples of Second Messenger

Cyclic AMP as Second Messenger

Among second messengers, cAMP plays an important role in numerous cellular functions. It is produced (synthesized) from ATP in response to the activation (G-protein-mediated) of adenylyl cyclase (AC) by the first messenger. Activation of the receptor by one molecule of ligand induces many signal transduction pathways by amplifying the signal intracellularly and can produce hundreds of cAMP molecules. The cAMP in turn activates intracellular enzyme protein kinase A (PKA) involved in cellular functions (Fig. 1.3). The cAMP pathway may involve increase/decrease in the concentration of cAMP in response to the first messenger leading to loss/gain of function. The cAMP is degraded by the enzyme Phosphodiesterase (PDE) which in turn, stops signaling process. This phenomenon of loss/gain function can be used as an opportunity for therapeutic intervention in treating diseases by designing agonist or antagonist for the second messenger.

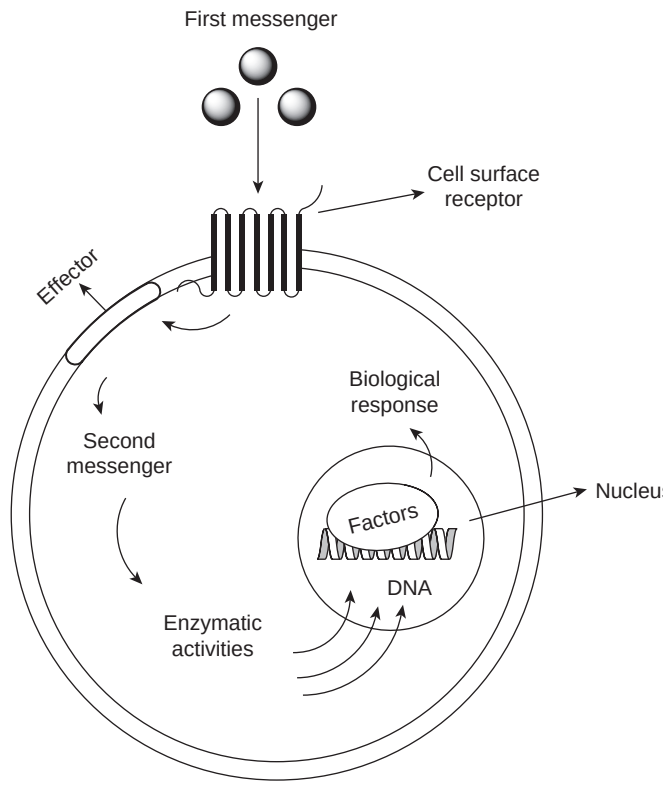


Figure 1.3 A typical signal transduction involving first messenger and second messenger.

Cyclic GMP as Second Messenger

It is yet another important class of second messengers that play an important role in numerous physiological functions. It is produced from guanine trisphosphate (GTP) in the cell in response to the activation of acetylcholine receptor (AChR) by acetylcholine (intravital ligand) as first messenger followed by

sGC/NO/cGMP signaling pathway. Alternatively, it can be produced by the activation of the membrane-bound Atrial natriuretic peptide (ANP) receptor A/receptor guanylyl cyclase-A linked to guanylyl cyclase (rGC) by ANP as first messenger followed by GC/cGMP signaling pathway (Fig. 1.4). Thus, there are two types of GCs involved in the production of cGMP from GTP: sGC forms present in the cell cytoplasm and rGC forms embedded in cell membranes.

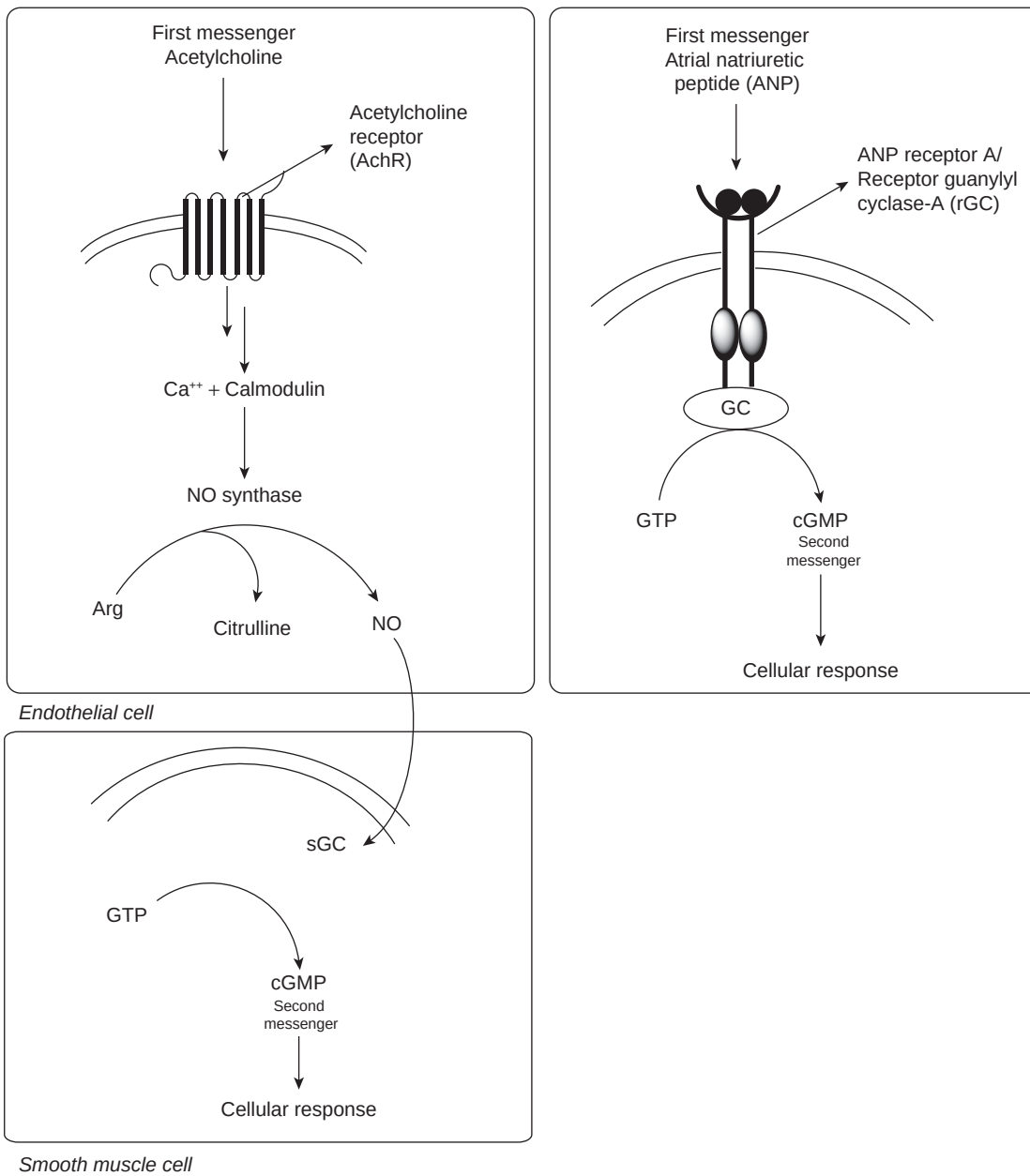


Figure 1.4 Two different pathways for the generation of cGMP.

CASE STUDY 1

Role (Therapeutic) of the Second Messenger cGMP in Disease Situations Such as Male Reproductive Dysfunction and Angina

Biological disorders resulting in a diseased situation may involve several biochemical pathways; they are all regulated by first and/or secondary messengers. In two clinical situations involving angina and erectile dysfunction, there is less supply of blood than is required in heart muscles and in corpus cavernosum, respectively. This predominantly results from the inactivation/degradation of second messenger cGMP. Formation of intracellular cGMP occurs following activation of adenylyl cyclase by systemic second messenger NO. The cGMP in turn, plays a key role in the modulation of vascular function maintaining flow of blood as required. However, termination of activity of cGMP by PDE enzymes blocks the supply of blood. Thus, PDEs are good target for therapeutic intervention (Fig. 1.5).

Sildenafil citrate drug (Fig. 1.6) acts as a substrate and inactivates the enzyme PDE which, in turn, blocks the degradation of cGMP. Thus, the second messenger cGMP remains in its native form and maintains vasodilatory effect for increased duration.

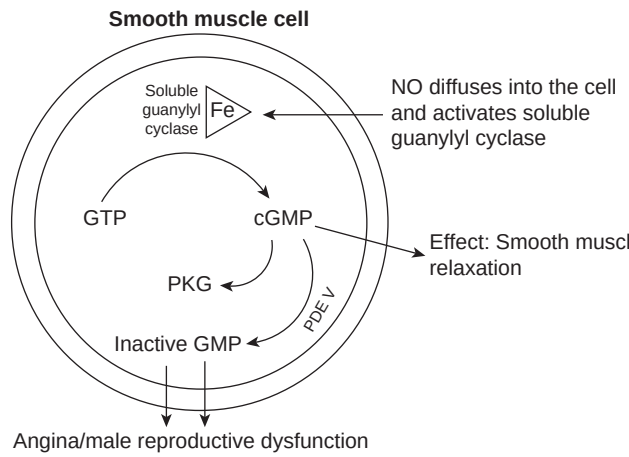


Figure 1.5 Hydrolysis of second messenger cGMP by PDE leads to reproductive dysfunction and angina.

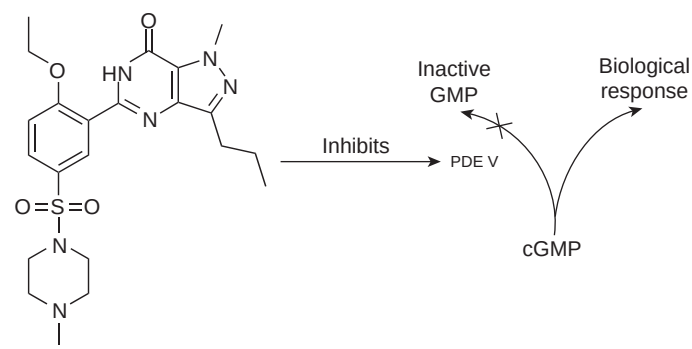
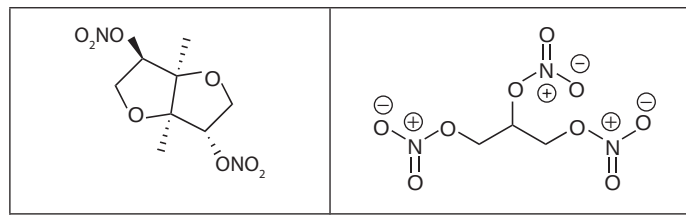


Figure 1.6 Sildenafil citrate prevents degradation of cGMP.

Nitric Oxide (NO) as a Second Messenger

NO is the only gaseous substance that acts as second messenger. It is produced in endothelial cells following deamination of Arg in the presence of the enzyme NO synthetase (NOS) and is involved in the modulation of vascular tone. The NOS is activated by acetylcholine/Ca^{++}/calmodulin pathway in the endothelial cells. Next, the NO moves out of the endothelial cells and diffuses into neighboring smooth muscle cells. After entering the cell, it activates the enzyme soluble guanylyl cyclase (in the presence of iron) which in turn produces cGMP, a second messenger, from GTP. Notably, soluble guanylyl cyclase as an intracellular enzyme is the only enzyme that acts as a receptor for the second messenger NO.[2] Decrease in the production of cGMP due to insufficient concentration of NO has been implicated in numerous diseases including cardiovascular diseases such as angina pectoris. Thus, administration of NO precursor nitroglycerine and sorbitrates (Fig. 1.7) as sublingual tablet has remained in practice for many years for an instant relief from angina.

Both the examples discussed above clearly suggest that defects resulting in signaling pathways can be corrected by administering drugs that can either act as ligands regulating signal transduction at receptor level or as substrates regulating enzyme activities.



Isosorbide dinitrate	Nitroglycerine

Figure 1.7 Structures of sorbitrate and nitroglycerin.

CASE STUDY 2

Role of Intracellular Signaling Network in Glucose Uptake in Cells

Insulin, a pancreatic hormone, plays an important role in the metabolism of glucose, protein, and lipid. In response to high level of glucose in blood circulation, insulin is released from pancreas, which binds with Insulin-receptor tyrosine kinase (IR) present on the surface of the many tissues including liver, muscle and fat cells. The binding of insulin with the IR triggers a series of intracellular signal events which ultimately allows GLUT 4 (glucose transporters) to translocate to the surface and open up to initiate uptake of glucose from surroundings. The intracellular signal events involve phosphorylation and recruitment of insulin-receptor substrate (IRS) proteins followed by the activation of Akt (also known as Protein Kinase B; PKB) and a PKC (Protein kinase C) signaling pathways (Fig. 1.8). Next, when the concentration of insulin and glucose in blood circulation goes down, GLUT4 stops glucose uptake and translocate back inside the cell. Among many causes for the development of insulin resistance, defects in intracellular signaling events at any stage (following binding of insulin to the receptor) can be one of the contributing reasons.

(*Continued*)

Study 2 (*Continued*)

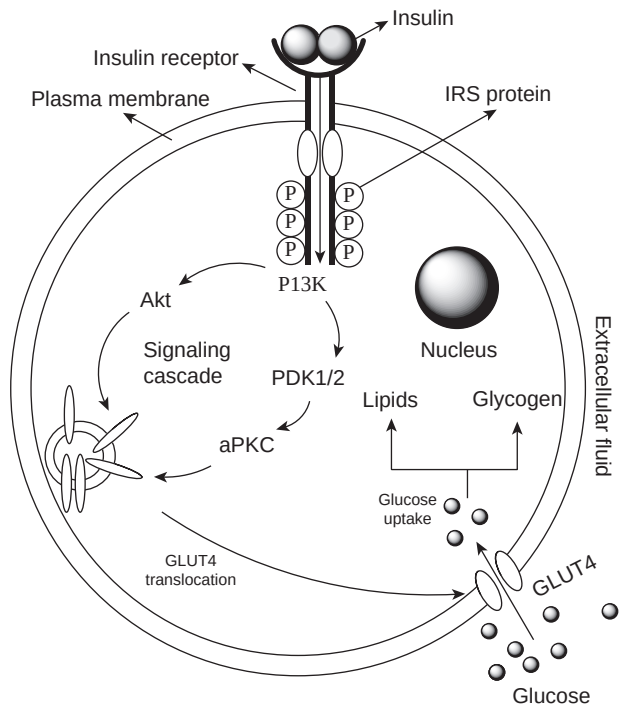


Figure 1.8 Role of second messengers in glucose uptake in cells.

CASE STUDY 3

Role of Second Messenger cAMP in Fat Cells

Opposing actions of insulin and glucagon in fat cells play an important role in the regulation of triglycerides (TG) levels. Glucagon signaling through cell-surface receptor (G-protein-coupled receptor; GPCR) activates AC and enhances production of cAMP followed by PKA activity, while insulin followed by its binding with IR (receptor tyrosine kinase) inhibits PKA activation by increasing the activity of PDE, which blocks cAMP and subsequently keeps PKA in its inactive form (Fig. 1.9).

The cAMP/PKA cascade following glucagon-mediated activation of AC is responsible for the HS-lipase-mediated breakdown of TG into glycerol and fatty acids. The latter are subsequently released into the bloodstream. In fat cells, insulin on one hand opposes this action and blocks the breakdown of TG by activating PDE, which in turn inactivates cAMP. On the other hand, it allows uptake of glucose which is used to produce glycerol. The latter is used to synthesize TG using free fatty acid from liver, thus allowing build-up of TG levels in fat cells. Although TG is needed to supply energy to our body between meals, excess TG in bloodstream can cause damage to the heart. Fibrates and statins are drugs of choice clinically used for the lowering of TGs.

(*Continued*)

Study 3 (*Continued*)

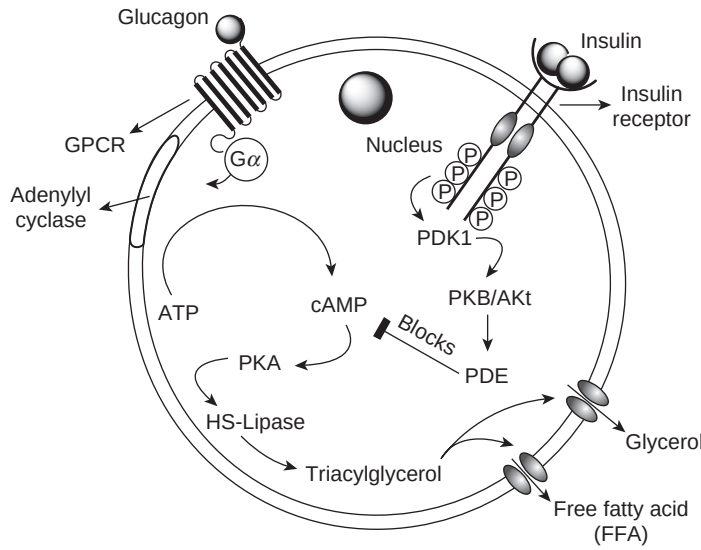


Figure 1.9 Graphical representation depicting opposing action of insulin and glucagon in fat cells.

1.3 Signal Transduction

Signal transduction involves a cascade of intracellular molecular events that begins with the stimulation of cell surface receptors by first messengers. The main objective of signal transduction is to trigger intracellular cellular functions through a network of biochemical pathways in response to external cues. The binding of external signals to cell surface receptors induces changes in the conformation of receptors which results in the generation of a signal. Latter is transmitted inside the cells through the intracellular component of the cell surface receptors leading to signal transduction cascade. Within cells the first category of molecules that respond to the signal are intracellular membrane bound cytosolic enzymes called effectors. These effectors generate multiple energy-carrying molecules (ATP/GTP) which then initiate a chain enzyme catalyzed biochemical event that in turn regulates the cellular/physiological functions such as metabolism, growth and proliferation (Details discussed in chapter 7). The cytosolic enzymes that participate in the downstream signaling leading to cellular functions are called signal transduction enzymes.

1.3.1 Signal Transduction Enzyme

A majority of cellular functions essential for the survival and cell cycle progression are regulated by a family of enzymes called protein kinases. They regulate the biological activity of cytosolic proteins via continuous activation and deactivation which in turn modulates cellular functions. To carry out these cellular functions, cellular proteins need to undergo activation and deactivation. One of the common phenomena for the activation involves phosphorylation of proteins which is catalyzed by signal transduction enzyme protein kinase A (PKA). In this reaction, the phosphate group is provided by either ATP or GTP. Cells have many kinases stimulated by numerous second messengers and phosphorylate different protein targets involved in different biochemical pathways.

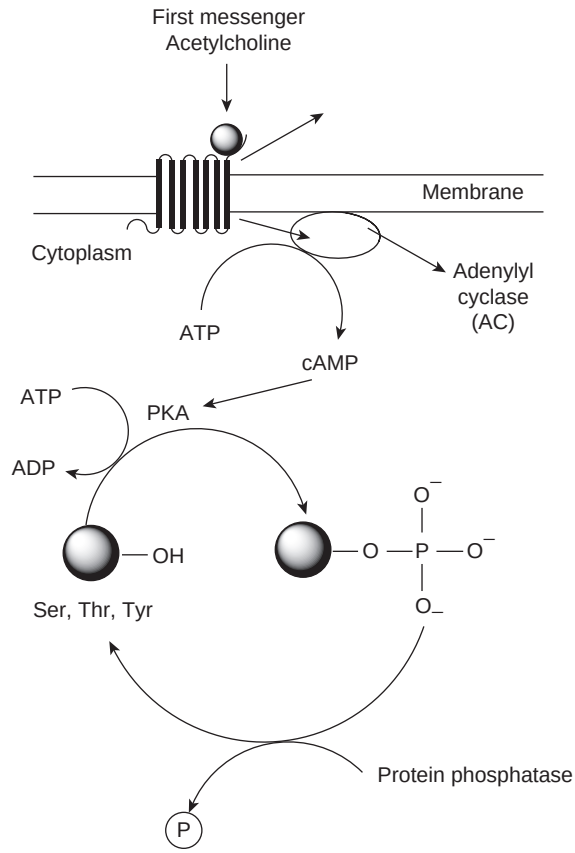


Figure 1.10 Signal transduction enzyme PKA.

Phosphorylation of proteins occurs in any of the three amino acids: Ser (Serine), Tyr (Tyrosine), and Thr (Threonine) having a hydroxy group in the side chain. Following phosphorylation of protein and its activation to perform the assigned cellular function, the process is reversed by yet other signal transduction enzyme phosphatase that triggers dephosphorylation of proteins (Fig. 1.10). In order to maintain these cellular functions, cells keep receiving signals and respond via several cytosolic signal transduction pathways (Fig. 1.11). This provides numerous opportunities for therapeutic intervention either at the receptor level or at enzyme (kinases) level for treating diseases arising due to impairment in signaling network. Protein kinases have been identified as therapeutic target for a number of diseases such as diabetes, cancer, etc.



Important Note

Protein kinases have emerged as one of the most frequently used drug targets for specific therapeutic interventions in a variety of clinical settings. Analysis of human genome has led to the identification of more than 500 different kinases, which regulate a variety of cellular functions such as, proliferation, apoptosis, metabolism, transcription, differentiation, survival etc. However, kinase inhibitors as anticancer agents do present challenges with the design of clinical trial protocols.

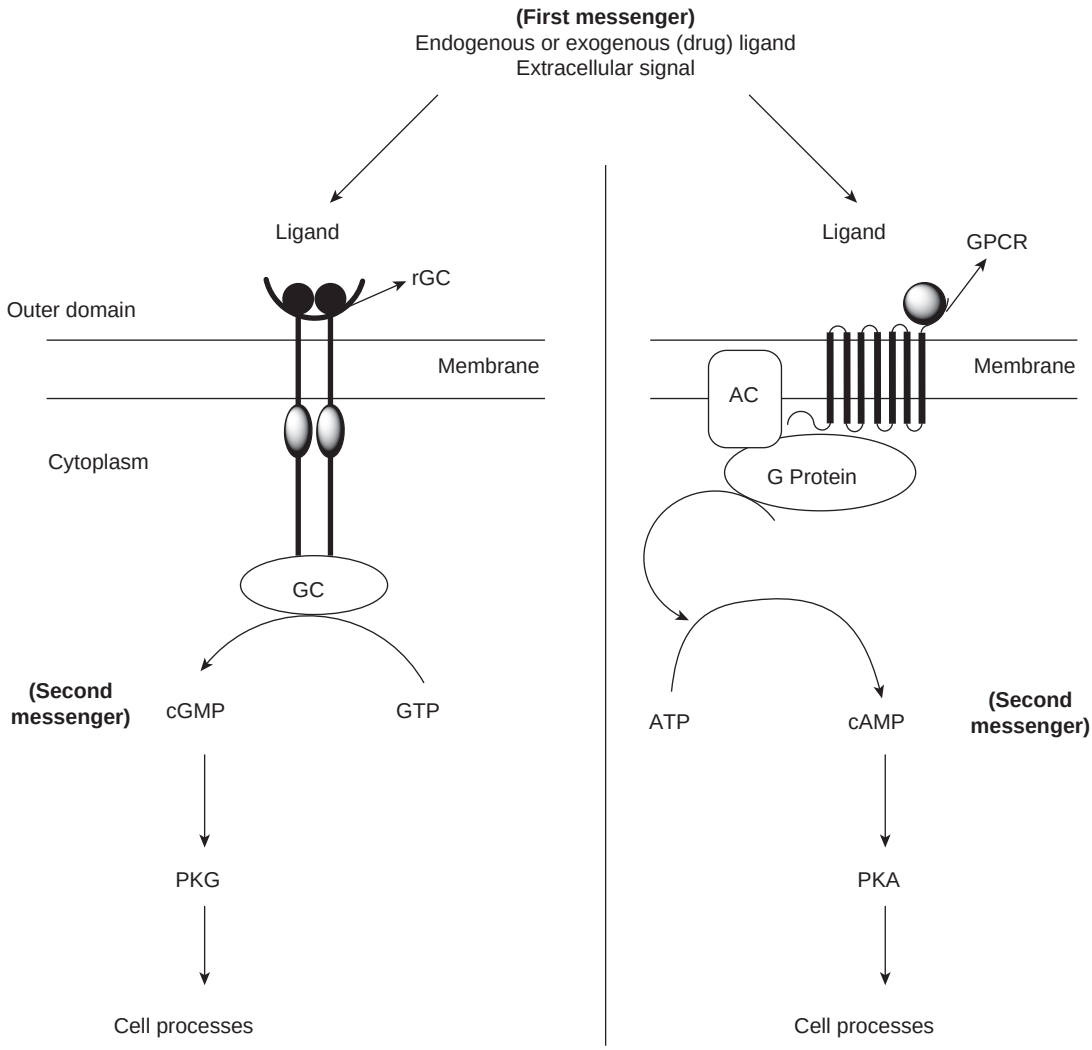


Figure 1.11 Diagrammatic representation of first/second messenger and effector proteins.

1.4 Effectors

Effectors are a group of membrane-bound cytosolic enzymes that alter biochemical processes in a cell in response to an external signal by decreasing or increasing the production of second messengers from ATP. Signals are produced following the binding of a first messenger (stimulus) to cell-surface receptors and transmitted inside the cells that activate the effectors for downstream signaling.

For G-protein coupled receptors (GPCR) receptors, the signal inside the cells triggers release of one of the trimeric cytosolic G-proteins that in turn bind with a membrane-bound effector molecule adenylyl

cyclase and catalyzes conversion of ATP into second messenger cAMP. In fact, the membrane-bound adenylyl cyclases are activated only after binding to ligands to GPCRs followed by its activation. Adenylyl cyclases are a group (family of nine) of best-characterized membrane-bound cytosolic enzymes except for a soluble AC (a ubiquitous enzyme) in cytoplasm that responds to bicarbonate. The adenylyl cyclase–cAMP-mediated downstream signal pathways are part of numerous cellular functions. Thus, adenylyl cyclase can be good therapeutic target for modulating the intracellular downstream signals. Although a variety of inhibitors and stimulators of AC have been reported but no clinical candidate for this path has yet been discovered.

The second example of effector molecules is phospholipase C (PLC), a phosphodiesterase, is another example of effector molecule that hydrolyses phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$ or PIP_2) creating two second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). These second messengers, in turn, regulate intracellular levels of free Ca^{++} and protein kinase C (PKC), which are responsible for cellular function and gene function (Figs. 1.12 and 1.13) respectively.

Yet another effector molecule involved in signal transduction is guanylyl cyclase, a lyase enzyme which catalyzes conversion of GTP into second messenger cGMP. There are two kinds to guanylyl cyclase, one linked with cell-surface receptor called guanylate cyclase-coupled receptor (Fig. 1.12) and second one is a soluble form called soluble guanylyl cyclase and is present in cytoplasm.

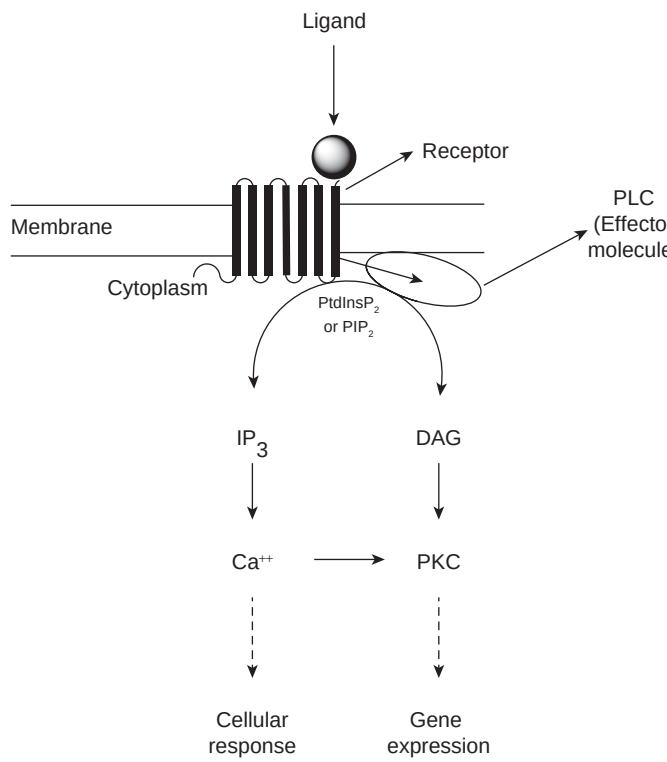


Figure 1.12 Phospholipase signaling interactive pathway.
(PLC, effector molecule; Ca^{++}/DAG/IP_3, second messengers; PKC, signal transduction enzyme).

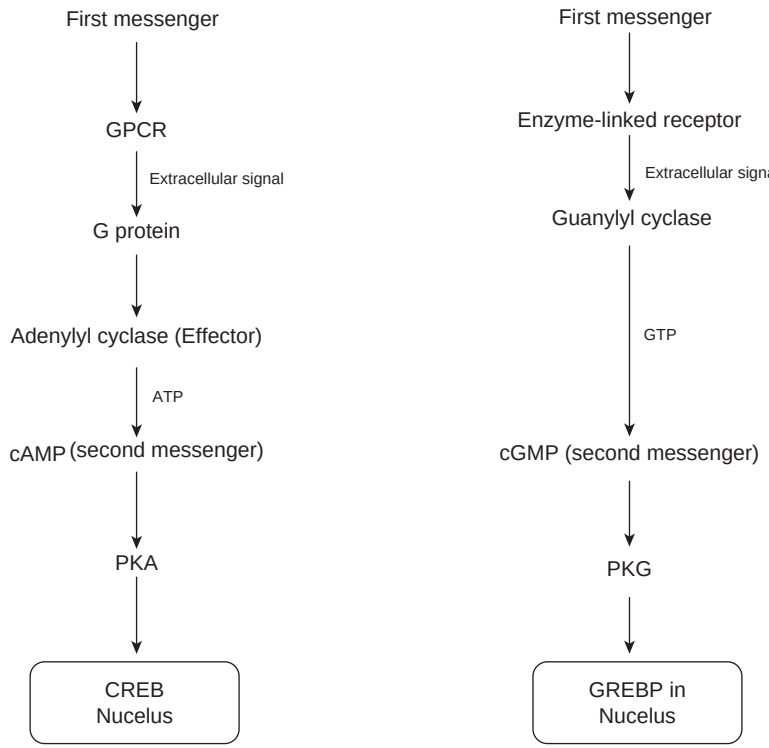


Figure 1.13 The chains of molecules that relay signals inside a cell leading to CREB (cAMP-responsive element-binding protein) and GREBP (cGMP-response element-binding protein).

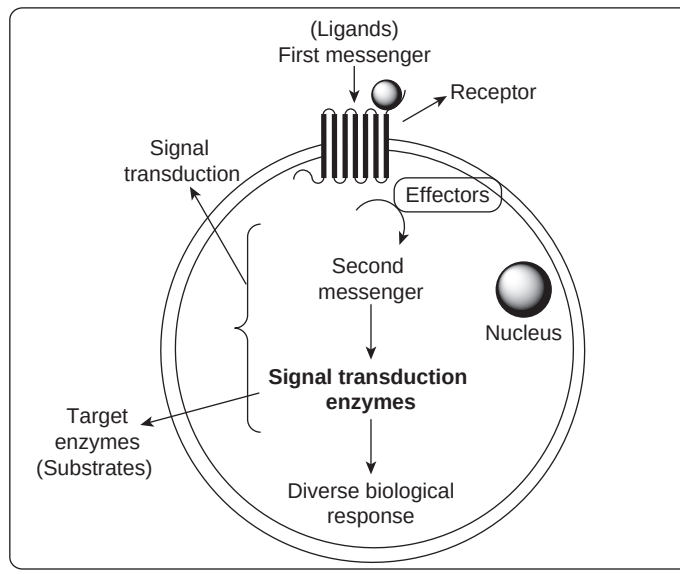
1.5 Signal Transduction Modulators as Drugs

Modulating signal transduction by small molecules has emerged as an exciting field of investigation in drug discovery. All differentiated cells are associated with specific functions that are regulated by cell-type specific signals. Interestingly, although individual cells respond to intravital or extravital cues and carry out their normal intracellular specific functioning, the same cells in groups (forming an organized sector of cells referred to as tissues), continue to communicate and coordinate among them via intercellular signaling. In other words, each cell depending on the task assigned to them not only communicate intracellularly through signal transduction but also communicate intercellularly. Thus, in multicellular organisms, there is a variety of signal transduction networks controlled by natural/endogenous ligands or by xenobiotics (exogenous cues) that either works in tandem or parallelly with the view to maintain the normal functioning of the entire organism and keeps it disease free. The cell signaling is initiated by three major classes of receptors: cell-surface receptors, intracellular/nuclear receptors, and ion channel receptors (discussed in Chapter 7).

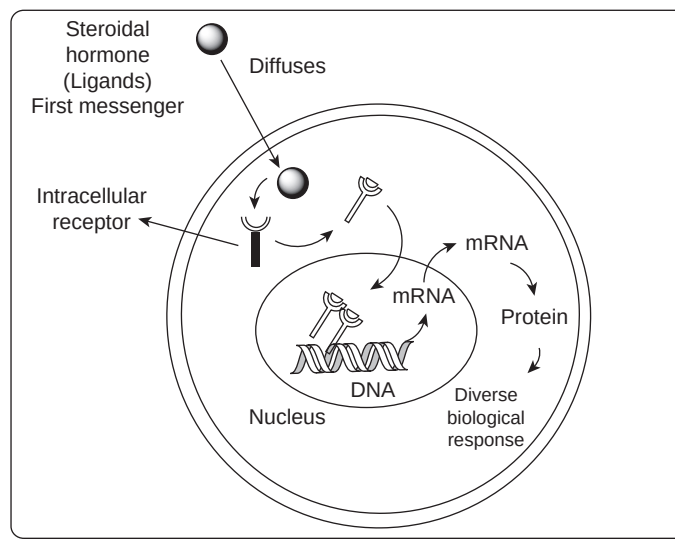
Any defect(s) in signaling pathway(s) can lead to a variety of disease(s). The defect in signaling pathway (communication) interferes with normal functioning of cells/tissues and in turn 'cells cry for help' which is manifested in the form of symptoms for any disease. The defect in signaling pathway can be either phenotypic or genotypic. Among extracellular cues originating from pathogenic organisms, the pathogens can disturb the normal functioning of the immune system by interfering with normal signaling events, thereby leading to

infection. Similarly, life-threatening non-infectious diseases caused by phenotypic aberrations of signaling pathways involve diabetes, cardiovascular diseases–hypertension and heart disease and CNS disorders: Alzheimer's and Parkinson's diseases.

Thus, signaling transduction pathways provide numerous opportunities for therapeutic intervention using small molecules as drugs either at the extra or intracellular receptor levels or at the enzyme level for treating diseases arising due to deviation in the normal signaling events (Fig. 1.14).



(a) *Extracellular receptor*



(b) *Intracellular receptor*

Figure 1.14 Two major therapeutic biological targets in the signal transduction.

There are two major drug targets in signal transduction (Fig. 1.15):

1. **Receptors:** Ligands as therapeutic drugs with agonist and antagonist properties that can bind with receptors and interfere with signals.
2. **Enzymes:** Substrates as therapeutic drugs with agonist and antagonist properties that can activate or inhibit intracellular signaling enzymes (transduction enzymes).

Thus, understanding the complex processes of signals involved between exterior and interior in a cell and among the cells can provide opportunities to discover novel therapeutic targets (enzymes/receptor) for drug discovery. For example, clinically used antidiabetic drugs display their activity by either targeting enzymes (Table 1.2) or receptors (Table 1.3) in different signaling pathways.

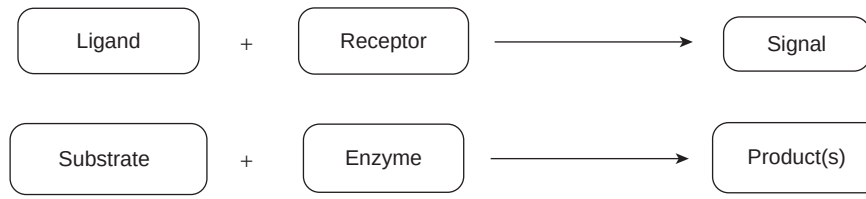


Figure 1.15 Two major drug targets in the signal transduction.

Table 1.2 Antidiabetic drugs (Substrates) that target enzymes

Drugs	Actions
Metformin	It binds with lipid phosphatase SHIP2 (a second messenger-mediated signaling enzyme) and inhibits its activity resulting in enhanced glucose uptake in muscle cells
α-glucosidase inhibitors	It inhibits extracellular α-glucosidase enzyme (located in the brush border of small intestine) in the intestine and interfere with digestion of carbohydrates
DPP-4 inhibitors	The drugs inhibit the extracellular enzyme present on the cell surface that degrades GLP-1, thus allowing GLP-1 to activate the receptor that signals release of insulin
PTP 1B inhibitors	Protein tyrosine phosphatase is an intracellular negative signaling enzyme for insulin. Its inhibitors are still under investigation

Additional Information

Phenotype

A phenotype is any visible trait or characteristic of a disease. The visible trait includes morphology, development, or behavior whereas characteristic of disease includes biochemical or physiological properties. The characteristics may be influenced both by its genotype and by the environment of the individual.

Genotype

A genotype is the set of genes that the organism carries. It refers to the two alleles (a variant form of a gene) an individual has for a specific trait (a) if identical, genotype is homozygous and (b) if different, genotype is heterozygous.

Table 1.3 Antidiabetic drugs (Ligands/First Messenger) that target receptors

Drugs	Actions
Insulin	Binds with insulin receptor tyrosine kinase and in turn triggers uptake of glucose from bloodstream
Sulfonylureas	Binds K_{ATP} channel receptor located on β-cells in pancreas and trigger secretion of insulin by closing the channel
Thiazolidinones	It binds with nuclear receptor peroxisome proliferator-activated receptor-gamma (PPRγ) in fat cells and regulates metabolism of glucose and lipid via transcription of corresponding genes
Meglitinides – nateglinide	They bind with receptors present on β-cells in pancreas and in turn closes K_{ATP} channel
GLP-1 agonists	Binds with GPCR receptor present on pancreatic β-cells and in turn trigger release of insulin

1.6 Intercellular (Between-Cells) Signaling

Besides, intracellular signal transduction dealing with communication between external cues and intracellular response, communication among cells/tissues occurs via intercellular signals. The latter helps cells to communicate with other cells around them (near and far) and respond temporarily to pass on the external cues. Thus, for the proper functioning of multicellular organisms, cells are constantly involved in a complex system of communication with each other. The intercellular communication via signals plays important role in numerous physiological functions such as propagation of signals during cell proliferation, propagation of impulse in neurons and heart, response by tissues to first messengers, propagation of signals during tissue homeostasis, etc. The intercellular signaling between cells is facilitated by circulating blood, which carries water, antibodies, macrophages, toxins, xenobiotics (including drugs), neurotransmitter, and hormones throughout the body. Required substances keep moving in and out of the blood capillaries into surrounding organs/tissues and perform the desired functions. The capillary walls through which the blood flows are made up of endothelial cells and act as barrier between the blood and surrounding tissues. Before we understand the intercellular cell-signaling process, it is important that we understand the complex structure of blood vessels comprising endothelial cells, nerves, and smooth muscle layer, fluid compartments, and extracellular matrix in multicellular organism.

Multicellular organisms have well-coordinated organized systems of organs that function in an integrated manner for the survival of the organisms. The integrated activity among organs is coordinated by the endocrine and nervous systems. The whole organization comprises different stages starting with cells that make up tissues and tissues that make up organs that carry out different functions. The tissues in organs are covered with a complex network of blood capillaries and nerves (Fig. 1.16).

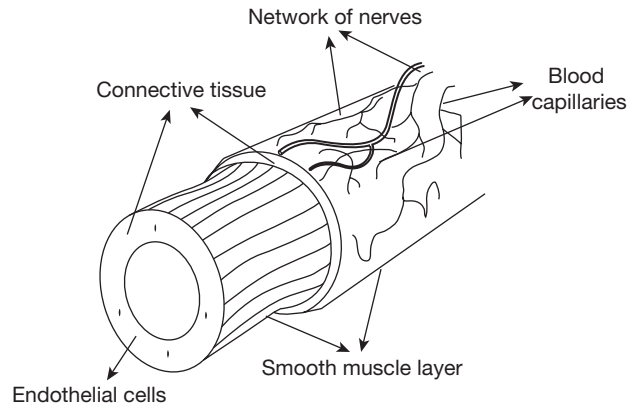


Figure 1.16 An integrated diagram of endothelial cells, blood vessels, network of nerves, and smooth muscle layer.

1.6.1 Fluid Compartment

As we all know that 60% of human body is made up of water. The aqueous fluid compartment in the body can be grouped into two regions: intracellular and extracellular. The two-thirds of the total body fluids are considered to be present within the cells while the remaining one-third of body fluid is present in the extracellular fluid compartment (ECF) outside the cells. The ECF can be further divided into two regions: aqueous fluid in blood plasma in capillaries surrounding the cells and the fluid in the space between cells called interstitial fluid (Fig. 1.17). The latter is the major component surrounding the cells and serves as an aqueous medium during the exchange (to and from) of nutrients, hormones, neurotransmitters, sugars, drugs, electrolytes, etc., across the membranes of endothelial cells lining the walls of capillaries. The phenomenon of exchange is regulated by the physicochemical properties of the substance and occurs via either of the following techniques: direct diffusion, transporter-assisted facilitated diffusion, fenestrated capillaries, and sinusoids or by endo/exocytosis.

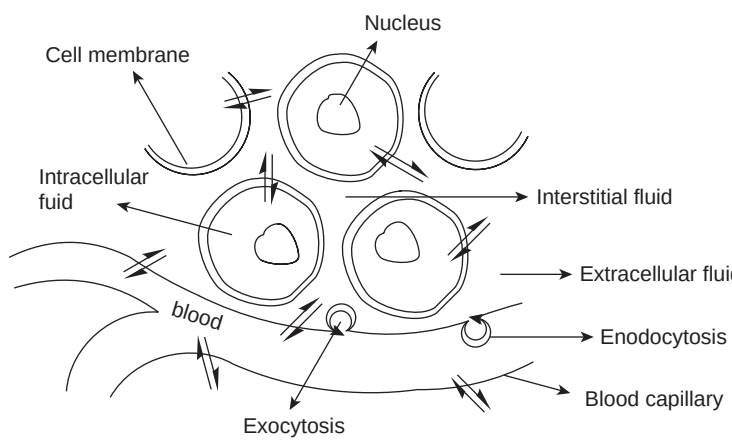


Figure 1.17 Extracellular and interstitial fluids.

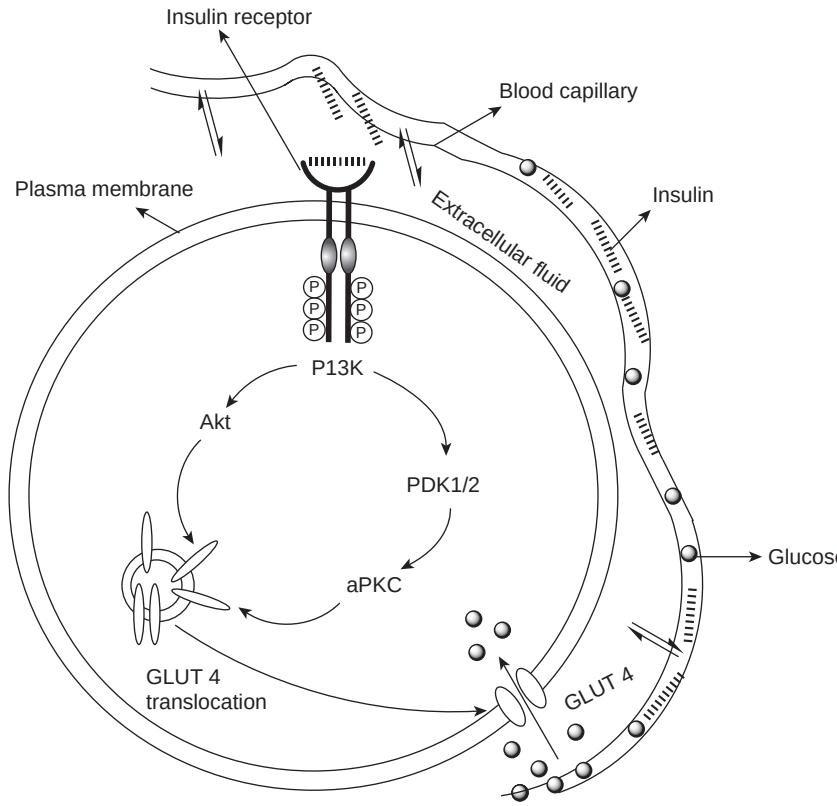


Figure 1.18 A diagrammatic representation of circulating insulin and glucose.

For example, pancreatic hormone insulin following its release in systemic circulation moves out of the blood capillaries into the interstitial fluids and binds with the cell-surface IRs present in surrounding muscle or fat cells. This in turn triggers uptake of glucose, which again moves out of systemic circulation into the ECM for uptake by GLUT present on the cell membrane (Fig. 1.18).

1.6.2 Extracellular Matrix

The Extracellular matrix (ECM) comprises a meshwork of polysaccharides and proteins in three-dimensional formats and plays an important role in the structural framework to tissues, organs, and the body. However, the amount of connective tissue varies with organs. They are a major component in cartilage and bone but are minor constituents in spinal cord and brain (Fig. 1.19). The ECM undergoes continuous remodeling (repair) in response to tissue homeostasis that regulates balance among components of ECM. Communication among cells in ECM occurs through chemical or electrical signals which may originate from internal or external cues and, in turn, regulate the composition of ECM. Impairment in the intercellular signaling leads to failures in the regulation of the ECM composition and has been implicated in numerous diseases such as neurodegenerative disorders, breast cancer, COPD, etc. One of the signaling molecules integrin is involved in the communication between ECM and cells, which occurs via cell-surface receptors. Integrin binds ECM and triggers intracellular signal transduction activating downstream signaling enzymes kinases that are involved in the regulation of adhesion, proliferation, and survival of cells.

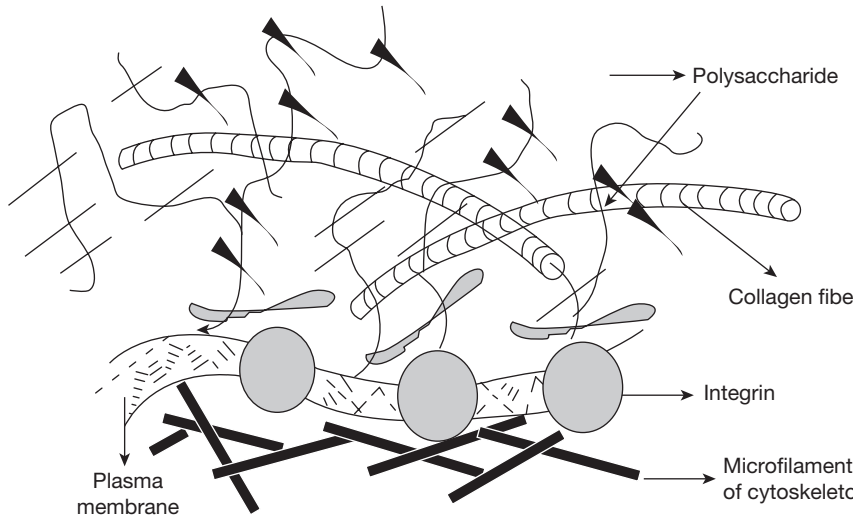


Figure 1.19 A diagrammatic representation of extracellular matrix.

1.6.3 Types of Intercellular Signaling (Communication) between Cells

In a multicellular organism, signaling (communication) between cells occurs either through release of chemical signaling molecules into interstitial fluids or by direct contact. The cells that release chemical signals (ligands) are sender cells and those that have receptors to detect the chemical signal are called target cells. It is to be noted that not all cells have ability to detect a particular chemical signal molecule; instead, they are recognized by specific cells with specific function. Once a signaling molecule is released by the signaling cell, it binds to the receptor on the target cells and induces intracellular signaling (Fig. 1.20).

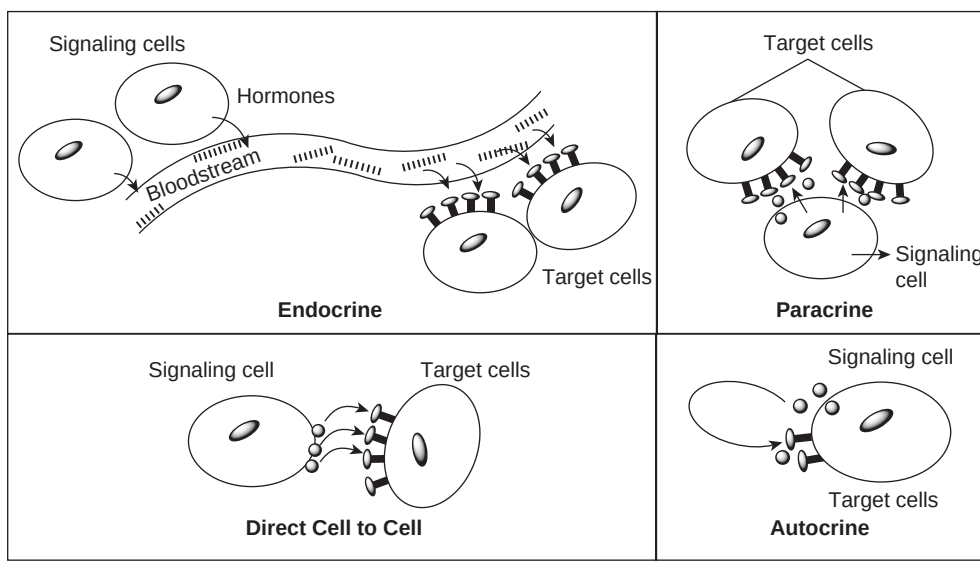


Figure 1.20 Variety of intravital signals inducers and signaling mechanisms.

Any miscommunication between cells arising due to errors in signaling pathways can lead to numerous diseases in a human body such as diabetes, cancer, etc., and can also form the basis for their treatment. Among many options to treat diseases, one option could be either blocking the endogenous signaling molecule or potentiating the signaling effect with an administered drug (possibly a mimetic of the signaling molecule). The four major types of intercellular signaling mechanisms that occur in organisms are paracrine, endocrine, autocrine, and direct signaling.

1.6.3.1 Endocrine Signaling

This involves a long-distance signaling via hormones secreted by endocrine glands directly into the blood. Through blood, the hormone as first messenger travels to target cells bearing specific receptors and binds to elicit the desired pharmacological response. The response arising from endocrine signaling is generally slower and long lasting (Fig. 1.21).

For example, the endocrine cells in the pancreas produce insulin (a hormone), which is directly secreted into the blood, from where it can travel long distance and act on target cells bearing the receptors specific for the insulin. Yet another example of circulating blood-borne hormones are renin, and angiotensin I and II that play a role in hypertension (Fig. 1.21) and are referred to as Renin–angiotensin system (RAS). As is evident from Fig. 1.21, rennin (hormone) is secreted by kidney, whereas angiotensinogen (hormone) is produced by liver cells, and both being endocrine hormones are directly released in the circulatory system and travel long distances to elicit their effect. During their circulation in blood, renin converts angiotensinogen into angiotensin I, which then enters lungs through blood circulation where it gets digested by angiotensin-converting enzyme (ACE) into angiotensin II. Finally, angiotensin II binds with angiotensin II receptor (AT_1R) present in the internal lining of arterioles, which leads to vasoconstriction and gives rise to hypertension. Sartans (ARBs) are used as antihypertensive agents that prevent the binding of angiotensin II to its receptor (AT_1R) and in turn inhibit vasoconstriction.

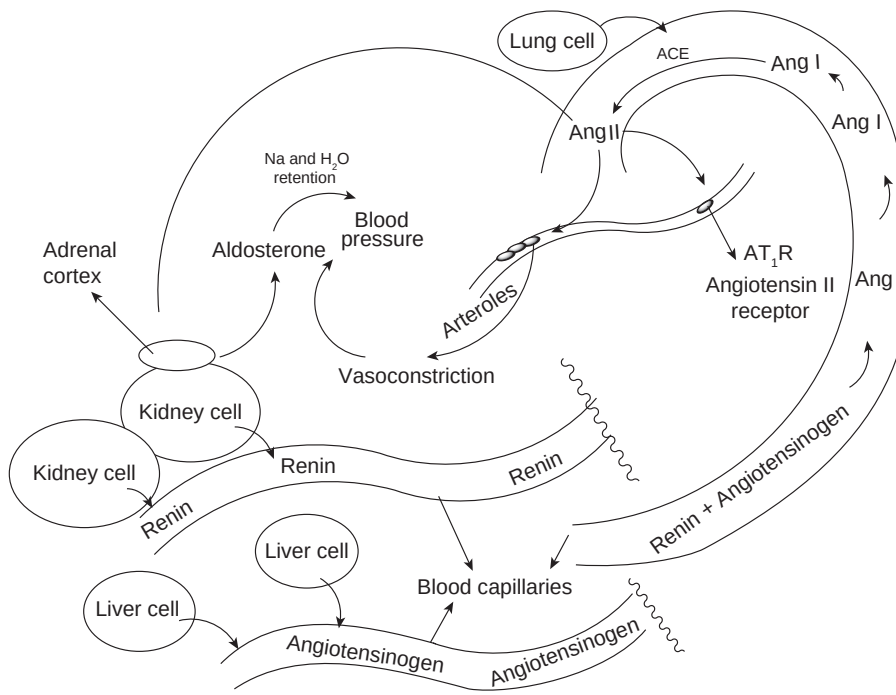


Figure 1.21 Endocrine signaling (blood-borne hormones renin, Ang I, and Ang II in circulation) in renin–angiotensin endocrine system (*only arterioles undergo vasoconstriction*).

1.6.3.2 Paracrine Signaling

It involves communication between local cells in which a cell produces a signal via chemical messenger (local mediators) to initiate changes in nearby cells around it (Fig. 1.20). The interstitial fluid assists in the diffusion of local mediators from one cell to another cell nearby. Unlike endocrine signaling, paracrine signals elicit quick response and for a short duration owing to the degradation of the paracrine ligands. Examples include NO, histamines, neurotransmitters, and prostaglandins. Histamine, a paracrine hormone secreted from enterochromaffin-like cells (ECL cells) in gut stimulates nearby parietal cell for the secretion of acid (Fig. 1.22).

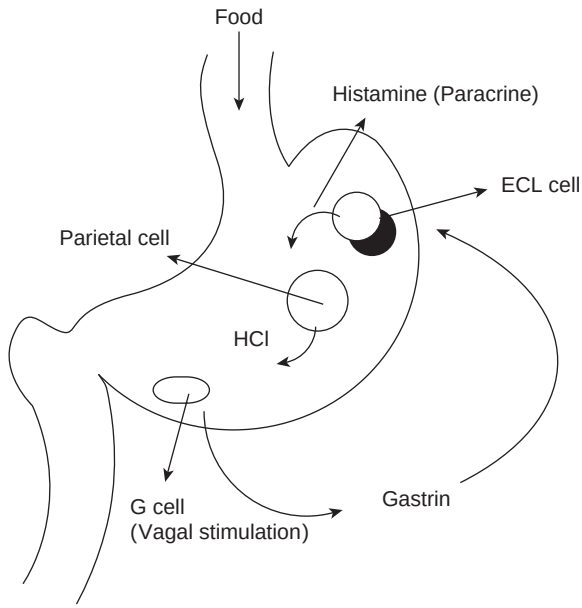


Figure 1.22 Histamine as a paracrine hormone.

Neuronal/Synaptic Signaling

Neuronal signaling is an example of paracrine signaling. It is a chemical and a unidirectional communication (a nerve impulse) process where neurotransmitters are involved as chemical signaling molecules. The neurotransmitters are stored in membrane-bound vesicles inside the axon terminal of the neurons. Following the transmission of electrical signal, the neurotransmitters from presynaptic neurons are released in the synaptic cleft and act as chemical messenger for transmission of the electrical impulse on to the postsynaptic neuron. Thus, neuron to neuron signaling occurs via neurotransmitters released by a neuron (the presynaptic neuron/secretory neuron) into synaptic space (Fig. 1.23). The released chemical messenger (neurotransmitter) in the cleft travels to the nearest postsynaptic neuron and binds to the receptors present on the cell surface. The reversible binding of a specific neurotransmitter causes particular ion channels on the postsynaptic membrane to open. A neurotransmitter continues to affect membrane potential as long as it remains bound to the postsynaptic receptor, which generally lasts few milliseconds before being terminated. Thus, signal molecules/neurotransmitters are responsible for generating and propagating unidirectional action potentials generated along the axon. The conduction of nerve impulses mediated by action potential is also known as firing of neurons.

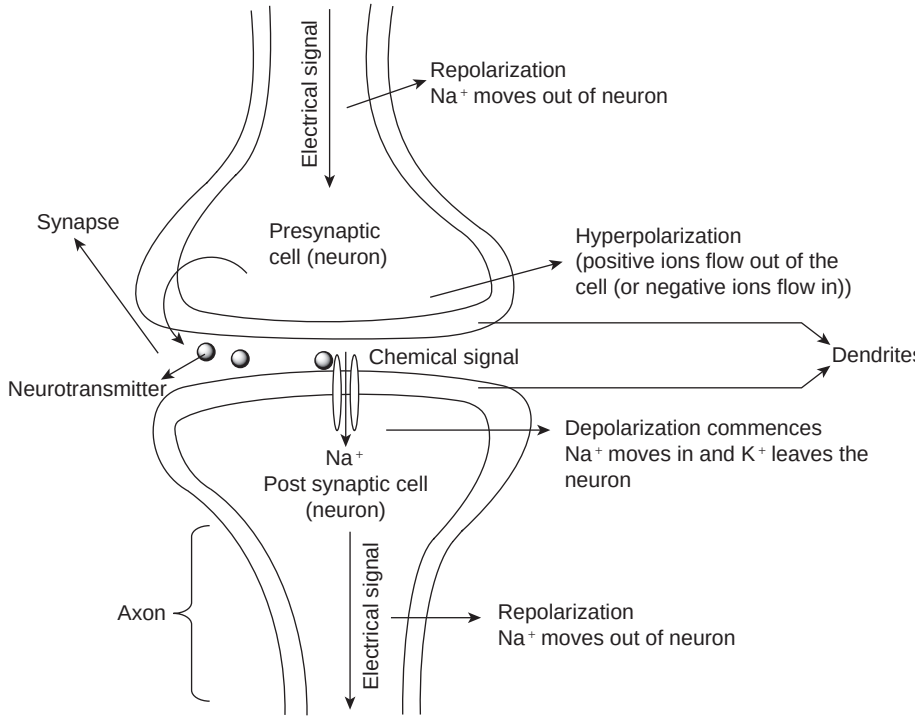


Figure 1.23 Neuronal/synaptic signaling involving unidirectional action potentials (firing of neurons).

Action Potential during Neuronal Signaling (Firing of Neurons)

The firing of neurons comprises depolarization, repolarization and hyperpolarization (Figure 1.24). The phenomenon involves change in membrane potential ranging from −90 to +55 mV. The change in membrane potential has been attributed to the difference in the electrical potential between the extracellular and intracellular regions of the plasma membrane. The concentrations of Na^+, K^+ and Cl^- ions on either side of the plasma membrane play a role in the generation of the electrical potential. Latter initiates the process of activation and inhibition of neurons (transmission of signals) which is governed by changes in the equilibrium between concentrations of oppositely charged ions across the membrane. Two major factors regulate the changes in the electrical potential: the sodium-potassium pump and the diffusion of K^+ ions through the potassium leak channel. Attraction between ions of opposite charges and repulsion between ions having same charges generates the electrical force while diffusion of ions involves movement of ions from higher to low concentrations.

In resting stage, intracellular region of neuron is negatively charged (−70 mV) due to the presence of K^+ and Cl^- in high concentrations. In contrast the outside of the neuron in synapse is positively charged due to the presence of Na^+ in relatively high concentration. The generation and maintenance of membrane potential at resting stage occurs through sodium-potassium pump and potassium leak channel. The membrane potential of −70 mV at resting is caused by movement of three Na^+ out of the cell for every two K^+ being pumped in. This leads to differences in the concentration of K^+ and charges (more negative inside than outside) across the membrane thereby creating chemical and electrical gradient. Thus, electrical gradient attracts K^+ back into the cell whereas concentration gradient allows K^+ to diffuse outside the cell. This ultimately, leads to equilibrium between the chemical and electrical gradient primarily due to potassium leak channel.

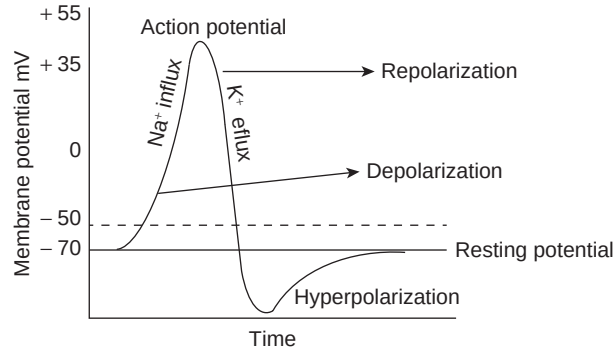


Figure 1.24 Action potential during firing of neurons.

During propagation of an electrical impulse, the neurotransmitter released from the presynaptic neuron binds with the membrane receptor on the postsynaptic neuron and triggers depolarization. This involves decrease of negative charge inside the cell or become more positively charged by allowing influx of sodium ion across membrane in the neuron from the surrounding. The conductance of Na^+ leads to depolarization of membrane potential and it changes from −70 mV to +55 mV. After reaching the peak, the Na^+ conductance decreases, and repolarization of membrane potential sets in at the peak of action potential. This is affected by increase in the conductance of K^+ which is accompanied by inactivation or closure of Na^+ channels. This trigger return of membrane potential to the resting potential, however during this course; the sodium conductance is reactivated once the membrane potential has reached −60 mV which in turn helps in the maintenance of the resting potential at −70 mV. At the resting potential of −70 mV, if the conductance to K^+ is increased, the membrane potential is hyperpolarized and moves to −90 mV.

Thus, at the synapse, the firing of an action potential in the presynaptic neuron causes the transmission of the electrical impulse via neurotransmitters (chemical messenger) to the postsynaptic neurons to fire its own action potential and continue with the propagation of impulse. Once the electrical signal moves on from the postsynaptic cell, the latter returns back to its normal resting potential, ready for new signals to arrive.

1.6.3.3 Autocrine Signaling

As the name suggests, the cell releasing chemical signaling messenger in response to external stimuli binds to the receptor (autocrine) present on the same cell in turn elicits response (changes in cellular function). In other words, both signaling and target cells are same (Fig. 1.20). An example of an autocrine agent is the cytokine interleukin-1 in monocytes that plays an important role in the pathogenesis and treatment of inflammatory diseases.

1.6.3.4 Direct (Juxtacrine) Signaling

Juxtacrine signaling is a type of cell-to-cell signaling in which two adjacent cells: emitting (releasing chemical signal) and receiving cells need to make physical contact in order to communicate (Fig. 1.20). It thus allows membrane-lined ligands on the emitting cells to reach their corresponding receptors on the receiving cells. Transferring of signaling molecules occurs via water-filled gap junctions (ECM) between neighboring cells. Alternatively, ECM may keep both the cells tightly localized near each other. Indeed, only small molecules can pass through this channel and macromolecules cannot fit through this channel. Thus, direct signaling allows a group of cells to coordinate their response with each other once any one of the cells may have received the signal. The direct signaling plays an important role in cell differentiation during embryonic stage.

CASE STUDY 4

Intercellular (Between-Cells) Signaling among Cells

Acetylcholine, an intravital neurotransmitter secreted from nerve cells causes vasodilatation and lowers blood pressure. The intravital signal inducer (first messenger) binds with GPCR found on endothelial cells lining blood vessels. This is followed by change in conformation of the receptor and activation of the NOS via intracellular signal involving influx of Ca^{++} ion. This results in the generation of NO and citrulline from arginine. The NO (signal inducer) then diffuses out of the cell and enters into neighboring smooth muscle cells where it binds with the Fe in GC. The activated guanylyl then produces cGMP (second messenger) from GTP. The cGMP in turn leads to smooth muscle relaxation and reduction in blood pressure (Fig. 1.25). This offers opportunity for therapeutic manipulation of cGMP and has been successfully exploited in angina pectoris, congestive heart failure, myocardial infarction, and hypertension either by NO donor drugs or by PDE inhibitors.

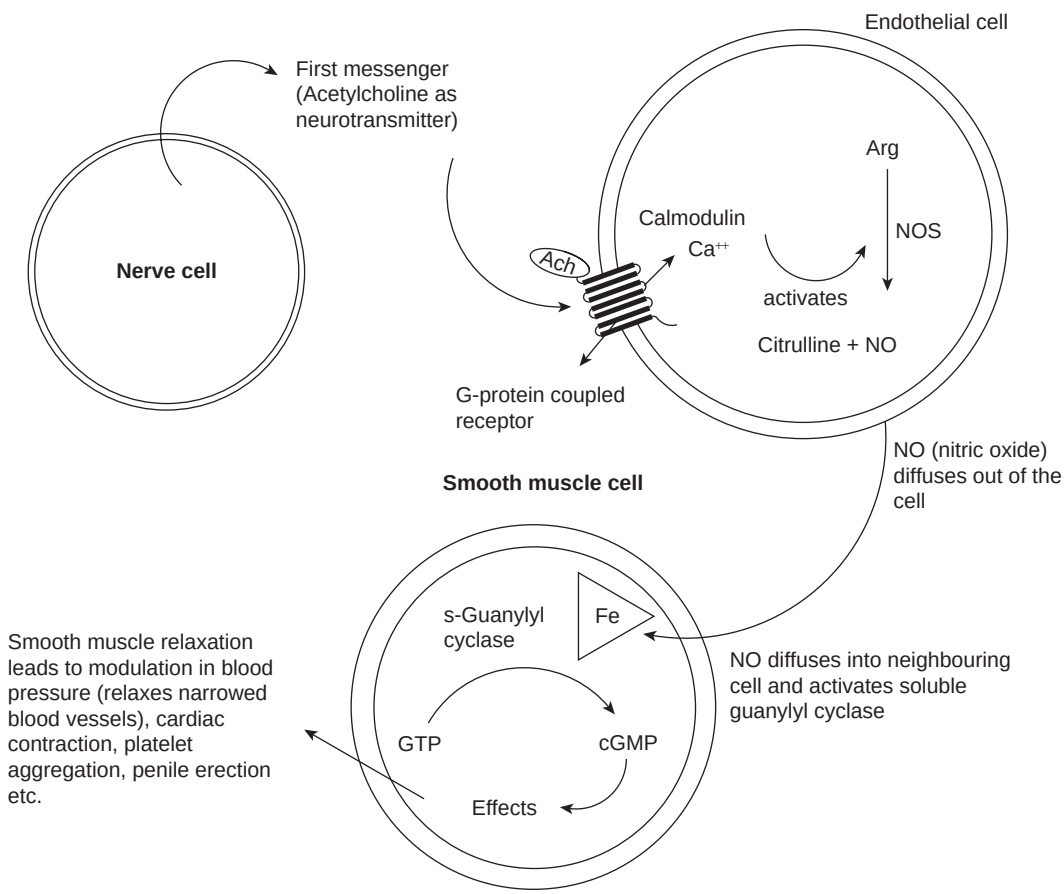


Figure 1.25 Intercellular (between-cells) signaling involved in the communication among nerve, endothelial and smooth muscle cells.

1.7 Homeostasis Regulated by Signaling Pathways

Homeostasis refers to the ability of an organism or cell to maintain stable state of equilibrium between two cellular/physiological functions despite changes in the environment. For example, a balance between cell growth and death is achieved through orchestrated signaling pathways mediated by complex functional interactions between components in the signaling axes. Similarly, intestinal homeostasis is regulated by a variety of signaling pathways that maintains a stable equilibrium between cell proliferation and differentiation. Other typical examples include maintenance of body temperature homeostasis, glucose homeostasis, body fluid homeostasis etc. At molecular level, maintenance of homeostasis involves interaction/communication between either neurotransmitters/hormones with receptors, or substrates with enzymes. In a given signaling axis, homeostasis occurs with forward activation and negative feedback regulations via signaling pathways (Figure 1.26).

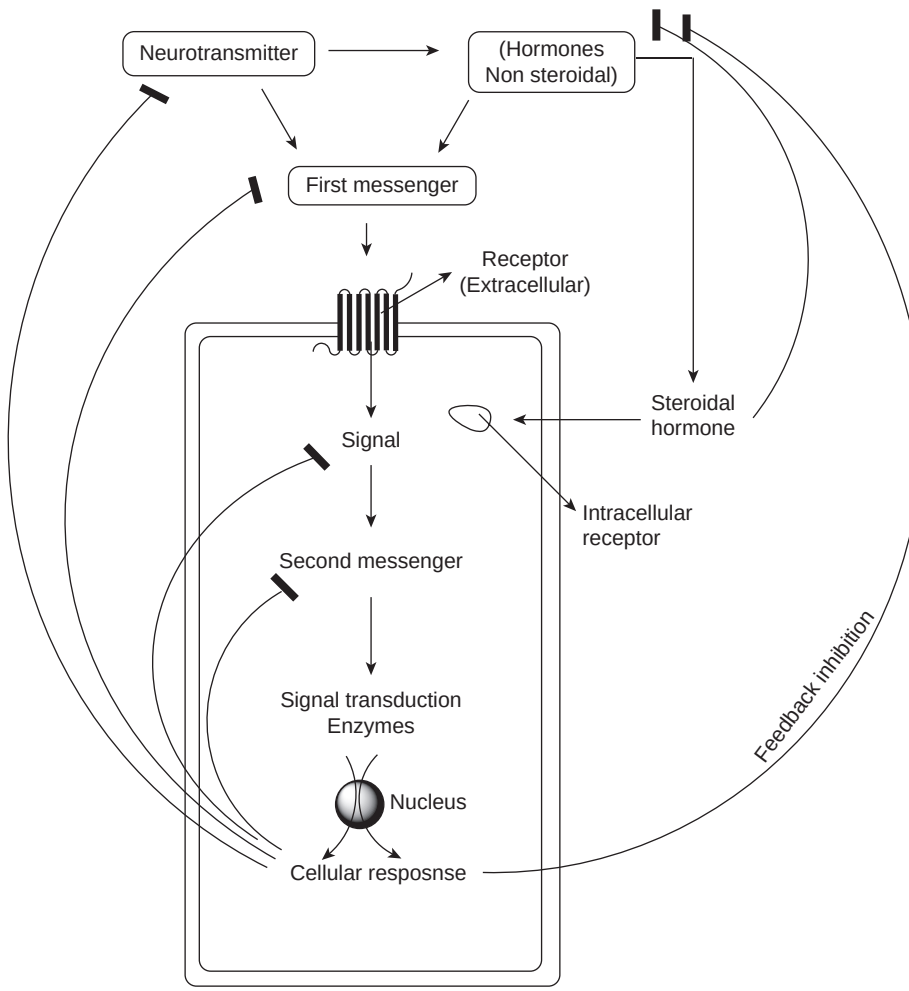


Figure 1.26 A diagrammatic representation of homeostasis with forward activation and negative feedback inhibitions.

CASE STUDY 5

Calcium Homeostasis

The presence of optimal concentration of calcium in blood circulation regulates many important functions in the body such as release of neurotransmitters, blood clotting, controlling heartbeat, muscle contraction, etc. However, decrease in calcium concentration can trigger bones to release calcium resulting in bone loss/osteopenia/osteoporosis. The homeostasis of the blood calcium level is regulated by two hormones: parathyroid hormone (PTH) and calcitonin secreted from parathyroid glands and thyroid gland, respectively. The increase in Ca^{++} levels on one hand triggers release of calcitonin that stimulates uptake of Ca^{++} by bones and on the other hand blocks secretion of PTH. On the contrary, decrease in Ca^{++} levels triggers release of PTH that stimulates release of Ca^{++} from bone in blood circulation (Fig. 1.27).

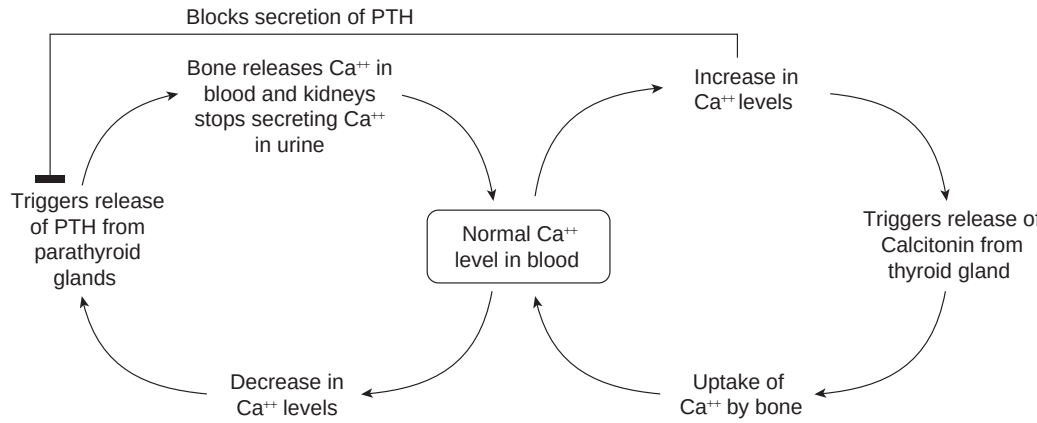


Figure 1.27 Calcium homeostasis involving PTH and calcitonin.

CASE STUDY 6

Feedback (Negative Feedback) Inhibition via Glucagon-Mediated Signal Transduction Pathway

During glucose homeostasis, low blood sugar levels trigger release of glucagon from α-cells in blood circulation. Glucagon acts as first messenger and signals liver and muscle cells to initiate glycogen breakdown into glucose (glycogenosis) followed by its release in blood circulation. Once glucose level rises in blood to a threshold level, it terminates the action of glucagon via negative feedback signal (Fig. 1.28). Another pathway involved in the maintenance of glucose levels in blood from dropping too low is gluconeogenesis. By this process, glucose is produced in liver from noncarbohydrate substrates such as fatty acid, lactate, and glucogenic amino acids. This generally occurs during heavy exercise, fasting, and in the absence of carbohydrate-rich diet and has been used as target of therapy for type II diabetes.

(*Continued*)

Study 6 (*Continued*)

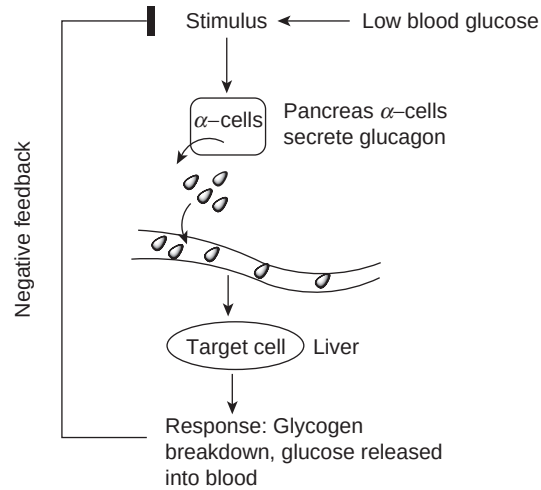


Figure 1.28 A negative feedback loop regulates glucose homeostasis.

CASE STUDY 7

Thyroid Homeostasis or Thyrotropic Feedback Control in the Hypothalamic–Pituitary–Thyroid Axis (HPT axis)

Maintaining a steady amount of thyroid hormones in bloodstream is essential for the vital cellular functions in the body. It important for the over-all health and plays a role in the functioning of many organs such as brain, kidney, heart, liver, etc. Regulation of thyroid homeostasis is under the direct control of neurons located in the hypothalamus. Neurons in the hypothalamus secrete thyrotropin releasing hormone (TRH), a tripeptide into hypophyseal portal circulation that carries TRH as signaling molecule to the anterior pituitary gland. The latter then secretes thyroid-stimulating hormone (TSH) which, in turn, is responsible for the synthesis and release of thyroid hormones T4 and T3. Once the levels of thyroid hormones exceed beyond the threshold level, it blocks pituitary and hypothalamus to release TRH and TSH by negative feedback loop (Fig. 1.29).

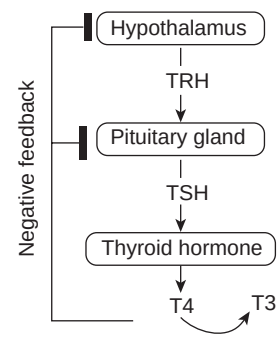


Figure 1.29 Feedback control in the hypothalamic–pituitary–thyroid axis (HPT axis).

1.8 Signaling in Cellular Metabolism

This plays an important role in the overall up keeping ('in-house keeping') of the organism through a set of biochemical reactions that occur inside cells. The signal mediated reactions carry out cellular functions through production of metabolites essential for the survival of the organism. The series of biochemical transformations in cells occur in a sequential and regulated manner also known as metabolic pathways. Some of the biochemical reactions involve biodegradation of ingested food, synthesis of building blocks such as amino acids/glucose and production of macromolecules such as proteins from amino acids. These controlled metabolic reactions are often linked together in chains or pathways and, in turn, plays a role in overall maintenance of physiological function in the organism.

At molecular level, the regulation of cellular metabolism (functions) requires interaction between enzymes and chemical metabolites via metabolite sensing and signaling mechanism. Any change in the environment of a cell leads to the modulation of their cell signaling, cellular metabolism and gene expression. Classical examples of regulators of cell metabolism are AMPK (5′ AMP-activated protein kinase)[4] signaling and mTORC1 (mammalian target of rapamycin)[5] signaling (both are kinases).The AMPK signaling deals with status of glucose (energy) and in a case of its shortage promotes glucose uptake (via GLUT) by cells through a series of downstream signaling. Whereas mTORC1 senses amino acids and triggers protein synthesis through a series of downstream signaling cascade. Both kinases have been identified as important therapeutic targets for controlling human diseases. Rapamycin, a drug used to prevent organ transplant rejection, inhibits mTOR signaling pathway.

1.8.1 Metabolic Pathway

Numerous biochemical reactions that occur inside a living cell are generally catalyzed by intravital biocatalyst such as enzymes. The enzyme-catalyzed biochemical reactions furnish products which in fact is the manifestation of a desired biological response and are generally called metabolic pathways (biochemical). Most of the enzyme-catalyzed reactions in cells occur in a sequential format and are regulated in a manner that product formed following one enzymatic reaction becomes substrate for the next enzymatic reaction. There are two general types of metabolic pathways: catabolic and anabolic. Catabolic reactions release energy to be used in an anabolic reaction. Thus, catabolism and anabolism form a constant recycling dynamic phenomenon involving degradation and reconstruction for most of the intracellular substances essential for life.

1.8.1.1 Catabolic Pathway (Catabolism)

This pathway involves break down of large and complex molecules into simpler molecules (may be building blocks) and in turn release energy and heat. For example, during cellular respiration, sugar is taken in by the cells and is broken down to generate energy (ATP) that allows us to live. In fact, breaking down of glucose to pyruvate, inside the cell, to form ATP (catabolic process) goes through a series of enzyme-catalyzed reactions as part of a biochemical pathway. Other examples of catabolic reactions are hydrolysis and oxidation reactions (Fig. 1.31).

1.8.1.2 Anabolic Processes (Anabolism)

The pathway (constructive metabolic pathway) involves formation of four classes of macromolecules: body-building materials proteins, polysaccharides, lipids, and genetic material nucleic acids (Fig. 1.30).

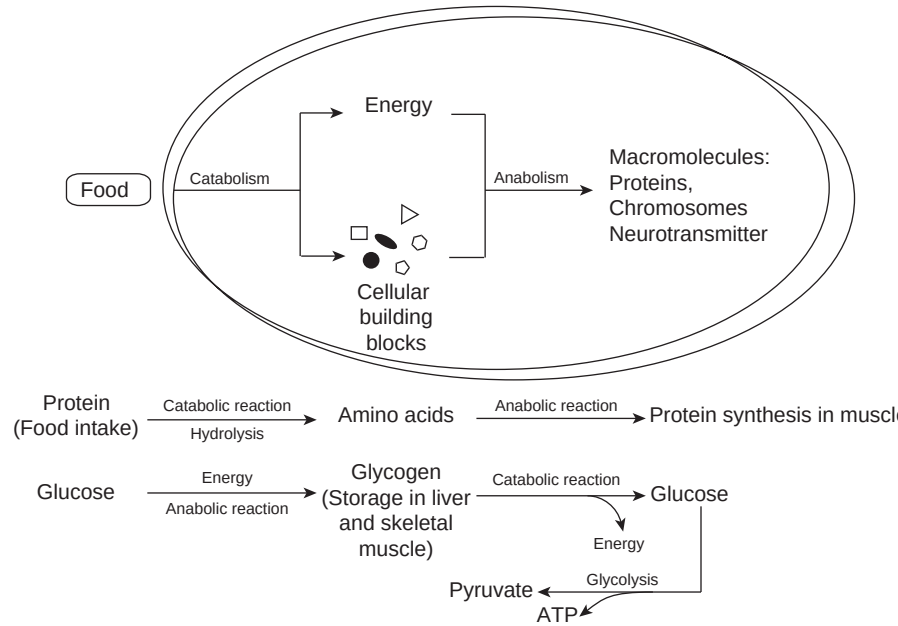


Figure 1.30 Catabolism and anabolism.

These molecules are important constituent components of living cells, such as membrane (bilayer), chromosomes (in nucleus), enzymes (proteins), receptors (proteins), antibodies (proteins), neuropeptides, hormones (peptidyl and steroidal), and neurotransmitters. These biochemical (metabolic) reactions are critical for the survival of cell and occur continuously. These reactions utilize ATP as a source of energy produced during anabolic process. In general, anabolism is responsible for cell differentiation and increase in body size, bone mineralization, and muscle mass.

1.8.2 Metabolites

During the enzyme-catalyzed biochemical reactions in a metabolic pathway in a living cell, the intermediate(s) formed during these transformations are called metabolites. The metabolites have been classifieds into two groups: primary and secondary metabolites (Table 1.4).

Table 1.4 Primary and secondary metabolites in plants

Primary Metabolites	Secondary Metabolites
1. Plants produce thousands of types of chemicals such as carbohydrates, fats, proteins, nucleic acids, chlorophylls, and hemes that have direct role in the growth of organism. 2. They are required for their basic metabolic processes and found throughout the plant kingdom. 3. These are produced in large quantities and can easily be extracted from the plants.	1. Many plants, fungi, and microbes of certain genera and families synthesize a number of accessory organic compounds that have no direct role in growth and development of plants. 2. They are not involved in primary metabolism (photosynthesis, respiration, and protein and lipid metabolism). 3. These compounds are produced in small quantities.

1.8.2.1 Primary Metabolites

The metabolites that are formed by the cell and are indispensable for their growth and survival are called primary metabolites. For example, amino acids, nucleotides, vitamins (B_2 and B_{12}), etc.

1.8.2.2 Secondary Metabolites

They are organic compounds produced by living organisms such as bacteria, fungi, or plants (80%) which are not primary metabolite and hence are not essential for their growth, development, or reproduction. Instead, the secondary metabolites for example produced from plants have a variety of important functions. For example, production of fragrances, dyes, pigments, drugs, etc. Some secondary metabolites produced by plants are used in their defense against herbivores and pathogens and attract animals for pollination and seed dispersal. Other important secondary metabolites from plants include atropine as muscle relaxant, vinblastine as antineoplastic, paclitaxel as anticancer agent, tubocurarine as muscle relaxant, caffeine as antispasmodic, etc. A large number of antibiotic secondary metabolites are of microbial origin and have been isolated from actinomycetes and streptomycetes. Thus, many of the drugs available today are derived from secondary metabolites.

Metabolic and physiological pathways interact in a complex manner in order to allow an adequate regulation and maintenance of homeostasis. The interaction/communication occurs between neurotransmitters, hormones, receptors, and enzymes with forward activation and feedback inhibition pathways. Thus, homeostasis for each physiological function involves an elaborate network of interconnected pathways involving multiple neural/hormonal signaling pathways in a regulated manner.

1.8.3 Metabolomics

Metabolomics[6] deals with the comprehensive analysis of low-molecular-weight metabolic substances formed inside the organism in response to pathophysiological stimuli/signal or genetic modification (Fig. 1.31). These metabolomes can be formed in response to a disease condition in cells/tissues/organs. This can also be referred to as 'finger printing' or 'small molecule profiles' of metabolomes produced following a cellular process that is specific for each cell. These molecular profiles can be used to compare with the molecular profiles following administration of drugs. The changes in the profile caused by the drug can help understand the action of drug at molecular level and in turn may lead to the identification of new biomarkers or avenues to increase the response to drugs.

Thus, the technology can be used to help unravel the metabolic phenotypes including the characterization of metabolic derangements (disturbance of normal bodily functioning) that underlie in the pathogenesis of a disease.

Metabolomics is an emerging technology that holds promise in identifying precision medicine at a number of levels, discovery of new targets, metabolic finger printing of a cellular process (function) and discovery of new biomarkers for the purpose of diagnosing diseases.

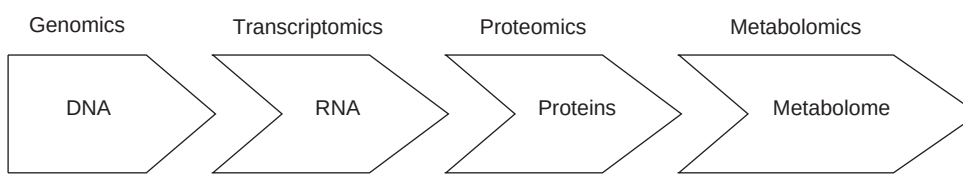


Figure 1.31 Metabolomics.

CASE STUDY 8

Neuronal/Hormonal Regulation of Acid Secretion in Stomach

Ingestion of food during cephalic (in mouth)/gastric/intestinal phase stimulates gastric secretions such as HCl, pepsinogen, etc. This follows stimulation of vagus afferent nerve present in both mouth and stomach and carries signal to brain (a sole link between brain and stomach). From brain, vagus efferent nerve brings signal to enterochromaffin-like cells (ECLs), D-, G-, and parietal cells in the stomach. Each secretory cell has ACh neurons that synapse with efferent vagal pathways.

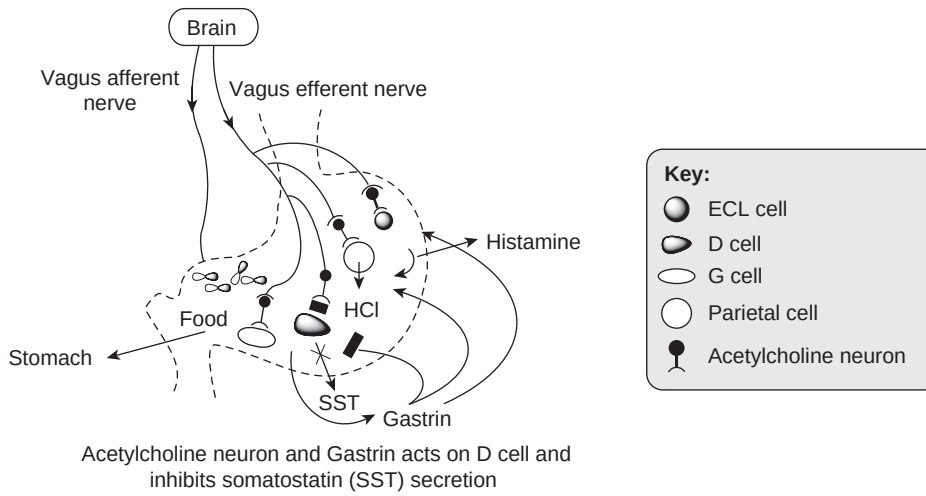


Figure 1.32 Positive and negative regulators involving neuronal (ACh) and hormonal interaction to stimulate gastric secretions.

Thus, besides indirect stimulation of secretion of histamine and HCl by gastrin, ACh neurons directly stimulate the secretion of histamine, HCl, and gastrin. However, gastrin and ACh both inhibit the secretion of somatostatin (SST) (Fig. 1.32). Thus, acid secretion in stomach has both direct and indirect stimulation.

Direct Neuronal Stimulation

Direct stimulation of acid secretion involves neuronal stimulation by the neurotransmitter ACh. The latter directly stimulates G-cells, parietal cells, and ECL cells (except inhibiting D-cells to release somatostatin).

1. ACh stimulates G cells to release gastrin, which in turn stimulates parietal cells to release HCl.
2. ACh stimulates parietal cell to release HCl.
3. ACh stimulates ECL cells to release histamine, which in turn stimulates parietal cells to release HCl.

Direct Neuronal Inhibition

ACh directly inhibits D-cells from releasing somatostatin, which in turn blocks gastric acid secretion by blocking histamine release from ECL cells and also directly blocking function of parietal cells.

Indirect (Hormonal) Stimulation

Gastrin (peptidyl hormone) secreted by G-cells stimulates acid (HCl) secretion by stimulating histamine release from ECL cells. Histamine in turn directly stimulates parietal cells to increase acid secretion.

(*Continued*)

Study 8 (*Continued*)

This causes drop in the pH of the stomach, which on one side triggers D cells in the stomach to stimulate the secretion of somatostatin, and on the other hand, low pH also inhibits the release of gastrin from G-cells by feedback loop. Release of somatostatin can have three-pronged effect:

1. Inhibiting secretion of acid from parietal cells.
2. Inhibiting secretion of histamine from ECL cells.
3. Inhibiting secretion of gastrin from G-cells.

Increase in somatostatin secretion is also mediated via gut-innervating sensory neurons such as VIP neurons on the D-cells in the antrum (Fig. 1.33).

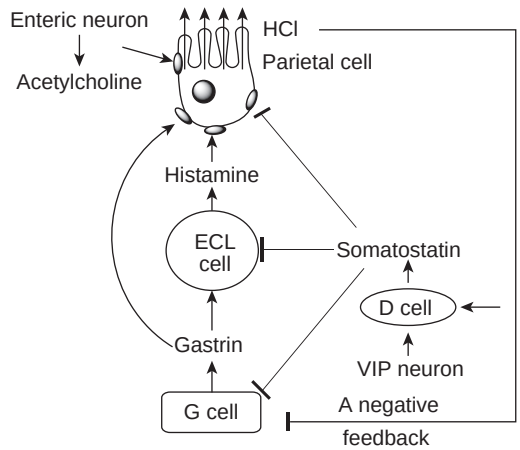


Figure 1.33 Gastrin release from G-cell is directly inhibited due to low pH (a case of negative feedback).

Thus, ACh, histamine, and gastrin are three regulatory molecules stimulating acid secretion in the stomach, while one regulatory molecule somatostatin inhibits the acid secretion. Somatostatin is secreted from D-cells following low pH and also by VIP neurons (Fig. 1.34).

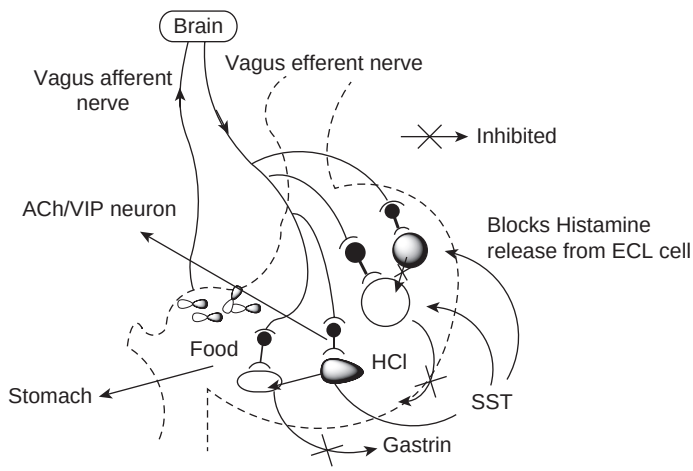


Figure 1.34 Negative regulators involving neuronal (VIP) and hormonal (somatostatin) regulation block acid secretion.

(*Continued*)

Study 8 (*Continued*)

Role of Receptors (GPCRs) in Neuronal/Hormonal Regulation of Gastric Acid Secretion

As discussed earlier, homeostasis for each physiological function involves an elaborate network of interconnected pathways involving multiple neural/hormonal signaling pathways in a regulated manner. The secretion of HCl from the parietal cell in gut is regulated following the binding of ACh, histamine, and gastrin to the cell-surface receptors (GPCR) on parietal cell as activators (for acid secretion), whereas binding of somatostatin and neural VIP (vasoactive intestinal peptide) to GPCR on parietal cell causes inhibition of acid secretion (Fig. 1.35).

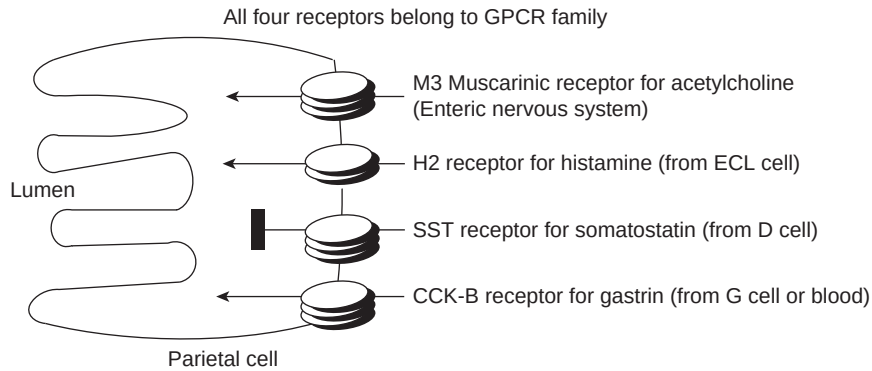


Figure 1.35 GPCRs present on parietal cells.

CASE STUDY 9

Clinically Used Antihistamine Drug Cimetidine as H2 Receptor Antagonist

Histamine is a paracrine hormone that stimulates the secretion of gastric acid from the nearby parietal cells. It stimulates the secretion by binding to GPCR referred to as H2 receptor on the parietal cells and releases H^+. The acid relapse by parietal cell is mediated by intracellular second messenger cAMP. However, excessive acid secretion may lead to gastroesophageal reflux disease. Therapeutic intervention for this disease is achieved by cimetidine (Fig. 1.36) that competitively inhibits histamine binding to histamine H2 receptors on parietal cells. The cimetidine acts as a histamine H2 receptor antagonist and blocks production of gastric acid. (In contrast, H1 receptor antagonist cetrizine is used as antihistaminic drug for the treatment of allergy and mast-cell-mediated disorders).

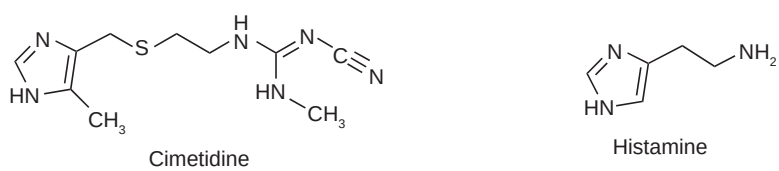


Figure 1.36 Cimetidine H2 receptor antagonist and histamine as a natural ligand (agonist) for H2 receptor.

CASE STUDY 10

Neuronal Regulation of Pancreatic Secretions by Glucose

Glucose homeostasis is regulated by two pancreatic hormones insulin and glucagon. The increase in glucose level triggers release of insulin whereas decrease in glucose level stimulates secretion of glucagon. However, the actual regulation of glucose sensing in blood circulation is neuronal that controls pancreatic secretions. The signal for the presence of glucose in circulation is sent by glucose-sensing cells present in mouth and hepatic portal vein. The signal is sent to brainstem and hypothalamus by afferent nerves. From hypothalamus and brainstem, the efferent nerves carry the activation or inhibition message back to pancreas either by sympathetic or parasympathetic nervous systems that innervate α- and β-cells (Fig. 1.37). The neurotransmitters secreted by parasympathetic innervate are ACh, VIP, etc., and by sympathetic innervate are NE, NPY, galanin, etc.[7]

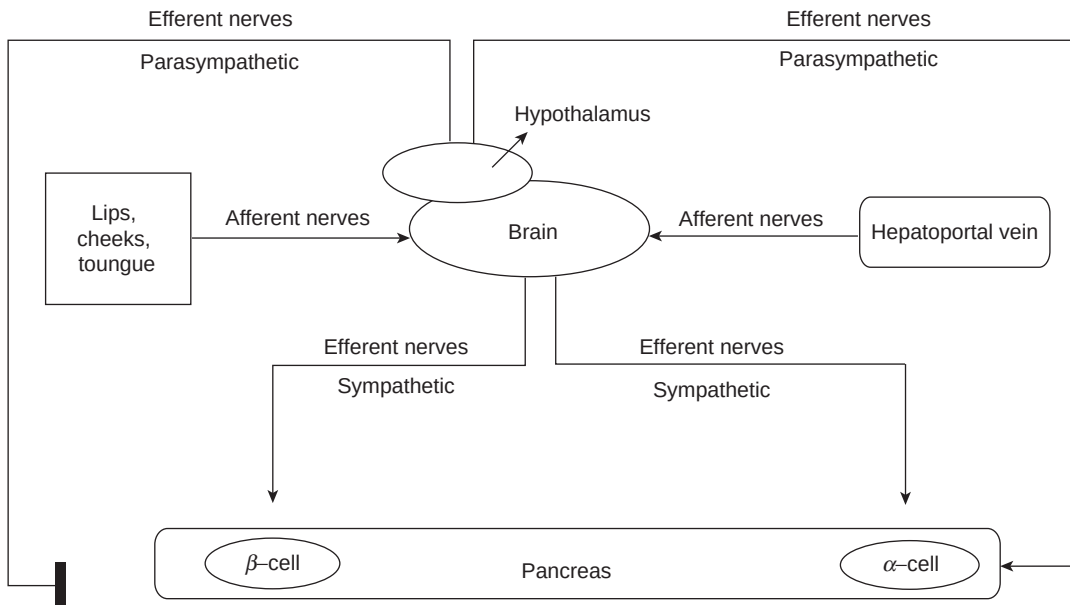


Figure 1.37 Regulation of hormonal secretions by α- and β-cells in response to glucose via parasympathetic (cholinergic and noncholinergic) and sympathetic nervous system (adrenergic and non-adrenergic).

Multiple Factors Regulate Insulin Secretion from the Pancreatic Beta Cells

The secretion of insulin by β-cells in response to glucose is stimulated following the binding of GLP-1 to its receptor (GPCR) present on β-cells. The binding triggers intracellular signal transduction and also allows uptake of glucose from circulation by transporter proteins. Inside the cell, glucose generates ATP via glycolysis which in turn prevents efflux of K^+ and causes depolarization of the membrane. This trigger opening of Ca^{++} channels, thereby increasing the concentration of Ca^{++} in cytoplasm. This eventually triggers the release of insulin in the presence of PKA formed via GLP-1/cAMP cascade (Fig. 1.38).

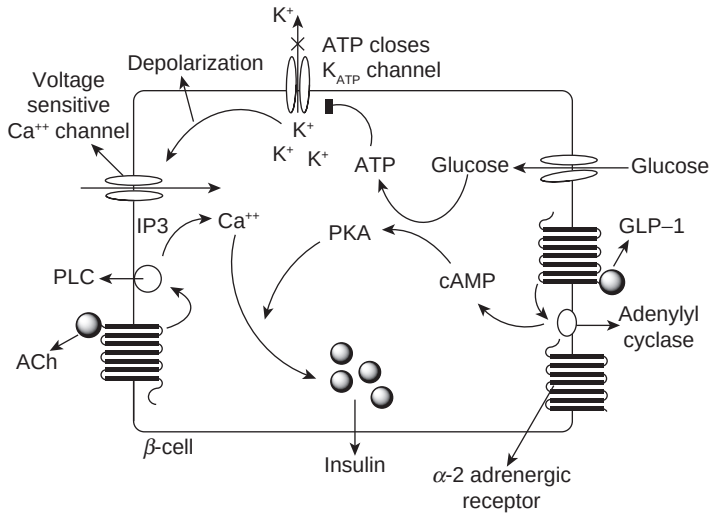


Figure 1.38 Multiple factors regulate insulin secretion from the pancreatic beta cells.

Inhibition of Insulin Release by Neurotransmitter Norepinephrine

Several agonists including NE inhibit insulin release from β-cells. Following the binding of NE to α_{2A}-adrenoceptor, the G-proteins dissociate and inhibit the action of adenylyl cyclase.[8] This in turn blocks the release of insulin by preventing the formations of second messenger cAMP and subsequent signaling molecule PKA (Fig. 1.39). Thus, the α_{2A}-adrenoceptor has been identified as an important regulator of blood glucose.

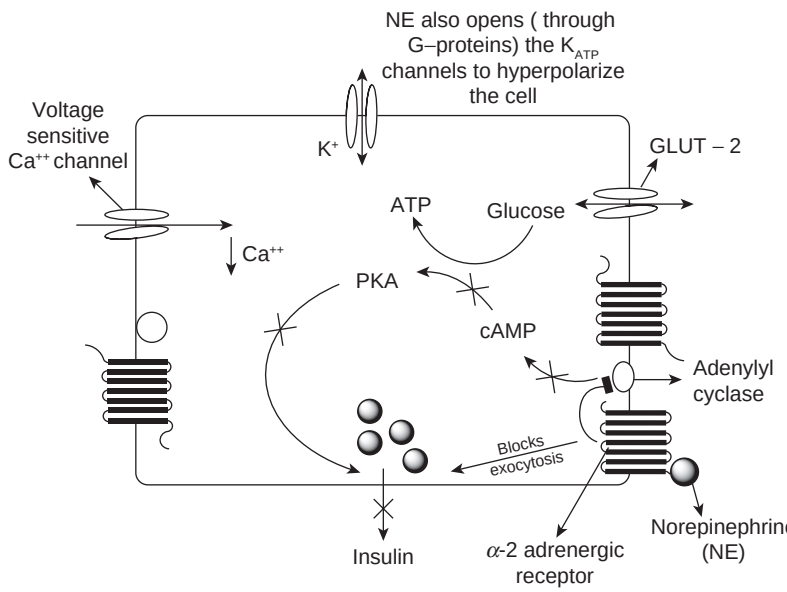


Figure 1.39 Inhibition of insulin release by NE has been attributed to the inhibition of AC.

1.9 Diseases

Cellular functions via numerous biochemical pathways are regulated by cell-type specific signals. Impairment in signaling pathways result in a disease that can occur either via the phenotypic changes of the signals so that their normal functions are subverted or via the genotypic changes. Thus, any deviation/anomaly/disruption that interferes with or disturbs the normal physiological functioning of the body is called a disease and is associated with certain signs and symptoms. At molecular level, it is the interference/disorder in cell–cell communication that leads to the disturbance in the normal cellular/physiological function and is manifested in the form of disease symptoms. A large number of diseases are caused by defects in signaling pathways. The nature of these defects and how they are induced varies enormously.

1.9.1 Main Causes of Diseases

Traditionally, the main causes of diseases have been attributed to cell damage, toxicity, nutritional deficiency, genetic disorder, or any other reason. However, the common underlying mechanism for the causes is due to disorder in cell-signaling pathways.

1.9.1.1 Cell Damage (Injury)

Disorder in the normal functioning of cell signaling pathways is the major cause for cell damage, which in turn is responsible for a large number of diseases. Each cell receives a complex combination of signals, which simultaneously triggers a cascade of different signaling pathways for regulating the intracellular activities.

1.9.1.2 Toxicity

There are two main types of toxicity that contribute to diseases:

Exogenous Toxicity

Exogenous toxins are chemical entities that after invading our bodies (through ingestion/inhalation/absorption) can harm host cells/tissues by interfering with their natural cell-signaling pathways. For example, smoking leads to inhalation of nicotine and carcinogens (polycyclic aromatic hydrocarbons) that causes addiction and lung cancer. Addiction is caused by nicotine that binds with receptors in brain and signals release of dopamine which is responsible for feel good factor (also called 'happy hormone'). When the concentration of dopamine goes down, the smoker develops withdrawal symptoms and craves for smoking (as receptors in brain crave for more nicotine). The lung cancer in smokers is caused by carcinogens present in the tar. The carcinogens interfere with the signaling pathway initiated by tumor-suppressing protein p53 following its binding to specific DNA sequences in cells. In fact, carcinogen such as benzopyrene present in the tar binds with tumor suppressor gene, due to which p53 is unable to signal the arrest of cell cycle growth or apoptosis, thereby leading to uncontrolled proliferation of cancer cells.

Pathogens such as bacteria/fungi can also be a source for toxins in the body that can cause disease by entering directly into cells/tissues. They cause injury to host tissues by disabling the immune signaling system.

Endogenous Toxicity

Most of the endogenous toxins are generated inside our body. They are produced either as waste products during metabolism (carbon dioxide, urea, and lactic acid) or by the trillions of microorganisms in the gastrointestinal tract. As part of our body's defense mechanism, these endogenous toxins are eliminated from the body either as gas or via metabolism. However, if our health is severely compromised, some endogenous toxins may make their way into our blood stream by travelling through the intestinal walls through which

they can access our cells and contribute to toxic burden. Studies have linked translocations/absorption of endogenous toxins from gut inside our body via blood circulation with number of diseases.

1.9.1.3 Deficiency of Nutrients

In multicellular organisms, cells are constantly surrounded by a variety of essential nutrients as the latter are needed for the survival, growth, and proliferation of cells. Notably, the uptake of nutrients is controlled by signal transduction pathways. A disorder in signal pathway can cause nutritional deficiency leading to a variety of health problems. The seven nutrient deficiencies widely common in humans have been summarized below:

1. Iron deficiency, which causes anemia due to the low level of red blood cells.
2. Iodine deficiency, which causes goiter.
3. Vitamin D deficiency, which causes bone diseases: rickets and osteoporosis.
4. Vitamin B_{12} deficiency, which causes nerve damage and can affect balance and coordination while walking.
5. Calcium deficiency, which causes osteoporosis.
6. Vitamin A deficiency, which causes blindness and fatal infections.
7. Magnesium deficiency, which causes celiac disease, diabetes, chronic diarrhea, etc.

1.9.1.4 Genetic Disorder

A defect/mutation in an individual's gene can cause medical condition called genetic disorder. The genetic disorders may arise either via mutations of genes (either randomly or due to environmental exposure) or can be hereditary in nature. The genetic inheritance can be grouped into the following:

1. Single gene inheritance
2. Multifactorial inheritance
3. Chromosome abnormalities
4. Mitochondrial inheritance

Single Gene Inheritance

This is also referred to as monogenetic or Mendelian inheritance. This involves changes in the single gene; particularly, sequence of DNA undergoes mutation. Examples include the following:

1. Muscular dystrophy: It is caused by mutation in Duchenne muscular dystrophy gene.
2. Huntington's disease: It is caused by mutation in Huntingtin gene.
3. Fragile X syndrome: It is caused by mutation in fragile X gene.
4. Cystic fibrosis: It is caused by mutation in cystic fibrosis transmembrane conductance regulator gene.
5. Sickle cell anemia: It is caused by mutation in the hemoglobin-β-gene found on chromosome 11.

Multifactorial Genetic Inheritance

This is caused by defects in multiple genes arising due to combination of many factors, thereby causing a birth defect. Two major factors that usually combine to produce multifactorial disorders are due to unknown environmental reasons as well as inheritance from parents. Examples include the following:

1. Diabetes
2. Obesity
3. Heart disease
4. High blood pressure
5. Alzheimer's disease
6. Arthritis
7. Cancer

Chromosome Abnormalities

Chromosomes located in the nucleus are made up of DNA and are carriers of genetic traits (hereditary information). Thus, any abnormality either in the number of chromosomes or DNA structure (via mutation) can result in the manifestation of disease under genetic disorder, thereby causing impairment in subsequent signaling pathways. Abnormalities in chromosomes typically occur as a result of defects in any one or more stages (such as mitosis/meiosis) during the cell division process. However, other factors could also cause chromosomal abnormalities. One such genetic disorder is 'Down's syndrome' disease where an individual carries 47 chromosomes, instead of the normal 46 chromosomes (23 pairs inherited from parents). The presence of extra chromosome causes impaired development, intelligence, and physical appearance.

1.9.2 Classification of Diseases

Diseases have been classified in a number of ways. Over the years different classification methods have been discussed in the literature for diseases.[9] The classifications of diseases have been continuously revised as knowledge in understanding the etiology of the diseases at molecular level progressed.

1.9.2.1 Diseases Classified under Four Major Classes

1. Diseases caused by the deficiency of essential nutrients (deficiency diseases)
2. Infectious diseases
3. Physiological diseases
4. Hereditary diseases

1.9.2.2 Diseases Classified under Two Major Categories

1. Infectious (communicable) diseases
2. Lifestyle (non-communicable) diseases

Infectious (communicable) Diseases

They are caused by acellular and unicellular organisms such as virus, bacteria, fungi, parasites, etc. These infectious diseases can be transmitted either from person to person, through inhalation from environment, through blood transfusion, insect biting, etc.

Lifestyle (non-communicable) Diseases

These diseases can be defined as 'self-imposed injury to health' as they are caused by person's individual choice to lead an unhealthy life on daily basis. The causative factors contributing to lifestyle diseases may

involve sedentary lifestyle (minimal or no physical activity), incorrect posture of sitting/sleeping, greasy foods, smoking, excessive consumptions of alcohol/beverages, ready-to-eat packed foods, mental stress including envying others, drug abuse, etc. Heart disease, hypertension, type-2 diabetes, obesity, asthma, depression, etc., are some of the widely prevalent lifestyle diseases in the world.

1.9.2.3 Other Classification Methods of Disease

This classification of disease involves causative factor responsible for change in the normal functioning of the body:

1. Infections caused by viruses, rickettsia, bacteria, fungi, protozoa, and worms.
2. Allergic diseases caused by antigens and foreign substances.
3. Metabolic disorders caused by defects in the body's ability to carry out normal reactions.
4. Cancer.
5. Toxic diseases caused by poisons.
6. Psychosomatic and mental diseases.

Diseases have also been classified based on their nature within the body:

1. Topographic appearance in a particular region of the body.
2. Anatomic basis, originating from defects in a particular organ/tissue.
3. Physiological basis, originating from defect in a biological function/pathway.
4. Pathological basis, originating from progression of a disease process.

1.9.3 Additional Disease Concepts

Symptoms for a disease are manifestation of observable characteristics caused by impairment in the natural messenger mediated physiological or biochemical process within cells/tissues. However, in many cases, the progression of a disease may result in the cascade of other symptoms by affecting other signaling pathways. Alternatively, several human diseases can be also attributed to a single dysfunction of cellular organelles that are part of signaling process in the cellular metabolism or arising from impairment in the cellular metabolism itself. Thus, etiology of many diseases can be also discussed under:

1. Systemic diseases
2. Metabolic diseases
3. Mitochondrial diseases

1.9.3.1 Systemic Diseases

This generally refers to a disease that affects many organs and tissues or the whole body at a time rather than a single organ or body part. Thus, under systemic disease one needs to take into account the manifestation of several medical symptoms arising due to chain of impaired events under a complex network effect. At molecular level, there is a systemic breakdown of physiological networks arising from the defects in multiple signaling pathways. Three examples of systemic diseases have been discussed below:

Diabetes Mellitus (an imbalance in blood glucose levels)

Basically, diabetes (type I and II) has been referred to as a metabolic disorder affecting glucose, lipid, and protein metabolism. It involves, a chain of events, starting with body's inability to process glucose (source

for energy) in the absence of insulin which, in turn, triggers metabolism of fat and protein for alternative source of energy. These chains of events are regulated by intercellular and intracellular signaling involving binding of ligands (insulin, glucagon, GLP-1) as first messenger to their corresponding receptors, which then triggers intracellular signal transduction. It is this intracellular signaling that through signaling molecules—second messengers, effector molecules, and enzymes—trigger cellular response in the form of impaired sugar/lipid/protein metabolism. However, despite managing (no cure available) the diabetic conditions with strict diet, physical exercises, and several state-of-the-art antidiabetic agents, gradually the disease starts affecting other biological functions and organs resulting in a chain of serious health issues. This systemic complication can be attributed to gradual glycosylation of proteins and lipoproteins leading to heart disease, stroke, damage to nerve, vision impairment, susceptibility to skin infection, kidney damage, digestive system, muscle weakness, etc. Thus, diabetes can be defined as a disease resulting from defect in signaling system that in turn triggers metabolic disorder and finally results in systemic effects and complications. In other words, diabetes is a cell-signaling, metabolic, and systemic disease, and may also include hitherto unknown factors, which still need exploration.

AIDS

The life-threatening disease is caused by HIV infection that destroys the immune system and leaves the body vulnerable to opportunistic secondary infections: tuberculosis and other diseases like HIV-associated cancers.

Flu

Seasonal attack of influenza virus is a highly prevalent disease that affects every single human being throughout their life. The infection is systemic in nature as it affects the entire body with host of systemic symptoms: running nose, sore throat, headache, fever, cough, fatigue, muscle, and joint pain.

1.9.3.2 Metabolic Diseases

Cell metabolism involves different enzyme-catalyzed biochemical reactions and processes necessary for the maintenance of life. Thus, any aberration during this enzyme-catalyzed normal biochemical reaction may interfere with normal cellular functions, thereby causing a metabolic disease.
There are many causes of metabolic disorders.

1. Metabolic disorder due to genetic change (inherited metabolic diseases/inborn errors of metabolism): All metabolic reactions are catalyzed by enzymes which are produced inside the cells by genes. A defective gene will lead to the formation of mutated enzyme causing disorder in the normal metabolic process. This results in the manifestation of disease due to genetic change/impaired enzyme activity. The mutated gene can either be inherited from parents to children or can just happen on its own. There are several known inborn errors of metabolism; examples include gout, sun sensitivity, and thyroid disease.
2. A metabolic disorder can also occur due to undesired chemical reactions that in turn may disrupt metabolic process. This may occur during the breakdown of amino acids, carbohydrates, or lipids. Examples of disorders that affect the metabolism of amino acids include non-ketonic hyperglycinemia, phenylketonuria, and maple syrup urine disease.
3. A diseased or damaged organ involved in the metabolism may also lead to metabolic disorder. For example, the damaged liver, pancreas, or thyroid can cause grave injury to metabolic disorder.
4. Autoimmune diseases can also be attributed to metabolic disorders, e.g. rheumatic diseases and type 1 diabetes.

Metabolic syndrome is a combination (group) of several metabolic disorders (cluster of risk factors) that occur together and increase the chance of developing heart disease, stroke, and type 2 diabetes.

1.9.3.3 Mitochondrial Diseases

The mitochondria are organelles found in the cells through our body. They are powerhouse responsible for producing 90% of the chemical energy to sustain life. The constant supply of energy in mitochondria is due to occurrence of many chemical reactions involved in cellular respiration. A notable feature of mitochondria is that mitochondria contain two kinds of DNA: cell's nuclear DNA (nDNA), and a separate mitochondrial DNA (mtDNA).

In general, the mitochondrial diseases can be attributed to malfunction in the mitochondria, thereby causing impairment with the production of energy. They are often inherited disorders that can be observed at the time baby is born or can occur at any age probably due to another disease or condition. Mitochondrial DNA heteroplasmy caused by the presence of more than one type of mtDNA (genetic variants) can result in major health issues involving neurodegenerative diseases. Mitochondrial diseases have been associated with neuromuscular disorders, blindness, deafness, weakness, cardiac failure, diabetes, renal dysfunction, cancer, liver disease, etc. In cancer treatment, mitochondria with impaired function can play a major role in prompting cell death.

1.10 Understanding Signaling Pathways in the Pathogenesis of Diseases

The process of drug discovery against any disease commences with the key step that involves understanding of causative factors for a disease. The basic objective is to restore the natural homeostasis (balance) surrounding the pathogenesis of the disease. The initiation or progression of most of the diseases involves a complex network of molecular signaling in biochemical pathways. Identifying and analyzing these pathways leading to the pathogenesis of a disease remains a challenging task. The pathways may commence with inter- or intracellular signal transduction involving receptors followed by enzyme-catalyzed metabolic reactions within cells that are responsible for the regulation of physiological functions. Notably, both receptors and enzymes are proteins and are linked with genetic make-up of the individual. Any impairment in the genetic pool may in turn impair the functioning of receptors and enzymes and result in the pathogenesis of disease. In the post genomic era, technologies based on proteomics and genomics are being extensively used to identify biomarkers involved in the progression of any disease. These biomarkers can be used either as diagnostic tool or as molecular targets for treating a disease. Thus, in general a pathogenesis of any disease may involve not only more than one pathway but a host of molecular components in the form of either DNAs/RNAs or proteins as possible therapeutic targets. Although gene therapy (introduction of new genetic materials to the cells within a body for therapeutic benefit) remains the best option to nip the disease at the bud stage, a major focus has been attributed to modulating the functions of proteins (receptors or enzymes) with drugs. Drugs, in general, affect the causative factors that are manifested in the form of symptoms for a disease.

Hypertension is one disease which probably has the largest number of well-understood pathways involved in its pathogenesis. Most of the drugs used clinically for treating this disease target either enzymes as substrates or receptors as ligands. Both enzymes and receptors are intermediate molecular components involved in the pathogenesis of hypertension in different signaling pathways.

Drugs targeting molecular components in one of the renin–angiotensin pathways are either inhibitors of enzymes such as ACE and renin or are blockers of Ang II receptor (ARB). Similarly, there are drugs that regulate relaxation of blood vessels by targeting receptors in different pathways such as calcium channel blockers (widens blood vessels), α-blockers (prevents noradrenaline-mediated tightening of walls of arteries, vasodilators (releasing NO as second messenger), and aldosterone receptor antagonists (enhances

bioavailability of endogenous NO). Yet another class of antihypertensive drugs act by blocking factors in the biochemical pathways responsible for increased rate of heartbeat, such as β-blockers and central-acting agents. Apart from this, diuretics are compounds that regulate hypertension without binding to any targets.

1.11 Unexplored and Emerging Intracellular Signaling Components as Therapeutic Targets

1.11.1 Peroxisomes

Peroxisomes (POs) are unique proliferating cytosolic ubiquitous organelles. They are involved in a variety of cellular functions such as lipid metabolism, reactive oxygen species metabolism, and enzyme-catalyzed biosynthesis of plasminogen (membrane glycerophospholipids), and bile acids. The non-metabolic role of PO involves signaling responses to cytosolic stress, cell aging, and pathogens. Among several enzymes and proteins present in the organelle, one of the initial enzymes isolated from PO was catalase responsible for the generation and degeneration of hydrogen peroxide (H_2O_2). The proliferation of PO is regulated by nuclear signaling chain of events and PPARα or other signaling pathways that could be instrumental in peroxisomes homeostasis. Thus, in response to external physiological cues, new peroxisomes keep replacing defunct organelles.

Removal of dysfunctional organelles occurs via macroautophagy/lysosomal degradation process. Impairment in the selective disposal of defunct peroxisomes has been linked with disorders due to aging and neurodegeneration. Research studies have also found link between the functioning of PO with mitochondria and endoplasmic reticulum.[10] Thus, the enzymes and proteins present in the PO and its communication through signals with other cytosolic organelles can be therapeutic target in the pathogenesis of disease; however, no success yet has been achieved with the discovery of drugs.

1.11.2 Lysosomes

Lysosomes are membrane-encapsulated cytosolic organelles responsible for the removal of extracellular/cytoplasmic junk/wastes. They carry all sort of enzymes (glycosidases, proteases, lipases, and nucleases) that can degrade the cytoplasmic wastes arising from defunct organelles, pathogens, and micro particles derived from foods and may comprise carbohydrates, lipids, and proteins. These waste materials can have both extra- or intracellular origins and following degradation may undergo recycling process or excretion. Substances of extracellular origin are taken up via endocytosis and the resulting endosomes then fuse with previously formed endosomes. After this fusion, the ingredients belonging to plasma membrane inside endosomes are recycled back to cell surface. The remaining endosomes fuse with hydrolase-containing transporter vesicles originating from Golgi apparatus, resulting in the formation of lysosomes. The latter carries out enzymatic degradation of dysfunctional/obsolete proteins in endosomes. Similarly, phagosomes resulting from phagocytosis (extracellular large particles) and autophagosomes resulting from autophagy (encapsulating cytoplasmic waste materials) fuse with lysosomes furnishing phagolysosomes and autophagosomes, respectively. Thus, targeting enzymes present in lysosomes[11] for treating age-related diseases Alzheimer and Parkinson can be a promising therapeutic potential.

1.11.3 Autophagy

The lysosomal digestion of materials in autophagosomes involves degradation of defunct and obsolete intracellular waste.[12] Thus, autophagy is a phenomenon that protects cells from stress arising from unwanted materials such as non-functional organelles, pathogens, etc. Any impairment in this process can lead to variety of diseases, particularly aging, and hence can be a good target for developing anti-aging drugs.

1.11.4 Proteasomes

Proteasomes present in cytoplasm of cells play an important role in protein homeostasis (synthesis and degradation). More than 80% of proteins in cells are degraded via ubiquitin proteasome pathways. They are protease complexes with multiple catalytic sites that can cleave amide bonds in proteins following hydrophobic and basic amino acids. Through protein degradation, proteasomes get rid of impaired, unfolded, and defunct proteins. In the last two decades, the role of proteasomes has been implicated in numerous cellular functions: intracellular signaling cascades, progression of cell cycle, immunogenic responses, etc. Any impairment in protein homeostasis has been implicated in cell death. Thus, proteasomes have been used as therapeutic target and their inhibitors have used for the treatment of few cancers.[13] Three drugs: bortezomib, ixazomib, and carfilzomib bind proteasomes and block the growth of cancer cells.

1.12 History of Drug Research

The roots of drug research relied mostly on traditional remedies that were derived from centuries of folk knowledge. Natural products derived from berries, leaves, bark and root were used for reducing fever and pain. On the contrary, synthetic drugs of 20th century have their origin in synthetic dyes. William Henry Perkin in 1856 discovered the first synthetic dye serendipitously and this led to the development of several synthetic dyes and also branch of organic chemistry as a subject. In 1891 Paul Ehrlich discovered certain dyes that selectively killed cells and organism. He coined the term chemotherapy and used synthetic chemicals to treat diseases. This triggered research activities in the area of drug being derived from synthetic dyes along with organic chemicals being isolated from the coal-tar (The first analgesic drugs phenacetin and acetanilide are derivative of aniline and p-nitro phenol which were derived from coal). However, in subsequent years, the focus of drug research shifted from dyestuff to synthetic medicines. Some important milestones in the history of drug research are:

1. In 1846, Morton demonstrated use of diethyl ether (now known as Ether dome) as general anesthetic.
2. In 1869, chloral hydrate, was discovered as the first synthetic drug and was introduced as a sedative-hypnotic;
3. In 1899, aspirin the first blockbuster drug was discovered by Felix Hoffmann at Bayer in Germany.
4. In 1921 insulin was first used in the treatment of diabetes by Frederick Banting.
5. In 1928, the first antibiotic penicillin was discovered accidentally by Scottish bacteriologist Sir Alexander Fleming.

Currently, the modern drug discovery relies on natural products, synthetic organic compounds, biologics and repurposing of drugs. However, even after the introduction of several modern drug discovery approaches, the drug development program still remains one of the most expensive and time-consuming process. On an average, it takes 12–15 years to launch a drug from initial discovery in chemistry laboratory to clinics. As far as cost of developing a drug is concerned, according to Tufts Center for the Study of Drug Development (CSDD) in 2016 it was found to be approximately $2.9 billion.[14]

1.12.1 Classification of Drugs Based on their Action on Signaling Pathways

Cellular/physiological processes are regulated via interaction between different inter- and intra-cellular signaling pathways. Pharmacological targets in these signaling pathways comprise either receptors or signaling enzymes. The receptors transmit signals while enzymes catalyze reactions during the process of signaling cascades thereby resulting in a specific cellular response. Most drugs used for treating

diseases exert their therapeutic actions by interfering in signaling pathways either by binding to receptors or enzymes. Among many available methods, the classification of drugs based on their pharmacological abilities to intervene in signaling pathways by targeting either receptors or enzymes has been discussed below:

1. Drugs for disorders related to autonomic nervous system
2. Drugs acting on peripheral (somatic) nervous system
3. Drugs affecting respiratory disorders
4. Drugs acting on central nervous system
5. Gastrointestinal drugs
6. Anticancer drugs
7. Drugs affecting blood and/or blood forming organs
8. Cardiovascular drugs
9. Drugs acting on kidney
10. Antibacterial drugs
11. Antifungal, antiviral, antiprotozoal, and anthelmintic drugs

Exercises

1. Classify pharmacological action of the drugs or theories of drug activity.
2. Describe cell-signaling disease and therapeutic targets.
3. Describe homeostasis with an example.
4. Explain intercellular cell signaling with one example for each signaling.
5. Describe briefly: (**i**) Systemic disease, (**ii**) Metabolic disease, and (**iii**) Mitochondrial disease.
6. Classify drugs based on their pharmacological action.
7. Discuss intracellular cell signaling with an example.
8. Describe role of first and second messengers in signaling process
9. Discuss difference between the following:
   - **(i)** Ligands and substrates
   - **(ii)** First and second messengers
   - **(iii)** Catabolism and anabolism
   - **(iv)** Primary and secondary metabolites
10. Write short notes:
    - **(i)** Autophagy
    - **(ii)** Lysosomes
    - **(iii)** Proteasomes
    - **(iv)** Peroxisomes
    - **(v)** Metabolic pathways
    - **(vi)** Toxicity
11. Discuss pathways involved in the pathogenesis of disease with an example.
12. Neuronal/hormonal regulation of gastric acid secretion
13. Describe neuronal signaling and action potential/firing of neurons

Competitive Exam Questions

1. One of the following statements for adenylyl cyclase is wrong. Identify it.

(a) It is a membrane bound enzyme.

(b) It is inactivated by phosphodiesterase.

(c) It catalyzes the AMP formations.

(d) It actives only when associated with G-protein.

[***GATE Pharmacy 1999***]

2. Gene therapy refers to the process of:

(a) identifying disease causing genes and activating them for therapeutic benefit.

(b) increasing expression levels of the set of genes involved in a given disease in affected cells through selective modulating agent.

(c) transfer of new genetic material to the cells of an individual for therapeutic benefit.

(d) removal of proteins corresponding to the disease-causing genes from the cells of the affected individual.

[***GATE Pharmacy 2007***]

3. An angiotensin receptor blocker useful in treating hypertension is:

(a) Valsartan. **(b)** Atenolol.

(c) Amlodipine. **(d)** Enalaprilat.

[***GATE Pharmacy 2008***]

4. The increase in concentration of second messengers (cAMP, cGMP, Ca^{++}, etc.) leads to:

(a) inhibition of intracellular protein kinases and protein phosphorylation.

(b) protein kinases activation and protein phosphorylation.

(c) blocking of interaction between a receptor and an effector.

(d) antagonism with endogenous.

[***GPAT 2013***]

5. Arginine serves as a precursor for which of the vasodilatory product?

(a) Bradykinin

(b) Atrial natriuretic peptide

(c) Nitrous oxide

(d) L-Citrulline [***GPAT 2013***]

6. Cyclic AMP (cAMP) is an important ·second messenger in signal transduction pathways. Which enzyme catalyzes the generation/accumulation of cAMP after a receptor-ligand interaction?

(a) Protein kinase A

(b) cAMP phosphodiesterase

(c) Guanylyl cyclase

(d) Adenylate cyclase [***GPAT 2015***]

References

1. Lo Vasco VR, The Crucial Roles of Enzymes Belonging to Signal Transduction Pathways, *Mol. Enz. Drug. Tar.,* 3e., **2017**, 105. doi: 10.21767/2572-5475.1000e105.

2. Gieseler F., Ungefroren H., Settmacher U., Hollenberg M. D. and Kaufmann R., *Cell Commun. Signal,* **2013**, *11*, 11.

3. John W. Denninger and Michael A. Marletta, *Biochimica et. Biophysica Acta (BBA)– Bioenergetic,* **1999**, *1411*, 334.

4. Hardie, D. G., Ross, F. A. and Hawley, S. A, Homeostasis, *Nat. Rev. Mol. Cell Biol.,* **2012**, *13,* 251.

5. Hay, N. and Sonenberg, N. *Genes Dev.*, **2004**, *18,* 1926.

6. Neel Jayesh Shah, Srinivasamurthy Suresh kumar and Deepak Gopal Shewade, *Indian J. Clin. Biochem.*, **2015**, *30*, 247.

7. B. Thorens, *Diabetes*, *Obesity and Metabolism*, **2011**, *13,* 82.

8. Susanne Straub G. and Geoffrey Sharp W. G., *Am. J. Physiol. Cell Physiol.*, **2012**, *302*, C1687.

9. Paul Thagard, *Communication and Cognition*, **1996**, *29*, 445.

10. Michael Schradre and Dariush Fahimi, *Biochimica et. Biophysica Acta (BBA)–Molecular Cell Research,* **2006,** *12,* 1755.

11. Ankur Sharma, Kalpesh Vaghasiya, Eupa Ray and Rahul Kumar Verma, *Journal of Drug Targeting*, **2018**, *3*, 208.

12. Beth Levine and Guido Kroemer, *Cell*, **2019**, *176*, 11.

13. Tiffany A. Thibaudeau and David M. Smith, *Pharmacological Reviews*, **2019**, *71*, 170.

14. Rick Mullin C., EN., **2014**, *92*, 6.

CHAPTER

2

An Overview of Drug Action

KEY THINGS TO LEARN FROM THIS CHAPTER

- Drug and type of pharmacologcal actions:
  - Structurally nonspecific drugs
  - Structurally specific drugs
  - Drugs that do not act on receptors
- Classification of drugs based on their origin: Plant products, synthetic molecules and biologics
- Drug actions involve Absorption (A), Distribution (D), Metabolism (M), Excretion (E), Toxicity (T), accessing target in tissues, affinity for target and therapeutic response
- Major physicochemical properties of drug affecting ADMET: Molecular weight, solubility, pK_a, logP, logD, polar surface area, H-bonding, etc.
- Major physiological barriers affecting ADMET
  - Route of administration
  - Binding of drugs to plasma protein binding and blood cells
  - First pass metabolism, Biliary excretion and enterohepatic cycle
  - Drug transport: Passive diffusion, Carrier Mediated transport, Transporter protein
  - Efflux of drug via P-glycoprotein

2.1 Definition of Drugs

Drugs are defined as key molecules of synthetic or semisynthetic or natural (animals or plants) origin that can rectify an impaired metabolic or signaling pathway specific to a disease or patho-physiological condition. At the molecular level, the drug exhibits its response either by activating (agonist) or inhibiting (antagonist) the macromolecular therapeutic protein targets (receptor, enzymes, etc.) involved in the pathway.

Drugs are generally used either to treat a disease or to reduce/minimize the pathogenesis of a disease (mitigation) or as prophylactics. For example, anti-infectious drugs are used to cure infectious diseases while antidiabetic, antihypertensive and hypolipedemic agents minimize progression of diabetes, blood pressure, and hyperlipidaemia respectively. Majority of drugs used for preventing the progression of a disease needs to be consumed on daily basis. Among other drugs, vaccines are generally used as prophylactics, however

small molecule drugs chloroquine, proguanil, mefloquine, and doxycycline are also used as suppressive prophylactics for the prevention of malaria while travelling to malaria-endemic regions. The chemical structures used most often as drugs are organic compounds can be broadly divided into classes: natural plant products (metformin), small synthetic molecules (captopril), and biologics derived from animal kingdom (hormone, monoclonal antibody, etc).

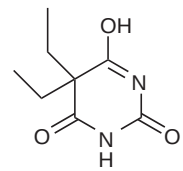
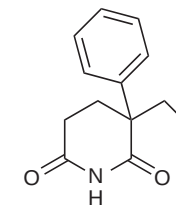
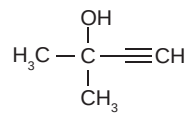
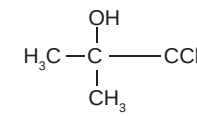
2.2 Types of Pharmacological Action of the Drugs (Theories of Drug Activity)

In general, a drug once administered in our body, elicits the desired pharmacological response for which it has been developed. Depending on the specificity of drugs, they can be classified as under:

2.2.1 Structurally Nonspecific Drugs

This class of drugs exhibit pharmacological activity which is solely governed by the physical properties of the compound alone with no perceptible relationship to their chemical structure. These structurally nonspecific drugs do not require specific sites of action in the organism, to display their activity and are usually administered in the body in relatively large doses. They elicit biological response in the organism by covering the entire area of certain cells in the form of a thin film. Fumigants (volatile insecticides), certain bactericidal, general anesthetics gases, depressants such as alcohol, chloral hydrate and antiseptics such as phenol and iodine are some examples of structurally nonspecific drugs (Table 2.1).

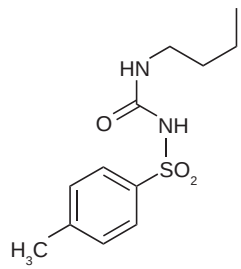
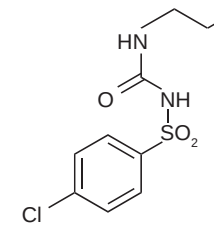
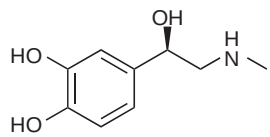
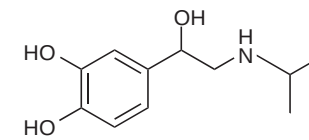
Table 2.1 Examples of structurally nonspecific drugs

Drug	Structure	Drug	Structure
5,5′ diethyl barbiturate		Glutethimide	
Acetylenic alcohol		Chlorobutanol	
Chloroform	$CHCl_3$	Ether	$C_2H_5OC_2H_5$

2.2.2 Structurally Specific Drugs

The pharmacological activity exhibited by this class of drugs is presumed to be primarily due to their physicochemical properties involving lipophilicity, pK_a, solubility and shape of the molecules, etc. Unlike nonspecific drugs required in large doses, specific drugs elicit desired biological effect in much smaller doses (fewer drug molecules). Moreover, specific drugs elicit therapeutic response by binding non-covalently (reversible manner) with macromolecular therapeutic protein targets such as receptor and enzymes (Table 2.2).

Table 2.2 Examples of structurally specific drugs

Drug	Structure	Drug	Structure
Tolbutamide short acting hypoglycemic		Chlorpropamide (long acting hypoglycemic)	
Epinephrine act on α, β-adrenergic receptor		(S)-Isoprenaline act on β-adrenergic receptor (hypotensive)	

2.2.3 Drugs that Don't Act on Receptors

There are three categories of drugs that elicit desired therapeutic response without binding to any therapeutic target.

2.2.3.1 Antacids

Antacids are a class of substances that neutralizes the acid in the stomach in order to relieve heartburn, indigestion or upset stomach. They contain ingredients such as aluminum, calcium, or magnesium which act as bases to counter the acidic pH in stomach.

2.2.3.2 Diuretics

A diuretic is any substance that promotes diuresis, that is, the increased production of urine in the kidney, thereby promoting the removal of salt and fluid from the body. They are widely used for treating hypertension, edema and glaucoma.

2.2.3.3 Alkylating Agents

These classes of therapeutic agents are predominantly used as part of chemotherapy for treating a variety of cancers. They inhibit proliferation of cells by damaging the DNA. The alkylating agents damage the DNA either by alkylating (covalent bonding) the DNA bases (guanidine) causing inhibition in the transcription of DNA. Nitrogen mustard gas derivatives are prototype of alkylating agents widely used as anticancer chemotherapeutic agents albeit with small therapeutic index.

2.3 Classification of Drugs Based on Their Origin

In general, therapeutic agents used clinically for the treatment of various diseases can be classified according to their origin:

2.3.1 Naturally Occurring Compounds as Drugs

Historically, natural products (secondary metabolites) have always remained a major source for pharmaceutical agents. Interestingly, even modern natural product chemistry methodologies continue to provide unique structural diversity of bioactive secondary metabolites both as lead candidate(s) or drug candidate(s). In coming years more and more therapeutic candidates are still likely to be identified since only less than 10% of the world's biodiversity (from terrestrial and marine sources) has been explored for potential pharmacological activities. Examples of naturally occurring therapeutic agents of both plant and animal origins include, macrocyclic antibiotics, alkaloids, neurotransmitters, anticancer agents, vitamins, hormones, biologics, amino acids, peptides, etc.). These therapeutic agents can be grouped under secondary metabolites derived from plant kingdom and biologics from animal kingdom.

2.3.1.1 Secondary Metabolites

The primary metabolites are compounds involved in primary pathways metabolism (breakdown/biosynthesis of proteins, fats carbohydrates and nucleic acids). Secondary metabolites are generally defined as chemical entities produced by bacteria, fungi or plants that have no role in their development, growth and reproduction. They are characteristic to an organism and serve as survival functions (as defense mechanism against predators) for the organism producing them or signaling moieties in symbiosis and ecological interactions. The structurally diverse secondary metabolites such as antibiotics, antitumor agents, immunomodulators, nutraceuticals, etc., have been used as both therapeutic agents as well as for improving health. Other applications of secondary metabolites involve pigments, flavoring agents, dye, pesticides, etc.

2.3.1.2 Biologics

They are referred to as a class of bio-pharmaceutical agents that are generated either via biological processes or via recombinant DNA technology. Basically, they are biological (intravital) products that are analogous to naturally occurring biochemicals such as antibodies, enzymes, or hormones. They either comprise proteins (monoclonal antibodies), gene products, polypeptides or living substances, such as cells and tissues.

Some of the categories of biologics as therapeutics are listed below:

1. Allergenic extracts (e.g. for allergy shots and tests)
2. Blood and its components
3. Gene therapy products
4. Devices and diagnostic kits
5. Human tissue and cellular products used in transplantation
6. Vaccines

Some of the categories of biological products mostly produced by biotechnology methods are:

1. Monoclonal antibodies (mAb) designed as targeted therapies in cancer and other diseases
2. Cytokines and Immunomodulators
3. Growth factors (proteins that affect the growth of a cell)
4. Enzymes
5. Hormones

Currently, applications of gene-based and monoclonal antibodies are at the forefront of biomedical research, and in near future it may be possible to treat a variety of medical conditions, including illnesses for which no other treatments are available.

2.3.2 Synthetic Compounds

Synthetic drugs are chemical entities synthesized either in a laboratory in small scale or manufactured in bulk quantities by pharmaceutical companies. They can be grouped into three categories:

1. Synthesis of natural products, such as atropine, steroid, peptide etc.
2. Synthesis of natural product derived structures or natural product derivatives including semi-synthetic compounds, such as penicillin).
3. Synthesis of small molecules with molecular weights less than 500, such as antidiabetic drug Metformin with molecular weight 129 g/mol, is generally recommended for a drug discovery program.

2.4 Drug Action

The understanding of drug action provides underlying mechanisms involved in the interaction of drugs with the physiological components of the organism. The drugs may be exogenous or even endogenous substances that can modulate cellular/physiological functions in the organism. Let us examine consequences that occur following the administration of a drug in the body. A drug once administered in our body has to do its job of treating the disease for which it has been developed. For this, the drug has to pass through several physiological barriers in the body in order to exert its action by ultimately binding to the specific pharmacological targets associated with the disease. In fact, when a drug is administered into the body, on one hand, our physiology treats the drug as foreign substance and resists its movement in the body and makes efforts to remove it from the system. On the other hand, depending on the ability (physicochemical properties) of the administered drug, it overcomes the physiological barriers and reaches its site of action. Thus, the drug action can be grouped under pharmacokinetics: summarizing actions of what body does to the drug while moving from the site of administration into blood circulation/tissues and pharmacodynamics: summarizing actions of what drug does to the body, involving interaction of drug with the biological targets and eliciting therapeutic response.

Generally, the drugs elicit biological response by interacting (binding) with highly specific areas within tissues called therapeutic targets. The targets made up of proteins may be intracellular or extracellular and can be grouped under enzymes and receptors. The pharmacological response may include the desired and/or unexpected/toxic response(s) following binding of drugs to the designated or undesignated (off-target) protein targets. The ability to access these targets selectively followed by binding has been attributed to the physicochemical properties of drugs lipophilicity, pK_a, molecular weight, polar surface area, solubility, etc. These properties arise from cyclic/acyclic molecular skeleton, the presence or absence of certain functional groups, 3D spatial relationships between groups (intra-atomic distance) and shape of the molecules. The L-amino acids lining the binding sites in the protein targets interact with drugs having specific structures in 3D format. The binding of drugs with targets involves several noncovalent reversible interactions such as H-bonding, Vander Waals interaction, ionic interaction, dipole interactions, and hydrophobic interactions.

Among physiological barriers, the prominent ones that resist entry of drugs by oral route are gut (where drugs are absorbed), liver (where drug gets metabolized), blood circulatory system (for distribution in tissues), Plasma protein binding (PPB; interfere with the distribution of drug), kidney (for excretion), and blood–brain barrier (BBB). However, when the drug makes its passage in the body from absorption to excretion, it has to also diffuse through several lipid and aqueous environments that pose yet another physiological barrier. Plasma membranes generally present lipid environment and the drug has to encounter them during absorption and distribution as it passes through gastrointestinal region via epithelial

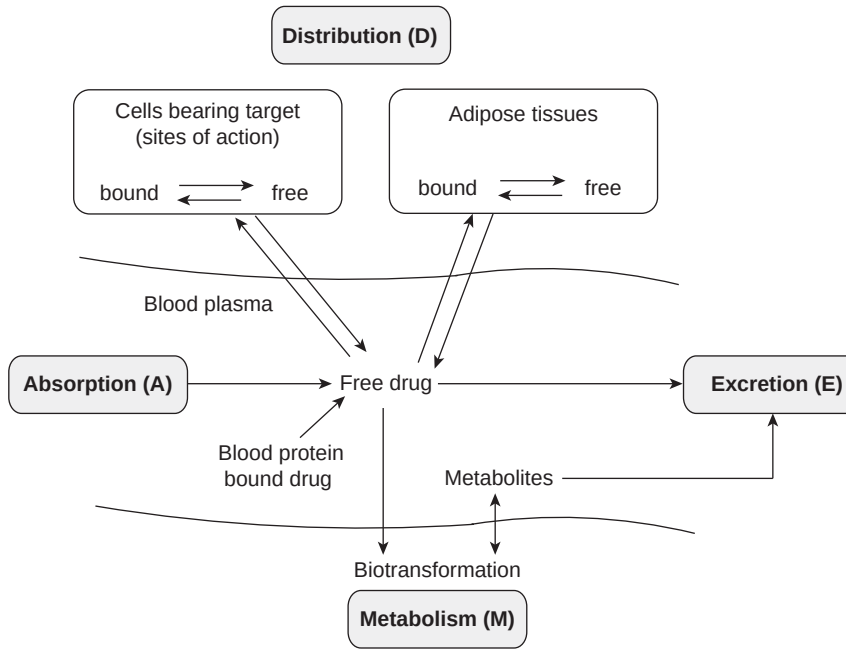


Figure 2.1 Action of drug following its administration depends on ADME.

cells, diffusion from blood capillaries into interstitial fluids (or extracellular fluids) via endothelial cells and finally diffusion into tissues containing active sites. Aqueous environment is encountered in the gut, in the blood, in the interstitial fluid (part of ECF) and in the cytoplasm within tissues and is also accompanied with variations in their pH values.

Thus, ability of drugs to elicit biological response, in general, is affected by five major parameters: the absorption (A), distribution (D), metabolism (M), elimination (E), and toxicity (T) of drugs (Fig. 2.1). Together, the factors are referred to as ADMET (now replaced with DMPK: Drug metabolism and pharmacokinetics) and are backbone of drug discovery. These five phenomena do not occur in sequence; instead, they occur simultaneously as parallel events and together regulate the lifespan of a drug in the body. The factors affect diffusion and distribution of drugs through several physiological lipid and aqueous environments, prevent accumulation of drugs (that may result in toxicity) through metabolism and elimination, and finally assist in accessing the targets for eliciting the response.

The phenomenon dealing with absorption (A), distribution (D), metabolism (M), elimination (E), and toxicity (T) of a drug together comprises drug action and has been described below. (Refer to Chapter 11 for detailed discussions on ADMET).

2.4.1 Absorption (A)

This involves administration of drugs from the site of administration inside the body, followed by uptake of drug into systemic circulation. Among many factors that affect the absorption phase, two major factors are physicochemical properties of drugs involving molecule size, lipid solubility, pK_a, and the routes of administration. Absorption is not applicable for drugs given by parenteral routes since they are administered directly into the bloodstream and therefore do not need to go through absorption phase. However, for drug administered by oral route (*p.o.*), the same has to pass through stomach, intestine and liver before being absorbed

in the systemic blood circulation. Absorption of test compound/drug is generally expressed as bioavailability and is represented by '*f*' (Details about absorption and its role in pharmacokinetics have been discussed in Chapter 11).

2.4.2 Distribution (D)

Following absorption of drug in systemic circulation, it needs to be distributed in the tissues to elicit its biological response (Fig. 2.2). Thus, there is a change in the concentration of drug in the blood plasma as it gets distributed into tissues. However, equilibrium prevails between concentration in blood plasma and tissues during distribution. Instant movement of drugs to tissues from plasma is not possible as there are several physiological barriers that affect distribution of drugs.

Two major factors (physiological barriers) that affect distribution of drugs from blood plasma are binding of drug to serum proteins and blood cells, and distribution of drugs from blood into tissues with/without target proteins. The other factors that affect distribution comprise membrane permeability of tissues, (affecting drug transport), lipophilicity of drugs, rapid excretion, and extensive metabolism of unbound drugs. Thus, distribution eventually leads to a decrease in concentration of plasma drug and can be approximated by a first- or zero-order kinetics. The binding of drugs to plasma proteins prevents the drug from eliciting its biological response as only the free drugs can undergo distribution in tissues. In fact, for reversible protein binding, equilibrium may exist between concentrations of protein-bound drugs and free drugs.

$$\text{Protein + drug} \rightleftharpoons \text{Protein-drug complex}$$

Distribution of drug can be measured and is expressed by a parameter called volume of distribution (V_d). The V_d, expressed in liters, represents the total concentration of drugs in plasma both protein bound drug and free drug. Large values of V_d reflect higher concentrations of drug in tissues, whereas lower values of V_d reflect higher concentration of drug in the plasma. The V_d values of 4L or less reflect no distribution of drugs

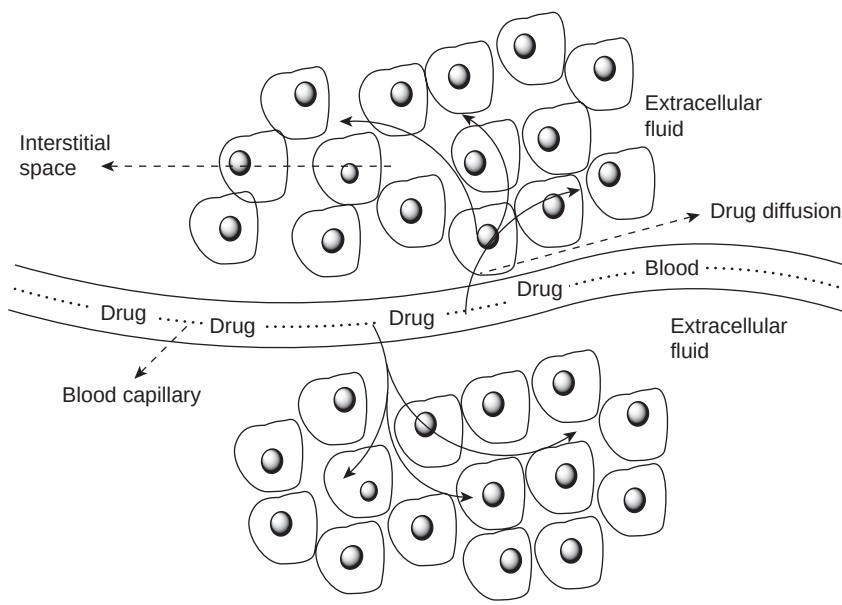


Figure 2.2 A diagrammatic representation of distribution of drugs via blood into tissues.

Table 2.3 Sites of concentration of drugs moving from blood plasma and volume of distribution

Tissues	Concentration of Drug in Tissues	Concentration of Drug Plasma	V_d value
Fat cells (Adipose tissue)	Accumulation of drugs in fat cells	Concentration of drug in the plasma is lowered	High
Bone tissues	Accumulation of drugs in bone tissues	Concentration of drug in the plasma is lowered	High
Tissue carrying site of action	Accumulation of drugs in tissues with therapeutic target (receptor/ enzyme)	Concentration of drug in the plasma is lowered	High

as it remains confined in the plasma; V_d value between 4 and 7L reflects distribution of the drug within the plasma including drugs bound to proteins and blood cells. However, if V_d is more than 42L (actual volume of body fluid), then it reflects drug distribution in all tissues within the organism. For example, metformin, an antidiabetic drug, exhibits V_d value between 300 to 1000L following single administration, which is more than 42L, thereby suggesting total distribution in tissues with minimum or no protein binding. There are three major sites where drugs are distributed from blood plasma (Table 2.3).

2.4.2.1 Drug Transport in Tissues

Permeability of drugs across biological membranes from blood to the site of action in tissues is yet another physiological barrier that affects distribution of drugs. The efficiency of drugs to penetrate cell membranes (barriers) in order to reach the site of action depends mostly on the drug's physicochemical properties (pK_a, solubility: logP, logD, molecular weight, etc.). Among these properties, lipophilicity of drugs plays an important role in the diffusion of drugs from plasma into tissues.

Structurally diverse drug molecules comprising small organic molecules and peptides get transported across cell membrane both in unidirectional and bidirectional movements. Membrane transporters often help in the transportation of larger and polar compounds such as sugars, amino acids, peptides, and nucleoside. It is important to note that only free drug (unbound) in bloodstream can traverse cell membranes or diffuse, and drugs bound to proteins in blood plasma cannot diffuse. Thus, among many strategies for the drug design, improving its permeability through cell membrane is an important parameter for improving the efficiency of a drug/test compound. Permeability of test compounds can be regulated by modulating the physicochemical properties of the molecules.

One of the primary physicochemical properties of test compounds that affect the diffusion of unbound drugs in tissues is lipophilicity. A higher lipophilicity of drugs/test compounds allows them to readily diffuse in fat cells (adipose tissues) and bone tissues. Drugs stored in fat cells sometimes remain there for a longer period of time or gradually leave the tissue and remain in blood circulation for days. Thus, following absorption of drug in the bloodstream, it is circulated throughout the body for distribution to tissues with site of actions. Generally, the average circulation time of blood is considered to be approximately 1 minute. Hydrophilic drugs tend to stay in aqueous environment such as blood or interstitial space surrounding the tissues and exert their effect by binding to the cell-surface receptors. In contrast, hydrophobic drugs depending on their ability may diffuse through plasma membranes in tissues. For example, clorazepate, an antianxiety drug with a logP value of 3.1 diffuses readily in fat cells. Similarly, rifampicin with logP value

of 3.7 is a highly lipid-soluble drug and readily diffuses through brain, whereas penicillin with logP of 1.41 is more of a hydrophilic and fails to diffuse.

Sometimes drugs may also get stored within certain organs such as lung, liver, heart, skeletal muscles, and kidney. This can have adverse consequences and damage the tissue of that organ. Alternatively, if drugs are stored in either of these tissues, they can be released again in bloodstream for binding with the target in tissues, thereby enhancing the lifespan of the drug in the body.

2.4.2.2 Binding of Drugs to Blood Plasma Proteins

A major portion of drug can bind with blood plasma proteins leading to protein-bound or unbound drugs in the blood plasma. The binding may be reversible or irreversible. Many drugs bind reversibly to plasma proteins, such as alpha1-acid glycoproteins bind primarily to basic drugs, whereas albumin binds primarily to acidic drugs. Irreversible binding may involve covalent bonding and could be toxic for the body. Notably, the protein-bound drugs can never display any activity and are therefore inactive.



Important Note

Zomepirac, an antipyretic drug was found to bind irreversibly with plasma proteins that led to its withdrawal.

Atenolol (Table 2.4), an antihypertensive drug, owing to its hydrophilic nature (logP = 0.23) remains in the aqueous environment and readily binds as competitive antagonist (inhibits sympathetic stimulation) to β(1)-adrenergic GPCR receptor present on the surface of the cell membrane of the heart and vascular smooth muscles. It exhibits bioavailability of 40–50% and only 6–16% binds to plasma proteins. On the contrary, atorvastatin (Table 2.4), a lipid-lowering drug being highly lipophilic (logP = 6.36) displays poor bioavailability and a very high level of plasma protein binding.

Table 2.4 Effect of logP on bioavailability and plasma protein binding of drugs

Drugs	logP	Bioavailability	Plasma Protein Binding
Atenolol	0.23	40–50%	16%
Atorvastatin	6.36	14%	>98%
Captopril	0.63	70%	25–30%
Chlorpromazine	3.17	30–50%	90%
Losartan	3.52	33%	98%
Ezetimibe	4.52	35–65%	99%
Cyclosporine	2.92	30	34%
Metformin	1.41	50–60%	Negligibly bound
Lidocaine	2.26	35%	60–80%

Effects of Plasma Protein Binding (PPB)

The binding of drugs to plasma protein significantly affects the biological response elicited by the drug. This is because the concentration of free drug in circulation is significantly reduced in comparison to the dose of drug administered in the body. Besides, the free unbound drug while in circulation also remains vulnerable to metabolic degradation and excretion resulting in further reduction in the concentration efficacy. This explains the significant difference observed between the concentration of drug administered and concentration of drug causing biological response inside the body. This can be attributed to ADMET of the drug caused by the physiological barriers. For example, in case of warfarin, an anticoagulant drug, 97% of the total drug in blood gets bound to plasma proteins. Only the remaining 3% of free drug exhibits the desired biological response and may be also accompanied by metabolism and excretion.

In some cases, the plasma protein-bound drug may act as reservoir and may release the unbound drug to maintain equilibrium in blood plasma between bound and free drug. This phenomenon not only enhances the duration of action of the drug but also affects the half-life of drug by protecting the drug (in the bound form) from getting metabolized and excreted.

A summary of implications of plasma protein binding are:

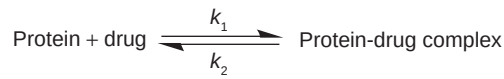
1. Delayed onset of therapeutic effect, since drug bound to protein is slowly released over a time period, which then elicits the desired biological response.
2. Delayed excretion as most drugs are bound to serum protein.
3. Competitive displacement of drugs/endogenous compounds (ligands/substrates) already bound to serum proteins.
4. Can decrease metabolism, clearance, and in turn prolong half-life and its action.
5. If due to physiological conditions, the serum protein levels are decreased, the effect of drugs with high affinity for protein is expected to increase, e.g. leflunomide, a drug used as immunosuppressant: 99.4% of the administered drug binds to serum proteins, and accordingly, $t_{1/2}$ of the drug is 14 days.
6. A variety of structurally diverse drugs and endogenous substances bind to serum proteins; however, they differ in their affinity and can be grouped under three categories:
   - **(a)** Drugs having high affinity, with >90% binding to protein, e.g., glimepride (antidiabetic), losartan (antihypertensive), prazosin (cardioprotective), etc.
   - **(b)** Drugs having moderate affinity, e.g., paracetamol 20%, theophylline 60%, aspirin 50%, etc.
   - **(c)** Drugs with poor binding affinity < 10%, e.g., metformin (antidiabetic), isoniazid (antitubercular), and metoprolol (antihypertensive).

Free Drug Hypothesis

Drugs bound to plasma proteins can never diffuse through bilayer cell membranes. Only unbound drugs can readily diffuse through membranes into tissues (with or without site of action) either through passive or active transport. Unbound drugs in systemic circulation are prone to metabolic degradation and elimination. Thus, reducing the duration of action of the drug, however, prolonged stay of drug in the body and may cause toxicity.

Thus, highly bound drugs to plasma proteins with slow dissociation rate can be restrictive (limitation), as this can limit the availability of drug from expressing its biological action. Similarly, drugs bound to plasma proteins with high dissociation rate can be permissive (allowed), as this will raise the concentration of free drug and allow it to exhibit its biological effect (Table 2.5).

Table 2.5 Effect of equilibrium and rate constant of plasma protein-bound drugs on biological activity *in vivo*



(K_d = Equilibrium dissociation constant and represents concentration of free drug at which half-maximal binding occurs)

% of Drug Bound (Equilibrium)	K_d (Dissociation Constant)	*In vivo* Effect
High	Slow	Restrictive
High	Fast	Permissive
Low	Slow	Permissive
Low	Fast	Permissive

Although dissociation rate constant of protein-bound drug plays a major role for the unbound drug to elicit is biological response and in the volume of distribution (V_d), concentration of the free drug in plasma has no bearing on volume of distribution. For example, in Table 2.6, hypothetical values for eight drugs A to H has been tabulated with the corresponding free drug concentrations in plasma and V_d values. As is evident from the Table 2.6, drugs A and F have almost same concentrations (3 and 4, respectively) of free drug in plasma; however, they exhibit tremendous difference in their volume of distribution (V_d). For drug A, the V_d = 0.2 L/kg and is restrictive, while for drug F, the V_d = 30 L/kg and is permissive. This suggests that drugs A and F despite having same concentration of free drug in plasma, exhibit different V_d values. This can be attributed to slow dissociation rate constant for plasma protein-bound drug A than protein-bound drug F, thereby suggesting that concentration of the free drug has no effect on V_d. Similarly, drugs G and H have same concentration of free compound in the plasma but have different V_d values and are permissive. Drugs B and C both have poor concentration of drugs in the plasma and also poor V_d values (Table 2.6).

Table 2.6 Impact of PPB of drugs on their volume of distribution

Drug	Free Drug in Plasma (%)	Volume of Distribution (L/kg)
Restrictive		
Drug A	3	0.2
Drug B	<2	0.17
Drug C	10	0.60
Drug D	<1	0.13
Permissive		
Drug E	8	45
Drug F	4	35
Drug G	30	40
Drug H	30	15

Important Note

Structural modification strategies that can reduce PPB:

- Reduce hydrophobicity (lipophilicity: logP and logD).
- Reduce acidity by enhancing pK_a of the acid.
- Increase basicity by enhancing the pK_a of the base.

2.4.2.3 Binding of Drugs to Blood Cells

Some portion of drug molecules may also reversibly bind with blood cells such as RBCs present in blood plasma and may act as reservoir for the drugs. Diuretic drugs acetazolamide and chlorthalidone have the highest affinities for the blood cells.

Blood–Brain Barrier

Blood carries water, antibodies, macrophages, toxins, xenobiotics, neurotransmitters, and hormones throughout the body. Required substances move in and out of the blood into surrounding organs/tissues and perform the desired functions. The capillary walls through which the blood flows are made up of endothelial cells and act as a barrier between the blood and surrounding tissues. In the normal blood capillary, throughout the body except brain, desired molecules from blood can readily move in and out; however, in brain this is not the practice, and there is a strong barrier in effect, called blood–brain barrier (BBB). The barrier prevents unwanted passage of substances from the blood capillary into the cerebrospinal fluid (CSF), in order to protect the brain. Prevention of unwanted substances through BBB regulates the composition of CSF. A comparison of normal and blood–brain capillary has been depicted in Figure 2.3 and explained in Table 2.7.

P-glycoprotein (P-gp/MDR1/ABCB1), an active transporter efflux protein, plays a major role in one of the most sensitive zones of the human physiology: BBB. The protein is present as transmembrane protein in the cells present in the BBB and acts by pumping drugs out of the cells, thus protecting the tissues of the central nervous system from getting exposed to xenobiotics, toxins, and drugs present in the systemic circulation.

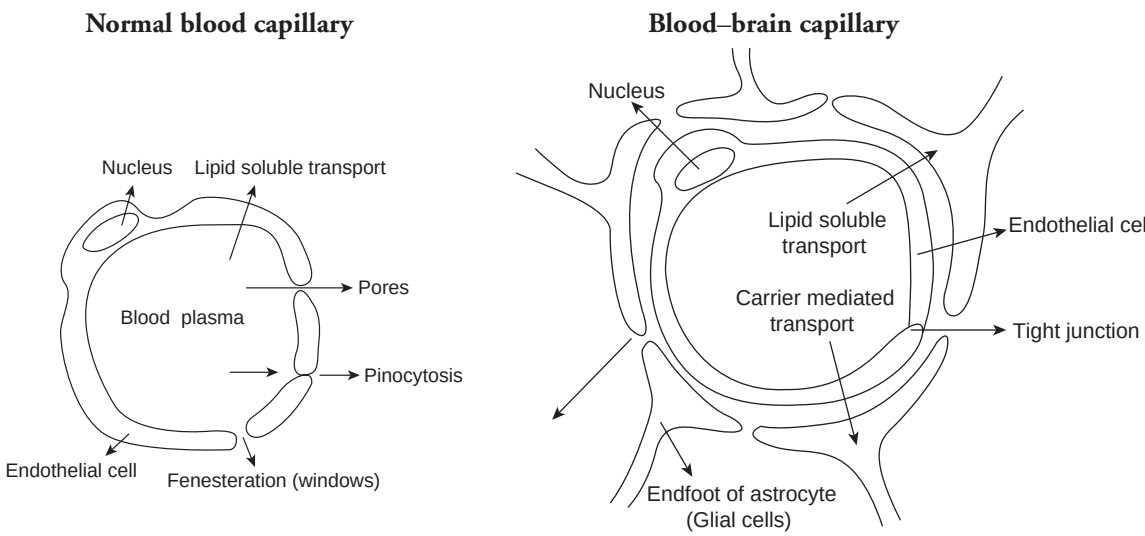


Figure 2.3 Comparison of normal and blood–brain capillary.

Table 2.7 Comparison of normal and blood brain capillary

Normal Blood Capillary	Blood–Brain Capillary
The capillary walls have a loose, leaky structure.	The capillary walls have uninterrupted lining.
Between the endothelial cells (that make up capillary walls) there are gaps and fenestrations (windows) making them leaky.	The adjacent endothelial cells (that make up capillary walls) are connected by solid and tight occluded junctions and donot have fenestrations.
Fenestrations are covered with a delicate porous membrane that facilitates easy movement of fluid and WBCs.	The basal membrane of each capillary is surrounded by glial cells (astrocytes) in brain that creates perivascular feet to surround the blood capillary wall. Thus, yet another tightly sealed cellular layer along with the capillary wall forms a strong BBB and prevents in and out movement of unwanted substances.
Small molecules from blood enter cells through pores present between the endothelial cells in blood capillaries. Alternatively, substances can pass directly through endothelial cells that make up the capillary wall or pumped in and out by specific carrier-mediated transport via concentration gradient.	Glucose, water, dissolved gases, and lipid-soluble substances such as alcohol, caffeine, nicotine, and anaesthetics readily diffuse through (pass through their intercellular clefts) the membrane and move into CSF. Other substances of interest required for brain moves in the CSF via carrier-mediated transport.

2.4.3 Metabolism

Drugs depending on their physicochemical properties are excreted from the liver either in its unchanged (native) form through the kidney (if water soluble) and bile/GIT/stool (if hydrophobic), or they may undergo chemical changes (to make it water soluble or hydrophilic) so as to allow them to get excreted through the urine. The process of undergoing chemical changes is called biotransformation or metabolism.

In the liver, metabolic enzymes cytochrome P450 catalyse the biotransformation of xenobiotics including drugs and endogenous substances following oxidation–reduction reactions or hydrolysis into more polar compounds. Liver cells carry out two types of enzymatic metabolism: Type I and type II, with the objective to terminate their biological activity and facilitating their excretion from the body.

2.4.4 Excretion

The excretion may involve either free drugs or its metabolites. By and large, hydrophilic drugs and their metabolites are excreted by urine while hydrophobic drugs and their metabolites are excreted in stool with exceptions (Fig. 2.4). Water-soluble drugs/metabolites are generally excreted through kidney and the presence of large pores in kidney glomeruli facilitates their filtration.

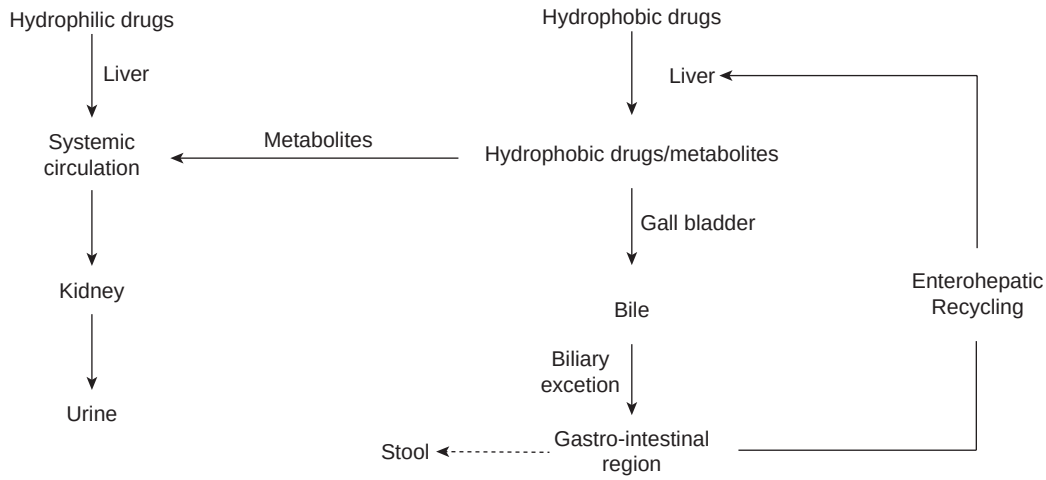


Figure 2.4 Excretion of hydrophobic/hydrophilic drugs (there are, however, numerous exceptions to this rule).

CASE STUDY 1

Drug Excretion Through Stool

Ezetimibe has been used for the lowering of cholesterol in the body, and it works by blocking the cholesterol uptake from the diet. It has a high logP value of 4.52 and pK_a 9.48 (acidic) and, accordingly, more than 90% of the drug is initially bound to plasma proteins. Besides, it undergoes extensive glucuronidation in the intestinal wall following absorption. Both ezetimibe and its glucuronide conjugate (part of metabolic process of drugs) undergo enterohepatic recirculation. Besides, high logP value may also be responsible for the major fraction of the dose, ~78% being excreted in stool and only 9% is excreted as water-soluble glucuronide (active metabolite) in the urine. Out of the 78% of the drug being excreted in stool, 68% comprises native (unchanged) drug.

2.4.5 Toxicity

The administration of drug in humans besides producing the desired pharmacological response may be also be accompanied by toxic effects involving unpredictable pharmacological effects (Adverse drug reactions [ADRs] or effects), secondary unwanted side effects, and allergic and non-allergic hypersensitivity reactions (Fig. 2.5). Widely prevalent ADRs, irrespective of subjects may be due to drug overdose (drug accumulation in cells), drug–drug interactions, and drug side effects, and subject-specific ADRs may be due to drug intolerance, drug allergy, pseudoallergic reactions or drug idiosyncrasy (genetically regulated). Thus, it is mandatory to establish therapeutic–toxic index called the 'therapeutic window' for drugs. The latter gives a comparison between the amount of drug that causes therapeutic efficacy and amount of drugs that causes toxicity. Some drugs exhibit small difference between therapeutic and toxic doses and are not recommended. Generally, a difference of 20-fold or higher is recommended between doses causing clinical efficacy and toxicity in animals.

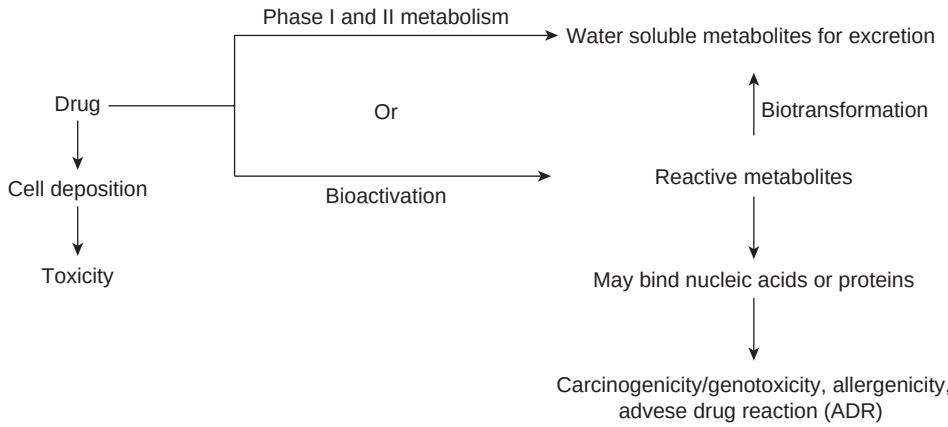


Figure 2.5 Toxicity due to cell accumulation of drug molecules or reactive metabolites.

2.4.5.1 Drug-Induced Adverse Reactions

ADRs in the organism include hepatotoxicity, nephrotoxicity, cardiotoxicity, neurotoxicity, teratogenicity, impaired vision, or hearing. The ADRs may not be common but can be serious and may lead to serious health issues or morbidity. Teratogenicity is a kind of ADRs that causes grave injury (birth defect) to the developing embryo or fetus. Some drugs that act as teratogens are ACE inhibitors, androgen, antibiotics, anticoagulants, antidepressants, etc. In 1950s, consumption of thalidomide (also known as horror drug) by pregnant women to treat morning sickness led to birth of babies with severe deformities.

2.4.5.2 Allergenicity

Administration of drugs/test compounds *in vivo* may often produce unpredictable reactions due to the immunological response of the organism. This is referred to as allergenicity (drug allergy) and comprises either immunoglobulin E (IgE)-mediated (immediate) or non–IgE-mediated (delayed) drug hypersensitivity reactions. Some examples of clinical manifestations of drug allergy involve symptoms of anaphylaxis exhibited by antibacterial and neuromuscular drugs, and pulmonary hypersensitivity reactions exhibited by amiodarone, non-steroidal anti-inflammatory drugs, and β blockers.

2.4.5.3 Genotoxicity/Carcinogenicity

Drugs that cause adverse reaction by inducing genetic (DNA) damage are called genotoxic/carcinogenic. The genotoxicity involves direct interaction of drugs with genetic materials, whereas carcinogenicity involves development of cancers by affecting gene expression, intracellular signal transduction, and cell proliferations.

2.5 Factors Affecting ADMET

2.5.1 Physicochemical Parameters of Drugs

The physicochemical parameters: pK_a, solubility, lipophilicity, polar surface area, etc., can directly affect pharmacokinetic (PK) characteristics involving ADMET (Fig. 2.6). The physicochemical parameters in drug design have been discussed in chapter 6.

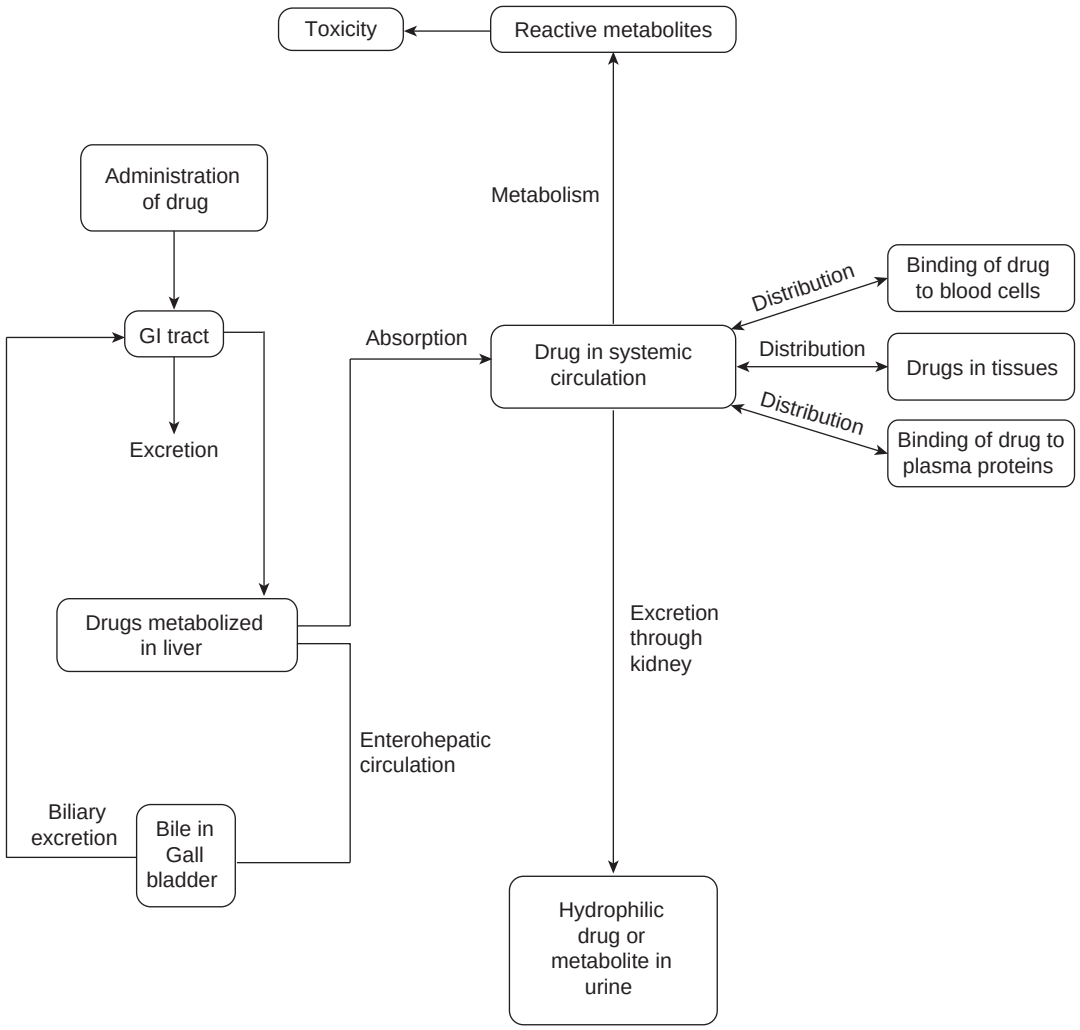


Figure 2.6 An overview of ADMET following oral administration of a drug in the body.

2.5.2 Physiological Factors

There are several physiological parameters affecting ADMET and have been discussed below under absorption, distribution, metabolism, and elimination.

2.5.2.1 Physiological Factors Affecting Drug Absorption

Absorption of drugs largely depends on the route the drug is administered in the body. The primary objective is to select a route that will produce therapeutic concentrations of drugs in systemic circulation from where the drugs can translocate in the tissue for eliciting response. It is represented by a parameter referred to as bioavailability. Thus, one of the physiological factors affecting drug absorption could be in selecting appropriate route of administration for drug. Factors that need attention while selecting a route of

administration for drug are:

1. Physicochemical properties of the drug
2. Physiological nature of the route
3. Drug metabolism
4. Systemic or local effect (side effects)
5. Onset of action
6. The pathological condition being treated
7. Patient compliance

2.5.2.2 Various Routes of Administration

The various routes of administration for drugs have been depicted in Fig. 2.7 and are summarized below.

1. Enteral routes (administration through gut)
   - **(a)** Sublingual (buccal)
   - **(b)** Oral
   - **(c)** Rectal
2. Parenteral routes
   - **(a)** Intravenous (IV) injection
   - **(b)** Intra-arterial (IA) injection
   - **(c)** Intrathecal injection (injection into spinal canal)
   - **(d)** Intramuscular (IM) injection
   - **(e)** Subcutaneous (SC) injection (vaccines and medications such as insulin)
   - **(f)** Inhalation
3. Topical application

Administration of Drug by Oral Route

This involves administration of drugs by mouth, or buccal or sublingual route. During the administration of drugs by mouth, they are absorbed through digestive tracts; in buccal administration, drugs diffuse through the internal mucosal lining of the cheek directly into the bloodstream and in sublingual administration, drugs enter the bloodstream via blood vessels under the tongue instead of digestive tract. Intake of food prior to administration and post administration of drugs often affects the oral absorption of drugs.

During oral administration, among many physiological barriers/factors that affect bioavailability of drugs, the four major factors are:

1. Single layer of intestinal epithelial cells that spans the entire intestinal interior acts as barrier for drugs (Fig. 2.8).
2. First-pass metabolism in gastrointestinal region.
3. Liver as major physiological barrier causing metabolism.
4. Efflux transporters in the intestine expelling absorbed drugs in enterocytes back into the lumen (Fig. 2.8).

Besides physiological factor, physicochemical properties of drugs such as pK_a, solubility, stability, etc., also affect bioavailability by *p.o.* route.

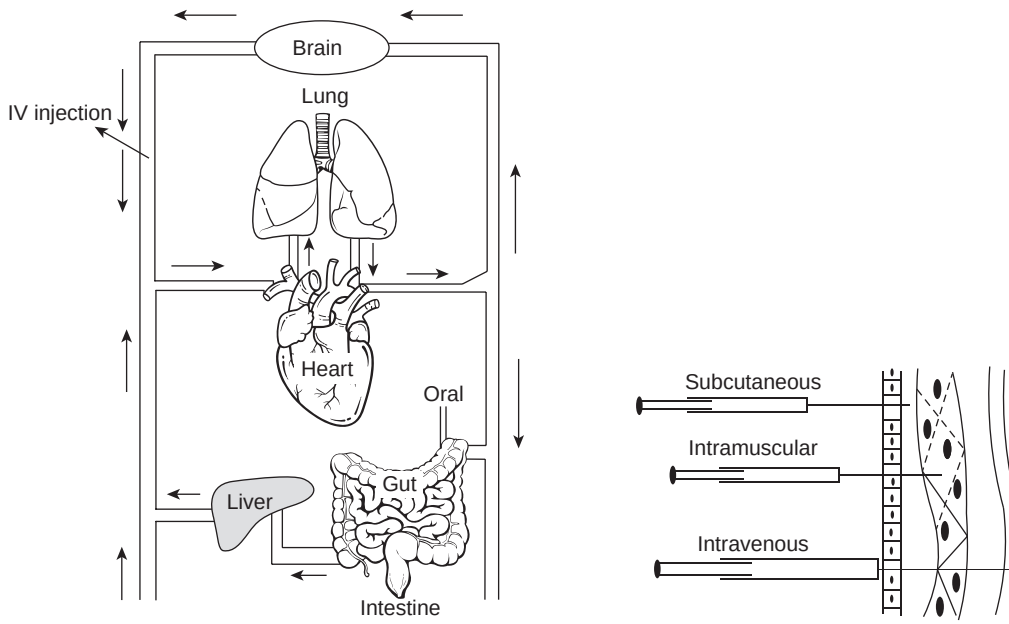


Figure 2.7 Routes of administration of drugs.

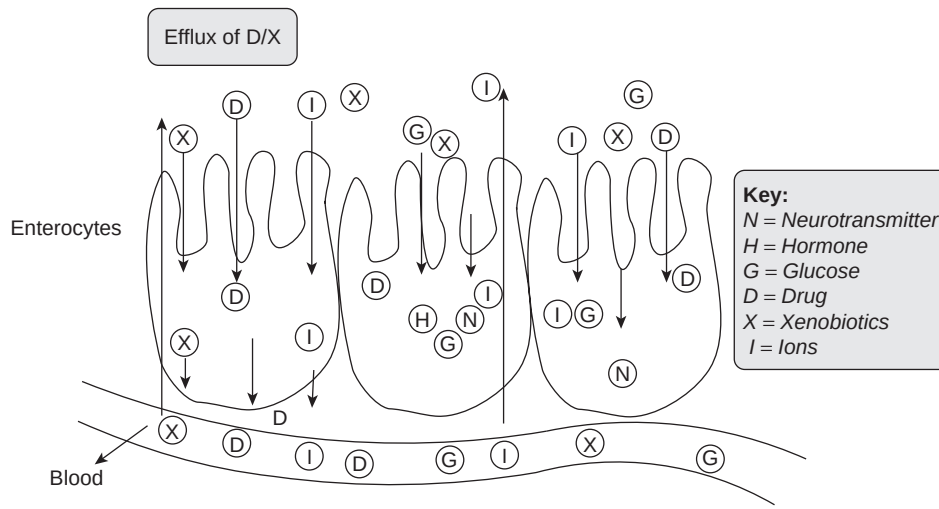


Figure 2.8 Orally administered drugs/foods absorbed through gut (absorptive gut epithelial cells/enterocytes) enter into blood circulation coupled with efflux of drugs by transporter proteins.

Epithelial cells in gut wall as barrier for drug absorption by p.o. *route*

There are two options for drugs to cross the gut wall: (a) the transcellular pathway involving diffusion/permeation through cell membrane, and (b) paracellular pathway that involves diffusion through water-filled pores among the tight junctions between the cells.

First-pass effect or metabolism following administration of drug by p.o. *route*

The first-pass effect or metabolism is the metabolism of drugs following their administration by oral route. The drugs are metabolized by cytochrome P450 (CYP) both in the intestinal epithelium (enterocytes or intestinal absorptive cells) as well as in the liver before reaching systemic circulation (Fig. 2.9). This results in significant reduction in the fraction of drug reaching bloodstream in comparison to the administered dose.

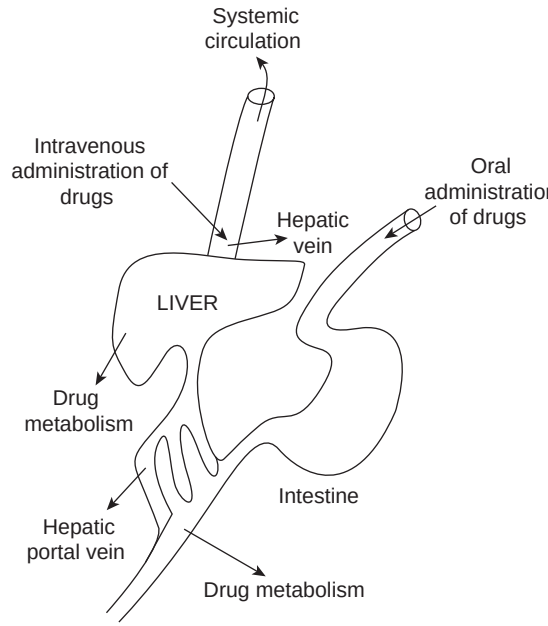


Figure 2.9 The first-pass effect or metabolism involves metabolic degradation of drug in gut and liver.

Gut and liver as major physiological barriers during absorption of drug by p.o. *route*

Drugs administered by oral route are absorbed into the bloodstream via the gut followed by liver, whereas those given intravenously directly enter the bloodstream. There are several factors that control drug absorption by oral route:

1. pK_a (ionization)
2. Lipophilicity/permeability (absorption) of drug through the GI membrane
3. Solubility/dissolution of drug through the GI milieu
4. First-pass effect/metabolism/stability
5. Efflux transporters
6. Gastrointestinal pH
7. Gastrointestinal blood flow
8. Gastric emptying (Drug absorption is often facilitated when there is faster gastric emptying. Thus, factors that slow down emptying process may impair absorption of drug. Hypothyroidism, presence of anticholinergics $Al(OH)_3$, etc., are factors that slow down gastric emptying).
9. The drug is largely metabolized in the liver after oral administration and, therefore, affects bioavailability.

Effect of efflux transporters on bioavailability by p.o. *route*

ABC transmembrane transporter proteins such as P-glycoprotein (P-gp) are major physiological barriers that can affect drug absorption and in turn reduce the concentration of drugs in systemic circulation and tissues. P-gp efflux proteins are widely distributed in many tissues; however, they play a major role in the intestine, BBB, and kidney. In the intestine, P-gp efflux proteins are present in the apical surface of epithelial cells and play excretory role by pumping the xenobiotics, including drugs, out of the epithelial cells, back into the intestinal lumen, and in turn lower bioavailability. This phenomenon is also a frequent cause of treatment failures in cancer patients. Inhibitors of P-gp can be co-administered with drugs to enhance the transport of drugs.

Yet another role of P-gp has been observed in excretion. In kidney, the transmembrane P-gp efflux proteins present in the luminal membrane of proximal tubule cells pumps the drug/metabolites from the inside of cells to the outside in urine against concentration gradient.

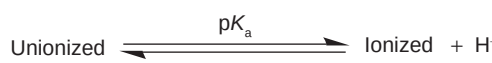
The P-gp transmembrane efflux proteins generally display their effect because the drugs/test compounds are able to activate the protein, but there are instances when some drugs interact and inhibit the action P-gp. For example, verapamil drug inhibits the action of P-gp and, therefore, may enhance the bioavailability of drugs beyond their concentration (may increase drug load). This can lead to drug–drug interaction, which is also observed during induction/inhibition of metabolic enzyme cytochrome P 450 (CYP). Fortunately, CYP is able to metabolize most of the drugs that are transported by P-gp.

Effect of pK_a *of drugs on bioavailability by oral route*

pK_a is considered to be one of the most crucial physicochemical parameters for a molecule during a drug development programme. A pK_a is defined as the pH where a drug exists in equilibrium between ionized and unionized species: in other words, 50% ionized and 50% unionized. The feature regulates absorption (diffusion/bioavailability) of drug through walls of Gastrointestinal tract (GIT) followed by binding with protein target in the cell. The unionized (water-insoluble) drug facilitates absorption (bioavailability) through lipid bilayer cell wall of enterocytes. Since the physiological pH of the GIT varies from 1.4 to 8.0, the pK_a of a drug can provide information about its absorption and bioavailability in GIT.

A correlation between pK_a and concentration of unionized drug at a physiological pH has been summarized in Table 2.8. As can be seen, in a given series of acidic compounds the pK_a of each compound varies; this in turn has its effect on their bioavailability. Accordingly, in the GIT at pH 2.5, the concentration

Table 2.8 A correlation between pK_a and concentration of unionized drugs (acidic) at a physiological pH in gut



pK_a	pH	Concentration of Unionized Drug (%)*	At pH in gut	Concentration of Unionized Drug (%)	Bioavailability
7.4	7.4	50	2.5	100	↑
4.4	4.4	50	2.5	98.8	
3.2	3.2	50	2.5	83.4	
2.8	2.8	50	2.5	66.6	

**Calculated using software available at https://www.manuelsweb.com/pka.htm*

of unionized form was found to decrease with decrease in pK_a of the compounds. The findings suggest that in a given series of acidic compounds, the ones with higher pK_a will have better and faster absorption/bioavailability during administration of drug by oral route. Similarly, in a given series of basic compounds, the ones with lower pK_a will have better and faster absorption/bioavailability during administration of drug by oral route.

Effect of solubility of drugs on bioavailability

In most cases, poor bioavailability has been attributed to poor solubility of drugs. If a biologically active molecule is not available in a solution form at the site of action, it cannot be developed into a clinically useful drug. Since the environment of gastrointestinal region is aqueous, the orally administered dug must have adequate aqueous solubility to dissolve in the gastrointestinal contents and also enough lipid solubility to permeate into the bilayer lipid membrane and then enter into systemic circulation. If a drug is to be absorbed through the mucosal lining of the GIT, then it must be in its lipophilic unionized form so that it is readily partitioned out of the aqueous medium. Thus, a relationship exists between aqueous solubility/lipophilicity and bioavailability/absorption rate of the molecule (Fig. 2.10).

Highly hydrophilic drugs may not be able to get absorbed through GI mucosa (bilayer membrane), and highly hydrophobic drugs may fail to solubilize in the aqueous GI environment. Thus, during drug development programme, one has to strike an optimal balance between the two conflicting parameters. LogP (partition coefficient) values that reflect lipophilicity of molecules can be used as an indicator for bioavailability. Compounds with lower logP values may have optimal balance of hydrophobicity and hydrophilicity and, in turn, may exhibit good bioavailability and distribution. On the contrary, compounds with higher logP or significantly low logP values may have poor bioavailability (Table 2.4). But this cannot be used as a thumb rule since there are many other factors that affect bioavailability and distribution. Generally, an optimal lipophilicity is required in the test compounds/drugs not only to facilitate diffusion through the lipid bilayer membrane but to also get out of the membrane on the other side and enter the cytoplasm in aqueous environment. Although clinically used drugs display large variations in their logP

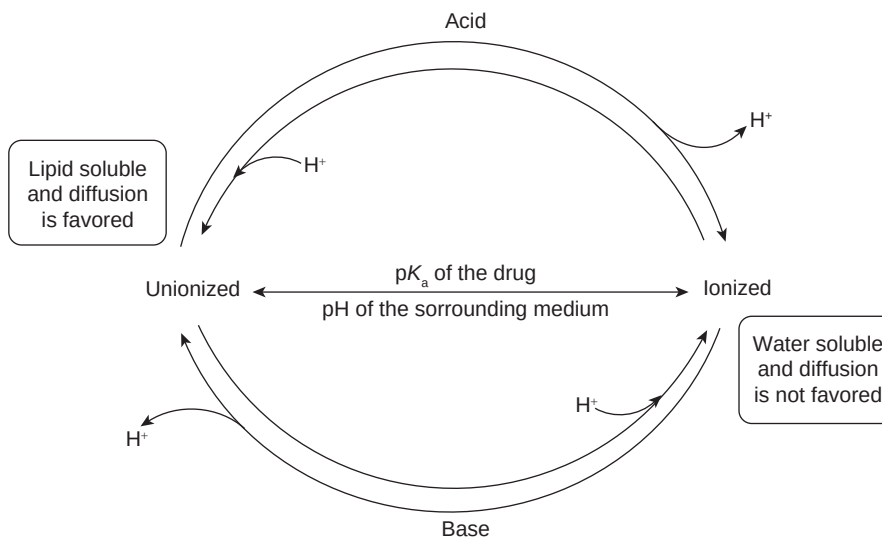


Figure 2.10 pK_a/ionization/pH/solubility of drugs affecting bioavailability.

values (lipophilicity), increasing lipophilicity of drug candidates is a commonly used approach to enhance their bioavailability. However, it should be borne in mind that modifying the chemical structure by adding hydrophobic residues may often result in compounds with poor PK profiles. Alternatively, one can overcome the drawback associated with solubility and bioavailability of drugs by converting them into appropriate salts. There are numerous examples where during drug design/lead optimization studies, the solubility, diffusion, route of administration, and efficacy of the drug have been successfully improved by employing salts of the active compounds. More than 40% drugs used clinically for treating diseases are used as salts. Among a variety of drug salts prepared, sodium and hydrochloride salts are highly prevalent.

For example, sodium diclofenac salt is used as extended-release tablets and two other salts of diclofenac epolamine and diclofenac potassium have also been developed and patented and may be released in near future. A diethylamine salt of diclofenac (commercial name Intagesic gel) is available as topical cream and is used as muscle relaxant.

Intravenous (IV) Route

Drug administration by IV route remains the fastest way to deliver drugs. Notably, oral drugs enter systemic circulation via stomach, intestine, and liver, while IV drugs enter systemic circulation directly (Fig. 2.9). Thus, the bioavailability of the IV medication is always taken as 100%. In a comparative study between oral and IV administration involving propranolol (an antihypertensive drug), the biological efficacy exhibited by 200 mg of drug administered by oral route was equal to 1–3 mg of IV injection for the same drug. Thus, the first-pass metabolism influences the activity of several drugs and hence they need to be administered in large doses by oral route or by another route of administration to bypass the first-pass metabolism by the gut and liver. This is because all of the venous blood from intestine enters the portal vein and then moves to liver (approximately, 75% of all blood reaches liver).

Subcutaneous (SC) Route

Subcutaneous (SC) administration of drug involves delivery as a bolus into the tissue layer between the skin and the muscle. Owing to poor network of blood vessels in subcutaneous tissue, drugs injected via SC route leads to slow and sustained rates of absorption in bloodstream. It is slower than IM injections but still faster than intradermal injections. For example, insulin injections are SC type, and medication given by this route is usually absorbed very slowly and sometimes extends up to 24 hours.

Intramuscular (IM) Route

Muscles have larger and huge number of blood vessels than subcutaneous tissue and hence drugs administered via IM injections usually have faster rates of absorption in bloodstream than SC injections or intradermal injections. Some examples of drugs that are commonly delivered by IM injections include antibiotics, hormones, and vaccinations.

Inhalation

Administration of drugs via direct inhalation in lungs is the most promising route of administration. Drugs in lungs are exposed to a large surface area that facilitates rapid absorption into systemic circulation. During drug delivery by oral route, the drug has to undergo first-pass metabolism, enzymatic biotransformation, and is effluxed back into the GIT lumen by efflux proteins. In contrast, in lungs, drug metabolism and activity of efflux transporters are significantly less than in other organs, e.g. gut and liver. Thus, bioavailability of drugs by inhalation is significantly enhanced than by oral route. This, in turn, brings down significant reduction

in the cost of treatment by reducing doses of drugs. By inhalation route, the drug is delivered in gaseous, aerosol, or ultrafine solid particle form. Drugs atomized in smaller droplets can be inhaled through mouth. For example, long-acting bronchodilator asthmatic drugs (salmeterol, formoterol, etc.) are administered using this atomized approach.

Topical Route

The three topical routes of administration of drugs involve topical formulations (creams, gels, ointments, lotions, etc.), transdermal patches, and hypodermis needles.[1] Topical formulations comprise administration of both topical and transdermal medicines. The topical medicines directly act onto the skin affected with a disease without entering the skin, whereas transdermal medicines are absorbed through the skin and enter systemic circulation. Antifungals are classical example of topical medicine used as antifungal cream, lotion, or spray. This non-prescription topical therapy is used for the treatment of superficial fungal infections of skin, hair, eye, and nails. The topical antifungals clotrimazole, econazole, and ketoconazole exhibit limited percutaneous absorption and act by killing the fungal cells by inhibiting either cell wall synthesis or nucleic acid synthesis, etc. Similarly, topical anaesthetic cream Numbex^{R} comprising eutectic mixture of local anaesthetics lidocaine and prilocaine displays its anaesthetic action by decreasing the firing of neurons (block conduction of impulses) of the free nerve endings in the dermis. This in turn blocks afferent nerves from sending pain signals to the brain.

The transdermal medicines can be also be administered via adhesive transdermal patches through which a specific dose of medicines is absorbed through the skin via diffusion process and enters bloodstream. The drug administered in the form of a patch initially penetrates through stratum corneum and then via epidermis and dermal microcirculation enters bloodstream. Two major pathways involved in the painless transdermal delivery of drugs involve transcellular and intercellular pathways. Transcellular route involves passage of drugs through phospholipids membranes and the cytoplasm of the dead keratinocytes that constitute the stratum corneum. Whereas intercellular route involve passage of transdermal drugs through the small spaces between the cells of the skin. Majority of transdermal drugs are absorbed through intercellular route. Scopolamine, a muscarinic antagonist used preoperatively to minimize secretions is administered via transdermal patch. The third route for topical administration of drug involves hypodermis needle (a hollow needle) which is used to inject drugs subcutaneously i.e. just beneath the skin (below the dermis and epidermis) in liquid form where it enters bloodstream. Since subcutaneous tissues have few blood vessels, the injected drug undergo a slow rate of absorption. Examples of drugs administered subcutaneously are insulin, morphine, goserelin (for treating cancer), etc.

The passage of drugs via topical route primarily involves three layers of skin: the epidermis, dermis, and hypodermis. The dermal layer has a network of dermal microcirculation through which drugs are directly absorbed in systemic circulation or lymph and free nerve endings responsible for pain sensation. The first layer of the epidermis is avascular and acts as a protective layer (barrier) of the skin and is called stratum corneum. It is made up of dead cells, flattened cells, and insoluble proteins keratin. The dermis is made up of connective tissue fibers and selectively allows entry of only water-soluble substances. Thus, just like the presence of aqueous and lipid barriers during the passage of drugs via oral route, the skin layers too exhibit differential affinity for hydrophilic and hydrophobic compounds. The stratum corneum being lipophilic allows permeation of hydrophobic drugs in their unionized form, whereas epidermal layer being predominantly aqueous displays affinity for hydrophilic drugs or ionized form of drugs. Thus, the first step in the topical application of transdermal drug involves partitioning between the vehicle in the cream and the lipophilic stratum corneum. This allows drugs to traverse through stratum corneum, and for this mostly

unionized state (basic nature) of the drug is ideal. Permeation through hydrophilic epidermal and dermal layers requires ionized state for optimal results and is therefore accompanied by partitioning into hydrophilic tissues at the interface of hydrophobic stratum corneum and the hydrophilic epidermis. This suggests that drugs must have optimal lipophilicity for diffusion via transcellular and intercellular pathways; however, other factors such as composition of the topical formulation involving solvent and penetration enhancers or inhibitors also affect delivery of drugs through the skin. The main base (solvent) in topical formulations is generally made up of oil mixed with water which assists in the penetration of drug through the lipophilic stratum corneum. The reason for the oily medium as vehicle in these topical formulations gets support from the fact that skins generally have oily (sebaceous) secretions.

The three major factors that affect absorption of drugs via topical formulations are physicochemical properties of drugs such as MW, solubility, pK_a, logP, physical properties or composition of formulations such as nature of oily medium, penetration modulators, etc., and physiological parameters of the skin. For example, a 1.16% emulsion of diethylamine salt of diclofenac exhibited significant increase in the skin diffusion in comparison to 5% gel of sodium salt of diclofenac. This can be attributed to the enhanced solubility of diethylamine salts due to their ability to readily form micelles, whereas sodium salt cannot form micelles.[2] Thus, it is not the concentration of drug alone, instead the excipients in these formulations (composition) together that affects drug solubility, dissolution rate, and absorption through the skin.

In the transdermal route of administration, the active pharmaceutical ingredients are delivered via transdermal patches through the skin. The advantage of transdermal administration involves prolonged duration of actions, reduction in doses, bypassing first-pass metabolism, termination of administration by removal of patches, etc. The patches include delivery of single or more than one drug such as nicotine for addiction, nitroglycerine for the treatment of angina pectoris, lidocaine for lower-back pain and steroids (estradiol, progesterone, and testosterone) for contraception and hormone replacement therapy. The first transdermal patch approved in 1979 involved systemic delivery of anticholinergics scopolamine for treating motion sickness. This was followed by development of 19 transdermal delivery systems for a variety of drugs. Currently, delivery of macromolecules and vaccines via yet another delivery route microneedling is undergoing clinical trials. The technique enhances delivery of drug by disrupting the stratum corneum barrier and creating micron-size pores, thus opening pathways for drugs to directly enter epidermis or upper layer of dermis from where they enter systemic circulation.

2.5.2.3 Bioavailability

It is a parameter used for the absorption of drugs in bloodstream and is defined as the fraction of an administered dose of unchanged drug that reaches the systemic circulation. Following administration of a drug by oral route, its concentration in serum peaks quickly and then slowly drops as the drug is degraded and metabolized by the liver and/or kidney. This has been demonstrated experimentally by plotting a curve involving concentration of drug in blood plasma against time (Fig. 2.11). As can be seen, the curve first ascends depicting absorption and then descends depicting metabolism/excretion. Thus, the Area under the curve (AUC) can be used as a tool to determine bioavailability of drugs (F). This is a very important parameter in drug discovery in defining how well the drug is absorbed and excreted and has been made mandatory by the FDA for the drug approval process.

Among different routes of administration, drugs taken by oral route have the most delayed T_{max} in comparison to other routes of administration for the same drug (Fig. 2.12). This may be attributed to the GIT as the major physiological barrier for drugs administered by oral route (*p.o.*). Administration of drug by IV route although gives quick response, its duration of action is short, whereas by oral route the response is delayed but the duration of action is enhanced.

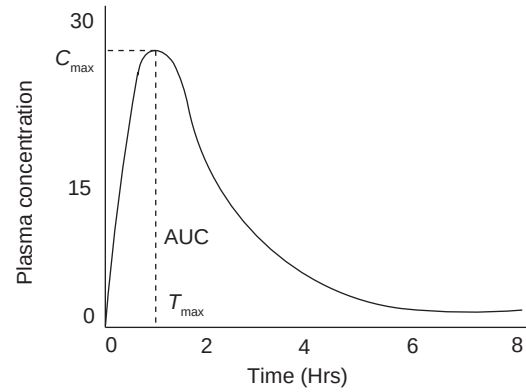


Figure 2.11 A typical AUC in drug plasma concentration versus time curve.

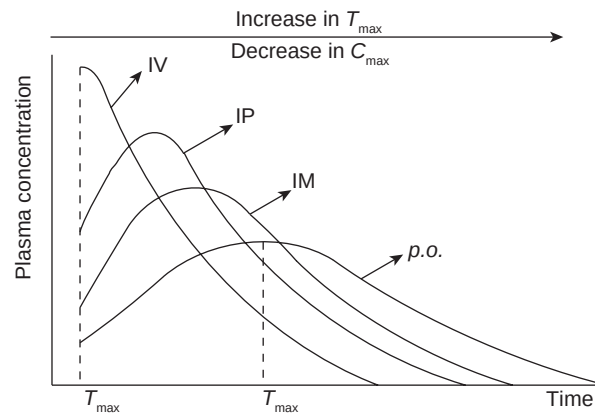


Figure 2.12 AUC following different routes of administration of drugs.

The Plasma Concentration–Time Curve

Besides calculating bioavailability, many more important parameters following administration of drugs by oral route can be derived (Fig. 2.13). (A detailed discussion of the concentration–time cure with reference to PK parameters has been given in the chapter 'Pharmacokinetics').

1. **Minimum Effective Concentration:** It refers to the minimum fraction of drug that is needed at the receptor/enzyme for the expression of biological activity.
2. **Minimum Toxic Concentration:** It reflects the concentration of drug required to just barely produce a toxic effect.
3. $\boldsymbol{C_{max}}$**:** The maximum plasma (serum) concentration (peaking) achieved following the first dose of administration of drug.
4. $\boldsymbol{T_{max}}$**:** The time taken to reach maximum concentration.
5. $\boldsymbol{t_{1/2}}$ **(Half-life):** This is an important PK parameter that reflects time taken by the drug for the C_{max} to be reduced by 50%.

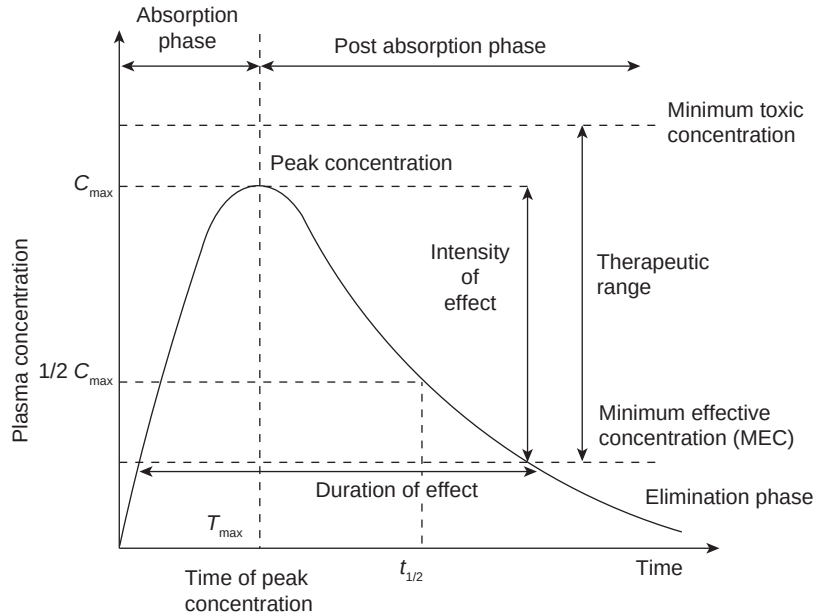


Figure 2.13 Various parameters derived from the plasma concentration–time curve.

Bioequivalence

In pharmacokinetics, bioequivalence is a term used to establish in vivo biological equivalence of a drug prepared by two different methods. This is carried out by assessing extent and rate of accessibility of drug to the desired protein target. In other words, bioavailability for both drugs should be same, and this can be assessed by evaluating three variables: AUC, C_{max}, and T_{max}. Based on international consensus, while assessing bioequivalence of drugs from two batches, a difference of more than 20% between the AUC and C_{max} values is not acceptable.

The International Council for Harmonization (ICH) defines bioequivalence as:

'The absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study'.

Bioequivalence is now routinely used to compare the bioavailability of generic brand of drug with brand name drugs, and FDA has made it mandatory data for the approval of generic drugs.

2.5.2.4 Physiological Factors Affecting Drug Distribution [D]

Distribution of drugs V_d primarily involves either binding of drugs to blood proteins or movement of drugs from blood to different tissues (sites of concentration). There are several factors that affect distribution.

Plasma Protein Binding

It has been discussed earlier in this chapter, under Section 2.4.2.2. A major portion of drug following administration can bind (in plasma) with blood plasma proteins leading to equilibrium between protein-bound and unbound drugs. The two major blood proteins involved in binding with drugs are albumin and β-globulin. Drug binds to these serum proteins at non-specific sites and prohibits the drug from binding to the receptor (by not allowing them to move into tissues) and thus inactivates the drug.

Binding of Drugs to Blood Cells

Besides, binding to plasma protein in blood, drugs can also bind to blood cells (~40%) present in the blood circulation. Notably, binding of drugs to blood cells is not taken into account while determining concentration of drugs in plasma. Drugs predominantly bind to RBC, a major component of blood cells, which carries three binding components:

1. **Hemoglobin:** A variety of structurally diverse drugs such as phenytoin, phenobarbitol, etc., can readily bind with it.
2. **Carbonic Anhydrase in RBC:** It is an enzyme present in RBC which converts carbon dioxide into carbonic acid. For example, acetazolamide is a diuretic and inhibitor of carbonic anhydrase.
3. **Cell Membrane of RBC:** Many drugs such as chlorpromazine and imipramine bind readily to the membrane of RBC. They are also potentially biocompatible carriers for a number of drugs.

Reports in the last decade suggest that platelets can not only store endogenous substances (serotonin and ADP) but also a variety of xenobiotics including drugs. In the blood, endogenous and exogenous substances are often recruited by blood platelets for the purpose of both storage and release from platelets. This process of uptake and extrude by platelet is regulated by the presence of specific transporters in the plasma membrane of platelets.[3]

Drug Transport in Tissues from Blood

This has been discussed earlier, under Section 2.4.2.1.

Mammalian Cell Membrane in the Transportation of Drugs

The plasma membrane not only acts as protective layer for the several intracellular organelles/components it holds but also acts as a physiological barrier by selectively allowing bidirectional movement of substances in and out of the cell. The basic structure of cell membrane comprises phospholipid bilayer impregnated with proteins (Fig. 2.14). The bilayer has two polar surfaces facing cytoplasm and extracellular matrix with nonpolar (hydrophobic tails) region sandwiched between the polar layers. Thus, the mammalian lipid bilayer cell membrane readily allows hydrophobic molecules to diffuse through the membrane following concentration gradients, and the process is called passive transport. On the contrary, the large polar molecules and ions diffuse through the membrane using energy-driven membrane transporters called active transport. Nonetheless, the efficiency of drugs to penetrate bilayer cell membranes (barriers) in order to reach the site of action depends largely on the physicochemical properties of drugs (pK_a, solubility: logP, logD, molecular weight, etc.).

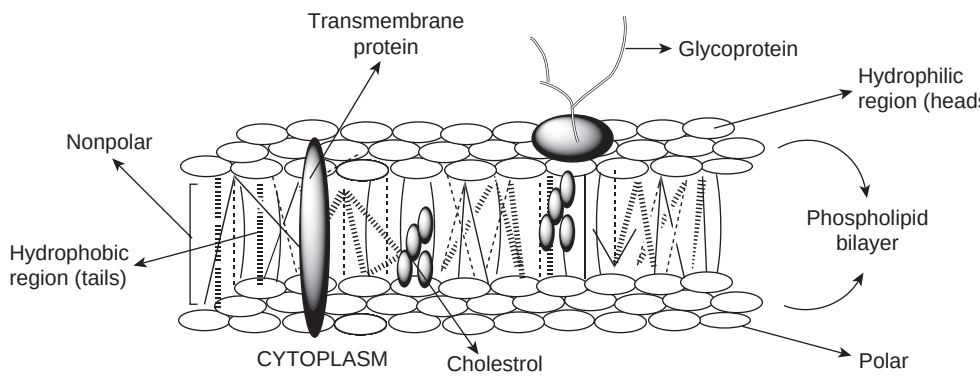


Figure 2.14 Diagrammatic representation of plasma membrane.

Membrane Transport Mechanism

This involves diffusion of drugs from one side such as blood/extracellular matrix to the other side such as cytoplasm via a biological barrier lipid bilayer cell membrane. The diffusion of drug is referred to as biotransport, and the mechanisms involved in the transpiration are called transport mechanisms. The four major transport mechanisms are as follows:

1. Passive transport involves simple diffusion regulated by hydrophobicity of drugs
2. Active transport and facilitated diffusion involve energy-driven carrier (proteins)-mediated transport
3. Ion channels
4. Pinocytosis or phagocytosis
5. Filtration and other methods

Passive transport (Diffusion for unionized/Hydrophobic drugs)

Lipid bilayer membrane owing to their polar and nonpolar regions in the membrane is selectively permeable to drugs. Lipid-soluble (hydrophobic), uncharged, and unionized compounds diffuse through the membrane more rapidly than lipid-insoluble (hydrophilic or water-soluble), charged, and ionized compounds. Thus, acidic/basic (pK_a) nature of drugs plays an important role in the diffusion through the lipid layer (Fig. 2.15). A weakly acidic drug in an acidic environment tends to remain in its unionized form which can readily diffuse through membrane. However, if the pH of the environment increases, the same drug becomes ionized (water-soluble) and becomes more basic (least permeable).

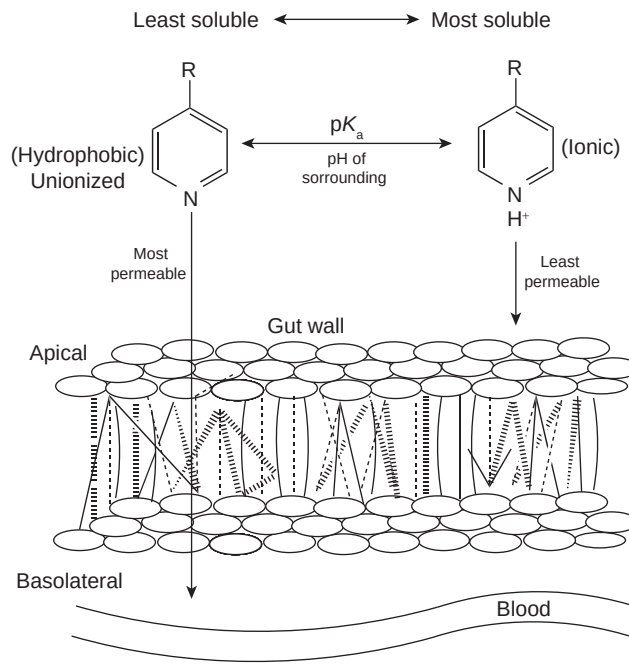


Figure 2.15 Diagrammatic representation of passive transport.

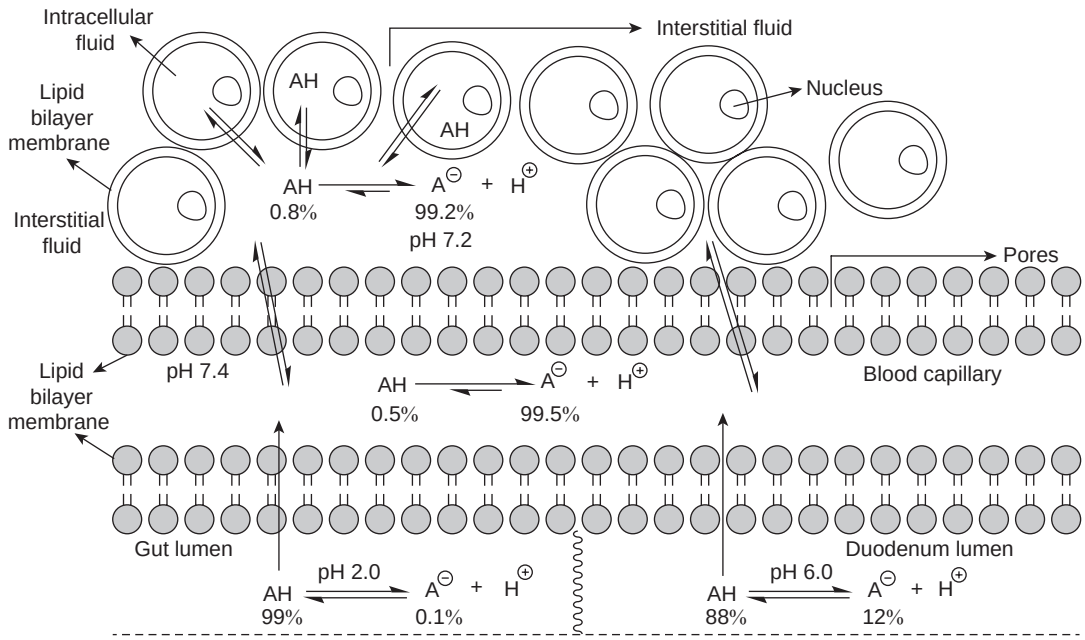


Figure 2.16 Absorption and distribution of a drug with pK_a 4.6 (ions can move out through pores in the walls of blood capillaries).

In general drugs do not undergo complete dissociation in aqueous medium. In fact, only a fraction of drug molecules ionize at a given physiological pH unless pH is equal to or close to pK_a. For example, a weakly acidic drug with pK_a of 4.6 remains predominantly unionized in the stomach and therefore gets readily absorbed through lipid bilayer membrane because of its lipid solubility.

However, the same drug becomes weakly ionized in the basic pH medium in the duodenumthus, reducing its absorption (Fig. 2.16). The fraction of unionized drug following absorption via GIT enters bloodstream. Next, the unionized fraction diffuses through lipid bilayer membranes of endothelial cells lining the capillaries into interstitial fluids. From this fluid, the drugs access receptors present on the cell surface of tissues or extracellular enzymes. Alternatively, unionized fraction of drugs from the fluid can yet again diffuse through lipid bilayer plasma membranes of tissues to access intracellular receptors or signaling enzymes. Thus, following administration of drugs by oral route, the absorption and distribution of drugs involve movement through atleast three lipid media (membranes lining GIT, blood capillaries, and tissues) and four aqueous environments (GIT lumen, plasma, interstitial fluid, and cytoplasm). It is pertinent to add that depending on the physiological pH of the environment, the ionization equilibrium of drugs shifts either in the forward or backward direction (Fig. 2.17). This justifies the significance of pK_a of test compounds as a major player during drug design.

pH Partition theory

The theory deals with transportation of drug across the biomembrane by passive diffusion. The extent of drug transfer (passive diffusion) or drug absorption is generally influenced by:

1. The dissociation constant (pK_a; ionized vs. unionized) of the drug.
2. The lipid solubility of the unionized drug.
3. The pH at the absorption site (GIT).

Effect of pK_a on passive diffusion

pK_a of the drug/test compounds and pH of the physiological environment play an important role in the passive diffusion of drug through lipid bilayer membrane. The fasting pH in the gut varies from 1–3 in stomach; 5–6.5 in duodenum; 6.5–7.0 in jejunum; 7–7.5 in ileum to 5.5–7.0 in colon. Higher is the concentration of unionized drug at absorption site, higher the permeation (diffusion) of drugs through membrane.

Effect of solubility of drug on passive diffusion

Drugs/test compounds must have optimal balance between pK_a and lipophilicity in order to facilitate its solubility in physiological environments involving aqueous and lipid media. Aqueous environment occurs in the GIT lumen, blood plasma, interstitial space and cytoplasm, whereas lipid medium is present in the lipid bilayer cell membranes in the epithelial cells in GIT, in the endothelial cells lining the blood capillaries, and tissues with site of action. In acidic environment in stomach, a weak base will remain in the ionized form, thereby reducing its absorption through lipid bilayer (Fig. 2.16). However, the same basic drug at higher pH (7.4) becomes unionized in the intestine and is thus readily absorbed through the lipid bilayer membrane. Thus, for both weakly acidic and basic drugs, depending on their pK_a and pH of the physiological environment, only the unionized form gets diffused through lipid bilayer membrane.

Effect of lipophilicity on diffusion

Lipophilicity (hydrophobicity) of molecules plays an important role in the diffusion of test compounds through the lipid bilayer cell membrane. Experimentally, lipophilicity is determined either by partition coefficient between octanol: water (logP) or by PAMPA assays. Data obtained by both experiments correlate well with diffusion of test compounds *in vivo*. Molecules with moderate hydrophobicity tend to diffuse into the cell while molecules with excessive hydrophobicity enter the lipid bilayer membrane but cannot exit the membrane and enter into cytoplasm. Weakly hydrophobic compounds fail to enter the lipid bilayer (Fig. 2.17). Paracetamol with logP value of ~0.5 and pK_a value of 9.5 is absorbed through gastrointestinal region through passive diffusion.

In yet another interesting example, cyclosporine A, prescribed as an organ transplant drug (immunosuppressant) despite its large size and being peptide (a hydrophobic cyclic undecapeptide; logP = 2.92) has been proposed to diffuse passively through lipid bilayer cell membrane.[4]

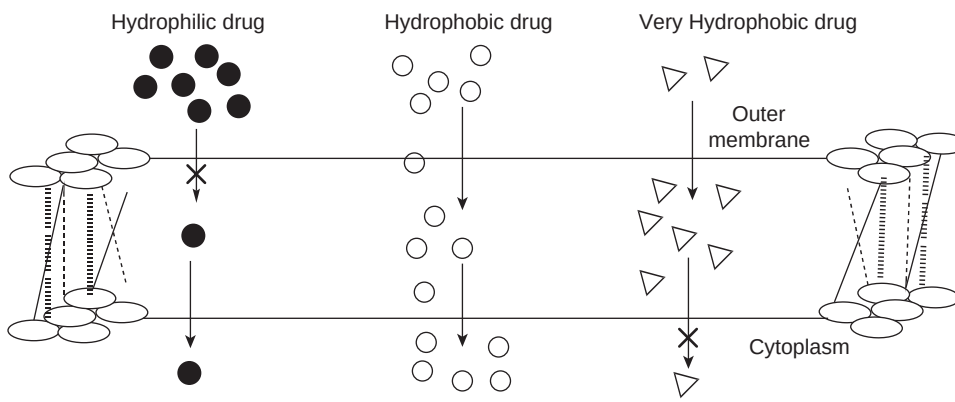


Figure 2.17 Drug transport across cell membrane: very hydrophobic drugs fail to cross the internal membrane, whereas hydrophilic ones cannot diffuse at all.

Carrier-mediated transport (for polar drugs)

Carrier molecules, usually proteins, combine with polar molecules (as they cannot penetrate by themselves): glucose, amino acids, and other polar drugs; they thus form a complex and cross the membrane. The drug is released and carrier molecules returns to the original side for reuse. The carrier proteins are located in cells of the liver, pancreas, GIT, kidney, BBB, and other organs. Generally, low-molecular-weight hydrophilic drugs such as mannitol move unidirectionally into the interstitial fluid.

1. **Facilitated Diffusion:** This involves energy (ATP)-driven and carrier protein-assisted diffusion of drugs from higher to lower concentration.
2. **Active Transport:** This involves energy-driven transmembrane protein-mediated (carrier proteins) transport of drug molecules from lower to higher electrochemical gradient. There are 49 transporter proteins distributed widely throughout the human body. These transporters can be categorized as influx or efflux transporters, which are based on the direction of the movement of the substance.
3. **ATP-Binding Cassette Transporters:** Molecules that fail to diffuse through bilayer membrane due to inappropriate hydrophobicity are transported by ABC transporters. The primary active transporters are involved in the export of drugs across membrane either from cytoplasm to the surrounding of the cell or from cytosolic organelle into the cytoplasm. Ions, amino acids, sugars, and polypeptides including hydrophilic molecules are exported across membrane using ABC transporters. One of the major causes for multidrug resistance in cancer chemotherapy has been attributed to the overexpression of ABC transporters on membrane. As a result, the chemotherapeutic agents are pumped (exported) out of the cell at a much faster rate than their diffusion inside the cell.

CASE STUDY 2

Facilitated Diffusion for Glucose

Uniporter (facilitated diffusion) transporter membrane protein mediates transport of a single solute across the membranes. GLUT 2(glucose transporter) is membrane protein (a glucose carrier) that carries glucose from bloodstream into cells and vice versa. Glucose uptake and release by mammalian cells from blood plays an important role in diabetes (Fig. 2.18).

GLUT 2, known as solute carrier family 2, are widely distributed in liver cells, pancreatic beta cells, small intestine (enterocytes), and kidney. It is a principal transporter involved in the rapid uptake and release of glucose between blood in liver cells. In kidney, it plays a role in the renal glucose reabsorption.

Similarly, GLUT 4 found in skeletal muscles, adipose tissues, and heart is involved in insulin-stimulated glucose uptake. Following translocation of GLUT4 via Akt/PKC cascade, the transporter allows diffusion of glucose (high to lower concentration) from interstitial fluid into the cell (Fig. 2.19). Once the concentration of glucose is reduced in the circulation, the GLUT 4 moves back into the cytoplasm. Yet another family of symporters, SGLT 1 (sodium–glucose cotransporters) found in small intestine and kidney is also involved in active uptake and reabsorption of glucose (Fig. 2.20).

Glucose transporters (GLUTs)—uniporter transport membrane proteins – are located in different parts of body cells like beta cells, hepatocytes, skeletal muscles, etc., and facilitate transport of glucose across cell membranes.

Sodium-glucose co-transporters (SGLT 1 and 2)—symporter transport membrane proteins – are located in small intestine and renal tubules of nephrons, facilitating the reabsorption of glucose back into circulation.

(*Continued*)

Study 2 (*Continued*)

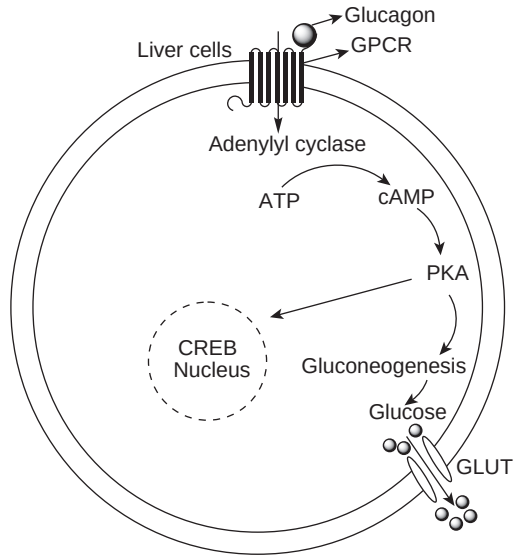


Figure 2.18 GLUT 2 in liver cells is the principal transporter for transfer of glucose between liver cells and blood (CREB: c-AMP response element binding protein).

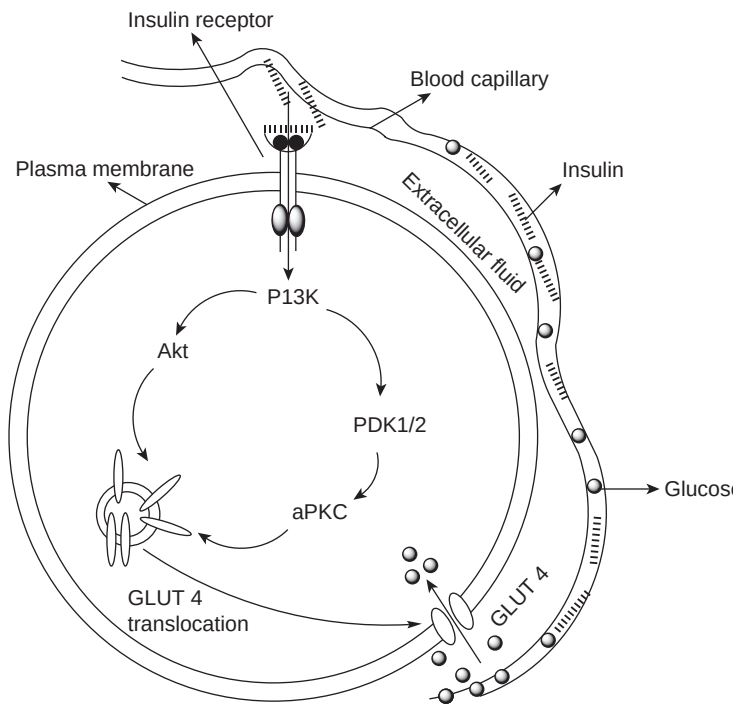


Figure 2.19 GLUT 4-mediated glucose uptake from blood by mammalian cells for energy.

(*Continued*)

Study 2 (*Continued*)

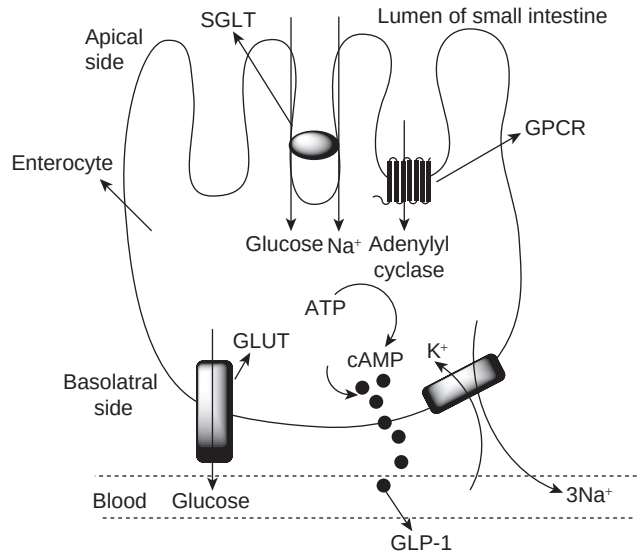


Figure 2.20 GLUT and SGLT 1-mediated glucose uptake and release in the small intestine.

In general, the transmembrane ABC transporters are made up of two distinct domains. The domain with 10–12 α-helices embedded in the membrane bilayer is referred to as TMD (transmembrane domain), whereas the cytosolic domain is referred to as nucleotide-binding domain (NBD). A typical ABC transporter protein has two TMDs and two cytoplasmic NBDs. The latter remains the site for ATP binding for energy-driven transport (Fig. 2.21).

Drugs while diffusing out from the cells through the plasma membrane in the tissues are recruited by the transporter protein from the lipid bilayer itself. Here, drugs bind to the substrate-binding site in the TMD region of the transporter (Step 1 in Fig. 2.22). Subsequently, ATP binds to the NBD region of the transporter and itself gets hydrolyzed into ADP and phosphate and, in turn, releases a large amount of energy (Step 2 in Fig. 2.22). This energy so produced is utilized in inducing fast conformational change in the protein by which it can efflux (pump) the drug out of cell (Step 3 in Fig. 2.22).

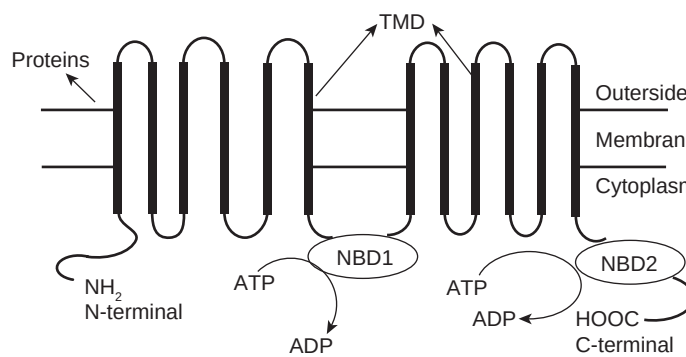


Figure 2.21 Diagrammatic representation of a general structure of ABC proteins.

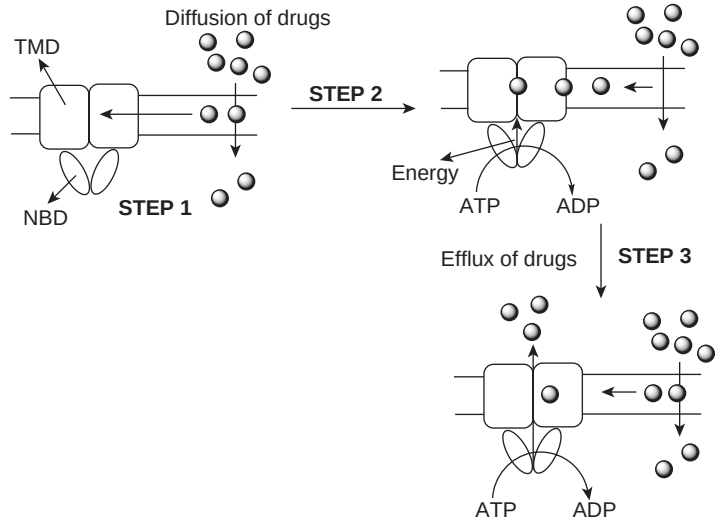


Figure 2.22 ABC protein-mediated efflux of drugs from cells using ATP as a source for energy (to move against concentration gradient).

CASE STUDY 3

Transmembrane P-glycoprotein Efflux Pumps in Intestine

They are also referred to as B-sub-family of ABC transporter proteins ABCB1 or Multidrug resistance protein 1 (MDR1). The efflux pumps are although widely distributed and expressed throughout the body, it is present in high concentration in the colon and intestinal lining and acts as major physiological barrier in the absorption of the drugs via oral route. In the intestine, Pg efflux protein pumps the drug back into intestinal lumen from the enterocyte cells and in turn, reduces the concentrations of drugs from entering the bloodstream. Thus, the basic role of P-gp is to protect tissues/organs from toxic compounds/xenobiotics by either preventing them to enter the cells or expelling them from the cytoplasm to the exterior.

A variety of therapeutic agents are substrates to P-glycoproteins, thus making the drugs redundant from eliciting biological response as they are extruded out from the enterocytes (the site for absorption of drug in the intestine).

A variety of structurally diverse drugs/test compounds can be recognized by the P-gp with molecular weight ranging from 250 g/mol (cimetidine) to 1202 g/mol (cyclosporine). This contributes to the efflux of drugs from enterocytes (intestinal absorptive cells) into lumen. Thus, efflux property of P-gp can ultimately lead to failure of the drug for therapeutic treatment, and therefore inhibition of P-gp could be an attractive target for drug discovery programme.

4. **Solute Carrier:** They are yet another class of transporter protein that facilitate bidirectional movement of endogenous substances (ions, amino acids, vitamins, etc.) to maintain cellular functions and also recognize exogenous drugs. The structure of Solute carrier (SLC) embedded in the membrane comprises 10–12 membrane-spanning segments (Fig. 2.23) and has been a therapeutic protein target for drug development.

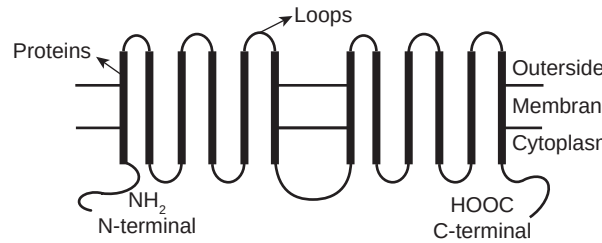


Figure 2.23 Diagrammatic representation of a general structure of SLC proteins.

CASE STUDY 4

SLC as Therapeutic Target (Selective Serotonin Transporter Inhibitor)

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter (a happy chemical in addition to dopamine, oxytocin, and endorphins) predominantly produced in brain which plays a role in regulating good mood, including emotions and affections. A diminished activity of serotonin pathways in the brain can lead to depression. Among many strategies used for treating depression, one strategy involves maintenance of adequate concentration of serotonin in the synaptic cleft by preventing its reuptake back into the presynaptic terminals by the transporter protein SCL6A4. Search for an inhibitor for the transporter protein target SCL6A4 led to the identification of a drug prozac as a selective serotonin reuptake inhibitor (SSRI)[5], which resulted in the inhibition of serotonin reuptake back into the presynapse from the synaptic cleft (Fig. 2.24).

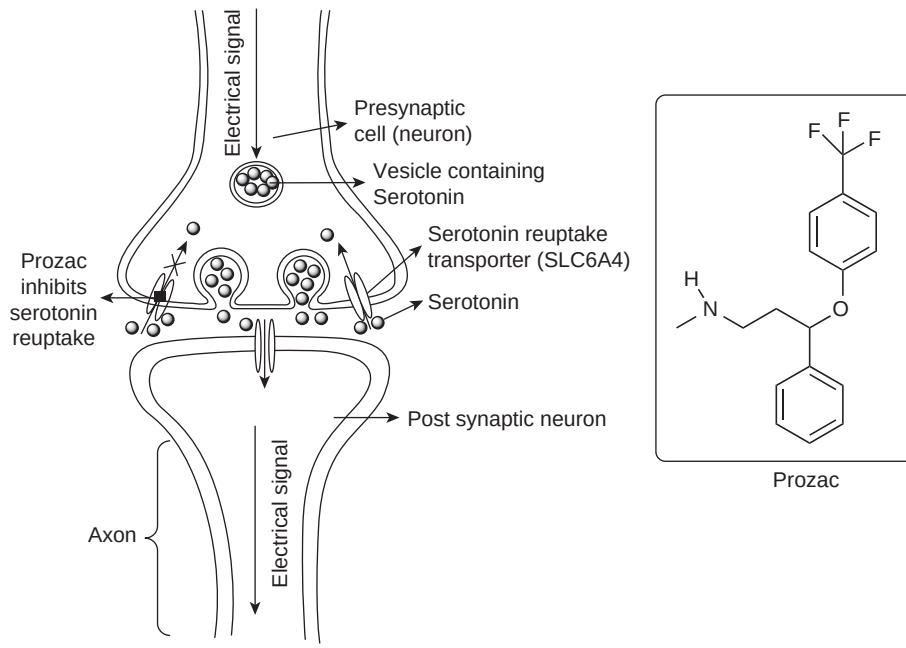


Figure 2.24 SLC as therapeutic target (selective serotonin transporter inhibitor SSRI).

5. **ABC and SLC Transporters in Platelets:** All drugs/signaling molecules (neurotransmitters) reach their sites of action in cells via the systemic blood circulation. In the blood, they are often recruited by blood platelets both for the purpose of storage and release of drugs from platelets. This process of uptake and extrude by platelet is regulated by the presence of specific transporters in its plasma membrane (Fig. 2.25). The specific transporters uptake proteins facilitating deposition of these molecules (drugs/signaling molecules) in platelets and, on their activation, the export-specific transporter proteins assist in their translocation across the plasma membrane.
6. **Role of Transporter Proteins in the Elimination of Water-Soluble Metabolite/Drug in Kidney:** Transporter proteins present in the tubular section in kidney assist in the efficient transfer of water-soluble metabolites/drugs from the blood into urine. The walls of blood capillaries allow soluble drugs/metabolites to permeate out which are then picked up by membrane-bound transport protein and translocated into tubules (Fig. 2.26).

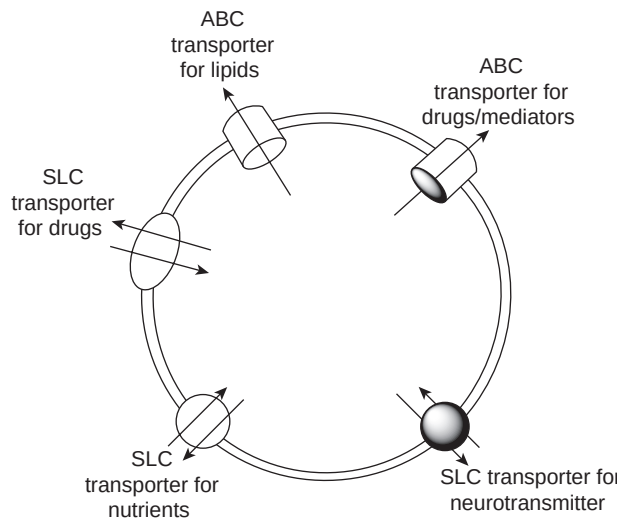


Figure 2.25 A variety of substrate(s)-specific ABC and SLC transporters in platelets.

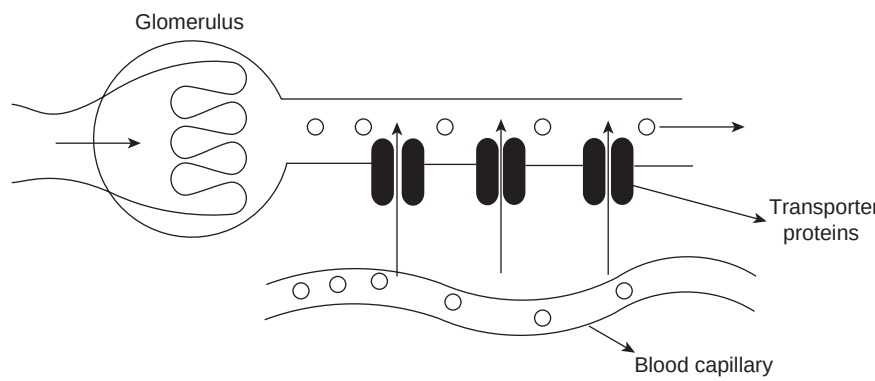


Figure 2.26 Role of transporter protein in the elimination of water-soluble drugs/metabolites in the kidney via urine.

In summary, diffusion of drugs from bloodstream into tissues via cell membrane can be grouped under passive and active transport. A brief comparison of passive and active diffusion has been summarized in Table 2.9.

7. **Ion Transport:** Ion channels are pore-forming glycoproteins that traverse the membranes of both cells and intracellular organelles (in the cytoplasm). They allow the flow of ions across cell membranes and are predominantly located in neurons. Notably, the membranes are as such impermeable to ions and it is the electric charges carried by the ions that generate membrane (action) potential (firing of neurons) which, in turn, triggers flow of ions. Although many ion channels (such as most Na^+, K^+, Ca^{++}, and some Cl^- channels) are voltage-gated (closure/opening), some ion channels are gated by second messengers and other intracellular and/or extracellular cues. (Details about the ion channels and its role in permeation has been discussed in Chapter 7, under Section 7.3.3).
8. **Pinocytosis and Phagocytosis:** Both phenomena of transport refer to ingestion of substances from the cell surroundings through the plasma membrane and together are called as endocytosis. The process involves progressive uptake of materials by a small portion of plasma membrane. The latter encircles the substance being transported and ultimately forms membrane-bound vesicles/droplets from the cytoplasmic side (Fig. 2.27). Both are energy-driven active processes and require ATP for the transport/uptake of materials (Table 2.10).

Table 2.9 A comparison between passive and active transport

Passive Transport	Active Transport
Passive diffusion involves solubilization of substances through lipid bilayer membrane. No energy is required for this process.	Active transport occurs at the expense of energy (ATP).
It is bidirectional slow transport.	It is a unidirectional rapid transport.
Molecules diffuse down the concentration gradient across the membrane: from high concentration to low concentration.	Energy (ATP)-driven movement of molecules against concentration gradient, for example Na^+/K^+ pump during firing of neurons, heart contraction, and function of kidney.
Passive transport occurs in liver, kidney, and lungs. The passive transportation in the body is observed in the exchange of oxygen and carbon dioxide.	Active transport in kidney assists in the reabsorption of essential substances from waste back into bloodstream in the nephrons.
Transportation of all the soluble molecules: sex hormones, lipids, gases, and water.	Large protein and complex carbohydrates, etc., can be transported.
Diffusion assists in the maintenance of the equilibrium.	Various substances are transported in the cells.

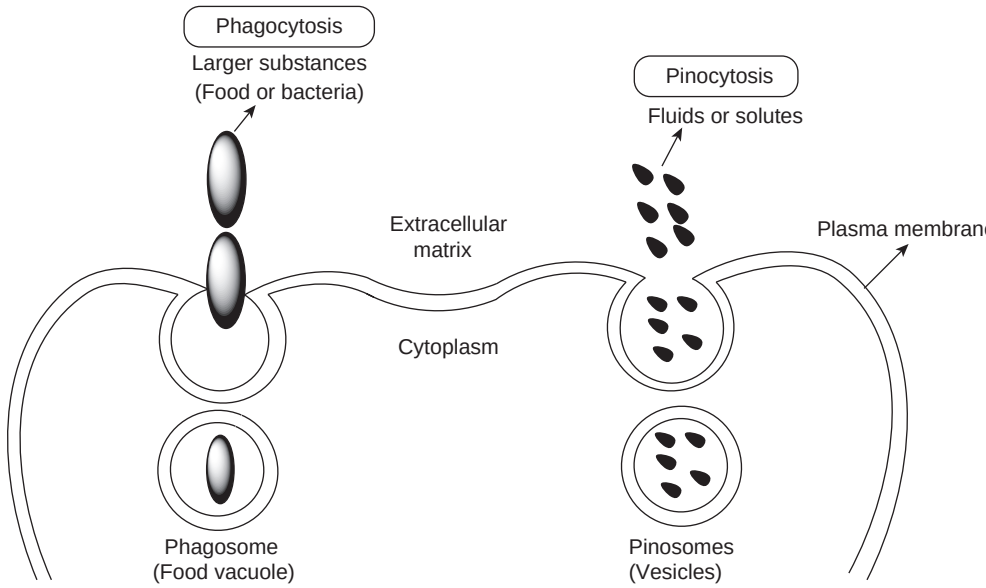


Figure 2.27 Diagrammatic representation of phagocytosis and pinocytosis.

Table 2.10 A comparison of phagocytosis and pinocytosis

Phagocytosis	Pinocytosis
Means cellular eating.	Means cellular dinking.
Is specific for the substances it transports.	Is non-specific for the substances that it transports.
The invaginated pinocytosis vesicles are much smaller than those generated by phagocytosis.	The invaginated phagocytosis vesicles are larger than those generated by pinocytosis.
Substance encircled membrane-bound vesicles formed after ingestion is called phagosomes.	Fluid-filled membrane-bound vesicles formed after ingestion are called pinosomes.
Large particles, such as cells, are taken in by a cell.	Fluids and nutrients are ingested by cells.

Phenomenon of endocytosis or phagocytosis occurs in GIT where M cells present in the Gut-associated lymphoid tissue (GALT) engulf molecules from the gut lumen. Engineered nanomaterials with ability to detect and treat complex diseases are known to enter human cells, often via active endocytosis.[6]

Antigen-presenting cells such as B cells/macrophages take up antigen either through phagocytosis and present them to lymphocytes such as T cells. The extracellular defunct/obsolete proteins are engulfed through endocytosis in the form of small bubble endosomes in the cytoplasm which then comes in contact with lysosomes and undergoes protein digestion (endolysosomal degradation). Endocytosis has also been reported in the internalization of ligand-bound cell-surface receptor within cytoplasm followed by endolysosomal (proteolyitc) degradation of endogenous ligands (receptors are sent back in its native form to cell surface).

Besides, endocytosis and exocytosis also occur in the cells and their important role is to assist in the release of enzymes (extracellular) from cytosolic secretory vesicles produced within cells.

2.5.2.5 Physiological Factors Affecting Metabolism (M)

The main objective of biotransformation of drugs/xenobiotics is not only to produce water-soluble metabolites for the purpose of excretion but also to terminate the action of signaling molecules (ligands/substrates) after they have completed their therapeutic roles.

Metabolism of xenobiotics is a cytochrome P450-driven enzymatic breakdown (biotransformation) into an active or inactive water-soluble metabolite(s). The phenomenon may affect the pharmacological activity of a drug by converting:

1. An active drug into an inactive drug metabolite.
2. An active drug into an active metabolite.
3. Biologically inactive prodrug into an active drug.

Although majority of tissues in the body participate in the metabolism of xenobiotics, liver and gut are principal sites for metabolism. Other organs involved in the metabolism occur in the lungs, kidney, and skin. In the proximal regions of the small intestine, metabolizing enzymes cytochrome P450 (CYPs) are present and are responsible for drug metabolism reactions. However, in the body, liver remains the major organ that carries a large number of metabolic enzymes CYPs and thus remains the principal site for degradation. In nutshell, kidney and the liver work together to remove toxic waste from the body.

The vast majority of small organic molecules are recognized by large number of CYPs available in our body, thereby facilitating metabolic transformation (Phase I and II) through chemical reactions. (Details about metabolism and its role in pharmacokinetics and pharmacodynamics have been discussed in Chapters 11 and 12). There are two major factors that affect the overall metabolism of any drug which indeed depends on routes of their administration.

Factor 1

The first-pass effect or metabolism: discussed under Section 2.5.2.2.

CASE STUDY 5

First-Pass Effect or Metabolism

Cyclosporine (CsA), a hydrophobic undecapeptide (logP 2.92) is used clinically in organ transplant patients to prevent rejection of the transplanted organ. The oral administration of the drug is followed by the metabolism (approximately 50%) in the intestine by cytochrome P450 (CYP3A isozymes, predominantly CYP3A4 and CYP3A5). The fraction of the unmetabolized drug is then absorbed by the

(*Continued*)



Additional Information

Xenobiotics

They are unsolicited chemical substances (not antigens) that exogenously enter the organism and are neither required nor are produced naturally by the organisms. They may include environmental pollutants, drugs, carcinogens (pesticides) and unhealthy chemicals through ingestion of food. They are subjected to metabolic process in the liver and converted into hydrophilic metabolites for elimination (it is different from antigens which are also foreign substances but give immunogenic response).

Study 5 (*Continued*)

GIT and reaches liver via hepatic portal vein where cyclosporine is yet again subjected to metabolism by cytochrome P450 (CYP3A4 and CYP3A5). Subsequently, after the first-pass metabolism of CsA, the remaining parent drug enters systemic circulation where it binds extensively to erythrocytes. The remaining 30–40% (bioavailability) unbound drug then enters lymphocytes (by passive diffusion) and exerts its main immunosuppressive effects.

Examples of clinically used drugs with low bioavailability due to high first-pass metabolism:

1. Morphine bioavailability 25% (oral)
2. Nifedipine bioavailability 45–56% (oral)
3. Imipramine bioavailability 43.6% (oral)
4. Propanolol bioavailability 20–40% (oral)
5. Neostigmine bioavailability 2% (oral)
6. Pentazocine bioavailability 18–25% (oral)
7. Nitroglycerine bioavailability <1% (oral)

CASE STUDY 6

Reducing the Hepatic First Pass (Metabolism) to Enhance Bioavailability

Paracetamol (also called acetaminophen) is a widely used analgesic (non-opioid agent) and antipyretic agent. In 2012, FDA laid down guidelines on the use of paracetamol and recommended a maximum daily dose of 3 g and a maximum single dose of 650 mg for adults. FDA also advised practitioners world-wide not to exceed single dose of administration to more than 325 mg. Paracetamol is well absorbed in the GIT and distributed throughout the body. Oral bioavailability (F = 70–90%) is dose dependent, with larger doses reducing the hepatic first pass (metabolism) effect due to limit of the enzyme (CYP) capacity in liver and therefore resulting in the increase in bioavailability. The analgesic activity is attributable to the small fraction that readily penetrates into the brain (CNS: primary site of action for paracetamol) through BBB. However, supratherapeutic doses of acetaminophen (more than 4 g/day) are also known to impair metabolism and cause liver injury (toxicity).

Factor 2: Metabolism Affected by Induction/Inhibition of CYP

There are two kinds of interactions reported between drugs and metabolic enzymes CYPs. Some specific drugs inhibit the isozyme CYP resulting in the increase in the concentration of drugs. This in turn may lead to toxicity due to the prolonged exposure of the organism to drugs. On the contrary, there are specific drugs that induce the production of isozyme and as a result speed up the metabolism and rapid clearance, thus impairing the biological response. This inhibition/induction of CYPs leads to major drug–drug interaction during combination/polytherapy.

Let us assume that two drugs A and B are administered as part of combination therapy to tackle a disease. If drug A, besides undergoing CYP-mediated metabolism, also inhibits the activity of CYP, this is bound to decrease the metabolism of drug B. This, in turn, will enhance the exposure of the body to the increased concentration of drug B and may result in toxicity. In a vice versa situation involving induction of CYP by drug A, the concentration of drug B will be reduced in the body, thereby decreasing the efficacy of drug B.

2.5.2.6 Physiological Factors Affecting Drug Elimination [E]

Xenobiotics including drugs are primarily eliminated from the body either through urine or stool. Generally, body carries out this phenomenon by degrading these compounds via enzyme-catalyzed metabolism. Predominantly, the drugs are metabolized into water-soluble metabolites for their excretion through urine. Some drugs due to characteristic physicochemical properties may or may not undergo metabolism and following their repeated enterohepatic circulation through biliary excretion enter intestine and may get excreted through stool either in their native form (unchanged) or as insoluble metabolite(s). Excretion of bile by-products through stool may also occur through GIT route.

Among routes available for the excretion of hydrophilic metabolites (saliva, sweat, milk, etc.), kidney remains the principal organ for elimination of water-soluble degraded products or unchanged form through the urine. The renal excretion involves three major routes: glomerular filtration, tubular secretion, and passive reabsorption. The glomerular capillary wall in kidney carries large pores (70–100 nM in diameter) through which excretion of hydrophilic metabolites or even unchanged drugs in some cases occurs. (Details about elimination, its role in pharmacokinetics has been discussed in Chapter 11).

Factors affecting elimination drugs primarily depend on the lipophilicity (logP and D) and dissociation constants for any weakly acidic or basic groups represented by pK_a of each group present in drugs. Metformin, an antidiabetic drug with logP value of 1.41 and pK_a values of 2.8 and 11.5 is predominantly excreted through the urine unchanged. This is because following its administration in the body, metformin while moving through different physiological pH always remains in its ionic form. Thus, the ionic form fails to undergo CYP-mediated metabolism and is excreted through urine (tubular secretion in kidney) in its native form (negligible metabolism). Similarly, captopril, an antihypertensive drug with pK_a values of 3.7 and 9.8 and logP of 0.546, is largely (over 95 percent) excreted through urine, out of which 40 – 50% is in its native form.

Biliary Excretion and Enterohepatic Cycle

After administration of drugs via oral route, it undergoes first-pass metabolism initially in the gut followed by absorption of free native drug by the GIT, from where it enters liver through hepatic portal vein. In the liver, drug again undergoes Phase I and II metabolism and from there the free native drug (or active metabolite in some cases) may enter systemic circulation or excreted into bile along with polar conjugate metabolites. After storage in the gall bladder, bile containing drug/conjugates is secreted back into gut. This phenomenon involving active secretion of drug molecules and/or their conjugate metabolites from hepatocytes into gut through bile is called biliary excretion (Fig. 2.28 and 2.29).

Following biliary excretion in gut, drugs may yet again undergo reabsorption process (highly hydrophobic drug owing to their poor metabolism may be excreted from gut by stools). In addition to this, in small intestine conjugated metabolites may be hydrolyzed by enzymes and the liberated native drugs may also undergo reabsorption. The reabsorbed drugs from gastrointestinal region re-enter portal veins and are then taken back into the liver. This cycle involving gut–liver–gall bladder–gut–liver is referred to as the enterohepatic cycle and may be repeated several times. This repeated cycling of drugs may not only enhance the duration of action of the drug in the body but may also lead to toxicity. Thus, both phenomena may significantly impact the systemic exposure, pharmacological effect, and toxicity for certain drugs (Fig. 2.28 and 2.29).

In case of tacrolimus, an immunosuppressant drug, more than 95% of metabolites are excreted through stool via biliary and enterohepatic route, while only 2% is excreted through urine. This may be attributed to its high logP value of tacrolimus 3.96 ± 0.83.

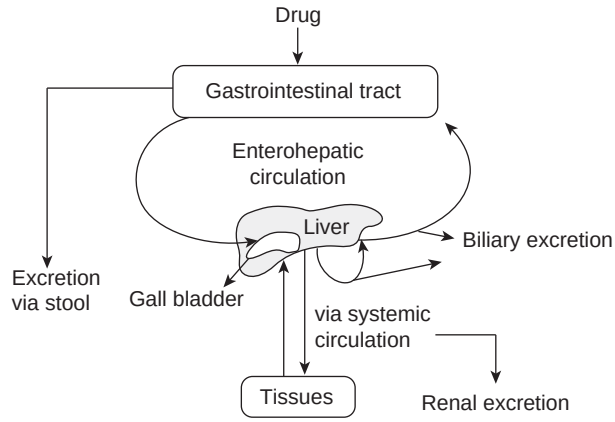


Figure 2.28 Biliary excretion and enterohepatic cycle.

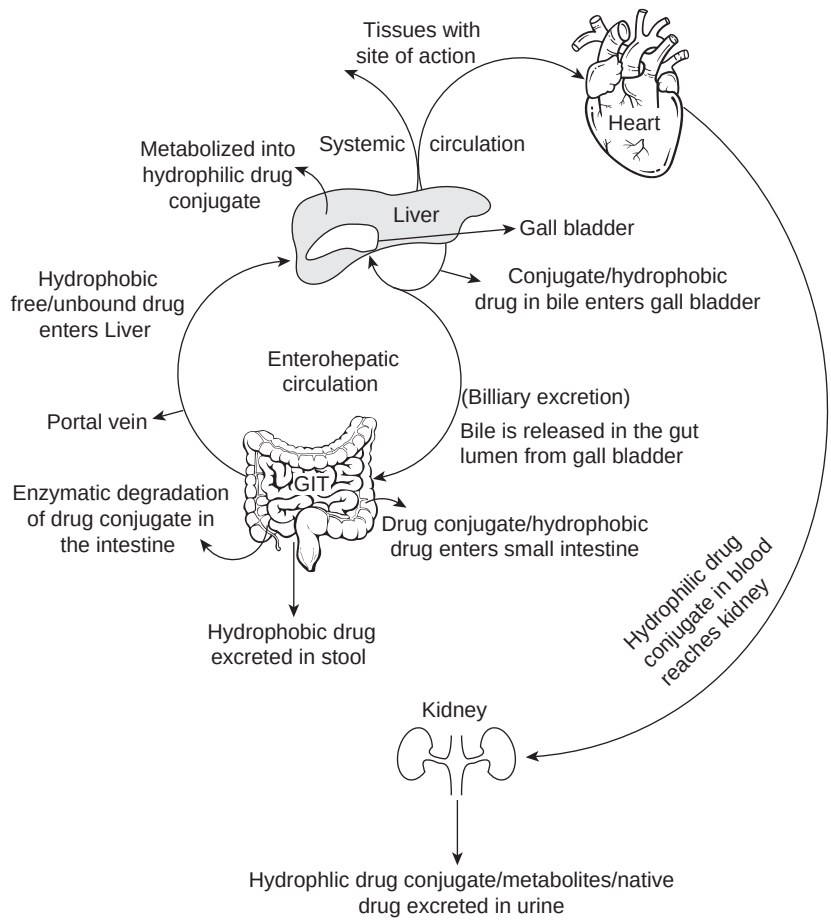


Figure 2.29 Excretion of drugs.

Similarly, metabolites of cyclosporine with logP of 2.92 are also predominantly excreted in the bile, with only around 3% of the drug being excreted through urine.

For Televas (telmisartan, an antihypertensive drug) with logP of 3.2, 97% of unchanged drug is excreted via biliary route/stool with urinary excretion accounting for <1% of the dose.

Losartan, an antihypertensive drug, despite very high logP value of 4 to 6 undergoes metabolism; thus, approximately 40% is excreted in urine while 60% is excreted in stool.[7]

Metformin, an antidiabetic drug owing to its hydrophilic nature is substantially excreted by urine. In fact, 90% of the absorbed drug is excreted within the first 24 hour of consumption of the drug.

Atorvastatin (Lipitor)—extensively metabolized by the liver, mostly during first-pass metabolism and is excreted in bile and stool without enterohepatic recirculation. Only <2% is excreted unchanged by the kidney.

Exercises

1. Describe the factors affecting absorption of drug by oral route.

2. Draw a flow sheet of ADMET following administration of drug in the body by oral route.

3. Describe BBB and write difference between normal and brain's blood capillary.

4. Explain PPB and its effect on drug distribution?

5. Describe effect of pK_a of drugs on bioavailability by oral route.

6. Describe the physiological factors affecting distribution of drugs from blood plasma.

7. What is P-glycoprotein (P-gp) efflux pumps in intestine and describe its role in drug absorption?

8. Discuss difference between followings:

(i) Phagocytosis and pinocytosis

(ii) Passive and active transport

9. Explain biliary excretion and enterohepatic cycle.

10. Write in brief:

(i) Xenobiotics

(ii) SLC transporters

(iii) Effect of lipophilicity on diffusion

11. Name routes of administration of drugs.

Competitive Exam Questions

1. The volume of distribution of a drug administered at a dose of 300 mg and exhibiting 30 microgram/mL instantaneous concentration in plasma shall be:

(a) 10 L **(b)** 100 L

(c) 1.0 L **(d)** 0.10 L

[***GPAT 2010***]

2. P-glycoprotein pump is responsible for which one of the followings?

(a) Transporting the drugs from the enterocytes into the gut lumen.

(b) Transporting the drugs from gut lumen into enterocytes.

(c) Transporting the drugs from oral mucosa into blood capillaries.

(d) Transporting the drugs from Peyer's patches into the gut lumen.

[***GPAT 2011***]

3. Which of the following parameters is/are important in determining bioequivalence?

(a) T_{max} **(b)** C_{max}

(c) AUC and C_{max} [***GPAT 2013***]

4. About 95% of transdermal drugs enter the skin through the which pathway?

(a) Intercellular **(b)** Transcellular

(c) Follicular **(d)** Exocrine [***GPAT 2015***]

5. If a drug is highly bound to plasma proteins, what might be its reason or consequence?

(a) It is most likely carried by α-glycoprotein.

(b) It has a high renal clearance.

(c) It has a large V_d.

(d) It is a likely candidate for drug interactions. [***GPAT 2016***]

6. Bioavailability of drug refers to:

P. The Ratio of drug excreted unchanged in urine to that excreted as metabolites.

Q. Fraction of the drug reaching the target to produce the action.

R. The length of time an administered drug is available for action.

S. Percentage of administered dose that reaches systemic circulation in the unchanged form.

(a) Only P **(b)** Q and R

(c) R and S **(d)** Only S [***GPAT 2016***]

7. In plasma phenobarbital is present as ionized and ionized forms in equal amounts because:

(a) it is weakly acidic drug.

(b) it is weekly basic drug.

(c) pH of plasma is 6.8.

(d) pK_a of phenobarbital is 7.4. [***GPAT 2018***]

8. Very weak bases having pK_a< 5:

(a) are ionized in the entire pH range of GIT.

(b) absorbed only in stomach.

(c) are unionized at pH values.

(d) none of these. [***GPAT 2018***]

References

1. Mark R. Prausnitz and Robert Langer, *Nat Biotechnol.*, **2008**, *26*, 1261.
2. Enam Khalil, S. Najjar and A. Sallam, *Drug Development and Industrial Pharmacy*, **2000**, *26*, 375.
3. Gabriele Jedlitschky, Andreas Greinacher, and Heyo K. Kroemer, *Blood*, **2012**, *119*, 3394.
4. Nicole J. Yang, Marlon J. Hinner, Nicole J. Yang, Marlon J. Hinner, *Methods Mol. Biol.*, **2015**, *1266*, 29.
5. Katrin Sangkuhl, Teri Klein and Russ Altman, *Pharmacogenet Genomics*, **2009**, *19*, 907.
6. Nuri Oh and Ji-Ho Park, *Int J Nanomedicine*, **2014**, *9*, 51.
7. Elizabeth Ripley and Ari Hirsch, *Int. J. Nephrol. Renovasc. Dis.*, **2010**, *3*. 93–98.

CHAPTER

3

The Modern Drug Development Process

KEY THINGS TO LEARN FROM THIS CHAPTER

Five major drug development process:

- Step 1: Drug Discovery and development: Identification and validation of targets, screening, search for new hit/lead molecule(s)
- Step 2: Identification of preclinical candidate(s) via lead optimization/drug design/ preclinical research programs
- Step 3: Clinical trials (Pages I–IV) and filing of IND
- Step 4: Filing of NDA for FDA approval
- Step 5: Post market monitoring of drugs for safety etc.

Initial steps in drug discovery process (Medicinal chemistry):

- Indentifying targets, druggability, target validation and assay development
- Generation of library of chemical compounds
- Screening of chemical library
  - *In vitro* screening of chemical entities
  - Parameters for measuring pharmacological activity
  - *In vitro* tests and advanced *in vitro* assays
  - *In vivo* assays and screening in model organisms

3.1 Introduction

The modern drug discovery involves design of a safe, effective, and commercially viable novel therapeutic agent (drug) with established mechanism of action. It is a long, time-consuming, and painstaking process involving the employment of a variety of state-of-the-art technologies and expertise in multidisciplinary research areas. For example, a group of medicinal chemists, biochemists, pharmacologists, formulation scientists, clinical pharmacologists and toxicologists, clinicians, bioinformaticians, and others have to pool their resources to carry out a drug discovery program.

In general, the drug discovery process against any disease remains a two-tier approach. On one hand it involves discovery of novel class of therapeutic entities with a new and validated mechanism of action.

On the other hand, it involves improvisation in the existing therapies available for the disease. In yesteryears, the discovery of new drugs was based on phenotypic observations of a biological effect produced by a natural product, usually obtained from a plant or microorganism. However, over the years, there has been a paradigm shift in drug discovery from screening of natural products to low-molecular-weight synthetic chemical compounds and currently it is screening of natural products, natural product like structures and synthetic molecules with drug-likeness under the umbrella of molecular structural biology. The goal is to find a clinical candidate with high efficacy, potency, selectivity, good pharmacokinetic (ADMET) profile.

Biological and technological advances for studying the mechanism of action of candidate drugs and in-depth understanding of disease development and progression has made it possible to understand the etiology of diseases at molecular level. Besides, the role of cell-signalling system in the pathophysiology of diseases has led to the identification of numerous macromolecular therapeutic targets (receptors/enzymes). The major components in cell signalling are neurotransmitter, hormones, receptors, effectors, and signal transduction enzymes involved in cellular/physiological functions. Each step in a signalling pathway provides an opportunity to therapeutically modulate the cellular function and cure a disease.

3.2 The Modern Drug Development Process Comprises Five Steps[1]

Step 1: Drug discovery and development: Identification and validation of targets, screening, search for new hit/lead molecule(s)
Step 2: Identification of preclinical candidate(s) via lead optimization/drug design/preclinical research programs
Step 3: Clinical trials (pages I–IV) (drugs are evaluated in healthy and diseased humans for establishing toxicity, safety, and efficacy, filing of IND)
Step 4: Filing NDA for FDA approval.
Step 5: Post-market monitoring of drugs for safety, etc.

These have been discussed below.

3.2.1 Drug Discovery and Development

The initial or early stages of drug discovery[2] against any disease involve build-up of a hypothesis by analysing the role of cell-signalling pathways in the progression of disease followed by target selection and screening of chemical libraries.

3.2.1.1 Understanding Etoilogy of the Disease

Before we commence with drug discovery program against any disease, it is important to analyse the etoilogy of disease. A large number of diseases have been attributed to impairment in signalling pathways. Both communicable and non-communicable diseases arise from subtle phenotypic changes in the natural signalling pathways.[3] Therefore, in the first instance one needs to analyse the biochemical/metabolic/signal pathways under normal conditions in order to study the factors leading to the disease. This exercise will assist in the determination of the cause(s) of the disease and understanding the mechanism that leads to the pathogenesis at the molecular level.

3.2.1.2 Analysing Existing Treatments Available

After studying the etoilogy of the disease, it is then mandatory to look into existing drugs if already available and also examine their mechanism of action. This can provide insight for blocking other signalling pathways responsible for the aberration in the cellular function.

3.2.1.3 Searching for New Insight into a Disease Process

Researchers continuously strive to discover novel drug candidates that can be used for treating a disease based on a new mechanism (signalling pathways) of action. For example, treatment of blood pressure comprises a plethora of drugs that acts by different mechanism: inhibitors of Angiotensin-converting enzyme (ACE), antagonists of calcium channels, inhibitors of rennin, diuretics, α- and β-blockers, etc.

3.2.1.4 Identifying a Therapeutic Target

A therapeutic protein target can be defined as the one that may be involved in metabolic/biochemical/signal pathways and whose functions when modulated pharmacologically by compounds can provide meaningful relief from specific human disease. In other words, the protein (macromolecular) target should be directly involved in the manifestation of disease process. Thus, understanding the pathogenesis of the disease is the key for the successful identification of a macromolecular therapeutic target (receptor or an enzyme).[4]

3.2.1.5 Druggability

This is one of the most crucial parameters during the target validation phase of drug discovery. While pursuing with the validation the target, it is mandatory that the target is also evaluated for its 'druggability'.[5] This means that small molecules/biologics should be able to bind with the active site (protein pocket) of the validated target and modulate its biological activity. A promising druggable therapeutic biological target should have following important features:

1. The role of macromolecular target is validated in the disease condition.
2. Restricted distribution of identified therapeutic target is preferred instead distribution pattern throughout the body.
3. Availability of the three-dimensional structure (crystal structure) of the identified therapeutic macromolecular target in protein data bank (PDB).
4. Development of an assay method to measure the biological activity of the macromolecular target while screening against chemical libraries.

3.2.1.6 Target Validation

The process[6] involves establishing and demonstrating the direct role of a protein target in the manifestation of the disease followed by its pharmacological modulation so that a therapeutic relief can be obtained from the pathophysiological (disease) condition. The first step after a target has been identified is to validate the same in animal models under disease conditions analogous to the human disease. Validation is simply defined as having gathered adequate scientific evidence for the direct association of the target with the disease and its druggability. This is achievable through numerous approaches such as monoclonal antibody approach, RNAi techniques, mutated human cell lines and transgenic animal models.

RNA Interference Technique for Target Validation

Messenger RNA, encoded with specific sequence of amino acids (mRNA; belongs to the family of RNA molecules) plays a crucial role in conveying genetic information from DNA to the ribosome for protein synthesis (gene expression). Thus, one of the methods used by cells as a natural phenomenon to regulate gene expression involves degradation of mRNA. This biological process of turning off a gene by way of mRNA degradation is known as gene silencing via RNA interference (RNAi). In cells, degradation of mRNA is carried out by double-stranded RNA (dsRNA), also defined as a signal in the form of gene-specific silencer.[7] Researchers used RNA interference (RNAi or siRNA) in drug discovery as an approach for the validation of therapeutic protein targets (Fig. 3.1).

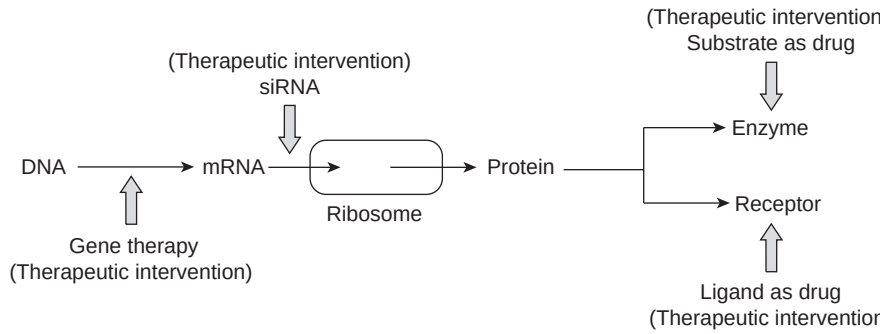


Figure 3.1 The three levels of therapeutic intervention for treating diseases.

In this approach, a gene knockdown animal model[8] is created by administering small double-stranded interfering RNAs (20 – 25 base pairs in length) known as short-interfering RNA or silencing RNA (siRNA) into the cytoplasm. This artificially induces the RNAi response by degrading the corresponding mRNA and in turn decreases the protein levels for the targeted gene. Thus, by silencing gene for the desired protein drug target, a transgenic animal model can be created that can mimic the human diseases. The target validation experiments give direct insight into the relationship of gene function with pathophysiological conditions.

CASE STUDY 1

Target Validation Using Transgenic Mice

Protein-tyrosine phosphatase 1B (PTP1B) is an enzyme that dephosphorylates tyrosine residues in insulin-receptor-linked proteins and has been implicated in inhibiting insulin signalling (leading to type II diabetes). In order to validate its role as therapeutic target in the prevention and treatment of type II diabetes, transgenic mice lacking PTP1B (therapeutic target) were created. These transgenic models due to the absence of PTP1B, in turn, lacked dephosphorylation of tyrosine residues in insulin-receptor-linked proteins. Accordingly, the mice exhibited increase in insulin sensitivity improved glucose tolerance, increased energy expenditure and remained lean (resisted obesity).[9]

Monoclonal Antibody Approach for Target Validation

It is well known that monoclonal antibodies when raised against macromolecular protein antigen can efficiently and specifically bind to the said protein. This approach has been used in drug discovery program as a tool for target (protein) validation in genetically modified mouse models with the objective of demonstrating direct association of the identified target with the disease.[10] The experiment involves creating mouse models that mimic human pathology. This can be achieved with either deletion (knocked out) or overexpression of gene analogous to human gene called mouse ortholog. This is then followed by antibody-based intervention of the therapeutic target in an animal mimicking human disease which in turn facilitates target validation. However, this also opens up avenues to exploit the utility of monoclonal antibody either as therapeutic agents under biologics or designing small molecules based on the binding characteristic between the protein and the monoclonal antibody.

3.2.1.7 Generation of Hits/Leads Against Validated Target

1. *In vitro* high-throughput screening (HTS) of compounds against validated targets/whole cell-based assays.
2. Analysis of screening data to select the best hits. (A 'hit' is a compound that exhibits weak to moderate activity against a target in μM level during preliminary stage of screening).
3. Hit validation. This involves screening of hit molecule in secondary/alternate functional assays to validate the activity and filter out false positives. Besides screening in alternative assays, purity of hit needs to be established and wherever possible it is recommended to resynthesize the compound and retest it as part of validation.
4. To establish the drug-like properties (drug-likeness) of the hit compound by analysing its physicochemical properties and ADMET (absorption, distribution, metabolism, excretion, toxicity) profile.
5. Some of the important physicochemical properties that play key role in the drug likeness of a compound are solubility, lipophilicity, permeability, logP, logD, pK_a, molecular weight, and Polar surface area (PSA).
6. Lead optimization. Hit molecule(s) with acceptable potency, physicochemical properties, ADMET properties, and no or poor hERG (the human Ether-à-go-go-Related Gene) inhibition (for cardiotoxicity) can be advanced through lead optimization with the view to get lead compound(s) with desirable biological efficacy and potency. (A 'lead compound' is defined as a structure that has some activity against the chosen target, but not yet good enough to be the drug itself.)

3.2.2 Preclinical Research

Molecules fulfilling the criteria of a lead molecule can go to preclinical research. The basic steps of preclinical research are as follows:

1. Extensive structure–activity relationship studies (SARs) are conducted to improve potency and selectivity (using well-chosen substituent replacements at different parts of the molecule).
2. Optimizing access to the selected therapeutic target (ADMET; pharmacokinetic parameters) both *in vitro* and *in vivo.*
3. Optimizing drug-target interaction.
4. Optimizing leads for no or poor hERG inhibition.
5. PK (pharmacokinetic)/PD (pharmacodynamic) studies of leads in animal models for optimizing dosing regimen design.
6. Screening of selected lead molecule(s) in animal models for primary and secondary pharmacological assays and preliminary toxicities at the dose derived from PK/PD index.
7. Multivariate optimization—simultaneous optimization of physicochemical properties, drug-likeness, and pharmacological properties—is often more efficient.
8. Development of commercially viable synthetic strategies for large-scale production of highly pure active compounds. The strategy should be also amenable to carry out synthesis of the compound using Good laboratory practice (GLP) regulations for future clinical trials.
9. Establishing mechanisms of action of the lead molecule.
10. Proof of concept studies for serious indications.
11. Toxicity studies (acute/chronic/regulatory toxicity) to assess the risks of unwanted drug effects.
12. Safety studies involve effects of drug/test compound on gastrointestinal systems, central nervous system (CNS), cardiovascular system (CVS), renal and pulmonary systems.

13. Addiction liability
14. Genotoxicity
15. Formulation and stability studies
16. Patent protection: Filing Investigational new drug application (IND)

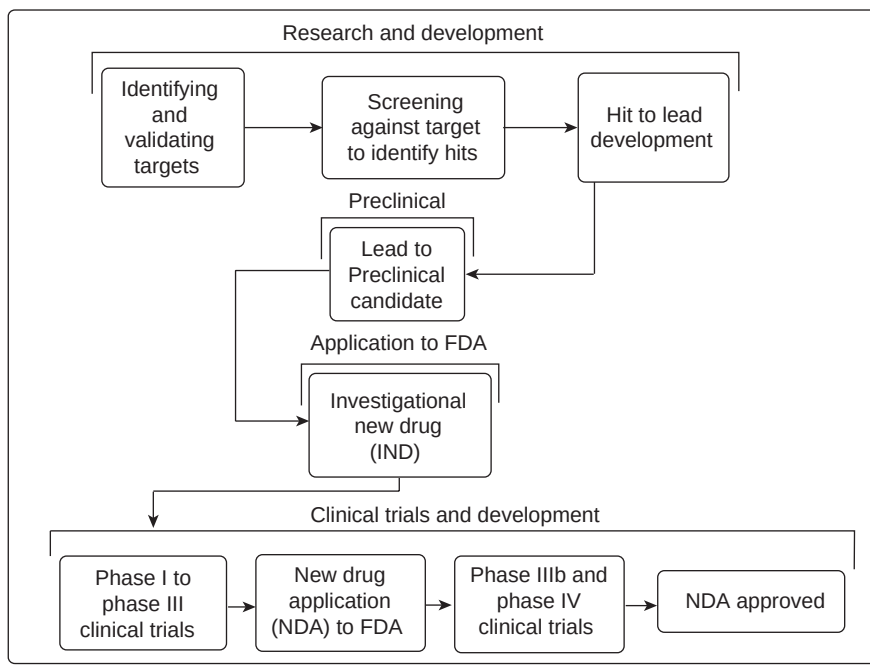
It is not a safe approach to pursue a single lead compound for lead optimization studies (no matter how potent it may appear); instead, back up lead molecules should be kept in the pipeline for any future failures during the drug development process.

3.2.2.1 Investigational New Drug

Once a preclinical candidate against a disease has been identified by researchers, an application of Investigational new drug (IND) is submitted to FDA to seek approval for starting human clinical trials. The application for IND has to be submitted by a physician who both initiates and coordinates the investigation of the candidate drug. Administration of an investigational drug in humans needs to be carried out under the direct supervision of a clinician. The application for IND approval comprises the following three major information pertaining to the preclinical candidate:

1. Toxicological and pharmacological studies in animal models.
2. Information about large-scale synthesis.
3. Methodology for carrying out clinical studies.

A window view to drug discovery program involving research and development, preclinical stage, application to FDA, and clinical trials has been depicted in Fig. 3.2.



(FDA = Food and drug administration; NDA= New drug application)

Figure 3.2 A window view to drug discovery.

3.2.3 Clinical Trials

Clinical trials involve three or four steps.

3.2.3.1 Phase I Clinical Trials

This involves safety trials of IND in healthy volunteers comprising 20 to 80 people at different doses. The objective is to establish the maximum dose of a drug that can be tolerated by humans without serious side effects. Besides, safety and dosing issues, clinicians also address issues pertaining to the route of administration of drugs.

3.2.3.2 Phase II Clinical Trials

This is carried out in a sample size comprising several hundred people suffering with the disease for which the IND has been developed. IND is administered by the same route and using the dose that was found to be safe and effective during phase I clinical trials. The participants are monitored by clinicians for several months to examine the side effects if any, due to the long-term exposure of the drug to humans.

3.2.3.3 Phase III Clinical Trials

This is carried out in a large number of disease patients with the objective to compare the efficacy and safety of the IND with existing therapies. The trials generally involve double blind and randomized process. In other words, neither the investigator nor the patients know who the recipients of IND are, and who are receiving the existing drugs. Besides, patients are randomly selected receiving either the IND or an existing drug. The trials often involve a sample size of 3000 participants and may last several years.

3.2.3.4 Phase IV Clinical Trials

This is carried out after FDA has given approval for the IND based on the data of Phase I, II, and II clinical trials. The Phase IV clinical trials again involve thousands of disease patients and can last for several years.

There are three IND types: An Investigator IND; Emergency IND, and treatment IND. There are two IND categories of IND: commercial and research (non-commercial).

3.2.4 FDA Review

FDA reviews new drug application for marketing. The new drug application (NDA) comprises a proposal submitted to the FDA by the institute/company/organization developing the drug for approval to sell and market the new drug. The proposal includes detailed information about the manufacturing, safety, and effectiveness carried out in humans during Phase I–IV clinical trials.

3.2.5 FDA Monitoring

Following release of drug in the market, FDA generally monitors the efficacy and safety of the released drug by picking up random batches.

3.3 Initial Steps in Drug Discovery Process (Medicinal Chemistry)

This involves stages from laboratory to preclinical stage and mostly comprises activities under medicinal chemistry. Typically, researchers discover new drugs by studying etiology of the disease and critically examining the underlying mechanism leading to the disease. This in turn gives insight to researchers to design a clinical candidate that would either block or reverse the effects of pathophysiological symptoms in a disease. The medicinal chemistry process commences with the screening of thousands of compounds against validated protein (receptor/enzyme) target followed by lead optimization studies.

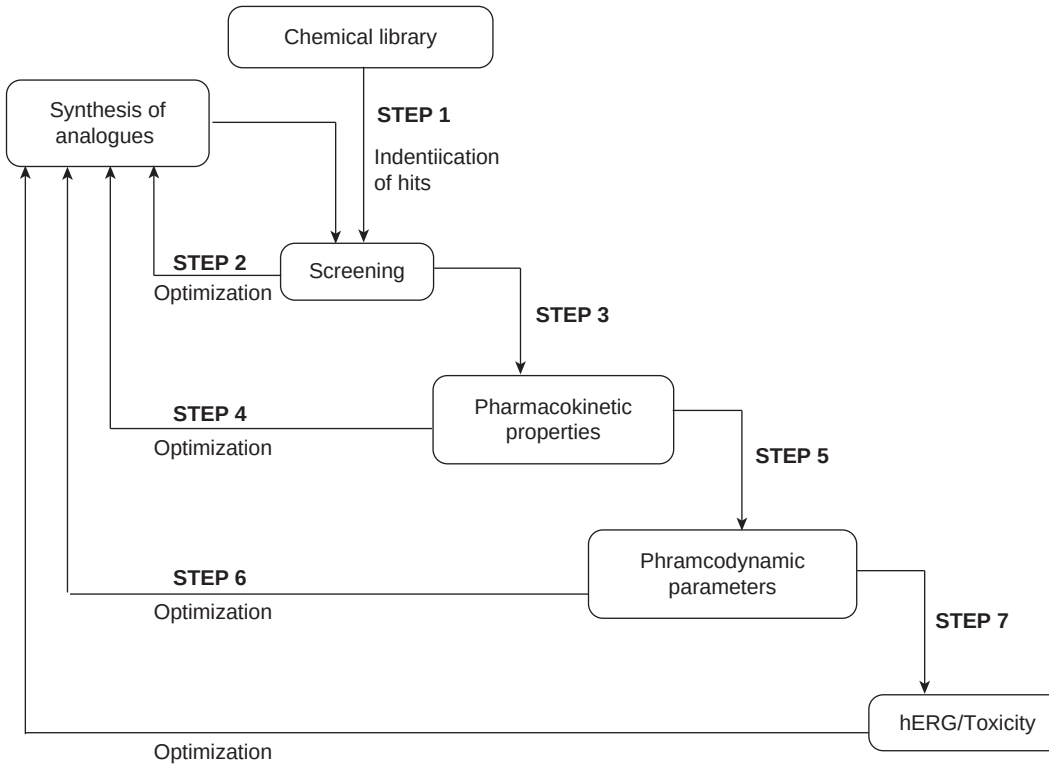


Figure 3.3 A typical medicinal chemistry protocol.

A typical medicinal chemistry protocol (Fig. 3.3) involves the following steps:

Step 1: Screening of library –*in vitro*/*in vivo* for hit/lead identification
Step 2: Hit to lead optimization
Step 3: Incorporation of PK properties
Step 4: Optimization of PK (ADMET) properties in lead molecule(s)
Step 5: Incorporation of PD parameters
Step 6: Optimization of pharmacodynamic properties
Step 7: Toxicity and hERG inhibition assays and optimization

3.3.1 Generation of Library of Chemical Compounds

Library of compounds required for the identification of drugs against disease target may involve screening of either random or focused chemical libraries. Random based screening also known as 'brute force technique' involves screening of non-biased library whereas in the screening of focused library, the size of the library is downsized using appropriate filters described below:

1. Using a commercial focused library comprising 1200 structurally diverse small molecule drugs, used clinically. It is an FDA-approved focused chemical library. These drugs not only display diversity in their pharmacological activity but also have good bioavailability and safety index data in humans.
2. Therapeutic target-specific libraries: such as focused chemical library against specific receptors or enzymes.

3. In-house collections of compounds stored at 4°C with drug-like properties based on Lipinski rule (focused library).
4. Focused libraries based on privileged structures.
   i. In 1980, Evans for the first time introduced the term 'privileged structure' based on his observation of diverse biological actives being exhibited by 1,4-benzodiazepine-2-one. Thus, privileged structure can be defined as 'a single molecular framework able to provide ligands/substrates for diverse receptors/enzymes. This gets support from the fact that some chemical libraries of organic molecules invariably have significantly higher hit rates in high-throughput screening results (focused screening).
   ii. Natural products too can display the 'privileged structure' characteristics. They are considered to contain scaffolds with the potentiality to be privileged structures because in many cases they are synthesized by biological systems to specifically interact with intravital protein targets.
5. Libraries based on natural product-inspired structures for high-throughput screening: Inspired by natural products, design, and synthesis of fused polycyclic structures have become integral part of drug discovery program. Besides, natural product inspired structures[11] carry following interesting features with relevance to medicinal chemistry:
   i. Molecular architecture involves rigidity and planarity.
   ii. Often two to three privileged structures are either annulated or tethered together.
   iii. Novel polycyclic motifs can be created by annulating privileged heterocyclic templates.
   iv. Libraries of almost unlimited generation of annulated heterocyclic structures can be synthesized thereby providing polycyclic skeletons with distinct physicochemical properties.

3.3.2 Screening of Chemical Libraries

Screening is a centrefold in a drug discovery process. It can be carried out *in vitro*, *in vivo,* and *in silico*. From initial 'hit' identification to 'lead optimization' to minimizing cardiotoxicity to accessing the target (PK parameters) to improving drug target interaction (PD parameters) all involve screening of compounds. Screening is generally carried out against a validated druggable biological target and for the purpose of lead optimization. Role of screening in the initial stages of drug discovery has been summarized in Fig. 3.4.

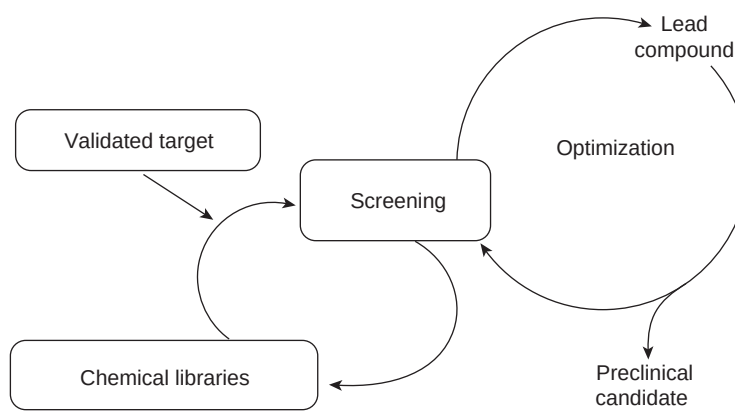


Figure 3.4 Screening in the initial stages of drug discovery.

3.3.2.1 Drug Targets for Screening

Therapeutic drug target is a macromolecular protein involved in the signalling or metabolic pathways either in the form of receptors or an enzyme. A defective signalling pathway in disease states can be attributed to either phenotypic change of the signals so that their normal functions are subverted, or genotypic changes. These changes can be attributed to aberration arising from three endogenous macromolecules involving DNA, RNA, and proteins involved in signalling pathways. Defects in DNA can be rectified using gene therapy; defects in RNA can be rectified using silencing RNA (siRNA) and aberrations in therapeutic proteins; receptors or enzymes can be addressed by administering chemical molecules (drugs) exogenously (Fig. 3.1).

The therapeutic targets in general can be classified under four categories:

1. Nucleic acids: DNA and RNA
2. Proteins:
   - i. Extracellular and intracellular enzymes
   - ii. Receptors (extracellular and intracellular)
   - iii. Transport proteins
   - iv. Structural proteins
   - v. Off-target proteins (e.g. hERG channel)
3. Lipids
4. Carbohydrates

Families of Receptor-Based Therapeutic Targets

Following the complete sequencing of the human genome encodes by the 'International Human Genome Sequencing Consortium'[12] in 2004, approximately 20,000 to 25,000 protein-coding genes were detected. Further, analysis led to the identification of 400 proteins as therapeutic targets that can be used for development of new drugs. In general, they can be divided into two major groups depending on their location.

1. **Cell-surface receptors:**
   - i. G-protein-coupled receptors
   - ii. Enzyme-linked receptors
   - iii. Ionotropic receptors
2. **Intracellular receptors:**
   - Nuclear hormone receptors

Details about the structure and function of receptors along with their application in drug discovery have been discussed separately in Chapter 7.

Out of these four families of targets, drug discovery relies predominantly on protein-derived therapeutic targets. A drug, following administration, exhibits its effect by binding to macromolecular proteins (receptors or enzymes). The drug–target interactions generally involve non-covalent and reversible interactions (in some cases covalent and irreversible interactions). The macromolecular therapeutic drug targets, used for the purpose of *in vitro* screening of test compounds, in general, address efficacy, toxicity, and pharmacokinetic (PK) and pharmacodynamic (PD) parameters (Table 3.1).

Table 3.1 Drug targets involved in PK and PD

Drug Targets for PK	Drug Targets for PD
Metabolic enzymes	Receptors
Transporter proteins	Enzymes
Plasma proteins	Ion channels
	Off target proteins

3.3.2.2 In vitro *Screening of Chemical Libraries*

This involves screening of compounds in 96 well or more microtitre plates to know their ability to elicit biological response. Generally, there are two kinds of *in vitro* screening methods:

1. Whole-cell-based *in vitro* screening
2. Protein target-based *in vitro* screening
   - i. Receptor-based screening
   - ii. Enzyme-based screening

Cell-based assays offer a pharmacologically relevant replacement to predict the phenotypic biological response of a drug in an organism. This remains one of the most valuable tools in the screening of structurally diverse compounds. In addition, they are used in other research areas of drug discovery program such as providing knowledge about therapeutic targets and about the biochemical pathways in the whole cells.

Both whole-cell (phenotypic) and target-based screens have sequentially dominated early stages of drug discovery program. While the former looks at the phenotypic effects that test compounds can produce in cells or tissues, the latter examines the effect of test compounds on a purified target protein via *in vitro* assays. Phenotypic screening involves modulating the phenotype of a cell or an organism by test compounds and has remained the foundation of drug development. Such screens can lead to the identification of compound that can alter disease either by acting on more than one biological target or on an unknown target. In the last decade, phenotypic screens have been successfully carried out using model organisms *C. elegans*, zebrafish, and drosophila. (Model organisms are *in vivo* models that bear genetic similarity with humans) The cells and tissues generally used during *in vitro* screening may comprise microorganisms such as bacteria/fungi, apoptosis estimation, cell viability or proliferation, and toxicity assays.

The target-based approach can effectively develop novel treatment methods with new mechanism of action. However, validation of therapeutic target remains a complicated process and remains associated with a high degree of uncertainty. The validated protein targets may be either receptors or enzymes. In a disease situation, the defects arising from signalling pathways can be corrected by administering drugs that can either act as ligands in order to regulate signal transduction following its binding with a receptor or act as substrate in order to regulate the activities of extracellular enzymes/hormones or intracellular enzymes involved in the downstream signalling cascades (substrates; that can activate or inhibit enzyme activities). Examples of drugs (Table 3.2) that bind to receptors/enzyme have been summarized below:

Table 3.2 Drugs targeting receptors and enzymes

Name of Drug (Ligand)	Name of the Receptor	Endogenous Ligand
Atropine (competitive antagonist)	Muscarinic receptors (Widely distributed throughout the body)	Acetylcholine
Cimetidine (Competitive antagonist)	H_2 receptors present in parietal cells in gut	Histamine
Atenolol (Competitive antagonist)	β1-adrenergic receptors present in the heart cells	Catecholamines

Name of Drug (Substrate)	Name of the Enzyme	Endogenous Substrate
Enalapril	ACE (extracellular) present in capillaries of the lungs	Angiotensin I
Lovastatin (competitive *or mixed* competitive inhibitor of enzyme)	HMG CoA reductase (intracellular signalling enzyme) present in endoplasmic reticulum cytosol	3-hydroxy-5-methylglutaryl CoA (HMG-CoA)
Indinavir (competitive inhibitor of enzyme)	HIV protease is a retroviral aspartyl protease (enzyme) in retroviruses	Gag-Pol polyproteins

CASE STUDY 2

Screening of Compounds Against RAS (Involving Extracellular Enzyme)

Screening of compounds for therapeutic intervention in a Renin–angiotensin system (RAS), a classic endocrine system leading to the hypertension, comprises both receptor and enzyme (involved in cell signalling) as validated protein targets.

In this system, in response to changes in renal perfusion (blood flow to kidney) – as its primary function is to restore perfusion pressure by increasing the blood pressure, kidney cells produce and release renin directly into the in bloodstream. The increase in blood pressure is caused by the RAS which involves release of angiotensinogen produced by the liver in blood circulation followed by its conversion to angiotensin I (an inactive decapeptide) in the presence of renin (Fig. 1.21). Next Ang I gets converted into angiotensin II (an octapeptide) by an enzyme called ACE (Fig. 3.5) and binds with receptor AT_1 and leads to vasoconstriction (hypertension). Thus, two therapeutic targets identified in RAS were enzyme ACE and receptor AT_1. Screening of compounds against receptor AT_1 led to the identification of sartans as antihypertensive drugs (ligand) that blocked the binding of Ang II to its receptor (ARB). Similarly, screening of compounds against ACE led to the identification of captopril that displayed antihypertensive activity by inhibiting the ACE. Thus, both receptors as well as enzymes can be used as biological (drug) targets for *in vitro* screening.

(**Caution:** *A combination therapy with two drugs affecting different stages of RAS is not recommended.*)

(*Continued*)

Study 2 (*Continued*)

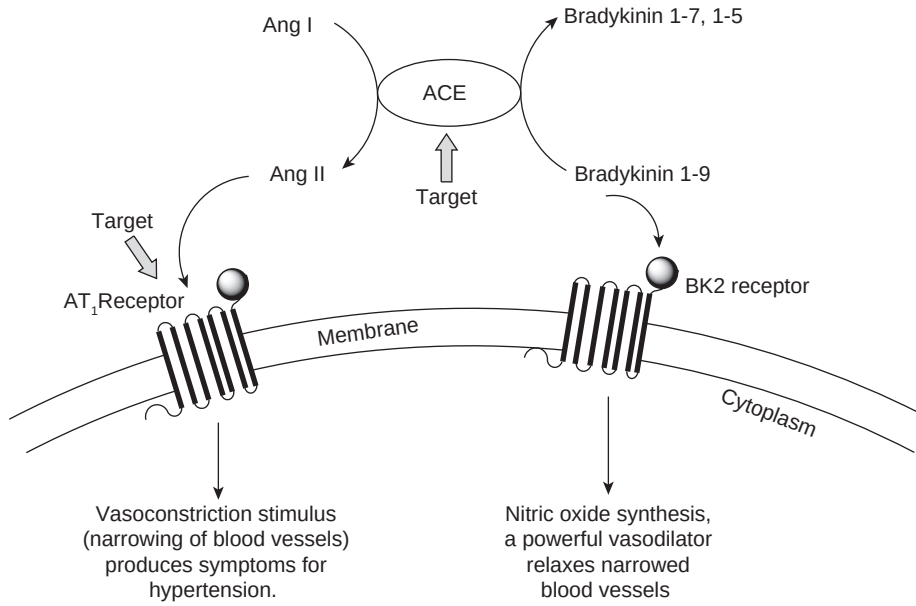


Figure 3.5 Renin–angiotensin endocrine system with both receptor and enzyme as therapeutic target.

Thus, a variety of receptor/enzyme-based assays for diseases are now available to carry out screening of compounds in high/medium-throughput screening mode. However, since the *in vitro* screening of test compounds against protein target often requires a large quantity of protein, recombinant DNA technology has been integrated with drug discovery program (for producing large quantity of protein) to meet this requirement. In case of receptors, it is rather difficult to determine antagonistic/agonistic properties using a traditional binding assay without performing further investigations. This is because enzymes react with substrate to yield products whereas receptor binds with ligand to produce signals.

CASE STUDY 3

Receptor for Neurotransmitters as a Therapeutic Target for Drug Discovery

The gut neurotransmitters are released by network of neurons lining the gut and it is so enormous that some scientists have nicknamed it as a 'second brain'. The sheath of neurons embedded in the alimentary canal from oesophagus to anus comprises enteric nervous system. The latter secretes more than 20 neurotransmitters that regulate (signals) grinding of food during digestion, breaking down of foods, absorbing nutrients, rhythmic muscle contractions, and secretion of juices from pancreas and gall bladder.

For example, serotonin is one of the neurotransmitters secreted by the enterochromaffin cells (ECs) in the intestinal mucosa in response to chemical and mechanical signal and within brain it is responsible for regulating anxiety, happiness, and our happy mood. In the intestine, serotonin can affect motility (peristalsis), secretory function of the intestine, and the tone of the intestine. Serotonin (5-HT) elicits its

(*Continued*)

Study 3 (*Continued*)

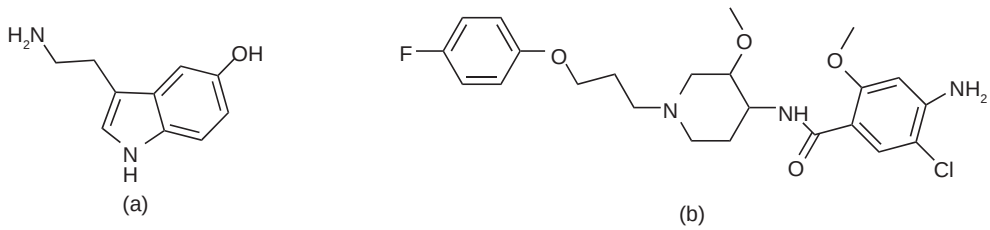


Figure 3.6 Structure of (a) Serotonin and (b) Cisapride.

biological response in the Gastrointestinal (GI) region following its binding to type 4 receptors (5-HT4 receptors) widely distributed in the intestinal mucosa. Thus, 5-HT4 receptor can be used as a therapeutic target for treating GI disorders. For example, cisapride, a 5-HT4 receptor agonist has been used for enhancing motility in upper GI tract. It binds 5-HT4 receptor and triggers release of the neurotransmitter acetylcholine in the enteric nervous system from postganglionic nerve endings (Fig. 3.6).

In the brain, low levels of serotonin can cause anxiety and depression. Among many methods used for treating depression, selective serotonin reuptake inhibitors (SSRIs) have been widely used to increase the levels of serotonins in brain (Fig. 2.24).

CASE STUDY 4

Receptor for Hormones as a Therapeutic Target for Drug Discovery

Hormones in gut (enteric endocrine system) are secreted by enteroendocrine cells (not glands) present in the lining of the entire digestive tract. They modulate gastrointestinal functions via autocrine and paracrine actions following their binding to specific cell-surface receptors. Some of these receptors have been identified as attractive therapeutic targets in drug development. For example, among many gut hormones, Glucagon-like peptide 1 (GLP-1) found in ileum has been found to increase insulin secretion in pancreatic cells. The GLPI-1 peptide hormone binds to the G-protein-coupled receptor GLP-1R present on the β-cells of pancreas and stimulates secretion of insulin. A peptidyl analogue of GLP-1 called exenatide[13] (a 36 amino acid peptide also resistant to DPP-IV enzyme) that binds GLP-1R is used clinically for the treatment of diabetes (Fig. 1.2).

CASE STUDY 5

Enzyme (Extracellular) as Therapeutic Target for the Treatment of Obesity

Fat (triacylglycerol, TAG) present in diet is absorbed in gut following its degradation into fatty acid (FA) and monoglycerides (MGs). This is catalysed in the presence of bile salts and an enzyme called lipase which is secreted in response to the fat by the pancreas. Next, MA and FA formed in the gut lumen forms micelles by combining with bile salts and phospholipids. The resulting micelles are then absorbed at the surface of enterocytes following which TAG is reconstituted inside enterocyte from MA and FA and packaged into tiny fat globules chylomicrons with the help of cholesterol and proteins.

(*Continued*)

Study 5 (*Continued*)

The fat globules then move out of enterocytes through exocytosis from the basolateral surface and instead entering directly into blood capillaries (due to large size), enter into lacteals (lymphatic capillaries) and then flow into circulation via lymphatic vessels (Fig. 3.7). Thus, one way to prevent absorption of fat from diet would be targeting the enzyme lipase. Orlistat[14] is a clinically used inhibitor of lipase that decreases the gastrointestinal absorption of fats (weight loss). The inhibitor (Fig. 3.8) is used for the treatment of obesity, which can subsequently result into type II diabetes and cardiovascular diseases.

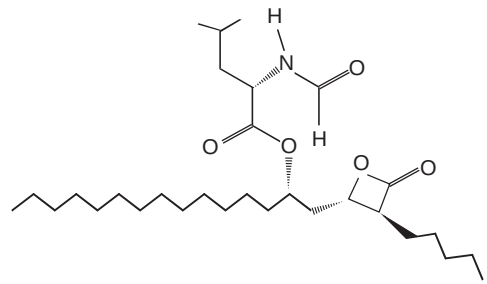


Figure 3.7 Lipase inhibitor orlistat.

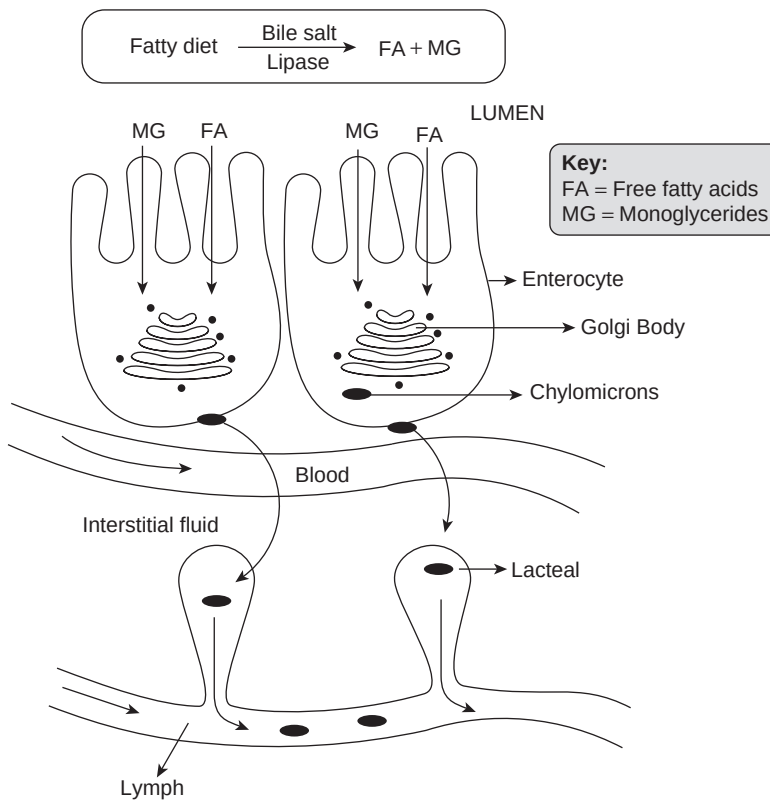


Figure 3.8 Absorption of fats present in diet in the lumen.

3.3.2.3 *Parameters for Measuring Pharmacological Activity of Test Compounds During* In vitro/In vivo *Screening*

Minimum Inhibitory Concentration

The results of *in vitro* screening of test compounds in whole-cell-based assay involving infectious microorganisms, cell viability tests, etc., are expressed as MIC. It is defined as the minimum amount of a test compound required to block the visible growth of the cell (being screened) *in vitro*. It is usually reported as ng/mL or nM concentration.

Inhibitory Concentration (IC_{50})

The results of *in vitro* screening of compounds in either whole-cell assays or against target-based assays are expressed as IC_{50} values. It is defined as the inhibitory concentration of an inhibitor where the pharmacological response results in 50% inhibition. It is usually reported as ng/mL or nM concentration.

1. IC_{50} is the concentration of the test compounds that gives half (50%) of the full biological response (Fig. 3.9).
2. In the initial stages of *in vitro* screening of chemical compounds, the biological response elicited by individual chemical compounds is distinguished using IC_{50} values.
3. IC_{50} values are used to compare the potency of compounds in a given series of compounds with the view to identify the most potent compound in the series.
4. IC_{50} values are determined through a series of experiments. In each experiment, the amount of inhibitor is steadily increased, and the observed biological response measured.
5. The inhibitory biological response at different concentrations of test compounds is an important pharmacodynamic parameter. A typical quantitative graph of biological response vs. concentration yields sigmoidal curve. However, some compounds depict non-sigmoidal curves such as bell-shaped or U-shaped curves.

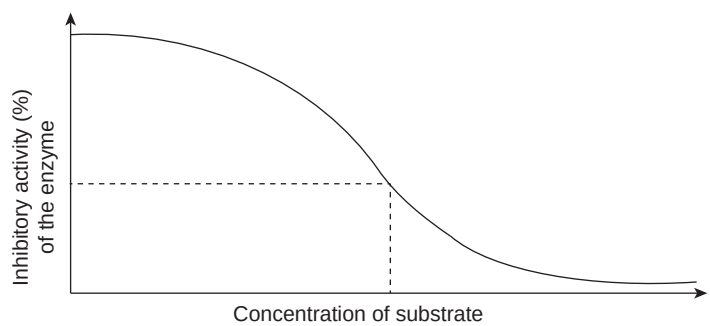


Figure 3.9 Determination of IC_{50} value.

An Absolute Inhibition Constant (K_i)

This is defined as concentration of an inhibitor under saturating substrate conditions, at which rate of reaction is 50% of the maximum reaction rate (V_{max}). This is reflective of binding affinity of the inhibitor to the enzyme. The absolute inhibition constant K_i can be calculated from IC_{50} values using the following equation.

$$K_i = IC_{50} + \frac{[S]}{K_m}$$

where, K_i is the binding affinity of the inhibitor,

IC_{50} is the functional strength of the inhibitor,

[S] is fixed substrate concentration and

K_m is Michaelis constant is the concentration of substrate at which enzyme activity is at half (1/2 V_{max}) of its maximal velocity (V_{max}). (Discussed in Chapter 12)

If K_i is small, it means binding is good, and it is a better inhibitor. A good inhibitor inhibits an enzyme in the nanomolar range.

IC_{50} vs. K_i

1. While IC_{50} and K_i are both measures of an inhibitor's ability to block the action of an enzyme; they are not equivalent.
2. K_i demonstrates binding affinity and can be used to express potency of an inhibitor.
3. IC_{50} values reflect functional strength of the inhibitor and vary since they depend on the substrate concentration.
4. IC_{50} values of the same inhibitor between different research laboratories can never be same; however, K_i being a constant value remains same.

pIC_{50}

Inhibition of enzymes can also be expressed in log units and is represented as pIC_{50}. It is the negative log of the IC_{50} value in molar concentration. As a word of caution IC_{50} value in molars (not micromolar) should always be converted to pIC_{50}.

$$pIC_{50} = -\log_{10}(IC_{50})$$

Unlike IC_{50} values, larger values of pIC_{50} reflect potent inhibitory activities.

EC_{50}

The EC_{50} is defined as the amount of agonist/test compound required to give 50% of its full observable response (effect) and is represented in molar concentration. In fact, it is the halfway response between the baseline (bottom) and full response (top). It is also used to express potency of a compound and is generally used to compare activities exhibited by two or more drugs (Fig. 3.10) for the same target.

ED_{50} Values

Effective dose 50 (ED_{50}) is a pharmacological term for the effective dose drug/test compound required to furnish drug-specific response in 50% of the subjects exposed to the treatment (Fig. 3.10). It can also be used to compare potency of more than one drug for the same disease.

LD_{50}

This is used to establish toxicity of the compound *in vivo*. LD_{50} (median toxic dose) is the dose of drug/test compound that causes 50% death in animals being exposed to the drug/test compound.

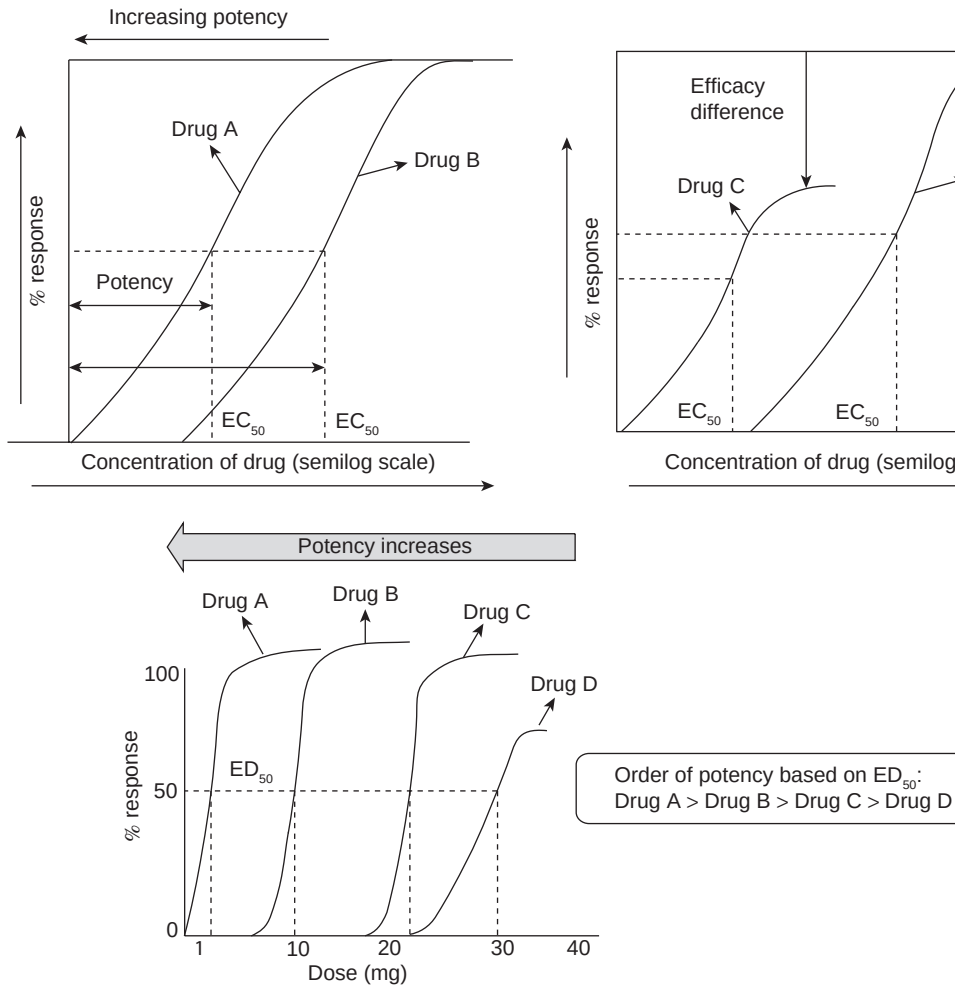


Figure 3.10 EC_{50} and ED_{50}.

3.3.2.4 In vitro *Tests*

The following are a battery of primary *in vitro* assays employed for a modern drug discovery program to identify active compounds.

1. Whole-cell-based assays
2. Target-based assays
3. Selectivity assays

3.3.2.5 Advanced In vitro *Assays*

This involves screening of active compounds (Hit/lead) for pharmacokinetic properties (ADMET) in the early stages of drug discovery program. Numerous assays to assess ADMET of active compounds have been discussed and are summarized in Table 3.3.

Table 3.3 A summary of some widely utilized *in vitro* assays to assess ADMET of test compounds.

Assays	Outcome
Absorption (A)	
Caco-2 permeability and PAMPA assays	Caco-2 colon carcinoma cell line and artificial membranes are used to estimate permeability.
Efflux protein transporters	MDCK-MDR1 cells are used *in vitro* to establish induction/inhibition of P-gp by test compounds.
Cell-to-cell communication assays.	Molecular permeability of gap junctional intercellular communication
Distribution (D)	
Plasma protein binding assay	To assess extent of binding to plasma proteins
Brain tissue binding	Since brain tissue binding is independent of species, brain tissues of rat are used as model.
Blood Plasma Partitioning	To asses *in vitro* clearance.
Metabolism (M)	
Cytochrome P450 inhibition/induction assays	Drug-drug interactions
Microsomal/metabolic stability	Due to the presence of metabolic enzyme CYP, it gives information about *in vitro* metabolic stability of test compounds.
Plasma Stability	To assess degradation of compound by blood plasma
Excretion (E)	
Analysis of bile, stool or urine samples as well as tissues.	To quantify levels of metabolites.
Toxicity (T)	
Ames test	To assess mutagenic potential
Cytotoxicity in suitable cell line	To asses cellular toxicity
Hep G2 hepatotoxicity	To assess toxicity on human liver
Genotoxicity	Gene mutations, clastogenecity and aneugenicity
Transformation assays	To assess carcinogenic ability
Inflammation assays	To asses inflammation/antiinflammation on macrophages
hERG inhibition	To assess cardiotoxicity
Adverse drug reactions	Basophil tests for immunoglobulin E (IgE)-as well as non-IgE-mediated hypersensitivity reactions, T-cellular assays for cutaneous adverse reactions, nephrotoxicity, neurotoxicity, teratogenicity, impaired vision or hearing.

In vitro Metabolic/Microsomal Stability Assays

In vitro metabolic assays are carried out with the view to understand the metabolism of the test compound or to identify the metabolites formed. Since liver is the major site for drug metabolism, liver microsomes, subcellular fractions containing membrane-bound cytochrome P450 system (phase I enzymes), are used for the assay. Incubation of microsomes with test compound provides *in vitro* intrinsic clearance of a compound over a time period (rate of disappearance/intrinsic clearance). Hepatocytes assays of test compounds provide full cellular metabolism involving phase I and II pathways.

Cytochrome P450 Inhibition/Induction Assays

There are two kinds of interactions reported between drugs and metabolic enzyme CYPs P450. On one hand, CYP induction may speed up the metabolism and rapid clearance of the drug; on the other hand, CYP inhibition may result in the increase in the concentration of drug. This inhibition/induction of CYPs can reflect major drug–drug interaction during combination/polytherapy. Numerous *in vitro* assays comprising either human derived cell lines/microsomes or cryopreserved human hepatocytes[15] are employed to establish test compounds mediated inhibition/induction of CYPs.

In vitro Plasma Protein-Binding Assays

One of the major factors that affect efficacy of drugs is plasma protein binding. This occurs when the drug reaches systemic circulation and binds with blood plasma proteins forming equilibrium between protein-bound drugs (drug–protein complex) and free drugs. Only unbound drug is available to bind to desired receptors and thus elicit biological response, albeit they may be also accompanied by metabolism/excretion. Bound drugs can neither elicit biological response nor undergo metabolism/excretion. *In vitro* protein-binding assays are carried out using proteins derived from plasma, brain, and liver microsomes. Using *in vitro* protein-binding assays, one can determine the availability of free drug concentration and study the consequences on PK and PD parameters of the test compounds. Among a variety of methods developed, ultrafiltration and equilibrium dialysis methods remain widely used tools for assessing drug–plasma protein binding. The outcome of the assays is expressed as percentage of unbound drugs (fu).

1. **Parallel Artificial Membrane Permeability Assay (PAMPA):** The assay[16] is carried out to establish the permeability of test compounds. It uses lipid-based artificial membrane between the donor and acceptor compartments mimicking the *in vivo* situations involving passive diffusion of drugs such as the blood–brain barrier, gastrointestinal tract, blood capillary exchanges and skin. More than 95% clinically used drugs are absorbed through passive diffusion. The assay is restricted to permeability mechanism by passive diffusion only and has nothing do with energy-driven active transport.
2. **Caco-2 Cell Assays:** Numerous studies have suggested that transporters (passive diffusion, active transport, and active efflux) play a role *in vivo* during drug absorption (bioavailability), disposition, therapeutic efficacy, and adverse drug reactions. *In vitro* assays therefore have been developed to assess permeability of test compounds. Caco-2 permeability assay is carried out *in vitro* to assess the absorption and bioavailability of test candidates via intestinal region, during the lead optimization process (early stage). It evaluates both passive and active transports and the absorption of orally administered drugs. The method examines the bidirectional permeability (active efflux transporters) of test compounds (from the apical to the basolateral side or vice versa). The Caco-2 cell line[17] is a human colon adenocarcinoma cell line which has been widely used as a general and routine screening of test compounds to predict the drug absorption of orally administered drugs.

In vitro Permeability Assay

Following administration of drug/test compound particularly by oral route (preferred one), physiological machinery makes a two-pronged attack simultaneously on the drug: on one hand it resists the absorption (bioavailability) of the drug into the blood stream and prevents it distribution in tissues, on the other hand it induces metabolism of the drug so as to facilitate its rapid excretion from the body. Thus, a drug/test compound has to face several physiological barriers and, in order to overcome these barriers, one needs to introduce appropriate modifications in the test compound. Both bioavailability and distribution involve diffusion of test compounds through several lipid bilayer cell membranes of tissues lining the gut, blood capillaries, and tissues carrying the site for action. Only difference between the two events is that during bioavailability drug is absorbed (diffuses) from gut lumen into bloodstream whereas during distribution, drug is diffused from bloodstream via capillaries into the interstitial fluid and finally into tissues.

The phenomenon of blood capillary exchange of substances across the membranes of endothelial cells (lining the walls of capillary) and the surrounding interstitial fluid/tissues is guided by the physicochemical properties of the substance. While lipophilic materials can diffuse directly across membranes, hydrophilic substances (ions, amino acids, glucose) may leave the blood either by facilitated diffusion using protein transporters or via intercellular clefts. However, large molecules including proteins may move out from blood via pores of fenestrated capillaries, sinusoids, or exocytosis. Water-soluble drugs can readily cross through the pore in blood capillaries. Thus, during drug design or lead optimization studies, test compounds can be screened for their ability to diffuse through cell membranes using *in vitro* model assays that will mimic (*in vivo*) physiological situations.

Induction/Inhibition Assay of P-gp

P-gp (ABC transporter proteins), a transmembrane efflux protein located in the intestine generally plays a role in pumping the drug/test compound from inside to outside of the cell, thus affecting the bioavailability of the drug/test compound and, in turn, affecting the desired concentrations of drug/test compound in systemic circulation and tissues. However, there are drugs that may inhibit the function of efflux protein, thereby causing drug–drug interaction. The efflux transporters are located in many tissues including the brain, intestine, and kidney. Thus, during early stages of drug discovery, it is important to assess the P-gp induction/inhibition properties in test compounds.

MDCK-MDR1 cells are used *in vitro* to establish[18] induction/inhibition of P-gp by test compounds. Identifying inhibitors of P-gp may provide an opportunity to enhance the bioavailability of drug and may find particular use in cancer therapy. This is because there is an overexpression of P-gp protein in cancer cells. Identification of inducers of P-gp may give insight into the possible pharmacokinetic drug–drug interaction during combination/polytherapy. One approach to counter P-gp inhibitory properties[19] in test compound could be to administer large doses of drugs (if safety parameters are within limit) as high concentration will bind and saturate P-gps and the remaining drugs can then diffuse through membrane and enhance bioavailability. However, in case a test compound exhibit P-gp inhibitory property, it cannot be delivered in high doses as it will fail to get absorbed and elicit response.



Important Note

Often a P-gp inhibitor is coadministered with the drug to enhance bioavailability

AMES Test for Mutagens

The *in vitro* Ames test is widely employed to assess the mutagenic potential (ability to induce mutations in the DNA) of test compounds. The assay involves use of bacteria as test organism against which test compounds are

screened for their ability to induce mutations in the DNA. A positive test suggests that the test compound is mutagenic and therefore may be a carcinogen, because cancer is often linked to mutation in DNA.

Cell Viability and Cytotoxicity Assays

Many *in vitro* cell viability and cytotoxicity assays are carried out with the view to assess the safety and cytotoxicity of test compounds. The cells used in these assays are either removed from the living organisms or from *in vivo* environments. Using this assay, one can assess apoptosis, cell viability, metabolic changes, and proliferation. Two of the widely used assays for this purpose are colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and Alamar blue *in vitro* assays.

Hepatotoxicity Assays

The assay is carried out to assess hepatotoxicity to human liver cells. In this assay, hepatocytes and HepG2 cells are exposed to test compounds followed by measurement of hepatotoxicity. Both acute and chronic effects of test compounds can be evaluated by exposing drugs to cell lines from 14 h to 10 days.

Genotoxicity Assays

The screening comprises a battery of tests for the identification of the three forms of genotoxicity:

1. **Gene Mutations:** ability to alter the DNA sequence that makes up a gene.
2. **Clastogenecity:** ability to break down chromosomes resulting in deletion, addition, or rearrangement of chromosomes.
3. **Aneugenicity:** ability to cause a daughter cell to have an abnormal number of chromosomes.

Transformation Assays

In vitro human cell transformation assay is carried out to assess the carcinogenic ability of test compounds. A cell transformation from normal to neoplasticity involves genetic mutations arising from replication errors. The assay generally involves agar soft formation to monitor the ability of test compounds to cause transformation into cancer cells.

Inflammation Assays

Ubiquitous macrophages have been implicated in the inflammatory responses to pathogens and tissue homeostasis. The macrophages defend against infections by secreting a series of signal molecules in the form of pro- and anti-inflammatory cytokines. In inflammation assay, test compounds are screened against the mouse macrophage cell line J774A.1 and evaluated for their ability to release pro- and anti-inflammatory cytokines using enzyme-linked immunosorbent assay (ELISA).

hERG Inhibition (Cardiotoxicity) Assays

In the last decade, numerous drugs were withdrawn from clinics due to sudden cardiac death. It was attributed to the impaired repolarization (responsible for action potential) of cardiac muscle cells which was evident from the prolongation of QT interval (LQT syndrome) in the ECG. LQTS leads to the induction of ventricular tachycardia resulting in sudden death. The ion channel that is responsible for repolarization (Fig. 1.24) of the cardiac membrane is hERG (*human ether-a-go-go related gene*) that allows K^+ out of the cell and any impairment during repolarization may affect heart beatings that are regulated by electrical activity originating from the action potential.

A variety of drugs were found to block voltage-gated K^+ channels encoded by hERG, thereby resulting in a fatal disorder called prolonged QT syndrome. FDA has, therefore, made it mandatory to carry out thorough preclinical and clinical cardiovascular safety measures. In recent years, the cardiotoxicity assays have been inducted during early stages of lead optimization studies. A classic example of drug being withdrawn is the antihistaminic drug terfenadine which after its release was found to be associated with severe arrhythmias and sudden death.[20]

Cell–Cell Communication Assays

In multicellular organisms, millions of cells/tissues despite being designated with specific functions keep communicating with each other in order to maintain the normal cellular/physiological functions necessary for survival. Intercellular and intracellular cell signalling through endogenous messengers plays a major role in cell–cell communications. Among many methods of signalling, gap junctional intercellular communication (GJIC) plays a regulatory role in the normal growth and differentiation of cells and has been implicated in many pathological conditions. GJIC provides a direct contact or channel between cytoplasm of adjacent cells, thus allowing flux of second messengers and nutrients via diffusion, a passive way of intercellular signalling. A number of assays have been developed to assess cell–cell communication[21] and can be grouped under two major techniques: by measuring dye transfer or electrical conductance.

3.4 *In Vivo* Screening Assays

In vivo functional assay needs to be carried out to establish efficacy, selectivity, and ADMET profile of lead molecules.

1. This involves screening of test compounds in diseased animal models (guinea pigs, rats) or genetically modified model organisms (*C. elegans*, *Danio rerio,* etc.). The test is carried out to assess both therapeutic response and toxicity.
2. Secondary pharmacological *in vivo* assays to study potential off-target or unintentional effects of leads.
3. *In vivo* ADMET profile of exemplar(s) from the lead series in order to assess potential liabilities (e.g. oral bioavailability, half-life, blood/plasma clearance, etc.).
4. **Safety/Toxicity:** This deals with toxic effects a test compound can have in animal models. *In vivo* screening of these adverse effects during lead optimization studies enhances the success rate of the selected preclinical drug candidates.
5. **Acute toxicity (*in vivo*):** The assay is carried out by exposing animal models to a single dose or multiple doses of test compounds for a short term and then assessing the subsequent harmful effects. In general, the studies are carried out in at least two different animal models. Sometimes, acute toxicity is represented by the lethal dose LD_{50}. Once lethal dose has been determined, further toxicity studies are carried out using a dose that is not lethal.[22] For this, it is always recommended that toxicity studies are done with doses which are two times the effective dose. In case the animals die, the dose is lowered.
6. **Chronic Toxicity (*in vivo*):** The assay is carried out to assess the ability of test compound to induce harmful effects via repeated or continuous exposure over an extended period.
7. **Therapeutic Index (safety):** During drug development, one parameter that is important for the safety and efficacy of the drug is Therapeutic index (TI). It is a quantitative measurement of the relative safety/therapeutic window of a drug by comparing the amount of drug required to produce therapeutic response and the amount that produces toxicity. A 20-fold or higher difference is generally recommended between doses of drug required to produce biological response and toxicity in animals. TI values for any drug can be calculated using ED_{50} and LD_{50} values.

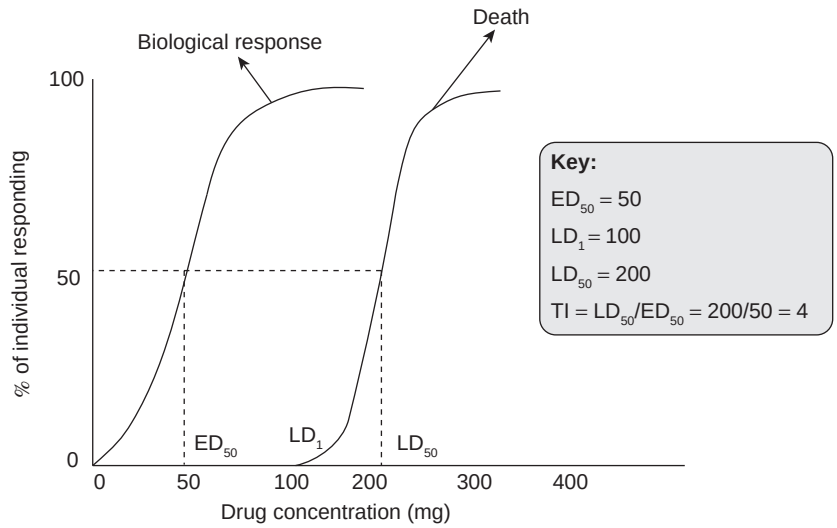


Figure 3.11 Therapeutic index.

In Fig. 3.11, two different dose–response curves ED_{50} and LD_{50} involving efficacy and mortality, respectively, for a drug has been depicted. The ED_{50} value (effective dose for pharmacological response in 50% of the people) is 50 mg and LD_{50} (effective dose causing mortality in 50% people) is 200 mg. The TI for this drug can be calculated by following the equation given below and was found to be 4. TI should not be less than 1. Ideally 5–10 is considered to be safe (anticancer drugs have TI of 2 or less).

$$\text{TI} = \frac{\text{LD}_{50}\,(\text{Lethal dose})}{\text{ED}_{50}\,(\text{Effective dose})}$$

Tacrolimus (immunosuppressant), warfarin (anticoagulant), seizure drugs, digoxin, theophylline, methotrexate, and amiodarone (antiarrhythmic) are some examples of drugs with narrow TI.

3.5 Screening in Model Organisms (Our Distant Fast Breeding Cousins)

Model organisms are considered as a good *in vivo* laboratory models (non-human) for screening of compounds against a variety of diseases. The typical features associated with them are their short life, fully characterized genomes which in some organisms bear a strong homology with humans, mature rapidly, easy manipulation of genetic traits, and can produce a large number of eggs/offspring for high-throughput screening. Choreography of the genes in these organisms has been recorded as development of adult organisms from eggs occurred within a short life span.

1. They have been widely used as *in vivo* models for human diseases involving genetic disorders and diseases like cancer, diabetes, CVS, Alzheimer's disease, etc.
2. The model organisms provide a unique opportunity to directly study various components of drug discovery against diseases, such as etiology of disease, identification and validation of target, screening of library, hit/lead identification and optimization, and toxicology.[23]

3. Larvae or adults have been used as vertebrate models for screening of library in a high-throughput manner.
4. They have particularly robust embryos and occupy a pivotal position in the evolutionary tree.
5. They can breed in large numbers.
6. Mutant model organism has been extensively used not only to study the etiology of diseases but also to screen compounds for regenerating abilities.

The three most widely used model organisms for the *in vivo* screening of compounds have been described below:

3.5.1 Danio Rerio[24] *(Zebrafish)*

1. It is a native to the himalayan regions and is a popular aquarium fish.
2. There exists a shared synteny between human and zebrafish genomes.
3. A unique transparent 0.7 mm embryo and short lifespan of ~12 weeks helps understand the disease progression and facilitates screening of test compounds.
4. Transgenic model organism can be readily produced which can be routinely used to assess the regenerative abilities of test compounds.

Screening: A library of structurally diverse compounds were screened against zebrafish embryo (5000) with expression of the *gridlock* mutation (puts break on heart growth). The studies led to the identification of one molecule, GS4012 (Fig. 3.12) that appeared the most effective in taking off the 'break' on heart growth.[25] The molecule may find clinical application following heart attack or stroke in promoting injury such as vascular growth, etc.

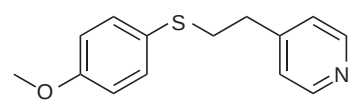


Figure 3.12 Suppression of the *gridlock* phenotype by compound GS4012.

3.5.2 Caenorhabditis Elegans[26]

1. It is a tiny little transparent organism which is non-parasitic in nature.
2. It measures about 1 mm in length and can survive in all kinds of soil conditions.
3. It has short lifespan of mere three days and is made up of a few thousand eukaryotic cells. This can be helpful in studying developmental biology dealing with formation of an adult organism from a single egg.
4. Excellent animal model to study as to how our brains are wired.
5. Applications to fundamental biological problems, human genetic disease, and bacterial disease.
6. The nematode carries 270 nuclear receptors; in comparison, humans carry < 50 such receptors.

3.5.3 Drosophila Melanogaster[27]

1. This is probably the first model organism identified for analysing genetics and developmental biology.
2. Has a life cycle of 12 days and female starts reproducing within 8–10 h.

3. The genome of fruit fly bears approximately 60% homology to that of humans, and approximately 75% of the genes causing disease in humans have homologs in flies.
4. Organism has been used to both study etiology of the disease and also for the purpose of screening compounds.
5. Fruit flies have been identified as next frontier model organism to unlock the mystery associated with diabetes[28] and cancer.[29]

Drosophila larvae or adults can be readily screened in a high-throughput manner to study the effect of orally administered compounds.

Exercises

1. Write in brief:
   - **(i)** Druggability
   - **(ii)** Target validation
   - **(iii)** Investigational new drug
   - **(v)** New drug application
   - **(vi)** Phase I to IV clinical trials
2. Describe the five stages in modern drug discovery program.
3. Draw flow sheet for three therapeutic interventions for treating diseases with two examples each of drugs binding to receptors and enzymes
4. Describe generation and screening of chemical libraries.
5. Describe parallel artificial membrane permeability assay (PAMPA).
6. Define: EC_{50}, ED_{50}, IC_{50} and Therapeutic index
7. Give names of advanced *in vitro* assays for pharmacokinetic properties.

Competitive Exam Questions

1. ELISA is based on:
   - **(a)** Antigen-protein interaction
   - **(b)** Lectin-antibody interaction
   - **(c)** Antibody protein interaction
   - **(d)** Antigen-antibody interaction

   [***GPAT 2019***]

References

1. Richard C. Mohs and Nigel H.Greig, *Alzheimer's and Dementia: Translational Research and Clinical Interventions*, **2017**, *3*, 651.
2. Hughes J. P., Rees S., Kalindjian S. B., and Philpott K. L., *Br. J. Pharmacol.*, **2011**, *162*, 1239.
3. Berridge, M.J. (**2012**) Cell Signaling Biology; doi:10.1042/csb0001012.
4. Monica Schenone, Vlado Dančík, Bridget K. Wagner, and Paul A. Clemons, *Nat. Chem. Biol.*, **2013**, *9*, 232.

5. Joanna Owens, *Nature Reviews Drug Discovery*, **2007**, *6,* 187.

6. Richard Kramer and Dalia Cohen, *Nature Reviews Drug Discovery*, **2004**, *3*, 965.

7. Jones S. W., Souza P. M., and Lindsay M. A.,*Curr. Opin. Pharmacol.*, **2004**, *4*, 522.

8. T. Rajendra Kumar, Melissa Larson, Huizhen Wang, Jeff McDermott, and Illya Bronshteyn, *Methods Mol. Biol.*, **2009**, *590*, 335.

9. Xue B., Kim Y. B., Lee A., Toschi E., Bonner-Weir S., Kahn C. R., Neel B. G., Kahn B. B., *J. Biol. Chem*., **2007**, *282*, 23829.

10. Catherine J. Mardsen, Sonia Eckersley, Max Hebditch, Alexander J. Kvist, Roy Milner, Danielle Mitchell, Juli Warwicker and Anna E. Marley, *Journal of Biomolecular Screening*, **2014**, *19*, 829.

11. Sunil Sharma and Bijoy Kundu, *Tet. Lett.,* **2008**, *49*, 6937.

12. International Human Genome Sequencing Consortium, *Nature*, **2004**, *431*, 931.

13. Robert Hood, Virginia Valentine, Susanna Mac and William H. Polonsky, *Diabetes Spectrum*, **2006**, *19,*181.

14. Ballinger A., Peikin S. R., *Eur. J. Pharmacol.*, **2002**, *440*, 109.

15. Walsky R. L., Boldt S.E., *Curr. Drug. Metab.*, **2008**, *9*, 928.

16. Reis J. M., Sinko B., Serra H. R., *Mini Rev. Med. Chem*., **2010**, *10*, 1071.

17. Sambuy Y., De Angelis I., Ranaldi G., Scarino M. L., Stammati A., Zucco F., *Cell Biol. Toxicol.*, **2005**, *21*, 1.

18. Sugimoto H., Matsumoto S., Tachibana M., Niwa S., Hirabayashi H., Amano N., Moriwaki T., *J. Pharm. Sci.,* **2011**, *100*, 4013.

19. Kale Mohana, Raghava Srivalli, Lakshmi P. K., *Brazilian Journal of Pharmaceutical Sciences*, **2012**, *48*, 353.

20. Vishal B. Siramshetty, Janette Nickel, Christian Omieczynski, Bjoern-Oliver Gohlke, Malgorzata N. Drwal, and Robert Preissner, *Nucleic Acids Res.*, **2016**, *4*, 44, D1080–D1086.

21. Muriel Abbaci, Muriel Barberi-Heyob, Walter Blondel, François Guillemin, and Jacques Didelon, *Bio. Techniques*, **2008**, *45*, 33.

22. S. Parasuraman, *J Pharmacol Pharmacother*., **2011**, *2*, 74.

23. Omprakash G. Bhusnurel, Jyoti M. Manel, Sachin B. Gholve, Sanjay S. Thonte, Padmaja S. Giram and Jaiprakash N. Sangshetti, *Pharm. Anal Acta.*, **2015**, *6*, 426.

24. Karl J. Clark, *Nature Education*, **2015**, *8*, 3.

25. Charles C. Hong, Quinn P. Peterson, Ji-Young Hong and Randall T. Peterson, *Curr. Biol.,* **2006**, *16*, 1366.

26. Titus Kaletta and Michael O. Hengartner, *Nature Reviews Drug Discovery*, **2006**, *5*, 387.

27. Nicholas S. Tolwinski, J. Dev. Biol., **2017**, *5*, 9.

28. Álvarez-Rendón J. P., Salceda R., Riesgo-Escovar J. R., *Biomed. Res. Int*., **2018**, 1417528.

29. Zhasmine Mirzoyan, Manuela Sollazzo, Mariateresa Allocca, Alice M. Valenza, DanielaGrifoni, Paola Bellosta, *Frontiers in Genetics*, **2019**, doi.org/10.3389/fgene.2019.00051.

CHAPTER

4

Strategies for Identifying Hit Molecules

KEY THINGS TO LEARN FROM THIS CHAPTER

- Definition of a hit molecules
- Characteristics of hits
- Strategies for identifying hits leading to drug candidates.
  - Serendipity in drug discovery
  - Endogenous sources for the identification of drug candidates
  - Traditional methods as source for hit identification from exogenous sources
  - Target-based screening for the identification of hit molecules
    - Quality of chemical libraries as source for hits
    - Fragment-based drug discovery (FBDD) for the identification of hit molecules
    - Virtual screening for the identification of hit molecules
  - Phenotypic screening to identify hit molecules
  - Repositioning of drugs

4.1 Hit Molecules

The first crucial step in the process of drug discovery against any disease is identification of a hit molecule.[1] A promising hit lays foundation for the subsequent generation of high-quality lead molecule(s) followed by its transformation into a preclinical candidate through lead optimization/drug design strategies. This forms basis for a laboratory to clinical translational research. Thus, hit molecules can be defined asstarting point (molecules with some biological response) identified during the initial *in vitro* screening of chemical libraries against validated biological targets/whole cells. The success of any medicinal chemistry program against a disease largely depends on the quality of a hit/lead molecule.[2] Initial hit against a disease can be obtained via *in vitro* screening of compounds using either of the formats described below.

4.1.1 *Random* in vitro *High-Throughput Screening of Chemical Libraries against Biological Targets/Whole Cells*

The high-throughput screening (HTS) involves a robotic-based platform for an automated and rapid *in vitro* screening of thousands of compounds in microtitre plates. With the introduction of HTS, millions

of compounds were randomly screened against a protein target without any rationalized approach or with no medicinal chemistry inputs. Neither the targets were validated nor did the compounds in chemical libraries had any drug-like properties (poor quality). With the result, the initial use of HTS was discouraging. Although HTS produced active compounds, the failure rates were high during their subsequent transformation to preclinical candidates primarily due to poor ADMET profile, promiscuous nature of compounds or invalidated targets.

4.1.2 In Vitro *High Throughput Screening of Focused Chemical Libraries against a Validated Biological Targets or Whole Cells*

The failure rates from random screening led to a paradigm shift in the screening of libraries. More emphasis is now laid on the validation of therapeutic targets and also on the quality of chemical libraries. Validation of target involves establishing direct role of protein targets in disease situations using transgenic animals or RNA interference techniques (Discussed in Chapter 3) and their draggability, whereas the quality of chemical libraries involves removal of compounds from libraries that are devoid of drug-likeness or the ones with reactive features. Additionally, target-specific chemical libraries are also built around a particular pharmacophore. Thus, the present-day identification of hits involves screening of rationalized or focused libraries of compounds against validated targets.

4.1.3 *Characteristics of Hits*

Hit molecules identified in the initial stages of *in vitro* screening against targets provide only preliminary information about its biological response/affinity (albeit weak), and are devoid of any other information pertaining to safety, drug-likeness, synthetic accessibility, etc. Thus, the next step following hit identification involves hit validation studies by rigorously assessing the quality of hit. This involves assessing the purity of compounds, confirmation of activity, selectivity, synthetic amenability, assessing drug-likeness, toxicity, etc. The hit molecules generally have following characteristics:

1. They may exhibit weak to moderate therapeutic response *in vitro* with IC_{50} value of test compounds in $<10\mu M$ range and with weak binding energy ranging from 6 to 10 kcal/mol.
2. Reconfirmed activity in a fresh sample of resynthesized structure with ≥99% purity (by HPLC).
3. Specificity for target as evident by *in vitro* assays with >10-fold selectivity against other undesired target(s) or isoforms.
4. May exhibit some preliminary structure-activity relationship (SAR) as apparent from available analogues within the library of compounds screened.
5. Hit molecule must be amenable to synthesis in few steps, thereby enabling development of SAR and lead optimization through synthesis of analogues.
6. Absence of reactive features in the hit. Often screening of compounds results in false positive hits (misleading results) as the biological activity observed may be due to undesirable mechanism. This has been attributed to some reactive chemical features in molecules that allow them to bind non-specifically to unrelated therapeutic targets and result in false hits. Some chemical features[3] responsible for promiscuous nature of compounds are acyl halides, aldehydes, and compounds lacking both oxygen and nitrogen, etc. This issue has been addressed by filtering out such promiscuous compounds from the library before subjecting the library to *in vitro* screening.
7. Hit molecule should have drug-like physicochemical properties and ADMET profile as evident from *in silico* (computational software) methods.

4.1.4 Strategies for Identifying Hits Leading to Drug Candidates

The various strategies for the identification of 'hit' molecule(s) can be broadly classified into two groups:

1. **Structure-Based Drug Discovery:** In the old concept of structure-based drug discovery, a compound with weak biological response is subjected to modification *vis a vis* pharmacological activity (with no knowledge of biological target) with the goal to identify a preclinical drug candidate.
2. **Target-Based Discovery:** In this strategy, initially a validated therapeutic macromolecular target is identified that could be involved in the manifestation of a disease (could be part of underlying mechanism for the said disease). The biological target may be either a receptor or an enzyme or another biologically relevant molecule involved in a disease pathway. Chemical libraries are then screened against this target for the identification of hit molecules. Both the structure-based (new concept) and target-based approaches are often used in conjunction depending on the success of the approach for identifying 'hits'.

4.1.5 Sources for Hits

There are various methods to identify hit molecules depending on the source which may have natural, biosynthetic, and synthetic origins. Natural sources may comprise terrestrial and marine plant products, microbial metabolites, and animals. Sources from plants comprise alkaloids, steroids, glycosides, etc., and their significance in drug discovery is evident from the fact that more than 40% best selling drugs[4] are either natural products or their derivatives. The path-breaking discovery of microbial product penicillin by A. Fleming led to the discovery of several antibiotics and other drugs from actinomycetes, fungi, and unicellular bacteria. Animal sources comprise polypeptides from snake venoms, hormones, enzymes, monoclonal antibodies (mAb), nucleic acids, etc., and are grouped under biotherapeutics or biologics. In general, the drug molecules irrespective of their origin can be grouped under two major categories: small organic molecules and biologics. The difference between these two categories has been summarized in Table 4.1.

Table 4.1 Difference between small molecule drugs and biologics

Small Molecule Drugs	Biologics
Low molecular weight	High molecular weight
Single chemical structure	Are very large, complex molecules or mixtures of molecules
Previous examples	Unique
Manufactured through chemical synthesis	Many biologics are produced using recombinant DNA technology
Well-characterized structure	May be undefined due to its complexity
Specific mechanisms	Pleiotropic mechanisms
Species-independent	Species-specific
Metabolized	Degraded
Maximal tolerated dose	Optimal biological dose



Additional Information

It is pertinent to add that 'structure-based drug discovery' discussed above is an old concept used for optimizing a chemical structure with weak response in order to get compounds with optimal biological response.

The modern 'structure-based drug discovery' approach involves identification of hit/lead compound by screening compounds against a known. The screening against therapeutic target is carried out using computer-aided in-silico *approach or* in vitro *studies using NMR/X-ray, etc. This in turn provides compounds that binds to the active site with a better fit. Captopril was the first drug developed using this approach.*

Various approaches (Fig. 4.1) used for the identification of hit/lead molecules have been summarized below:

1. Serendipity ('a fortunate accident')
2. Endogenous sources (derived from humans or animals)
3. Traditional methods (exogenous natural sources: ethnobotany)
4. Target-based screening
5. Phenotypic screening
6. Repositioning of drugs

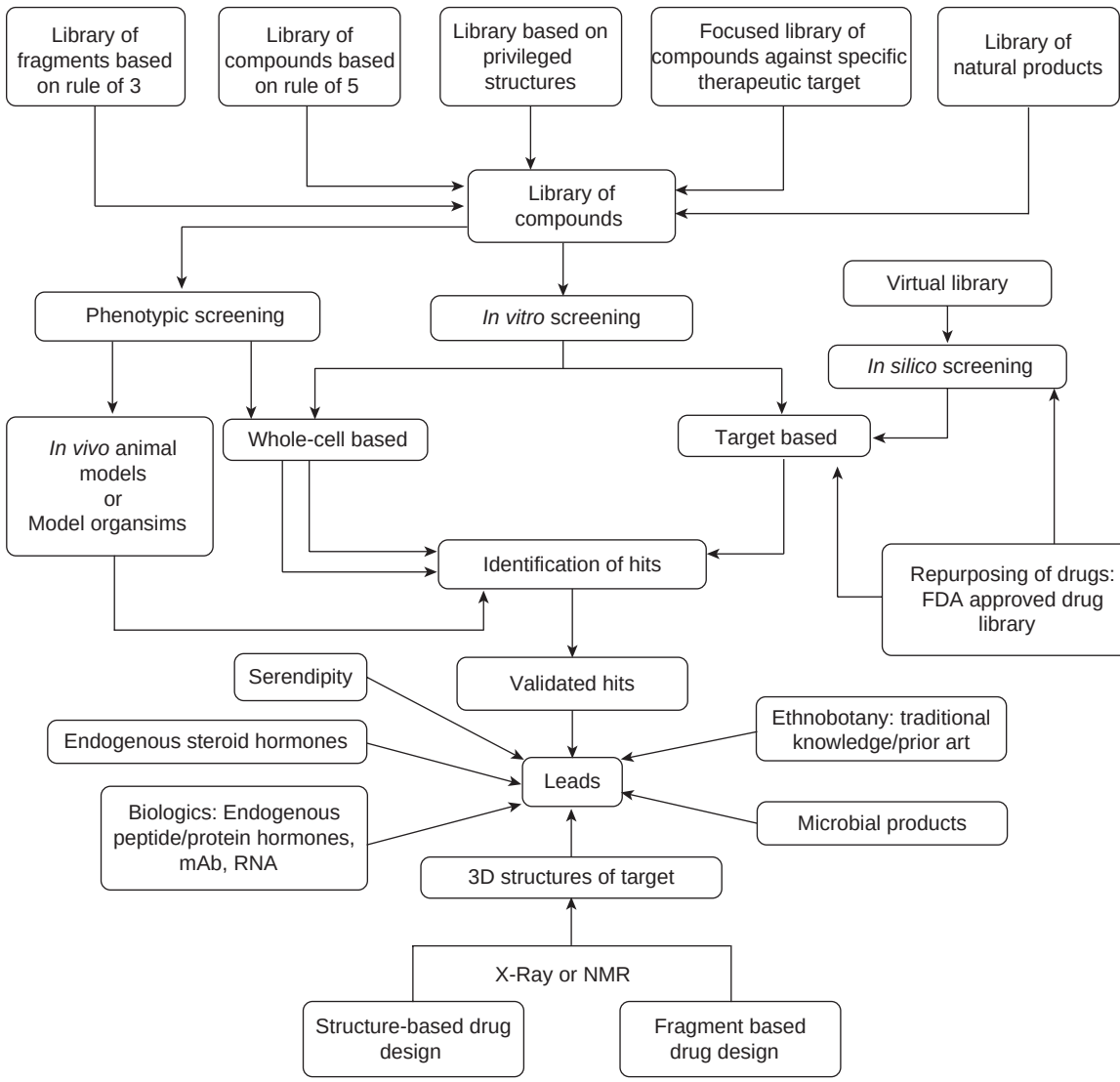


Figure 4.1 Various approaches for identifying hits/leads.

4.2 Serendipity in Drug Discovery

This strategy of accidental or chance discovery has played a major role throughout the history of drug discovery. It has generally been based on the observations made by researchers while they were performing experiments with an objective. The observations led to unexpected results that they were not searching and were very different from the original objectives.[5]

4.2.1 Chlorpromazine

The 1950s saw the emergence of several new classes of psychiatric drugs including the antipsychotics. With the discovery of chlorpromazine as the first antipsychotic, a large number of chemically diverse antipsychotics were identified.[6] Development of chlorpromazine originated from an accidental discovery, while researchers were working with this class of compounds with antihistaminergic activities on the central nervous system. Following its selection as preclinical candidate, assessment of this compound in human subjects was found to induce calmness without any sedation, and this led to its development as psychiatric drug (Fig. 4.2*a*).

4.2.2 Minoxidil

The drug was originally developed for treating hypertension. However, during its clinical trials in humans,significant growth in bodily hair was observed. This led to the marketing of this drug (Fig. 4.2*b*) as a 5% topical formulation for growth of hair particularly for patient with baldness.[7]

4.2.3 Aspartame

A sweetening substance with 200 times more sweetness than sugar was accidentally discovered by a scientist at G. D. Searle while working with peptides as anti-ulcer agents (Fig. 4.2*e*). Accidental licking of his finger led to the discovery and marketing of this compound as a sweetening agent.

4.2.4 Viagra (Sildenafil citrate)

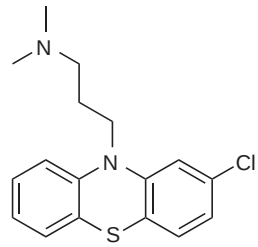
An anti-impotence drug marketed in 1998 was discovered accidentally during phase I clinical trials of sildenafil as antihypertensive drug (Fig. 4.2*c*). The finding concluded that the drug had more effect on libido than on blood pressure.

4.2.5 Warfarin

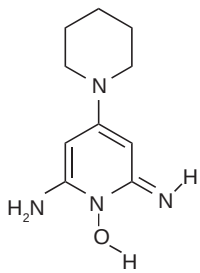
In 1920s, hundreds of cattle started to die of internal bleeding (hemorrhaging, named 'sweet clover disease') after eating moldy silage made from a sweet clover plant. Later, the compound responsible for deaths in cattle was identified as dicoumarol as an anticoagulant. Due to its ability to have effect on internal bleeding, this was developed and marketed as rat poison. However, it was not until 1951 when a man attempted to commit suicide by consuming this rat poison and he could be saved by administering vitamin K, as it led to the reversal of anticoagulant effect.[8] Warfarin (Fig. 4.2*d*) has since then become mainstay drug in the treatment (dissolve) of blood clots.

4.2.6 CNS Drugs

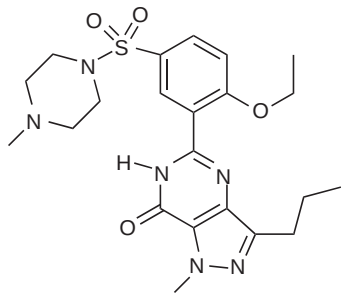
The first advances in the treatment of CNS disorders were based on serendipity. In 1950s, iproniazid (Fig. 4.2*f*, N-isopropyl derivative of isoniazid), initially discovered for treating tuberculosis, later was found to be associated with mood altering activity. This led to its development as antidepressant agent and was subsequently approved in 1958 for clinical use but was withdrawn a few years later in 1961 due to a high incidence of hepatitis.



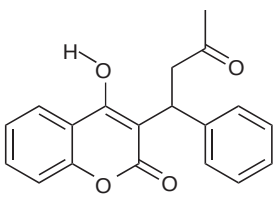
(a) Chlorpromazine



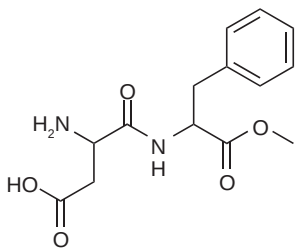
(b) Minoxidil



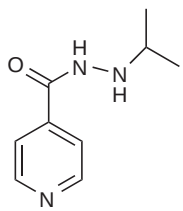
(c) Viagra



(d) Warfarin



(e) Aspartame



(f) Iproniazid

Figure 4.2 Drugs discovered through serendipity.

4.3 Endogenous Sources (Derived from Humans or Animals) for the Identification of Drug Candidate(s)

Intravital molecules (endogenous) present in our system or animals have been a source for numerous drug candidates. They can be steroid hormones (intravital gonad hormones), corticosteroids (man-made drugs that closely resemble cortisol, an intravital hormone produced naturally by adrenal glands), polysaccharides, peptides/proteins hormones, neurotransmitters, enzymes, and genes/nucleic acids, etc. Treatment of diseases using gene (DNA/RNA) therapy, therapeutic proteins/peptides hormones, mAbs, and vaccines are grouped under biotherapeutics (biologics). Thus, biologics can be defined as therapeutic chemical entities of human/animal origin that are used clinically either in its natural form or as their truncated fragments/derivatives. Protein-derived biologics are also produced using recombinant DNA technologies. Discovery of some of the biotherapeutics from endogenous sources have been described below.

4.3.1 Endocrine Hormones and their Therapeutic Applications

Endocrine hormones are a class of signaling molecules (chemical messenger) produced by the endocrine glands (ductless glands). Following their release directly into the bloodstream, they are distributed throughout the body's distant parts. The physiology and function of various organs are regulated by these

hormones. There are two major classes of endocrine hormones: Peptide/protein hormones and steroid hormones (additional types of ***endocrine glands*** as they are part of endocrine system). Steroid hormones are made up of cholesterol while peptide hormones are made up of amino acids. (*Besides endocrine glands, there are* ***exocrine glands*** *secreting substances such as sweat, salivary, mammary, and mucous onto an epithelial surface by way of a duct.*)

Interestingly, both liver and pancreas act as exocrine and endocrine glands. The exocrine and endocrine secretions from liver involve bile through ducts into intestinal lumen and ductless release of insulin-like growth factor-1 (IGF-1) in the blood stream, respectively. On the contrary, exocrine and endocrine secretions for pancreas are pancreatic juice released through pancreatic duct into duodenum and two ductless hormones: insulin and glucagon, secreted by islet cells directly into bloodstream. Insulin (biologics), a 51 amino peptide, is used clinically for the lowering of blood sugar in diabetic patients.

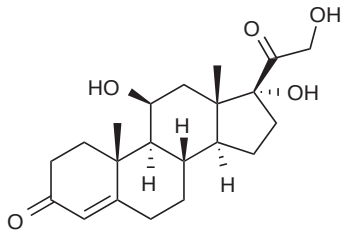
In vertebrates, all endocrine secretions are regulated by the hypothalamus and is generally governed through an axis (connects the nervous and endocrine systems by way of the pituitary gland). Three examples of axes are hypothalamic–pituitary–adrenal axis (HPA), hypothalamic–pituitary–gonadal axis (HPG) and hypothalamic–pituitary–thyroid axis (HPT). In these axes, release as well as inhibition of different hormones along the axis is regulated via feedback loop. The hormones secreted by endocrine glands primarily act as chemical messengers in cell signaling and coordinate a variety of physiological functions via cell-cell communication. Major endocrine glands present in vertebrates are as follows:

1. Hypothalamus: present in brain
2. Pituitary gland: present in brain
3. Pineal gland: present in brain
4. Thyroid gland: present in neck
5. Parathyroid: present in neck
6. Thymus: present in between lungs
7. Pancreas: present just behind the gut
8. Adrenal glands: present on the top of kidneys

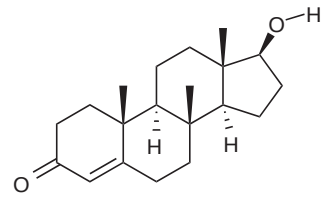
Aberrations in the secretion of endocrine glands have been linked to numerous diseases such as hyper/hypothyroidism, obesity, growth hormone (GH) deficiency, low testosterone, osteoporosis, Cushing syndrome, diabetes, etc. Thus, these endogenous endocrine hormones due to their direct involvement in pathogenesis of diseases serve as hits/leads for treating several endocrine-related disorders.

4.3.2 Steroid Hormones as Endogenous Source for Drugs

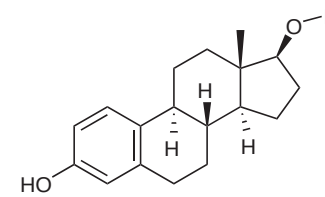
In humans, there are three steroid glands that are source for steroid hormones: the adrenal cortex, testes, and ovaries (also secreted by placenta during pregnancy). In general, they are lipid-soluble substances and act by readily diffusing (solubilize) through the plasma membrane in cells. Once in the cytosol, they bind with intracellular steroid hormone nuclear receptors (typically cytoplasmic or nuclear) and enter nucleus where they modulate gene expression. Adrenal cortex produces two main groups of corticosteroid hormones: glucocorticoids and mineralocorticoids. Among these hormones, hydrocortisone (a glucocorticoid) also known as cortisol (Fig. 4.3) is used for treating skin-related disorders. Steroid hormones secreted by gonads are sex hormones and have been extensively used as lead molecules for treating reproductive disorders including cancers.



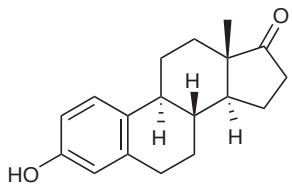
(a) Hydrocortisone



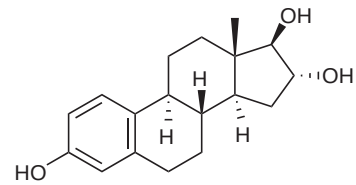
(b) Testosterone



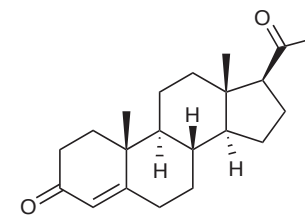
(c) Estradiol



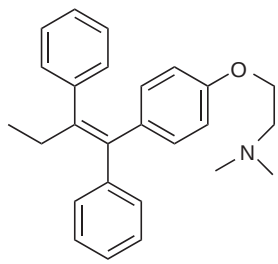
(d) Esterone



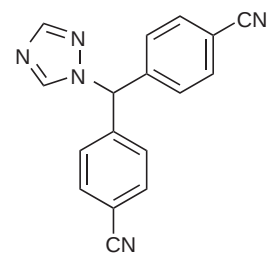
(e) Estriol



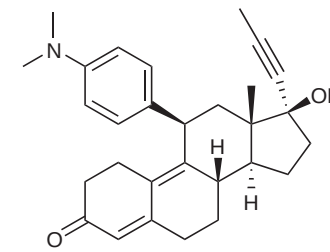
(f) Progesterone



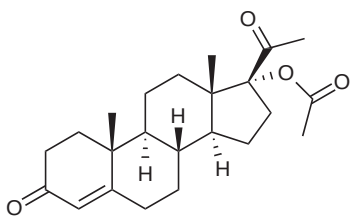
(g) Tamoxifen



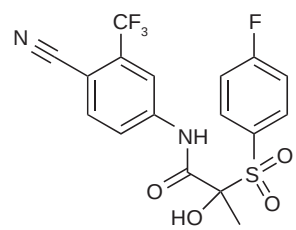
(h) Letrozole



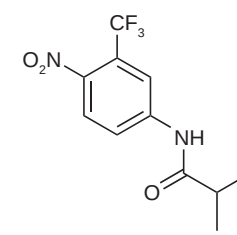
(i) RU-486



(j) Progestin



(k) Bicalutamide



(l) Flutamide

Figure 4.3 Steroid hormones and their analogues as endogenous source for drugs.

4.3.2.1 Male Sex Hormones

They are also referred to as androgens and comprise testosterone (Fig. 4.3*b*) and its metabolite androsterone. In men, testosterones are primarily produced in the testes, and in small quantities it is also produced in the adrenal glands. The production of testosterone in the body is regulated by hypothalamus and pituitary. In males, the testosterone regulates fertility, libido, red blood cell production, and muscle mass and strength.

Oestrogens (Female Sex Hormones) in Males

Estradiol (Fig. 4.3*l*) plays a critical role in male sexual function. In men, it is essential for modulating erectile function, libido, and spermatogenesis (critical role in male sexual function). Since men lack female anatomy for the production of estrogen, testosterone is transformed into estradiol via aromatization in the presence of an enzyme called aromatase. An increase in estrogen levels and low levels of testosterone in men can contribute to prostate cancer, fatigue, low libido, and erectile dysfunction. Notably, although testosterone is transformed into estrogen, the reverse does not happen.

4.3.2.2 Female Hormones

The two female sex hormones are estrogens [estradiol (predominant form), esterone, estriol] and progesterone (Fig. 4.3*d*, *e*, *f*). The progesterone, secreted by the female reproductive system, regulates the condition of the inner lining (endometrium) of the uterus to receive the fertilized egg (embryo). The progesterone plays a crucial role in the menstrual cycle and maintenance of pregnancy and is secreted by the ovaries, placenta, and adrenal glands. However, a sudden drop in the levels of progesterone levels after ovulation (the luteal phase) may trigger recurrent miscarriages and inability to conceive. This has led to the development of numerous synthetic progesterone-based oral contraceptives for females.

Testosterone (Male Sex Hormone) and its Effect in Females

Testosterone is also produced in a woman's ovaries in small amounts and the rest is produced in the adrenal gland, (and during pregnancy by the placenta). The presence of male sex hormone testosterone plays a role in woman's overall health in general, libido, and higher energy levels. However, high testosterone level has a negative impact such as frontal balding, infertility, and polycystic ovarian syndrome (PCOS). The level of testosterone diminishes naturally with age/menopause or due to impaired functioning of ovaries or pituitary or adrenal glands.

4.3.2.3 Regulation of Sex Hormone Release

The hypothalamic-pituitary-gonadal (HPG) axis (Fig. 4.4) clearly depicts the link between peptidyl and steroidal hormones and their effect in the regulation of reproduction. Initially, hypothalamus secretes peptidyl endocrine hormone GnRH (signal molecule) that moves to the anterior pituitary through blood circulation and binds with receptors. This in turn triggers release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in blood circulation. The protein hormones LH and FSH subsequently triggers release of testosterone in males (production of sperm) and estrogens/progesterone in females (the maturation and release of an egg during each menstrual cycle) and maintain their reproductive functions. An increase or decrease in the levels of androgens and estrogens accordingly regulates the production of gonadotropin-releasing hormone and LH/FSH by feedback mechanism.

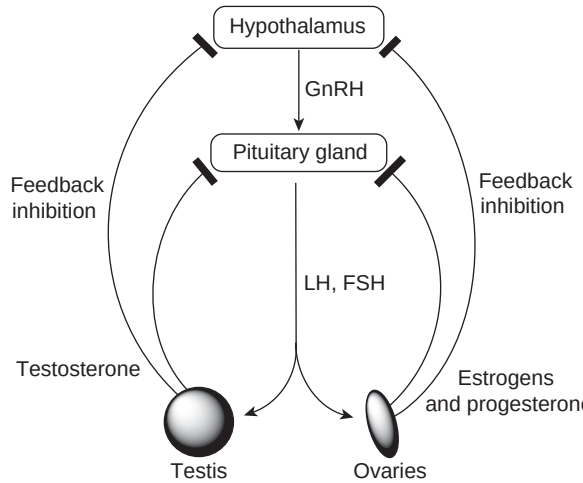


Figure 4.4 Hypothalamic-pituitary-gonadal axis.

Dual Role of Estrogen in Shutting Down Testosterone Concentration in Males

The concentration of testosterone levels in males is reduced by estrogen via two different routes. In one of the routes, the testosterone in males gets transformed into the estrogen through aromatization, and in the second route, the estrogen so generated shuts down the production of testosterone by inhibiting hypothalamus and pituitary via feedback inhibition to release GnRH and LH/FSH, respectively. High estrogen levels in men lead to the pathogenesis of prostate cancer.

4.3.2.4 Sex Hormones and Their Antagonists as Therapeutics

Hormonal imbalances of testosterone and/or estradiol levels can lead to greater incidences of degenerative disease and death. For example, mortality is high among men with low levels of estradiol and testosterone, compared to men having levels in the permitted physiological range. Similarly, in females, although estrogen assists in menstrual cycle, the same feeds breast cancer cells to proliferate establishing direct link between breast cancer and estrogen. The most common type (~80%) of breast cancer diagnosed today is estrogen receptor-positive (ER-positive) breast cancer. 'ER-positive' cancers have estrogen receptors (ERs) on the surface of the cell that bind to the estrogen. Treatment for ER-positive breast cancer involves hormonal therapy (medications) that can either:

1. Reduce the concentration of estrogen in the body.
2. Block estrogen (by blocking receptors using anti-estrogens) from feeding breast cells to proliferate.

Blocking Estrogen Production as Therapy

The concentration of estrogen can be reduced by using a class of drugs called aromatase inhibitors that can block the transformation of testosterone to estrogen. Indeed, they are recommended in women who've already gone through menopause. The examples of aromatase inhibitor drugs include anastrozole (Arimidex), exemestane (Aromasin), and letrozole (Fig. 4.3*h*).

Use of Anti-estrogens (Estrogen Antagonists) as Therapy

Tamoxifen belongs to a class of drugs that prevents estrogens like estradiol from mediating their biological consequences in the body. They act by occupying the ER, thereby blocking the binding of estrogens. For the last

40 years, tamoxifen (Fig. 4.3*g*) remains the only drug of choice to treat patients with estrogen-related (ER) positive breast tumors. The drug is also known as selective estrogen receptor modulator (SERM), because it acts like an anti-estrogen in breast cells, but like an estrogen with agonistic effect in other tissues such as the uterus and the bones.

Steroidal Hormonal Contraceptives

The synthetic version of steroid hormone progesterone with activities similar to natural progesterone is referred to as progestins. The progestin (synthetic medication) alone or in combination with estrogens is widely used as hormonal birth control and menopausal hormone therapy. Progestogen-only pills act by preventing the release of eggs from the ovaries (ovulation) and thickening the cervical mucus and the lining of the uterus. Thickening of endometrium prevents sperm from reaching the egg. Yet another class of commonly used birth control pill involves combination pills of two hormones – estrogen and progestin (Fig. 4.3*j*).

Progesterone Antagonists as Abortifacients (Antiprogestogens)

These are class of drugs that prevent progesterone from exhibiting their pharmacological response in the body. The progesterone antagonists bind with the progesterone receptor (PR) and block the action of progesterone and thus find application as abortifacients/emergency contraceptives and in the treatment of uterine fibroids. Examples of antiprogestogens include the PR weak partial agonist/pure antagonist mifepristone (RU486; Fig. 4.3*i*).

Antiandrogens and Their Therapeutic Application

Androgens comprising testosterone and its active component dihydrotestosterone produced in testes are required for normal spermatogenesis and secondary sex characteristics. Besides, they have also been implicated in the proliferation of prostrate cells both normal and cancerous by binding with androgen receptors. The treatment for prostate cancer involves either decreasing the level of male hormone androgen or blocking the androgen receptors present on cancer cells. One such therapeutic approach involves use of anti-androgen drugs (Fig. 4.3*k*, *l*) such as flutamide (Eulexin), bicalutamide (Casodex), and nilutamide (Nilandron) that blocks the androgen receptors on cancer cells and in turn block the effect of androgens.

4.3.3 Peptide/Protein Endocrine Hormones as Endogenous Source for Drugs

In general, all peptide/protein hormones are produced and processed by the cytosolic organelles, ribosomes attached to endoplasmic reticulum and Golgi apparatus, and stored in secretory vesicles for export outside cells. They can be secreted by either of the two pathways:

1. **Regulated secretion:** The cell creates and stores hormone in secretory granules and releases them in 'bursts' following stimulation.
2. **Constitutive secretion:** The cell instead of storing hormone secretes it as and when synthesized.

These endogenous hormones (first messenger) being hydrophilic in nature exert their pharmacological effect by binding with receptors present on cell surface instead of diffusing through lipid bilayer cell membrane. Some of the major endocrine glands secreting peptide hormones include the pituitary gland, pancreas, thyroid gland, parathyroid gland, hypothalamus, etc.



Additional Information

Men need progesterone too. Notably, progesterone is also a precursor to the male sex hormone testosterone. The masculinity in males is regulated by female hormone progesterone which also aids to counteract the effects of estrogen on the male body. However, as men age, the testosterone levels begin to go down with consequent increase in the levels of estrogen and with sharp fall in progesterone levels.

4.3.3.1 Peptide Hormones Produced by Hypothalamus

There are numerous neurons in the hypothalamus responsible for releasing a variety of peptide/protein hormones.

1. Thyrotropin-releasing hormone (TRH)
2. Gonadotropin-releasing hormone (GnRH/LHRH)
3. Growth hormone-releasing hormone (GHRH)
4. Corticotropin-releasing hormone (CRH)
5. Somatostatin

All of these hormones following their direct release from the hypothalamus into the blood stream (typically released in periodic spurts) reach the anterior lobe of the pituitary to exert their effects. Although many peptide hormones released from hypothalamus have found clinical application, therapeutic application of one of the widely used peptide hormone GnRH has been described below.

Therapeutic Role of GnRH Analogues

Gonadotropin-releasing hormone, a decapeptide plays an important role in HPG axis (Fig. 4.4). It triggers synthesis and release of gonadotropins LH and FSH, which in turn regulates ovulation and corpus luteum maintenance in females and spermatogenesis in males. GnRH-derived analogues have found application both as an agonist and as an antagonist. Leuprolide, a potent nonapeptide amide analogue of GnRH with agonistic properties is used for the treatment of cancer in males (prostrate) and females (endometriosis). Owing to its agonistic properties, leuprolide initially triggers the secretion of FSH and LH by stimulating pituitary gonadotrophs. This is then followed by secretion of sex hormones (estrogen/testosterone) from gonads. This sudden surge in the concentration of sex hormones eventually inhibits pituitary gonad axis by the feedback loop. On the contrary, GnRH antagonist degarelix directly suppresses pituitary gonadal axis by blocking the secretion of LH and FSH. It is used in patients suffering with advanced hormone-sensitive prostate cancer where it causes reduction in the levels of testosterone.

4.3.3.2 Peptide Hormones Produced by the Pituitary Glands

The pituitary gland has two kinds of lobes called anterior and posterior pituitary. Each of the lobes has different types of cells and produces different kinds of endocrine hormones with different functions.

Anterior Pituitary

It releases the following hormones:

1. Adrenocorticotropic hormone (ACTH)
2. Thyroid-stimulating hormone (TSH)
3. Luteinising hormone (LH)
4. Follicle-stimulating hormone (FSH)
5. Prolactin (PRL)
6. Growth hormone (GH)
7. Melanocyte-stimulating hormone (MSH)

Posterior Pituitary

It releases the following hormones:

1. Anti-diuretic hormone (ADH) or vasopressin
2. Oxytocin

Although most of the peptide hormones released from pituitary have found clinical application either as therapeutics or diagnostics, therapeutic application of one of the widely used peptide hormone oxytocin.

Naturally, oxytocin is produced in hypothalamus; it is a nonapeptide with a disulfide bridge and has been used as drug (injectables) in its native form in the following clinical settings:

1. During labor induction, oxytocin promotes uterine contractions to induce labor or strengthen uterine contractions.
2. Oxytocin can be used to stop postpartum hemorrhage.
3. During an incomplete or threatened miscarriage, oxytocin can facilitate abortion by stimulating uterine contractions.
4. May influence social behavior, emotions, and love.[9]

4.3.3.3 Peptide Hormones Produced by Parathyroid and their Clinical Use

There are four parathyroid glands present as tiny little glands in the neck behind the thyroid glands. The protein hormone produced by these glands is called parathyroid hormone (PTH) and is released in response to the low levels of calcium in blood. In fact, the parathyroid glands keep round the clock watch on calcium levels in blood. Just like a thermostat, PTH maintains calcium levels within required physiological range in the body. Optimal levels of calcium in circulatory blood are crucial for the normal functioning of bones, heart, nervous system, and kidney.

A synthetic form of PTH (biologics) corresponding to the 1–34 N-terminal peptide of natural PTH has found clinical application under PTH therapy for osteoporosis. The synthetic PTH 1–34 named as teriparatide (Forteo) is used as injectables to treat patients who are at high risk of fractures, particularly postmenopausal women. The daily injection strengthens bones and reduces the risk of bone fracture.

Other Examples of Therapeutic Peptide/Protein Hormones

Based on the mechanism of action, clinically used biologics[10] can be grouped under two categories. One class of biologics acts as signaling molecules (first messengers) following their binding to receptors. They are used either in their natural form or designed as mimetics with agonistic properties. The second class of biologics does not act as first messenger; instead, it inhibits or activates enzymes involved in the regulation of cellular/physiological functions. A variety of peptide/protein biotherapeutics have been derived from endogenous hormones as lead molecules. Some examples have been given below:

1. **Salmon Calcitonin:** It is a 32 amino acid peptide (also produced in thyroid by humans) used clinically to treat osteoporosis (bones become weak and brittle) in postmenopausal women. It acts as first messenger and binds with GPCR calcitonin receptor located on osteoclasts, and triggers cAMP (second messenger)-mediated inhibition of bone resorption. It promotes deposition of Ca^{++} in bones and reduces the concentration of Ca^{++} in blood circulation.
2. **Sermorelin:** It is a 29 amino acid amide, a pharmacologically active fragment of GHRH. It is a polypeptide of 44 amino acids corresponding to the N-terminal region of GHRH that mimics natural hormone and binds GHRH receptors to trigger release of GH. It is used clinically for the treatment of children with GH impairment.
3. **Exenatide:** A 39 amino acid synthetic peptide is an enzyme-resistant analogue of glucagon-like peptide 1 (GLP-1) (also known as incretin mimetic agent) and is used in combination with other hypoglycemic drugs (metformin/sulfonylureas) to regulate sugar level in diabetic patients. It mimics natural GLP-1 and binds as first messenger to GLP-1-receptor present on β-cells in the pancreas and triggers release of insulin.

4. **Menotropins:** It consists of crude LH and FSH hormones extracted from the urine of menopausal women or can also be produced by recombinant DNA technology. It is used clinically for the treatment of female infertility. It binds with LH/hCG/FSH receptors in ovary and elicits its biological response.
5. **Bivalirudin:** A 20-amino acid direct thrombin inhibitor peptide (substrate) is used clinically as an anticoagulant. It is a synthetic congener derived from of the naturally occurring drug hirudin, a peptide present in the salivary glands of blood-sucking leeches with anticoagulant property. It binds with thrombin (a serine protease enzyme) in a unique manner and inhibits its action. The N-terminal region (D-Phe-Pro-Arg-Pro) of bivalirudin binds to the catalytic site of thrombin while the C-terminal region binds with the anion-binding exosite.
6. **Streptokinase:** Streptokinase isolated from streptococcal bacteria is a protein (enzyme)-based thrombolytic agents. It is used as drug to dissolve the fibrin matrix of the clot/thrombus in some cases of heart attack (myocardial infarction), pulmonary embolism, and arterial thromboembolism. It acts by binding with circulating plasminogen to form 1:1 complex which then activates another molecule of plasminogen to form the serine protease plasmin. The latter displays its thrombolytic action by dissolving the clot.
7. ***t*-Plasminogen Activator:** It is also a serine protease present in the endothelial cells lining the wall of blood vessels. It acts by activating plasminogen into plasmin responsible for dissolving clot. It is used as medication for the treatment of ischemic stroke and has to be administered within 90 minutes of onset of symptom.

4.4 Monoclonal Antibody Therapy

Traditionally, the term antibodies meant proteins that can fight infections by neutralizing the antigens. The traditional antibodies are polyclonal and are produced by different clones of plasma B cells, and they bind to the different epitopes in the same antigen. mAbs or moAbs are produced from a single B-cell (immune cell) clone and bind to a unique epitope (binding site) in a single antigen (monovalent affinity). Generally, mAbs bind with functionally important cell-surface proteins such as receptors or other proteins present on the surface of normal and cancer cells that influence cellular activity. The advantages of mAbs over polyclonal antibodies are their stability, purity, homogeneity, and consistency.

The traditional process for the production ofmAb involves generation of hybridomas (mAb-producing cells) by fusing desired antibody-producing splenocytes (e.g. B cells) of mice origin with myeloma cells. There are four types of mAbs based on their origin, which are differentiated by the suffix in their name:

1. **mAbs with suffix –*omab*:** These mAbs are derived entirely from a murine source. However, they can lead to allergic reactions in humans.
2. **mAbs with suffix –*ximab*:** They are chimeric in nature and comprise the variable regions of murine joined with the constant regions from human. However, these too may cause an allergy. FDA-approved basili**ximab** (Simulect) is an immunosuppressant mAb used clinically to prevent organ rejection in people who have received a kidney transplant. Another FDA-approved drug is Abciximab that is used as a type of 'blood thinner' to prevent blood clots.
3. **mAbs with suffix –*zumab*:** They are mostly derived from a human source except for the part of the antibody, which binds to its target. FDA-approved mAb alemtu**zumab** (Campath) is used clinically for the treatment of certain type of leukaemia and multiple sclerosis.

4. **mAbs with suffix –*umab*:** These mAbs are derived in totality from human source. FDA approved mAbs of human origin include belim**umab** (Benlysta), bezlotox**umab** (Zinplava), and canakin**umab** (Ilaris).

4.4.1 Monoclonal Antibodies in Diagnostics

mAbs can also be used as diagnostic tool, for example determining pregnancy and HIV infection. The objective is to detect the presence of particular protein content in bloodstream/urine that results from pathogenesis of a disease or due to a biological change(s) in the organism. The pregnancy is tested by checking the presence of human chorionic gonadotrophin (hCG) in urine using its corresponding mAb. hCG is a hormone (protein) normally produced by women during fetal development, and thus its presence in the urine has been used to establish pregnancy.

4.4.2 Treating Disease at the RNA Level with Oligonucleotides

The objective of this treatment is to prevent the formation of non-functional proteins causing the disease at the genetic level. The formation of functional proteins in cells is generally regulated by DNA which specifies the encoded sequence of mRNA molecules followed by production proteins with specific sequence. There are two major steps involved during the journey from gene to protein: transcription and translation. Synthetic oligonucleotide fragments have been used as drugs to interfere with the production of faulty proteins during the translation of genes to disease-related proteins. The therapeutic oligonucleotides are generally made up of 12 to 30 nucleotides and are designed in a manner so that it can bind to a specific region in messenger RNA (mRNA) responsible for the production of non-functional protein. Following parenteral administration, the oligonucleotide drug enters the cell, binds to the complementary mRNA, and triggers its degradation, thus blocking production of the disease-related proteins.

4.5 Drugs of Microbial Origin

Microorganisms such as actinomycetes (intermediate between filamentous fungi and bacteria), fungi, and bacteria have remained a source for life-saving drugs/leads. They have been primarily used as antibiotics, anticancer agents, and immunosuppressants.

4.5.1 Drugs Isolated from Actinomycetes

1. **Streptomycin:** Isolated from *Streptomyces griseus.*
2. **Chloramphenicol:** Isolated from *Streptomyces Venezuela.*
3. **Aureomycin:** Isolated from *Streptomyces aureofaciens*.
4. **Rifamycin:** Isolated from *Amycolatopsis mediterranei.*
5. **Terramycin:** Isolated from *Streptomyces rimosus*.

4.5.2 Drugs Isolated from Fungi

1. **Lipid Lowering Lovastatin:** Isolated from *Aspergillus terreus*
2. **Penicillin:** Isolated from *Penicillium notatum*
3. **Cephalosporins:** Isolated from *Cephalosporium acremonium*
4. **Cyclosporine:** It is a naturally occurring fungal peptide isolated from *Tolypocladium inflatum Gams.* It is a lipophilic (logP = 2.3) cyclic undecapeptide, which belongs to a class of drugs known as

immunosuppressants. The peptide is used clinically as organ transplant drug to prevent rejection of the newly transplanted organs. It acts by binding to the intracellular receptor cyclophilin-1 followed by inhibition of calcineurin (a serine/threonine protein phosphatase) which in turn regulates intracellular signaling enzymes and prevents rejection of organs.

4.5.3 Drugs Isolated from Bacteria

1. **Gramicidin:** Cyclic undecapeptides isolated from *Aneurinibacillus*.
2. **Polymyxin:** Isolated from *Paenibacillus polymyxa*.

4.6 Traditional Methods as a Source for Hit Identification (Exogenous Sources)

Ethnobotany, which deals with traditional knowledge about plants for the purpose of therapeutic use and practiced by communities with different ethnic background has been a major source for lead compounds/drugs. The communities, in general, use plant extracts or concoctions for treating diseases. This inspired researchers to navigate the chemical space in the domain of natural products and even today remains one of the most successful and powerful approaches for the identification of hit molecules. The traditional approach involves screening of natural compounds both as crude extracts and as purified compounds for the activity (trial and error testing) in cultured cells or animal models. In fact, the natural product research deals with extraction, isolation, and finally elucidation of pure structures. Once a potential compound in the form of either 'hit' or 'lead' is identified, lead optimization studiesare carried out by synthesizing a series of congeners with the view to develop clinically useful candidates. Indeed, the screening has although produced a vast majority of existing drugs used in clinical settings, certain drawbacks including environmental issues made researchers to look for alternative strategies as well.

4.6.1 Paclitaxel (Taxol)

The discovery of paclitaxel as a natural product for treating variety of cancers involved a long journey of approximately 30 years from laboratory to clinics. It started with the screening of numerous extracts of natural products for antineoplastic activity. During this screening, a crude extract of the bark of the Pacific yew, *Taxus brevifolia* exhibited cytotoxic activity against cancer cell lines. Isolation and characterization of active component from the extract resulted in the identification of paclitaxel. It is used clinically for the treatment of a variety of cancers: ovary, lungs breast, cervical, etc.

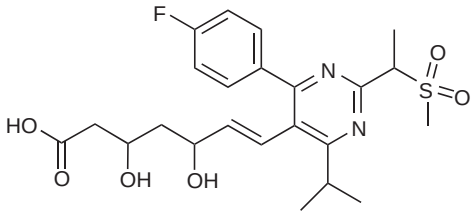
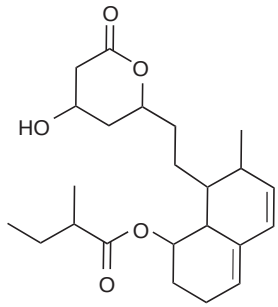
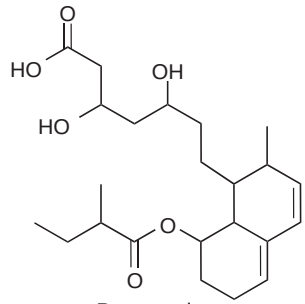
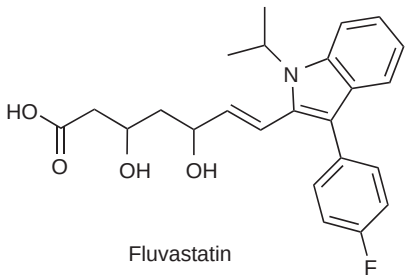
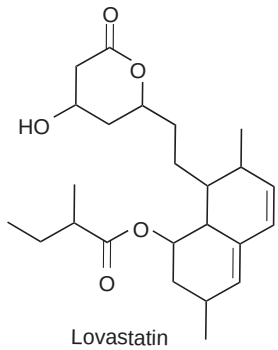
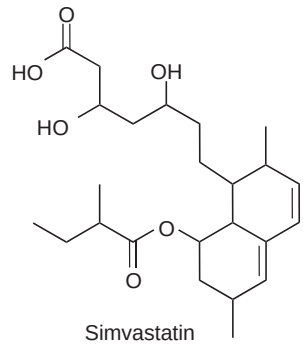
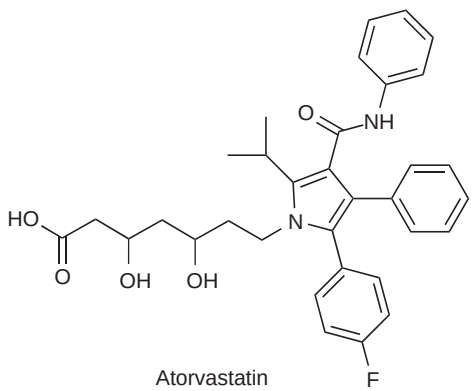
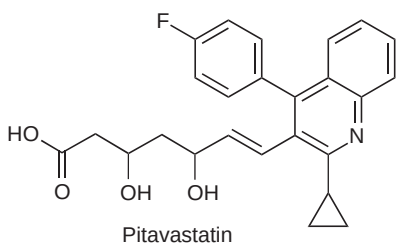
CASE STUDY 1

There are several statins used clinically for the treatment of hypercholesterolemia, reducing LDL cholesterol levels. Originally, statins (Table 4.2) were derived from natural sources; however, later synthetic variants of natural statin and synthetic statins were also introduced in the market. Lovastatin and compactin are the two naturally occurring statins isolated from *Aspergillus terreus* and *Penicillium citrinum*, respectively. Simvastatin and pravastatin are variants of natural statins and remaining four are synthetic statin-based ('Me too') drugs: fluvastatin, atorvastatin, rosuvastatin, and pitavastatin.[11]

(*Continued*)

Study 1 (*Continued*)

Table 4.2 Clinically used statins

Synthetic Compounds	Natural Compounds	Semisynthetic Compounds
 Rosuvastatin	 Compactin	 Pravastatin
 Fluvastatin	 Lovastatin	 Simvastatin
 Atorvastatin		
 Pitavastatin		

Compactin[12]	IC50 23 nM
Lovastatin	IC50 3.4 nM
Simvastatin	IC50 11 nM
Pravastatin	IC50 5.6 nM
Rosuvastatin	IC50 5.4 nM
Fluvastatin	IC50 28 nM
Atrovastatin	IC50 8.2 nM
Pitavastatin	IC50 5.8 nM

All statins compete with HMG-CoA an endogenous substrate and binds to HMGR to block its enzymatic activity.

CASE STUDY 2

Discovery of Metformin (An Antidiabetic Drug) from Plants

French plant lilac *Galega officinalis* extracts were used to treat painful and frequent urination in diabetic patients. Researchers isolated the active constituent galegine (I; Fig. 4.5) that was put to use in the 1920s but was withdrawn due to toxicity. Structural modifications in the galegine (I) led to the identification of diguanidines derivative synthalin (II) and was approved for clinical use. However, due to severe toxicity issues II was yet again withdrawn in 1940s. Subsequent lead optimization studies of II led to the identification of antidiabetic drug metformin (Fig. 4.5).

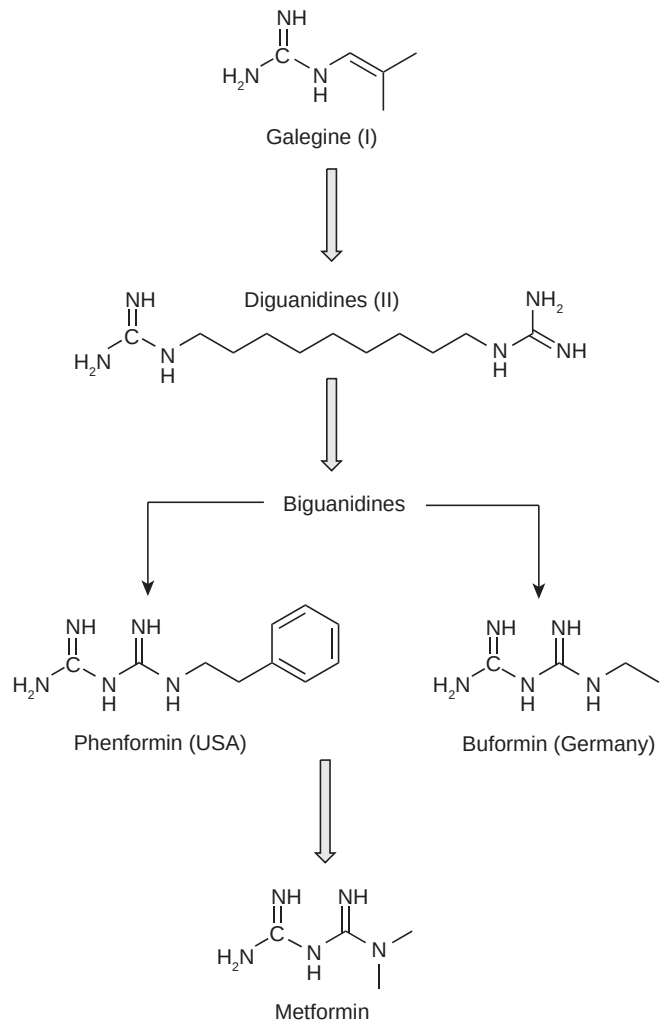


Figure 4.5 From galegine to diguanidines to metformin.

4.7 Target-Based Screening as a Rationalized Approach for the Identification of Hit/Lead Molecules

The vision of rational drug design came into existence through the understanding that drugs exhibit their pharmacological activity following their binding with the active site present either in the receptor or an enzyme. Thus, in the rational drug design approach, chemical libraries of high quality are screened *in vitro* against validated therapeutic targets. Alternatively, compounds can be screened against therapeutic protein targets using NMR or X-ray techniques to find a hit that can bind to the active site in the protein targets. Since the approach involves screening of compounds against a purified and validated therapeutic target, the selection and quality of chemical libraries play an important role in the success of the strategy.

4.7.1 Quality of Chemical Libraries

Identification of hit molecules depends on the quality of compounds used for screening against the targets. This is because biological activities exhibited by compounds in general are regulated by the physicochemical properties of test compounds. In fact, a direct relationship has been established between drug-like properties and physicochemical properties of test compounds such as solubility, lipophilicity, pK_a, molecular weight, etc. Thus, it is recommended that only test compounds with drug-like properties in a library are submitted for screening against targets.

4.7.1.1 Chemical Libraries with Drug-like Properties

Among many methods for identifying a hit/lead molecule, screening of chemical libraries comprising compounds with drug-like features is emerging as a good option. However, a poorly designed library lacking sufficient diversity and drug-likeness may not result in hit molecules. In fact, before screening, compounds with drug-like features need to be separated from compounds with undesirable molecular features using computational software-driven filters. Thus, two important parameters for a good chemical library should be collections of chemically pure compounds (99%) with drug-like properties and chemical diversity. Some of the selection criteria used for the purpose of filtration of drug-like properties in a chemical library involve molecular weight (MW), lipophilicity (logP and logD), hydrogen bond donor/acceptor (HBA/HBD) polar surface area (PSA), Lipinski guidelines, etc. Filtration of chemical library also involves removal of compounds in a library that are unlikely to progress beyond preliminary drug development stage due to the presence of a reactive group. Compounds with reactive groups are prone to yield false-positive activity due to their ability to bind non-specifically to proteins. The filtration process of chemical libraries (also called databases of compounds) is generally carried out using *in-silico* (computational tools) approach by applying Lipinski rule of 5. Lipinski's rule states that, in general, an orally active drug has no more than one violation of the following criteria: not more than 5 hydrogen bond donors, not more than 10 hydrogen bond acceptor, a molecular mass less than 500 Daltons and an octanol-water partition coefficient log P not greater than 5.

4.7.1.2 Focused Libraries of Compounds as a Source for Hits

A medicinal chemistry programme against a disease requires identification and validation of disease therapeutic protein (receptor/enzyme) targets and a high-quality rationalized chemical structure that can modulate the activity of the protein target. When the 3D structural information (including X-ray structure) of the therapeutic protein target or target family is known, therapeutic protein-based focused libraries can be designed based upon the structure and function of the target protein of interest. These focused libraries

Table 4.3 Examples of focused libraries

Focused Library	Screening
GPCR library	Screened against GPCR receptors to identify hit molecules binding to the orthosteric site.
Allosteric GPCR library	Screened against GPCR receptors to identify hit molecules binding to the allosteric site.
Kinase library	Screened against enzyme kinases to identify hit molecules binding to the active site.
Allosteric kinase library	Screened against enzyme kinases to identify hit molecules binding to the allosteric site.
CNS library	The compounds in this library have physiochemical properties that allow hit molecules to diffuse through BBB.
RNA targeted library	Screened against RNA to identify hit molecules (For undruggable therapeutic proteins, it is recommended to target the RNA responsible for the production of therapeutic protein).

comprise few hundred/thousand tailor-made collections of compounds with bias for a particular protein target. The library can then be screened against the protein target *in vitro* or using *in-silico* approach for the identification of hit/lead molecule. Examples of some focused libraries comprising small molecules have been summarized in Table 4.3.

Commercially Available Focused Chemical Libraries

There are several vendors who sell focused chemical libraries for drug development, which are collections of compounds that either are based on drug-like features or have affinity for a specific therapeutic target. Following are examples of some focused commercial libraries:

1. **Maybridge Library of Compounds with Drug-likeness:** The focused library also called Hit FinderTM Collection comprises 14,400 premier compounds with >90% purity. Each compound in this library has drug-like properties as per Lipinski rules (rule of five).
2. **ChemBridge Library of Compounds with Drug-likeness:** The focused library comprises a huge collection of 10,000 structurally diverse, drug-like, low-molecular-weight small organic molecules.
3. **FDA-Approved Drug Library of Compounds with Drug-likeness (Prestwick Chemical):** This focused library contains 1200 small molecules, all of which are marketed drugs. The library can be used under repositioning/repurposing of drugs approach. The library comprises collection of clinically used drugs with significant structural and pharmacological diversity. This library is sold either in DMSO solution or in the powder form. Finding a preclinical candidate from this library will not only reduce the cost of development but can bypass phase I clinical trials and can directly enter phase II clinical trials once the activity has been established.
4. **Libraries of Fragments:** There are several focused libraries based on fragments sold by vendors. These libraries find application in fragment-based drug discovery approach using NMR or X-ray

spectroscopy. All fragments in these libraries are based on rule of three criteria. Rule of three criteria for fragments involve molecular weight < 300, the number of hydrogen bond donors ≤ 3, the number of hydrogen bond acceptors ≤ 3, and the clogP ≤ 3. Some of the suppliers of fragment-based libraries are Zenobia therapeutics, Maybridge, UK, and ChemBridge, USA.

Libraries Based on Privileged Structures

In 1980, Evans for the first time introduced the term 'privileged structure', based on his observation of diverse biological actives being exhibited by a single class of scaffold 1, 4-benzodiazepine-2-one. Thus, privileged structures[13] can be defined as 'a single molecular framework able to provide substrates/ligands against diverse enzymes/receptors' with differential activities. This also explains as to why some chemical libraries of organic molecules invariably furnished significantly higher hit rates than other libraries. Examples of some privileged heterocyclic structures have been depicted in Fig. 4.6.

Natural products too exhibit 'privileged structure' characteristics, with one scaffold being present in many natural products and associated with diverse biological activities. An example in this class is naturally occurring benzopyran scaffold, since it has been found to be present in more than 4000 structurally diverse structures isolated either naturally or introduced in synthetic compounds. Diverse pharmacological activities reflected by benzopyran-based compounds suggest that these scaffolds can interact with both extracellular as well as intracellular therapeutic targets. Recently, a third generation of benzopyran-based compounds has been found to be associated with anticancer activity.[14] Indole is yet another class of naturally occurring privileged scaffold that has been found to be present in four naturally occurring drugs with diverse therapeutic activities (Fig. 4.7). Other examples of drugs[15] based on a privileged structure triazolonapththyridines and with diverse biological activities have been depicted in Fig. 4.7. Thus, libraries based on privileged structures can be successfully screened against a host of therapeutic targets to identify hit molecules.

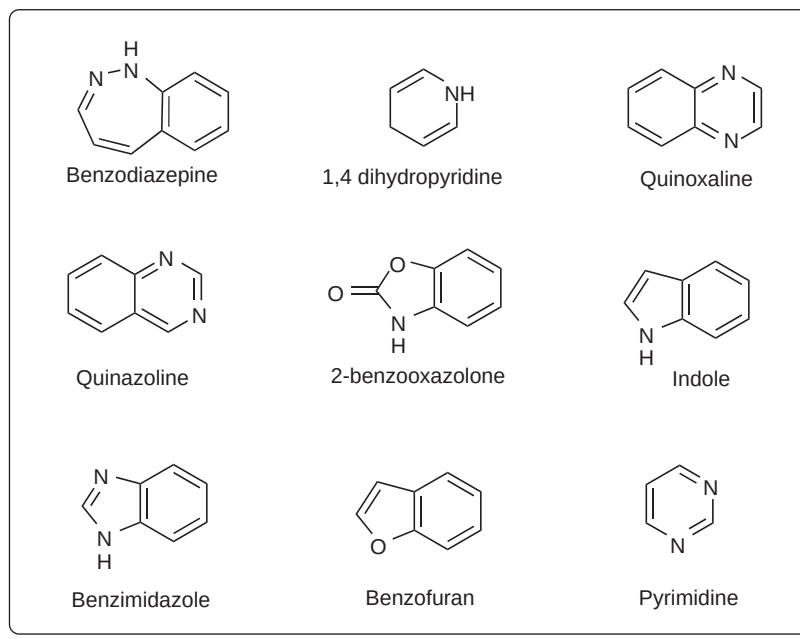


Figure 4.6 Synthetic privileged structures based on heterocycles.

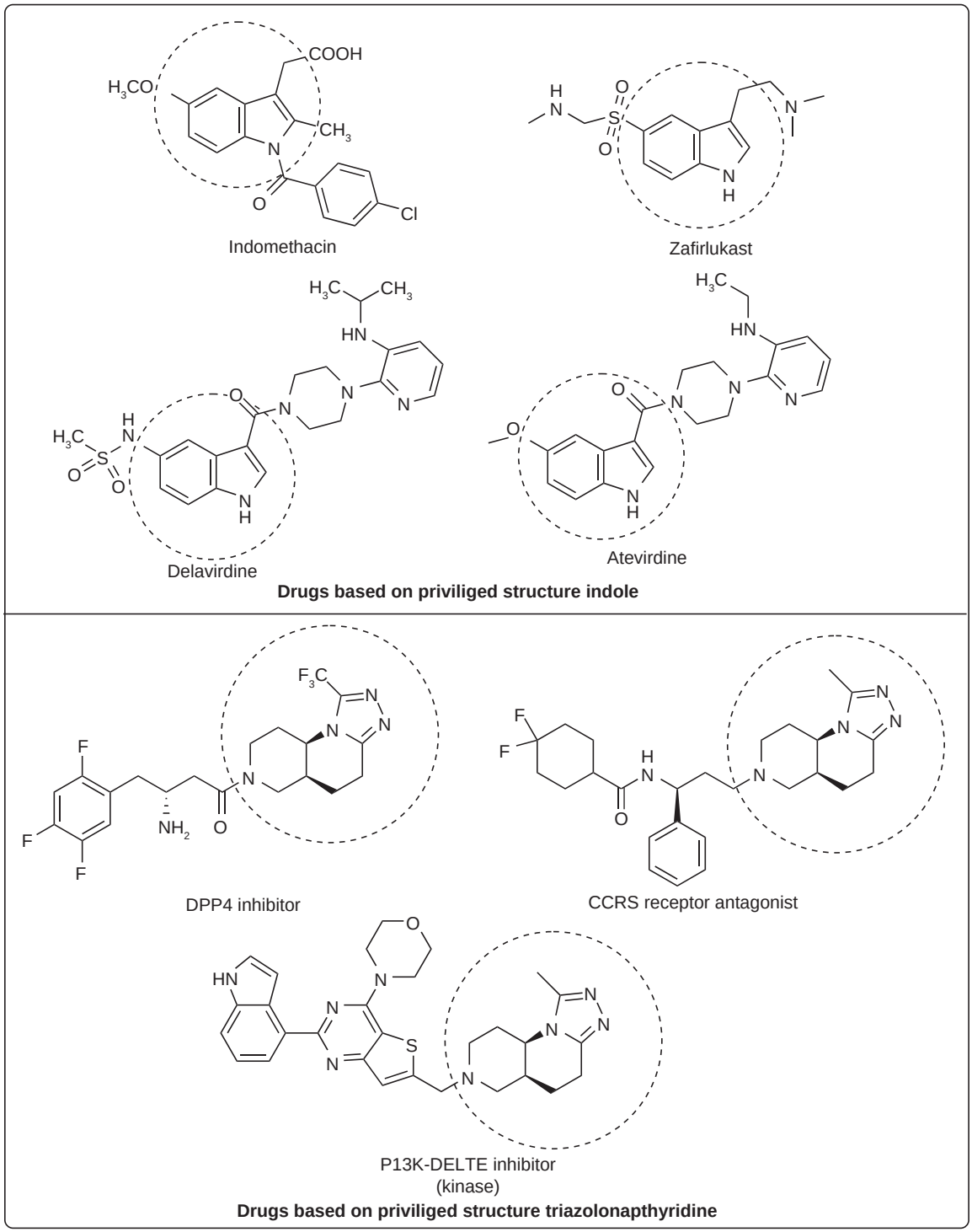


Figure 4.7 Drugs based on privileged structures.

4.7.2 Fragment-Based Drug Discovery for Identifying Hits

This has emerged as a revolutionary tool for *discovering drug*s against validated therapeutic targets using X-ray[16] and NMR spectroscopy.[17] It commences with the identification of moieties of low-molecular-weight fragment as a starting point or hit against a protein target. Attempts are made to dock this fragment or their derivatives in one of the pockets (subsites) in the binding site of the target with full occupancy. Although initially, the fragment may exhibit weak binding affinity, refinement via structural modifications of the fragment furnishes a better fit. This results in the increase in the binding affinity till the fragment completely occupies the subsite. Next, similar efforts are made to identify a second fragment which completely occupies the second binding pocket. Once both the fragments that fit in the pockets have been identified, they are then covalently joined together to get a lead compound which when screened against the said target exhibits potent activity in nM range (Fig. 4.8).

NMR methods are capable of robustly detecting the binding of fragments as test compounds to macromolecular protein targets over an extraordinarily broad affinity range.[18] The NMR-technique can also be used to

1. Validate the initial hit identified through *in vitro* screening.
2. Derive structural information about the compound–protein complex, which in turn may assist in lead optimization studies (Fig. 4.8).[19]

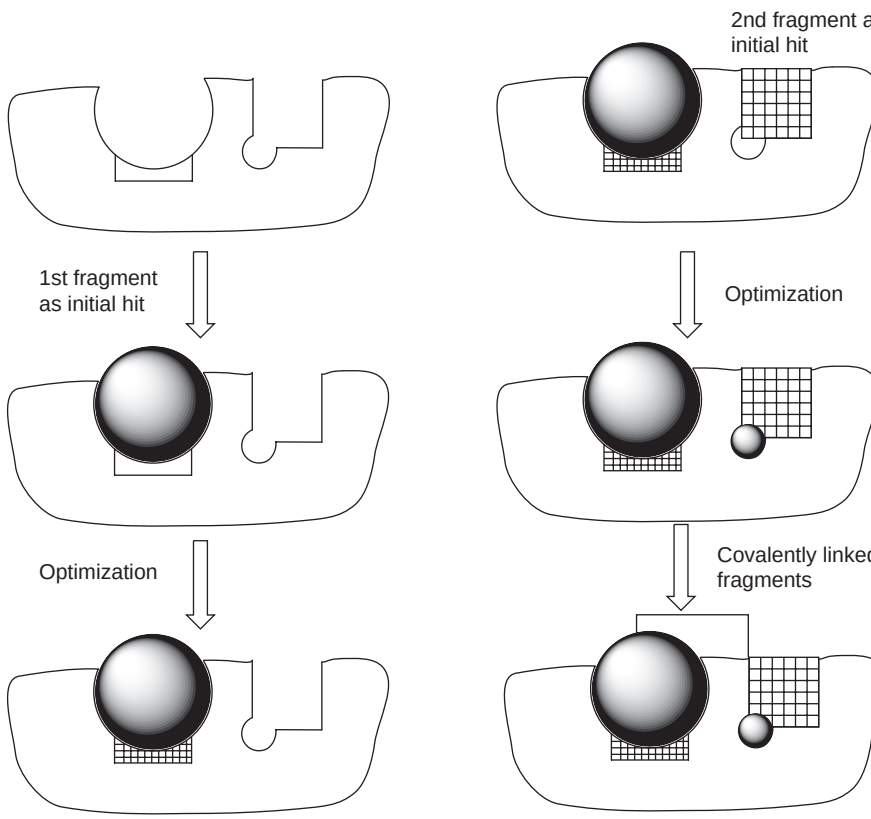


Figure 4.8 A diagrammatic representation of fragment-based drug discovery.

CASE STUDY 3

FBDD

Researchers have carried out NMR-based screening of test compounds against matrix metalloproteinase enzyme with the view to design inhibitors of the enzyme.[20] Matrix metalloproteinases (MMPs) are endopeptidases that degrade both matrix and nonmatrix proteins in the extracellular environment of tissues such as extracellular matrix (ECM). Thus, it plays an important role in cell proliferation, apoptosis, and angiogenesis. Deregulation of MMPs causes pathological conditions leading to arthritis, cancer, and ulcers, and MMP inhibitors exhibit great therapeutic promise.

The FBDD approach commences with the identification of starting pointscomprising low-molecular-weight fragments (substrates) that are about half the molecular weight generally recommended by Lipinski rule, i.e. ~500 Da. These fragments are then screened for their ability to bind with the different pockets in the active site(s) of the enzyme metalloproteinase. The initial hit(s) so obtained are then optimized (expanded) till the fragments fully occupy the active site(s) present in the enzyme. These active low-molecular-weight *fragments* are then covalently joined to get a lead molecule with IC_{50} value of 15 nM (Fig. 4.9). In yet another study, vemurafenib, a kinase inhibitor, was developed using a fragment-based drug discovery strategy for the treatment of melanoma. It is the first marketed drug developed using FBDD[21] (translation from hit to FDA-approved drug) approach.

Application of X-ray in FBDD

In this strategy, crystals of therapeutic proteins are soaked with a cocktail of 3–10 low-molecular-weight fragments. Next, the structure of fragment specifically bound to the target is established by electron density map obtained from X-ray analysis of fragment-bound protein crystal. A set of approximately 100 diffractions can be carried out to screen a library of fragments comprising of few hundred compounds. After the completion of experiment, protein crystal-bound fragments can be expanded into lead molecules.

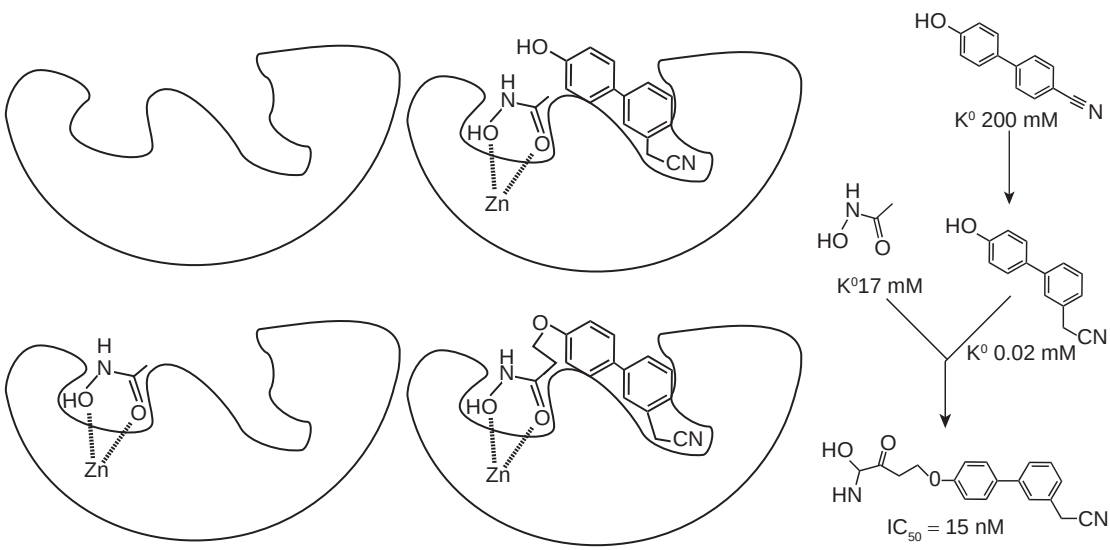


Figure 4.9 A diagrammatic representation of fragment-based drug discovery against matrix metalloproteinase.

4.7.3 Virtual Screening for Identifying Hits

This is also referred to as computer-aided drug design (CADD) approach and provides numerous options for searching hits. Virtual screening involves *in-silico* screening of virtual library comprising 3D structures of test compounds against a 3D structure of therapeutic target. In absence of the availability of 3D structure of therapeutic target, other methods involving similarity search of ligands or pharmacophore matching are applied.

4.7.3.1 Virtual Libraries of Compounds

The library comprises collection of several thousand chemical structures in 3D format generated using computational software and is called virtual library. Screening of these virtual libraries is generally carried out using *in-silico* screening methods; however, if compounds are available, they can be subjected to *in vitro* biological assays. However, before screening, the virtual libraries are filtered for structures with drug-like properties using computational software. Some applications (Fig. 4.10) of virtual libraries in hit identification have been summarized below:

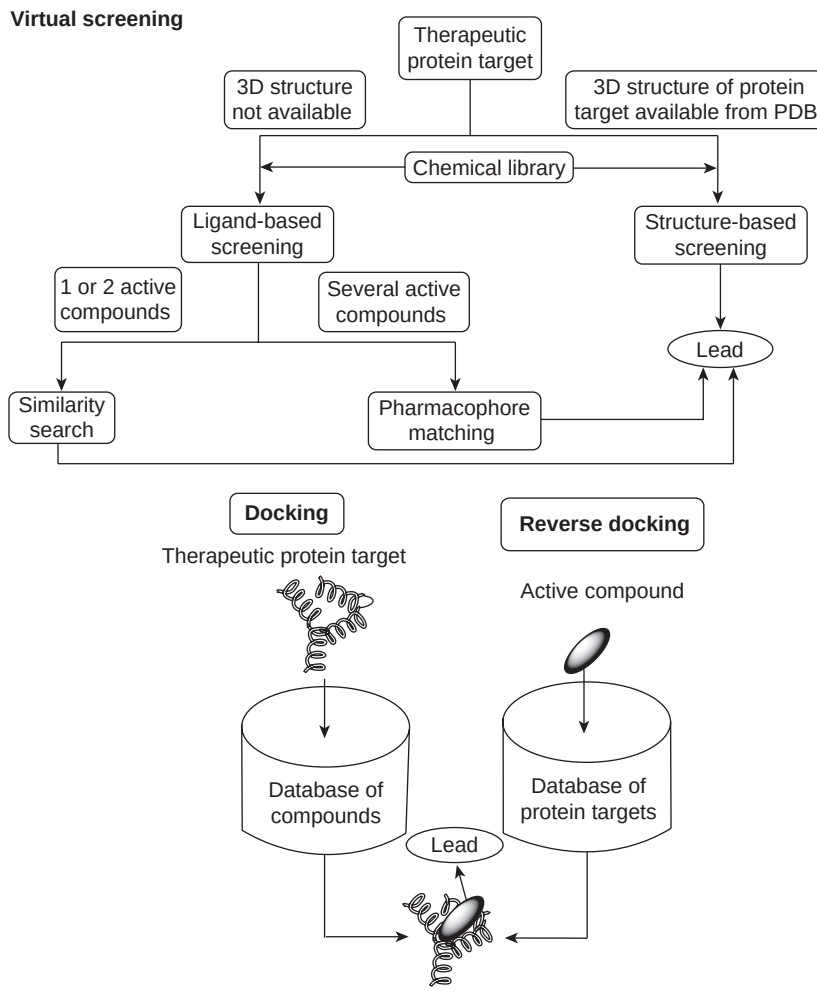


Figure 4.10 *In-silico* or virtual screening.

4.7.3.2 Structure-Based Virtual Screening

This involves *in silico* screening of drug-like chemical library of compounds against a validated protein target(s) by computational methods (Fig. 4.10). It represents a fast and cost-effective tool for the novel hits/leads.[22] The *in-silico* screening method commences with the downloading of 3D structure of a protein target from protein data bank (PDB) followed by search for small molecules that can bind (dock) with the active site of the protein target, referred to as docking. The extent of binding/docking is established by assigning score to each compound that are designed to predict the biological activity through the evaluation of interactions between test compounds and the protein target. At the end of the *in-silico* screening, the compounds with highest scores are experimentally screened *in vitro* (wet screening) against the same protein target to identify prospective hit candidates.

4.7.3.3 Ligand-Based Virtual Screening

This technique[23] is used when information about 3D structure of the desired protein target is not known. In this situation, a set of active and inactive molecules is used to generate information about the pharmacophore responsible for the binding and pharmacological response. There are two different strategies used to carry out ligand-based virtual screening:

1. **Development of a Pharmacophore Model as Query for Virtual Screening:** Using a set of structurally diverse compounds that exhibits biological activity against a target, a hypothetical 3D model of the target can be created by putting the information together derived from the above set of active compounds. This 3D model also known as pharmacophore model specifies the spatial relationship between the groups which may be involved in binding with the target. The 3D pharmacophore model so generated can be used as query for retrieving potential leads (pharmacophore matching) from structural databases of chemical compounds for the identification of hits.
2. **Similarity Search-Based Virtual Screening:** It is a popular approach wherein 2D or 3D shape of a pharmacologically active molecule (in the absence of a target) is subjected to similarity search by screening a database of test molecules. Using computational tools, test compounds are allowed to overlap 2D or 3D shape of the active molecules and compounds exhibiting high order of overlapping are subjected to *in vitro* assays (Fig. 4.10).

4.7.3.4 Reverse Docking

Reverse or inverse docking[24] is yet another powerful tool in CADD. The technique (Fig. 4.10) involves docking of a small molecule with established therapeutic efficacy. The docking experiments are carried out with a set of therapeutically relevant validated macromolecular protein targets such as G-protein-coupled receptors or kinases, etc. This is then followed by ranking of protein targets based on the tightness of their binding with the active compound. The method is particularly useful for identifying novel molecular targets for an active compound with the view to either identifying its new application in treating diseases or establishing mechanism of action.



Additional Information

Definition of Pharmacophore

IUPAC defines a pharmacophore *to be 'an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular (noncovalent) interactions with a specific biological target and to trigger (or block) its biological response'. The noncovalent interaction types can be better understood by looking into the 3D structures of both bioactive molecule and the therapeutic protein target. In other words, pharmacophore can be either whole molecule or a part of a molecular structure that binds via noncovalent interactions with the active site in protein target and is responsible for a particular biological or pharmacological response.*

4.7.4 Phenotypic Screening for Identifying Hits

Before the emergence of target-based drug discovery, phenotypic screening strategies remained the cornerstone of drug discovery. Historically, cells, isolated tissues (*in vitro*), or animal models (*in vivo*) were used in phenotypic screening as it provided a direct relevance with physiological functions but had nothing to do with targets or mechanism of action. In the 19th century, a majority of drugs were discovered using phenotype-based drug discovery.[25] Screening of test compounds in animal models provides information by combining cell signaling, cellular functions, physiological consequences, safety, and toxicity. However, the specificity of the drug for the desired target and indeed the mechanism of action in phenotype-based screening remains elusive. Nonetheless, integrating *in vitro* validated target-based screening followed by phenotype-based screening may speed up identification of preclinical candidate. In the last two decades, with the discovery of genome sequence followed by identification of several therapeutic targets, phenotype-based screening went out of favor as target-based screen-dominated drug discovery. However, re-emergence[26] of phenotype-based screen can be seen particularly in view of concepts involving repositioning of drugs and emergence of model organisms (*C. elegans*, zebrafish, *Xenopus laevis*, and *Drosophila melanogaster*). These model organisms bear strong resemblance in their genomes with humans. Screening of test compounds against embryos of these model organisms can be carried out in high-throughput mode.

4.7.5 Repositioning of Drugs for Identifying Hits

It is also referred to as repurposing, mean searching for a new therapeutic use for a drug which is either in clinical use or that failed in clinical trials. In either situation, we have the whole dossier about the drug's efficacy, toxicity, DMPK profiles, etc., available at our disposal. Thus, this may reduce the time frame of drug discovery from 12−15 years to 3−5 years with significant saving in R&D expenditures coupled with reduced risk for failures. Although drugs discovered in yesteryears through serendipity may have analogy with repurposing, significant difference exists between serendipity and repurposing. In fact, repurposing approach is more rationalized as it can be combined with CADD and screening of compounds in model organisms. In contrast, serendipity is more of a nonrationalized approach and is primarily based on mere observations. Aspirin is a classic example of repositioning as it has found several new clinical applications.

4.7.5.1 Wonder Drug–Aspirin

Aspirin, or acetylsalicylic acid (ASA), a century-old drug, has been widely used as a pain reliever for minor aches and pains, and to reduce fever. However, over the period of time, aspirin has been repurposed for indications summarized below:

1. Taking aspirin daily may cut odds of developing the polyps that can lead to colorectal cancer, according to a new study.
2. Consumption of aspirin on daily basis can minimize the risk of heart attack.
3. Consumption can reduce stomach cancer risk.
4. Aspirin combats liver problem.
5. Daily consumption can reduce mortality in older women.

4.8 Difference Between Fragments, Hits, and Leads

The various approaches discussed above for drug discovery involved use of fragments, hits, and leads (Discussed in Chapter 5). These three prototypes in the initial stages of drug discovery lay foundation for

the discovery of preclinical candidates. They differ in their physicochemical properties as well as in their biological response, as depicted in Fig. 4.11.

Fragments are initial hits with low molecular weights ranging from 100–300 Da and may exhibit weak *in vitro* biological response (1mM to 100 μM). They generally obey the rule of three (discussed in Chapter 6); in contrast conventional hit/lead molecules obey the rule of 5 and have slightly higher molecular weights, ranging from 250 to 600 Da and may exhibit moderate *in vitro* biological response (100 μM–1 nM). A comparison between fragments and hit/lead molecules has been described in Table 4.4. Lead optimization studies with fragment as hits and conventional hits are carried out with the view to improve the *in vitro* biological response to nM level.

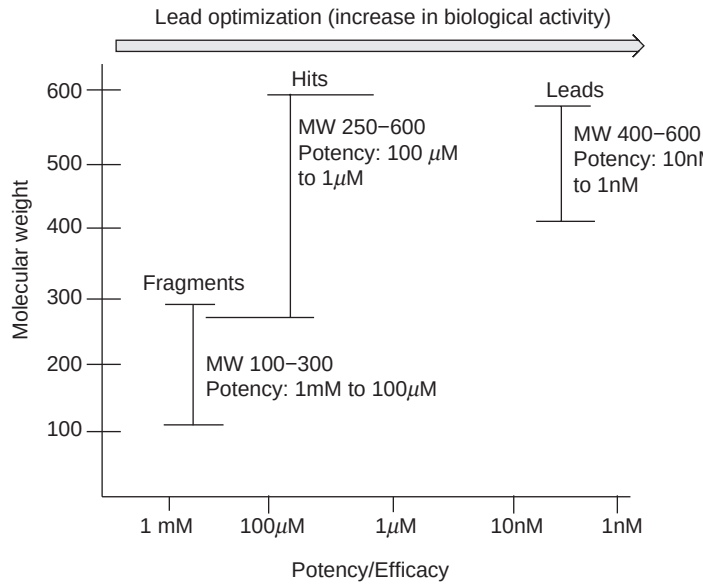


Figure 4.11 From fragments to hits to leads.

Table 4.4 Difference between fragments and hits/leads

Features of Fragments[27]	Features of Hits/Leads
They generally follow rule of three	They generally follow rule of 5
Molecular weight: 100 to 300	Molecular weight: 250 to 600
In vitro biological response: 1mM to 100 μM	*In vitro* biological response: 100 μM to 1nM
H-bond donors less than 3	H-bond donors less than 5
H-bond acceptors less than 3	H-bond acceptors less than 10
clogP less than 3 (a measure of lipophilicity of a compound)	clogP less than 5 (a measure of lipophilicity of a compound)
Polar surface area less than 60Å^2 (a measure of permeability through the cell membrane)	Polar surface area less than 140 Å^2 (a measure of permeability through the cell membrane)
Transformed into hits/leads by adding more fragment(s)	Preliminary *in vitro* ADMET assays

Exercises

1. Define a "hit" molecule and its characteristics.
2. Describe serendipity with two examples.
3. Discuss steroid hormone and their therapeutic applications.
4. Describe role of peptidyl and steroidal hormones in hypothalamic-pituitary-gonadal axis.
5. Describe effect of pK_a of drugs on bioavailability by oral route.
6. Discuss role of therapeutic hormones and peptides as signaling molecules.
7. Discuss fragment-based drug discovery.
8. Write in brief:
   - **(i)** Pharmacophore
   - **(ii)** Biologics
   - **(iii)** Structure based virtual screening
   - **(iv)** Privileged structures
   - **(v)** Library of compounds with Drug-likeness
   - **(vi)** Repositioning of drugs
   - **(vii)** PTH therapy
   - **(ix)** Dual role of estrogen in shutting down testosterone concentration in males

Competitive Exam Questions

1. Which one of the followings is the most likely positive sign of pregnancy when detected in urine?
   - **(a)** Estrogens
   - **(b)** Progesterone
   - **(c)** Human Chorionic Gonadotropin (HCG)
   - **(d)** Corticotropic Hormone

   [***GPAT 2012***]

2. Identify hormone that stimulates production in testis and ovulation in females.
   - **(a)** Prolactin
   - **(b)** Luteinizing hormone
   - **(c)** Follicle stimulating hormone
   - **(d)** Adrenocorticotropic hormone

   [***GPAT 2018***]

References

1. Gyo rgy M. Keseru and Gergely M. Makara, *Drug Discovery Today*, **2006**, *11*, 741.
2. Tudor I. Oprea, Tharun Kumar Allu, Dan C. Fara, Ramona F. Rad, Lili Ostopovici and Cristian G. Bologa, *J. Comput. Aided Mol. Des.*, **2007**, *21*, 113.
3. Robert F. Bruns and Ian A. Watson, *J. Med. Chem.*, **2012**, *55*, 9763.
4. Mouhssen Lahlou, *Pharmacology and Pharmacy*, **2013**, *4*, 17.
5. Ban T. A., *Dialogues Clin. Neurosci.*, **2006**, *8*, 335.
6. Leucht S., Wahlbeck K., Hamann J., and Kissling W., *Lancet*, **2003**, *10*, 1581.
7. Olsen E. A., Whiting D., Bergfeld W., Miller J., Hordinsky M., Wanser R., Zhang P., and Kohut B., *J. Am. Acad. Dermatol.*, **2007**, *57*, 767.

8. https://pdf.hres.ca/dpd_pm/00047189.PDF.

9. Inna Schneiderman, Orna Zagoory-Sharon, James F. Leckman, and Ruth Feldman, *Psychoneuroendocrinology*, **2012**, *37*, 1277.

10. Jolene L. Lau and Michael K. Dunn, *Bioorg. and Med. Chem.*, **2018**, *26*, 2700.

11. Akira, ENDO, *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.,* **2010**, *86*, 484.

12. Jamsem and Mckenney, *Clin. Cardiol.*, **2003**, *26* (Suppl. III), III-32–III-38.

13. Petra Schneider and Gisbert Schneider, *Angew. Chem. Int. Ed. Engl.*, **2017**, *56*, 7971.

14. Alexander J. Stevenson, Eleanor I. Ager, Martina A. Proctor, Dubravka Škalamera, Andrew Heaton, David Brown and Brian G. Gabrielli, *Scientific Reports*, **2018**, *8*, Article no. 5144.

15. Gaochan Wu, Tong Zhao, Dongwei Kang, Jian Zhang, Yuning Song,Vigneshwaran Namasivayam, Jacob Kongsted, Christophe Pannecouque, Erik De Clercq, Vasanthanathan Poongavanam, Xinyong Liu, and Peng Zhan, *J. Med. Chem.*, Just Accepted Manuscript, Publication Date (Web): 03 May 2019.

16. Badger, J. *Structure-Based Drug Discovery, Methods in Molecular Biology*, **2011**, *841*, *161.* doi:10.1007/978-1-61779-520-6_7.

17. Daniel A. Erlanson, Stephen W. Fesik, Roderick E. Hubbard, Wolfgang Jahnke, Harren Jhoti, *Nature Reviews Drug Discovery*, **2016**, *15*, 605.

18. Lepre, C. A., *Methods Enzymol.*, **2011**, *49*, 219.

19. Campos-Olivas R., *Curr. Top Med. Chem.*, **2011**, *11*, 43.

20. (a) Haiduk, P.J. et al., *J. Amer. Chem. Soc.,* **1997**, *119*, 5818. (b) Puerta, D.T., Lewis J.A., *J. Amer. Chem. Soc.*, **2004**, *126*, 8389.

21. Terry V. Hughes, Ian Baldwin, Ian Churcher, https://www.biocompare.com/117128-b-Fragment-based-Drug-Discovery-From-Hit-Discovery-to-FDA-Approval-b-Lessons-Learned-and-Future-Challenges/*Posted*: November 14, 2011.

22. Li Q., and Shah S., *Methods Mol. Biol.*, **2017**, *1558*, 111.

23. Adel Hamza, Ning-Ning Wei and Chang-Guo Zhan, *J. Chem. Inf. Model.*, **2012**, *52*, 963.

24. Kharkar P. S., Warrier S., and Gaud R. S., *Future Med. Chem.*, **2014**, *6*, 333.

25. David C. Swinney and Jason Anthony, *Nature Reviews Drug Discovery*, **2011**, *10*, 507.

26. Mihaly Szabo, Sara Svensson Akusjärvi, Ankur Saxena, Jianping Liu, Gayathri Chandrasekar, and Satish S Kitambi, *Drug Des. Devel. Ther.*, **2017**, *11*, 1957.

27. (a) Ertl, P. et. al. Congreve, M. et al. *Drug Discov. Today*, **2003**, *8*, 876. (b) *J. Med. Chem.*, **2000**, *43*, 3714.

CHAPTER

5

Drug Design Strategies

KEY THINGS TO LEARN FROM THIS CHAPTER

- Definition and Characteristics of lead molecules.
- Drug design strategies:
  - Pharmacophore identification, Extensive SAR, Functional group and structural modification.
  - Structure-based drug design.
  - Bioisosterism and stereoisomerism drug design.
  - Enhancing absorption, preventing degradation of drugs, targeting drugs.
  - Prodrug, soft drug and "Me too" strategies for drug design.
  - Structure property relationship: Optimizing (regulating) pK_a.
  - Allosteric modulators.
  - Antimetabolite strategy for drug design.
  - Role of DMPK/ADMET during lead optimization studies.
- Timeline of a pharmaceutical drug development—from laboratory to clinics.

5.1 Introduction

The main objective of drug design strategy is to transform the initial hit molecules into lead compounds via lead optimization studies with improved activity, selectivity, and minimized toxicity. Even while lead optimization studies remain in progress, simultaneous efforts are made to incorporate desired pharmacokinetic (PK)/pharmacodynamic (PD) parameters by optimizing the physicochemical properties of the lead molecule by taking solubility, pK_a, lipophilicity, target-binding abilities, etc., into account. The entire objective of drug-design strategy/lead optimization studies is to get a preclinical candidate having drug-like features and that can be considered fit for clinical trials. It is worth mentioning that since a large number of drug-like properties need to be incorporated during drug design process in the hit/lead molecule, optimization of many features/properties is executed simultaneously.

5.2 Hit to Lead

Once a 'hit' molecule has been identified, the next step is to transform the hit into a lead molecule with drug-like properties. 'A lead compound can be defined as the starting point from which a clinically useful preclinical candidate can be developed'. Alternatively, it can also be defined as starting point with therapeutic significance (better than hit) around which congeners (with chemical features added or deleted) can be designed and synthesized using drug design/lead optimization strategies. Lead compounds, although exhibit high order of therapeutic response than their corresponding 'hit' molecule, are often not suitable for clinical use. This could be either due to their poor PK profile in terms of ADME and/or may be toxic (T) owing to selectivity issue.

5.2.1 Characteristics of Lead Molecules

A confirmed lead series should ideally display the following characteristics *in vitro*:

1. IC_{50} value of ≤1 μM (preferably in nM level) in functional assays
2. >30 - fold selectivity against isoform targets and key undesired target(s)
3. Confirmed mode of action
4. Demonstrable structure – activity relationship (SAR)
5. Experimentally derived *in vitro* ADMET profile, e.g. CYP P450 inhibition, ideally IC50 >10 μM; (in rodent and human microsomal clearance); aqueous solubility >10 μM; permeability; pK_a, logP/logD (a lipophilicity measure), etc.
6. hERG inhibition (binding to study cardiac effect/cardiotoxicity), ideally IC_{50} >10 μM
7. Favourable intellectual property right protection

5.3 Characteristics of Preclinical Candidates

The whole objective of transforming initial 'hit/lead molecules' into a preclinical candidate is to incorporate following drug-like features in the molecules such as

1. Desirable solubility (controlled by logP/D, pK_a, etc.)
2. Survive a range of pH in gastrointestinal tract (GIT)
3. Absorption across intestinal membrane (bioavailability)
4. Minimize first-pass effect
5. Efflux from intestine is minimized
6. Minimal binding with blood serum proteins
7. Survive liver metabolism by cytochromes
8. Avoid biliary excretion
9. Avoid excretion by kidney
10. Partition into target organs
11. Avoid partition to undesired tissues leading to off-target binding (toxicity)
12. Binds with desired target (selectivity) in tissues with high affinity (interaction)

5.4 Lead Optimization/Drug Design Strategies

The lead optimization strategies are applied to generate structure–activity and structure–property relationship (SAR and SPR) (discussed later in the chapter) studies for improving activity, selectivity, PK (ADMET) and PD (accessing target and affinity) parameters. Thus, the primary objective of lead optimization studies is to design a molecule that can overcome (survive) physiological barriers following administration via oral route and reach the desired biological target in the tissues for eliciting biological response. In general, various lead optimization/drug design strategies can be summarized under optimization of pharmacokinetic (PK) and pharmacodynamic (PD) properties of test compounds.

1. **Optimizing Pharmacokinetic (PK) Properties:** PK parameters refer to the lifespan of drug within the body from administration to complete excretion. It is in fact represented by concentration of drug versus time course, commencing from the site of administration to the site of action in tissues for eliciting biological response. Following administration of drug/test compounds by oral route, physiological barrier resists the absorption (A; bioavailability) of the drug into the bloodstream and its distribution (D) in tissues. The resistance during absorption involves first-pass metabolism, biliary excretion, action of efflux transporters, numerous gastric conditions, etc., whereas distribution is affected by plasma protein binding and binding to blood cells. The other two barriers involve metabolism (M) in liver along with elimination of drugs/metabolites from bloodstream. These physiological barriers in turn change the concentration of drugs/test compounds in the body, and this has been attributed to the physicochemical properties of compounds which in turn affect absorption, distribution, metabolism, excretion (E), and toxicity (T). In other words, drugs/test compounds with poor ADMET profile often fail to elicit desired therapeutic response and thus remain a leading cause of failures for preclinical drug candidates during clinical trials. Thus, the sole objective of optimizing PK (ADMET) parameters of a test compound is to regulate the physicochemical properties of compounds (SPR). An investigational test compound with good ADMET profile should be able to access tissue-based biological targets (receptors/signaling enzymes) or extracellular enzymes after moving through these barriers in sufficient concentration to elicit the desired therapeutic response.
2. **Optimizing Pharmacodynamic Properties:** Pharmacodynamics (PD, biological effect vs. time) deals with the optimization of the concentration of unbound drug available at the site of action in tissues vs. biological response following interaction (binding parameters) of the drug with protein target. The whole objective of improving PD parameters is to enhance the ability of drug to access its target in sufficient concentration and bind with high affinity. This can again be regulated by optimizing physicochemical properties (to access target) including structural features (to enhance affinity) in the test compounds.

   The above PK/PD properties need to be optimized in the molecule simultaneously, along with other structural modifications during early stages of lead optimization strategies. However, these modifications in the test compound may or may not enhance the biological activity. Notably, the role of analogues that exhibit poor response or no activity is as important during lead optimization[1]/drug design studies as those analogues where activity is enhanced or retained. Using these activity profiles of analogues, a structure–activity and SPR can be established that can then assist in combining the favorable features together in order to get a molecule (preclinical candidate) with optimal biological response, drug-like characteristics, and good ADMET profile. Compounds with good ADMET profile should be able to enhance absorption and distribution and minimize metabolism, excretion and toxicity.

There are various methods used for lead optimization/drug design strategies have been outlined below:

1. Pharmacophore identification
2. Extensive structure activity relationship (SAR) studies to improve potency and selectivity
3. Functional group optimization
4. Structural modification to increase potency
   - **(i)** Variation of alkyl substituent
   - **(ii)** Extension of structure
   - **(iii)** Ring variation
   - **(iv)** Ring fusion
   - **(v)** Simplification of structure
   - **(vi)** Conformational blockers/design of rigid congeners
5. Structure-based drug design
6. Bioisosteric replacements
7. Stereoisomerism: separating enantiomers
8. Strategies to improve absorption
9. Preventing degradation of drugs
10. Strategies for targeting drugs
11. Prodrug design
12. Structure-property relationship (SPR)
13. Allosteric modulators
14. Me too strategies
15. Antimetabolite strategy
16. Role of DMPK in the selection of preclinical candidates

5.4.1 Pharmacophore Identification

During lead optimization studies, it is important to delineate the part of the hit/lead molecule that acts as a pharmacophore. Pharmacophore is a collection of groups within the molecule that interact with the receptor or the enzyme and is responsible for therapeutic response. It identifies the important groups and their relative positions in the molecule that facilitate binding (drug–protein binding parameters), thereby providing preliminary knowledge about the probable active conformation of the pharmacophore. Examples of relative pharmacophore components include ionic groups (carboxylate or ammonium ions), polar groups (carbonyl, hydroxy, amines) as hydrogen bond donor/acceptor and hydrophobic features derived from alkyl or aromatic moieties. Once the pharmacophore features in a test compound are established, a 3D model of molecular structures can be built by joining the spatial location of features responsible for the activity (Fig. 5.1).[2] The spatial location of groups that are taken as points is then linked in a geometric manner. The distances between the points referred to as inter-feature distance gives an insight about the topography of the binding site in target proteins. Although experimentally, this information can be derived either by X-ray or NMR of target-bound compounds, often it is not possible to carry out such experiments.

Armed with this geometric information about the pharmacophores, the 3D model can be used as a template for building more effective drugs with better binding abilities. This can be carried out either by chemical modifications or by computational tools. The latter involves searching of databases of 3D structures of small molecules with drug-like features.

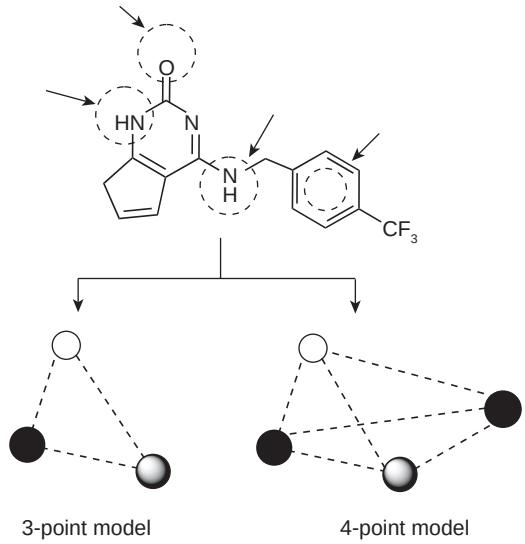


Figure 5.1 Pharmacophore models for a test compound.

5.4.2 Extensive Structure Activity Relationship to Improve Potency and Selectivity

SAR is a cornerstone for any drug discovery programme. During lead optimization studies, it is very important to synthesize numerous congeners with distinct physicochemical features in order to achieve a good relationship between structural features and biological activity (SAR). The structural modifications may address PK, PD parameter issues, and issues pertaining to pharmacophore identification, etc. Besides, SAR also identifies functional groups that are essential for binding and activity. This is achieved by altering, removing, or masking a functional group and screening the resulting analogues for activity. The biological activity data of congeners can be generated following *in vitro* screening of compounds against the protein target (enzyme/receptor). If removal or modification of a group results in drop or diminution of *in vitro* activity, then the group has no role in the activity, and if the activity is unaffected or enhanced, then the group is essential for activity.

Armed with these results, finally, SAR is generated by correlating the biological activity exhibited by compounds with its structural features. Using this SAR, one can derive information with regard to structural features/properties that leads to the enhancement in the biological activity followed by identification of the pharmacophore. The information can then be used to further refine the structure by introducing structural modifications in the molecule in a manner that would ultimately afford congeners (or analogues) with optimal biological activity and drug-like features. Thus, for a medicinal chemist, SAR remains a daunting task as one has to strike a balance between optimal biological response and appropriate physicochemical properties. Salient features of SAR studies are as follows:

1. The main objective is to identify part(s)/group(s) of the molecules essential for the expression of biological activity.
2. The activity may increase or decrease following removal/alteration of functional groups, which may be used to establish their role in biological activity. Thus, biological activity all analogues should be compared with the parent compound along with internal standard.

3. If possible, using X-ray crystallography and NMR techniques, important binding interactions between the drug and the active site should be established.
4. Often it may be possible to modify/alter the pharmacophore to enhance the biological activity.

CASE STUDY 1

SAR of Sulphonamides

The sulfonamides[3] are antimetabolites of *p*-amino benzoic acid (PABA) that competitively inhibits dihydropteroate synthase and, in turn, interfere with bacterial folic acid synthesis. Originally, sulfonamides were synthesized in Germany as azodyes, as antibacterial agents by a man named DOMAGK, who later received Nobel Prize for his pioneering work. In 1930s, these were developed as life-saving drugs called era of sulfa drugs with an illustrious history, but currently they are rarely used in clinics.

The discovery of sulfonamides commenced with the identification of the first lead molecule Prontosil. It exhibited *in vivo* antibacterial activity but was found to be inactive *in vitro* assays. Later, it was found to act as a prodrug, as it was metabolized *in vivo* into sulfanilamide (Fig. 5.2). This was probably the first antibacterial introduced in the market for clinical use against numerous bacterial infections.

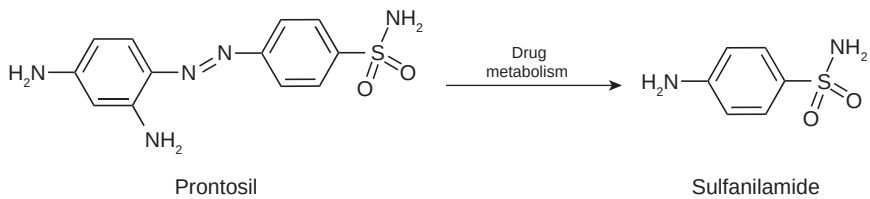
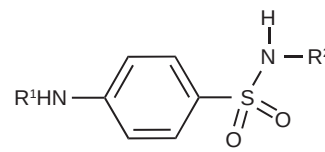


Figure 5.2 Lead molecule and first synthetic antibacterial sulfonamide drug.

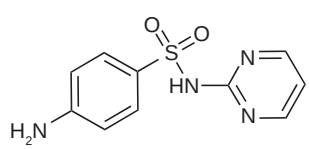
Extensive SAR was carried out by introducing substitutions in the two amino groups and in the aromatic ring, and by replacing sulfone with analogous moieties. This led to some interesting findings and eventually led to the synthesis of varieties of analogues of sulphonamides with different biological and PK properties. The prominent features of SAR that resulted in the identification of four sulfonamide drugs have been depicted in Fig. 5.3.



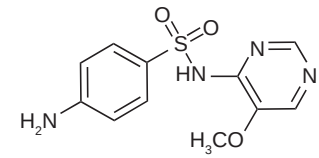
1. Unsubstituted (R^1= H) aromatic amine is essential for biological activity.
2. Acylation of aromatic amine furnished prodrug.
3. Removal of aromatic ring furnished inactive compounds hence aromatic ring is essential for activity.
4. Removal of *p*-amino group resulted in loss of activity; hence *p*-substitution is essential for activity.
5. Replacement of Sulfonamide group furnished inactive compound hence sulfonamide needs to be retained in the molecule.

(*Continued*)

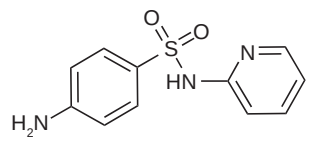
Study 1 (*Continued*)



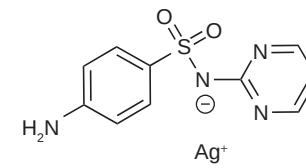
Sulfadiazine



Sulfapyridine



Sulfalen



Sulfazin silver salt

Figure 5.3 SAR on sulfonamide furnished several new drugs with antibacterial activity.

Based on SAR studies following sulfonamide drugs with different properties were generated:

1. **Short action:** Sulfadiazine, etazol, sulfazin urosulfan
2. **Long-acting:** Sulfapyridine, sulfadimethoxine
3. **Super-long action:** Sulfalen
4. **Topical drugs:** Sulfatsil sodium and sulfazin silver salt

CASE STUDY 2

SAR with Morphine

Seed capsules of poppy plants are a major source for morphine (Fig. 5.4) and have been used as analgesic. The structure comprises a phenol (aromatic ring), ethereal oxygen, an alcoholic functional group, a double bond, and tertiary nitrogen. SAR studies afforded a series of congeners with different therapeutic efficacies as well as with loss of activity. All congeners of morphine (synthesized during SAR studies) possessing the basic pentacyclic skeleton exhibited both analgesic activity as well as addiction liability.

A molecule of morphine (Fig. 5.4) has the following structural features:

1. The basic skeleton comprises an annulated tricyclic phenanthrene with rings A, B, and C, and two additional rings: a piperidine ring D and a dihydrofuran ring E.
2. The presence of basic tertiary amine functionality at position 17.
3. The presence of ether linkage resulting in dihydrofuran ring E is formed between C4 of ring and C5 of ring B.
4. The presence of one double bond between C7 and C8 in ring C.
5. The presence of two hydroxyl groups: a C3-phenolic OH in rings A and a C6-allylic OH in ring C.
6. The presence of five chiral centres (C5, C6, C9, C13, and C14).

(*Continued*)

Study 2 (*Continued*)

Modification in the Ring A of Morphine

The analgesic activity of morphine has a direct link with the presence of 3-hydroxyl group in the ring A as its removal (3-deoxymorphine) led to reduction in the analgesic activity by 10-fold (**I**, Fig. 5.4). Etherification of C-3 OH reduced narcotic analgesic activity (**II**), whereas etherification of the C-3 OH furnished derivatives with modest increase in activity (**III**). In order to establish the role of hydroxyl groups in the expression of activity, acetylation of both the hydroxyl (3- and 6-OH) groups incidentally furnished heroin (a banned substance), with significant enhancement in lipophilicity and potency (**IV**). The naturally occurring codeine, a C-3-O-methylated derivative resulted in drop of analgesic activity (**V**); however, it was found to be associated with very useful antitussive activity and is available commercially for pain and cough.

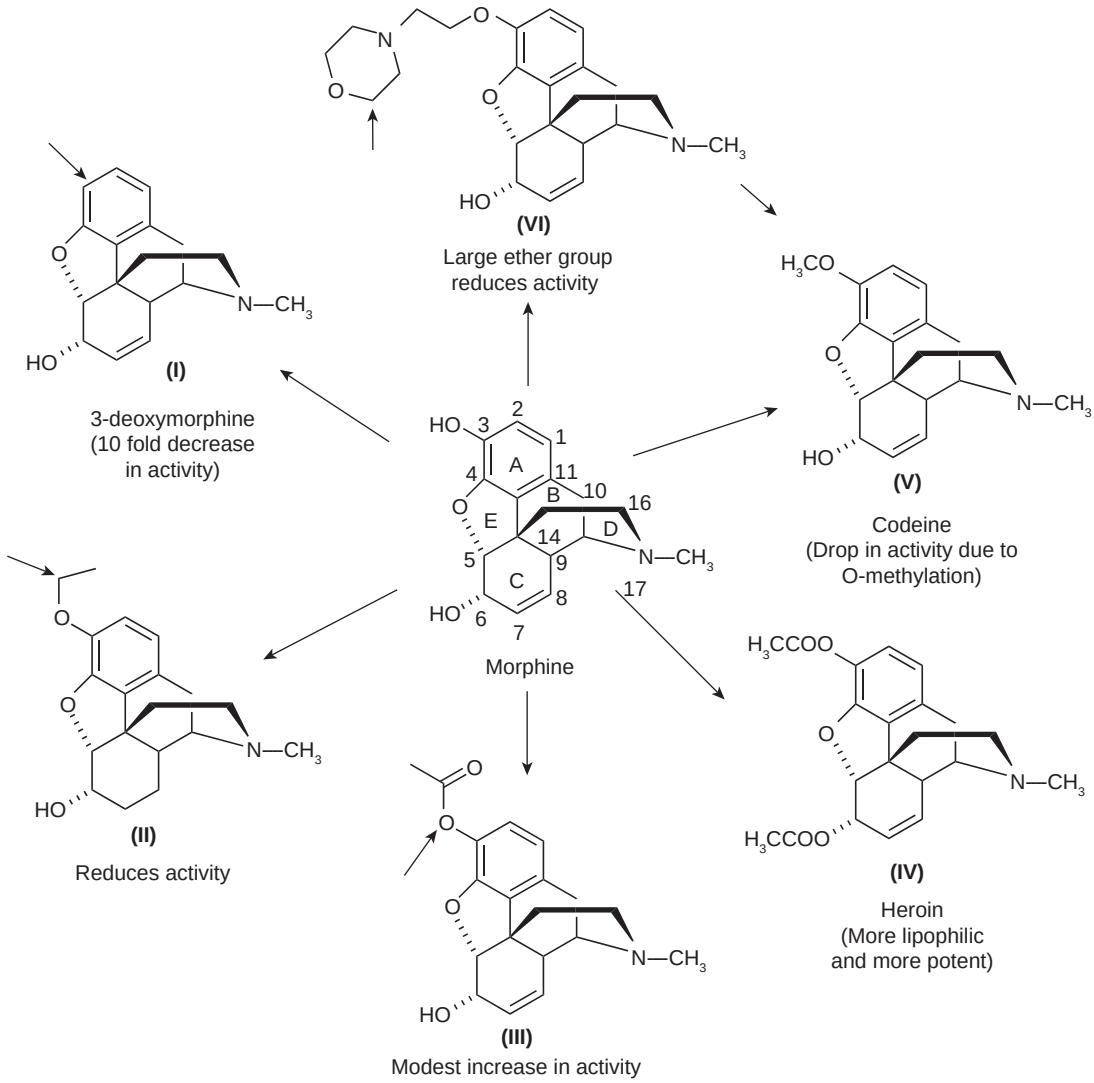


Figure 5.4 Effect of modification in the ring A of morphine on analgesic activity.

(*Continued*)

Study 2 (*Continued*)

Modification in the Ring C of Morphine

The 6-OH was not found to be essential for activity as its replacement resulted in 6-deoxymorphine with significant enhancement in the activity (**VII**; Fig. 5.5) and methylation of 6-OH furnished **VIII** with increase in activity. Similarly, reduction of double bond at C7–C8 had no effect on the biological activity (**IX**). The reduced form of morphine dihydromorphine and oxidation of 6-OH in morphine exhibited no change in the activity (**X**). Interestingly, oxidation of 6-OH in dihydromorphine furnished hydromorphone with enhanced analgesic activity (**XI**). Oxymorphone (hydroxy-dihydromorphinone), a semisynthetic analogue resulting from the introduction of a β-OH group at C-14 in the hydromorphone, exhibited significant improvement in the analgesic activity (**XII**). This opioid medication was although released in the market for managing pain but was later withdrawn due to its abuse.

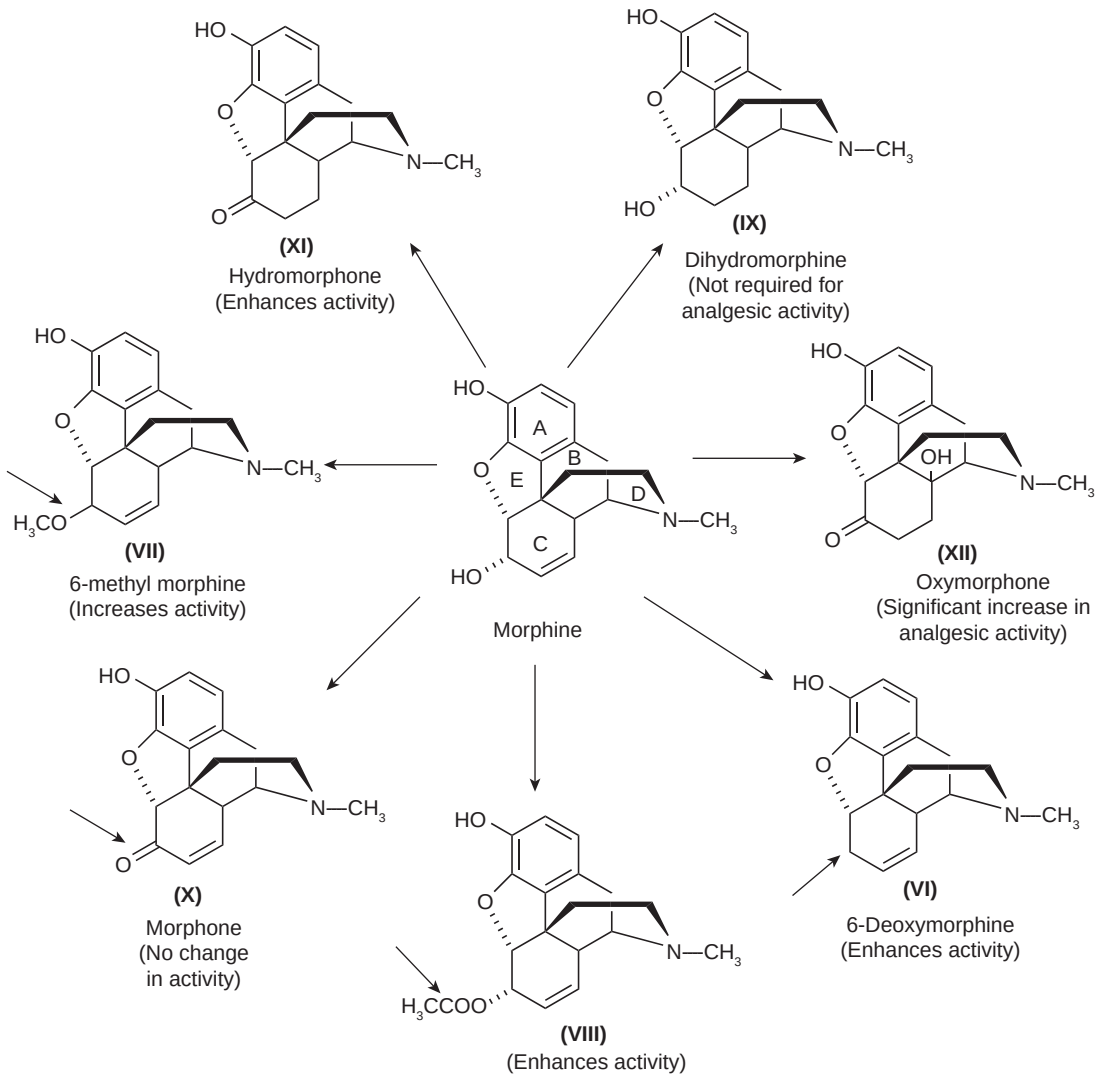


Figure 5.5 Effect of modification in the ring C of morphine on analgesic activity.

(*Continued*)

Study 2 (*Continued*)

Extension of SAR Findings of Morphine to Codeine (Modification in the Ring C of Codeine)

Codeine is utilized as a central analgesic, sedative, hypnotic, antinociceptive and is also recommended for coughing. Structural modifications in ring C of codeine (**XIII;** Fig. 5.6) produced similar SAR data as obtained by the modifications in ring C of the morphine structure. Reduction of 7, 8-double-bond afforded dihydrocodeine with relatively enhanced analgesic activity in comparison to codeine (**XIV;**). Oxidation of the 6-OH of dihydrocodeine furnished hydrocodone with further increase in the activity (**XV**), whereas introduction of a −OH group at C-14 afforded oxycodone with significant improvement in the analgesic activity than hydrocodone (**XVI**). Hydrocodone is clinically used as analgesic and as an antitussive drug (available as the bitartrate salt in tablets and syrups). It is used in combination with a non-opioid pain reliever acetaminophen to relieve moderate to severe pains; however, it is not recommended for use in children.

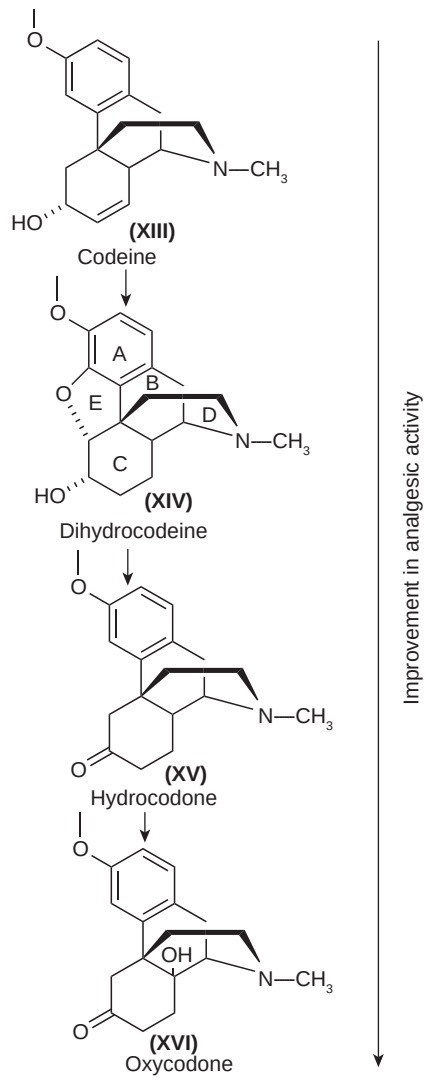


Figure 5.6 Effect of modification in the ring C of codeine on analgesic activity.

(*Continued*)

Study 2 (*Continued*)

Modification in the Ring E of Morphine (Morphinans)

Deletion of the ring E comprising ether bridge from the structure of morphine afforded morphinans with 5-fold increase in the analgesic activity (**XVII;** Fig. 5.7). However, opening up E ring (the ether linkage) furnished the catechol-type morphine derivative with significant decrease in the activity by 90% (**XVIII**). The morphinan skeleton has been structurally modified to obtain compounds with agonistic and antagonistic properties and selectivity towards different populations of opioid receptors (μ, κ, and delta-receptor). For example, levorphanol is used as drug to treat severe pain and levallorphan is used as an opioid antagonist similar to naloxone. The latter is sold as levallorphan tartrate (Fig. 5.7) and is used as an opioid analgesic and opioid antagonist/antidote. Cyclorphan, yet another derivative of morphinan family, is an opioid analgesic that provides relief from strong pain but is not marketed because of hallucinogenic side effects (Fig. 5.7).

Modification in the Ring D and in N-Methyl Residue

Demethylation of morphine's N-methyl group (normorphine) or replacing methyl group with larger alkyl groups led to significant drop in the analgesic activity; however, substituting methyl group with aralkyl groups significantly enhanced the analgesic activity as in N-phenethylnormorphine (**XIX**; Fig. 5.8). The most interesting finding during SAR studies was opiate receptor antagonist activity exhibited by analogues when N-methyl group was replaced with an allyl group (**XX**), a methylcyclopropyl group (**XXI**) or a methylcyclobutyl group (**XXII**).

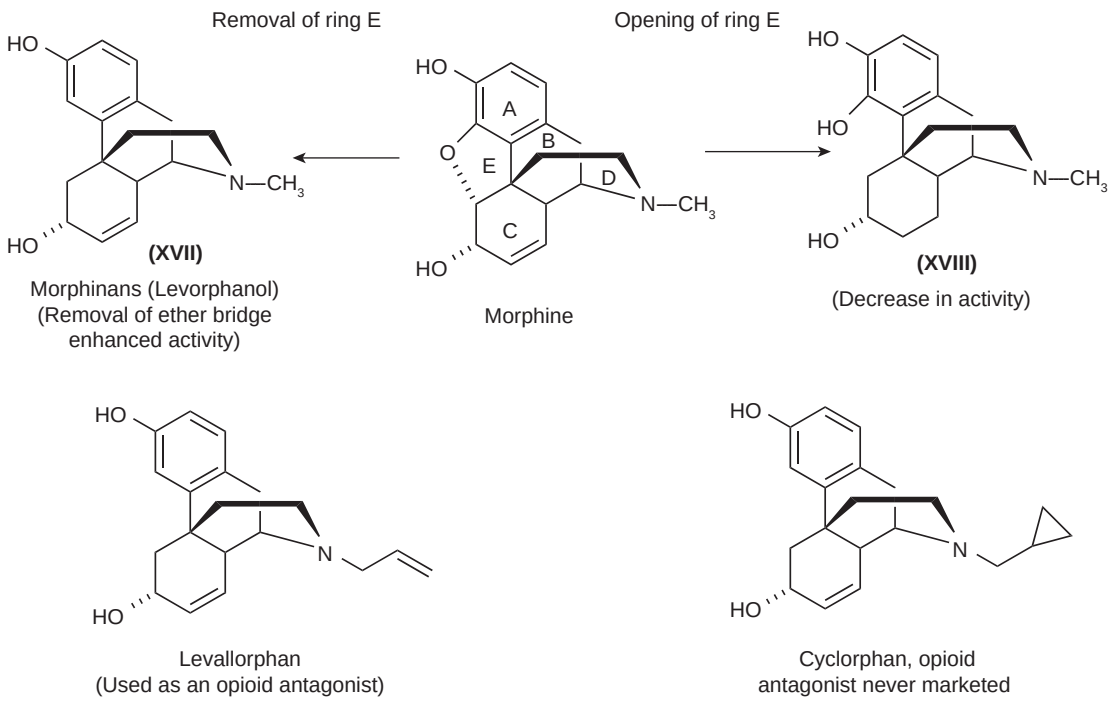


Figure 5.7 Modification in the ring E of morphine and morphinan based-drugs.

(*Continued*)

Study 2 (*Continued*)

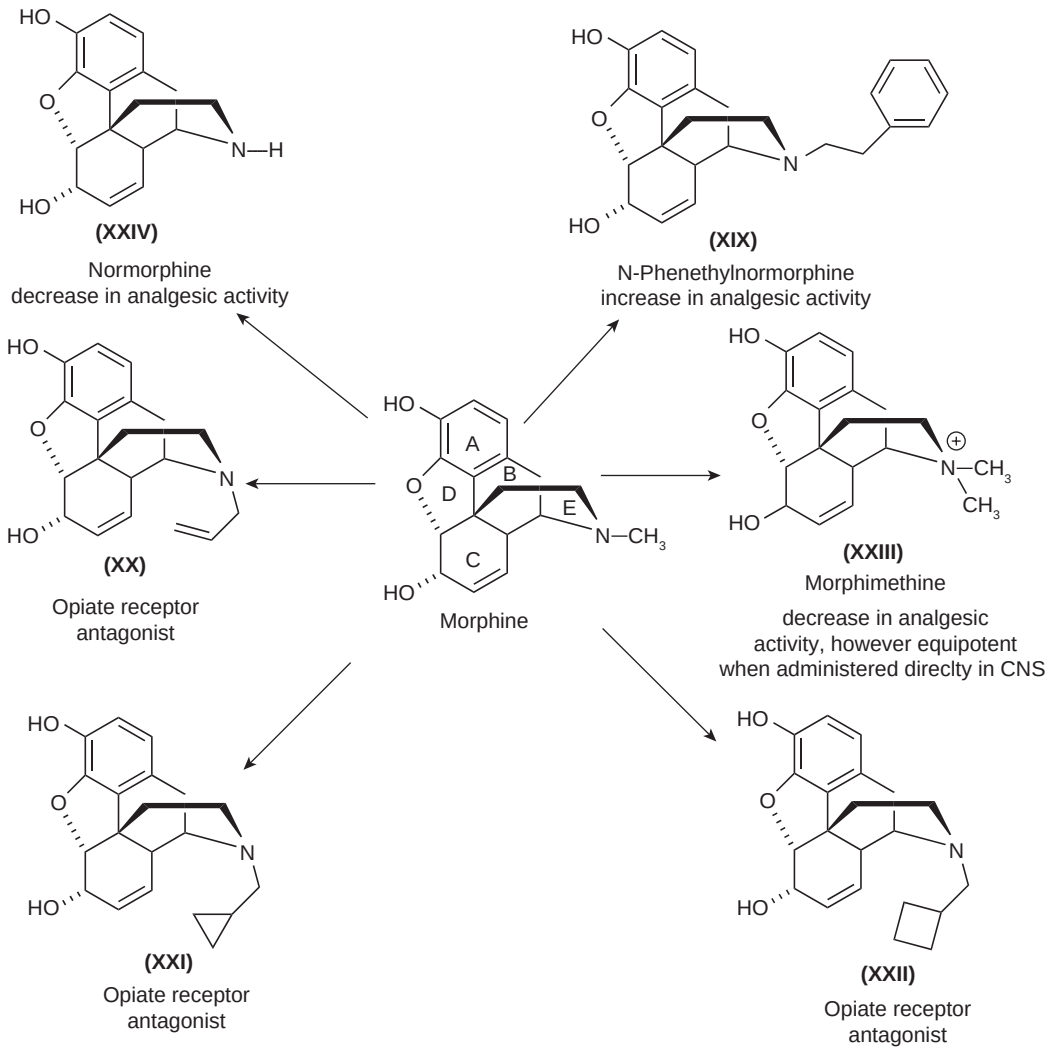


Figure 5.8 Effect of modification in the ring D and in the methyl residue linked to nitrogen on analgesic activity.

Combining Potent Agonistic with Antagonistic Properties

The most interesting part of SAR studies is to combine the structural features that resulted in the enhancement of biological activity into a single molecular framework with the view to design congeners with enhanced activity. Thus, the N-alkyl chains that led to the enhancement in the antagonistic properties of morphine (**XX**, **XXI**, **XXII**; Fig. 5.8) were introduced in the oxymorphone skeleton (**XII**; Fig. 5.5), which was more active than morphine and then evaluated for their analgesic activity. Accordingly, substituting N-methyl group in the potent narcotic agonist oxymorphone with chains such as allyl group (**XXV**), a methylcyclopropyl group (**XXVI**) or a methylcyclobutyl group (**XXVII**)

(*Continued*)

Study 2 (*Continued*)

led to compounds with potent opiate G-protein-coupled receptor (GPCR) antagonistic response at mu, kappa, and delta receptors. Some of these compounds – naloxone HC1 (Narcan) and naltrexone HC1 (Trexan) are clinically used to reverse the responses induced by opioid agonists (Fig. 5.9).

A summary of SAR findings on morphine and its derivatives possessing the basic skeleton has been summarized in Fig. 5.10. These data can be used to design compounds with better efficacy, selectivity, and reduced toxicity.

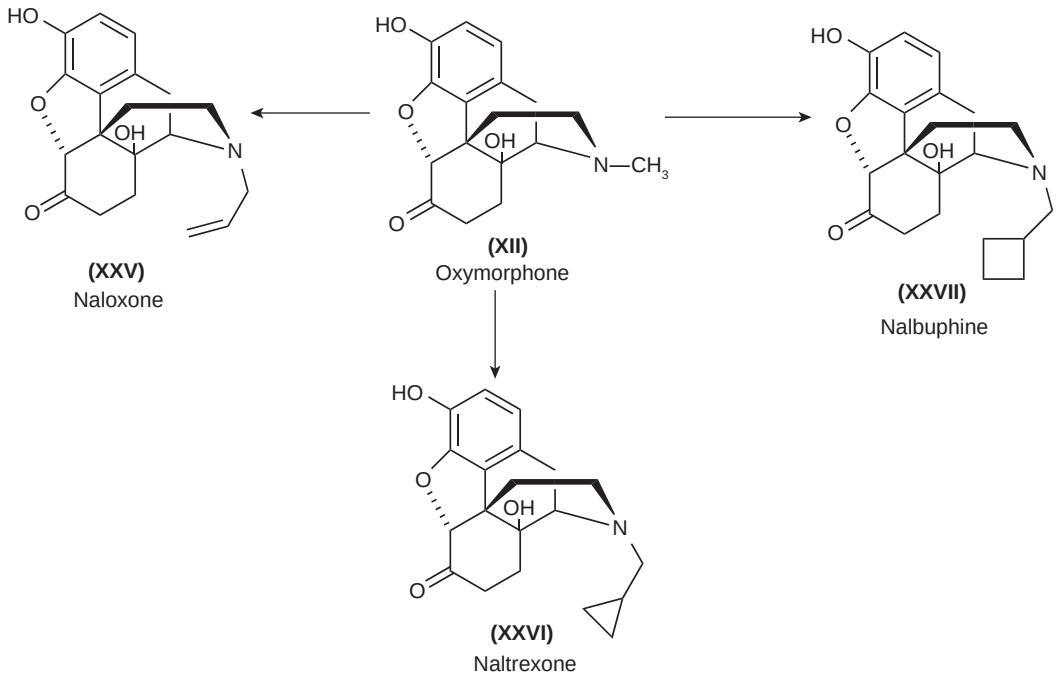


Figure 5.9 Combining potent agonistic with antagonistic properties in morphine.

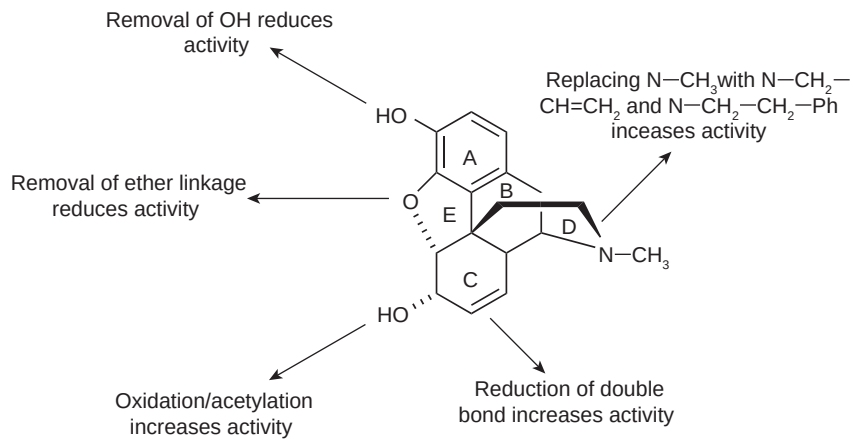


Figure 5.10 A summary of SAR data on morphine.

(*Continued*)

Study 2 (*Continued*)

Design of Super Agonist Analogue of Morphine from SAR Data

The above SAR data summarized in Fig. 5.10 was applied to design a super agonist of morphine by combining and introducing additional features with the view to obtain analogue with enhanced analgesic activity. This led researchers to synthesize a series of congeners and evaluated them for their analgesic activity. The studies culminated in the identification of an oripavine derivative called etorphine as a super agonist which was found to be approximately 1000 times more potent than morphine (Fig. 5.11). However, owing to its high order of potency, it was not recommended for human use, instead used as a tranquilizer for large animals.

Benzomorphans as Non-natural Analogues of Morphine Lacking C and D Rings

Naturally occurring (–)-morphine, although an extremely potent analgesic, suffers from a major side effect such as addiction. Efforts have been made to dissociate the analgesic from side effects by introducing structural modifications. The studies led to the identification of certain unnatural derivatives: benzomorphans[4] with significantly reduced addicting effect. The benzomorphans bear strong structural resemblance to the morphine, except for the lack of the C and D rings. SAR studies on benzomorphans similar to the one carried out for morphine furnished therapeutically useful drugs: pentazocine and phenazocine[5] clinically used (Fig. 5.12) as analgesics and in the treatment of cocaine addiction.

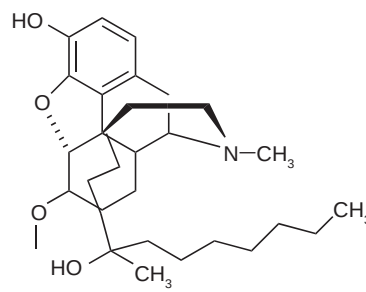


Figure 5.11 Etorphine (super agonist analogue).

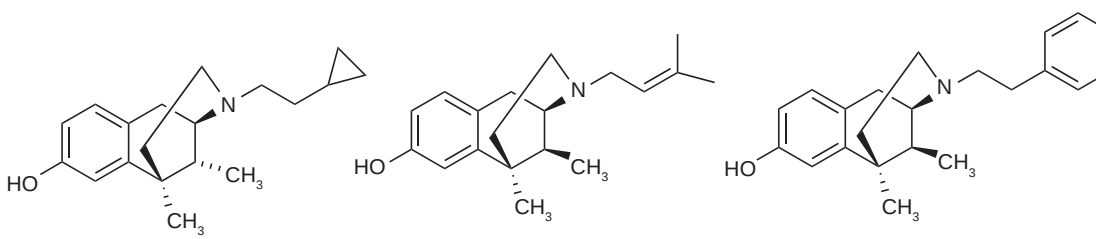


Figure 5.12 Benzomorphans as unnaturally occurring analogues of morphine.

5.4.3 Functional Group Modification

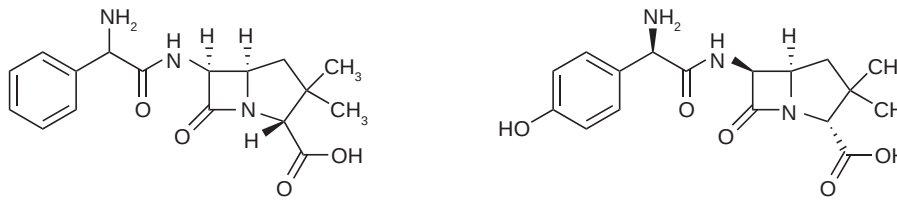
In the 19th century, pharmacological activities exhibited by several natural products and their derivatives laid platform for the significance of SAR in drug discovery. In general, pharmacological activity exhibited by a compound may be due to specific structural unit/part called pharmacophore and functional groups linked

to this pharmacophore. Often modifications in the functional groups have led to significant increase in their pharmacological activities. Notably, introduction and removal of functional groups can also affect the physiochemical properties including binding abilities of compounds.

CASE STUDY 3

Introduction of Hydroxyl Group on Bioavailability

Ampicillin and amoxycillin both belong to class of antibiotics widely used for treating a variety of bacterial infections. Ampicillin exhibited bioavailability of 40%, but introduction of *p*-hydroxyl group (Fig. 5.13) in the aromatic ring improved bioavailability to >90% by oral route.



Ampicillin Amoxycillin

Figure 5.13 Introduction of OH group at *para* position of phenyl group improved bioavailability in amoxycillin.

5.4.4 Structural Modifications to Increase the Activity

The structural modification during lead optimization of hit molecule is carried out with the objective to design a compound with enhanced biological activity and acceptable physicochemical properties. This will allow survival of test compounds while moving through physiological barriers, enough to access target to elicit optimal biological response. Notably, it is due to action of physiological barriers on the administered drugs that only fraction of unbound drugs reaches the target. Therefore, the preclinical candidate(s) must exhibit high order of affinity for the target as well as enhanced potency, preferably in nM level.

5.4.4.1 Variation of Alkyl Chains/Substituents

The modification in the length and size of the alkyl substituent is carried out with the view to fill up the unoccupied hydrophobic space at the binding site. This will enhance the interaction of drugs with the target and, in turn, improve biological activity and selectivity of test compounds. Any heteroatom in the molecule carrying alkyl substituent can be modified or shifted to facilitate drug–target interactions. For example, replacement of *N*-methyl group in adrenaline with *N*-isopropyl and *N*-*t*-butyl groups resulted in isoprenaline and salbutamol receptively. Salbutamol is clinically used for the treatment of asthma and acts as β-2-receptor agonist. Isoprenaline is a non-selective β-adrenergic agonist and is used for the treatment of heart blockade. This can be attributed to the presence of a bulky isopropyl group that provided a better fit at the hydrophobic binding site of the receptor (Fig. 5.14).

Alternatively, extension or reduction of a chain length in the hit/lead molecule can be carried out with the view to allow better interaction with the binding site in the target. For example, increasing the distance between the proline ring and SH group by one methylene unit transformed the lead molecule into clinically useful antihypertensive drug captopril. Thus, increasing the distance between two binding moieties in a

pharmacophore can maximize the binding between the drug and target (Table 5.1). Similarly, mefloquine, an antimalarial drug, was discovered by introducing changes in the chain length in quinine and chloroquine (Fig. 5.15).

In an interesting example, chlorpromazine, an antipsychotic drug, was obtained while improving the activity of diphenhydramine, a first-generation antihistamine drug. The development of chlorpromazine as neuroleptics occurred by combining the features of diphenhydramine and promethazine, H_1 antagonist, along with chain elongation from two to three carbons (Fig. 5.15).

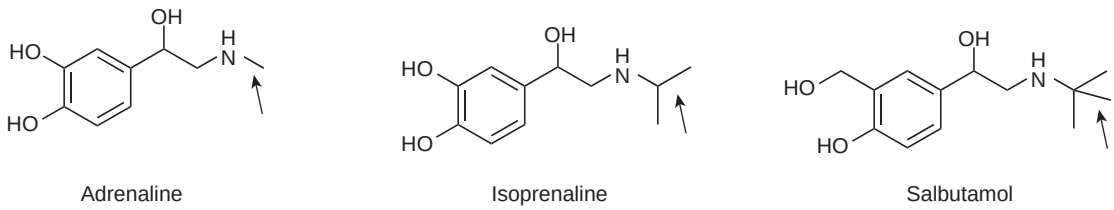


Figure 5.14 Introducing alkyl substituents.

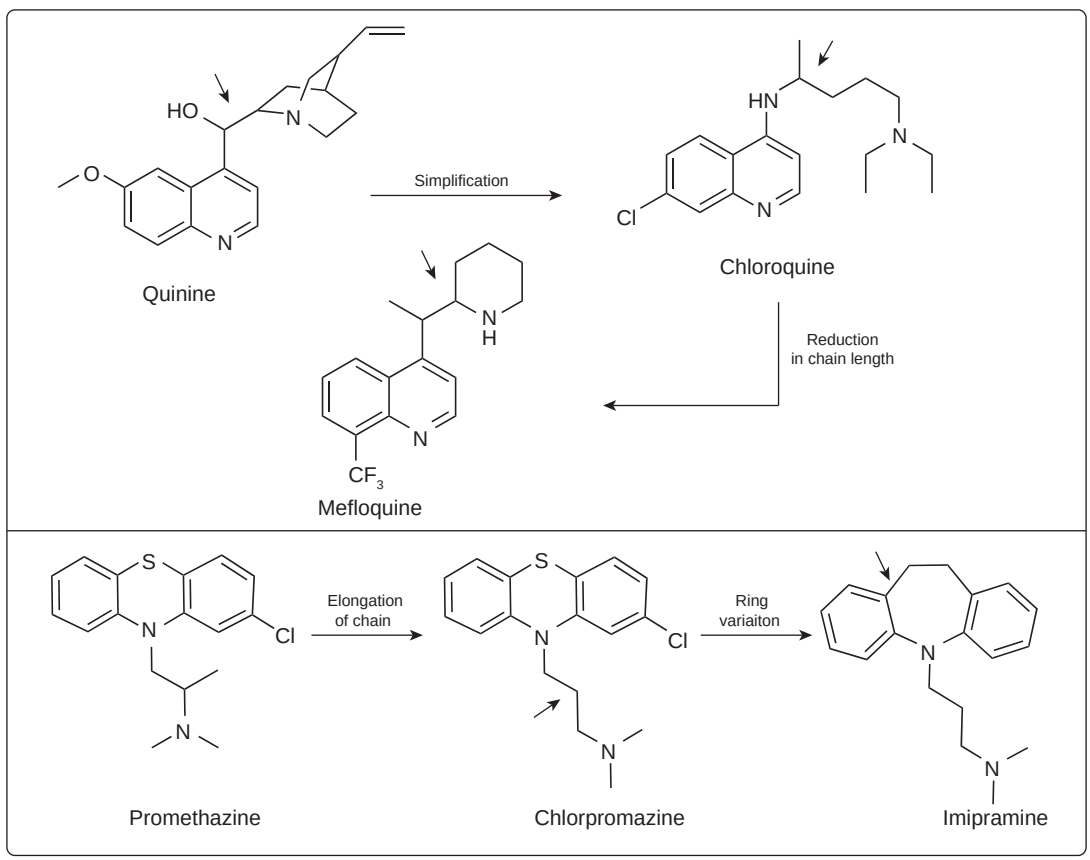


Figure 5.15 Variation of alkyl chains.

5.4.4.2 Extension of Structure

Using this strategy, additional polar or nonpolar functional groups are added with the view to further enhance the binding of the test compound with therapeutic targets.

Captopril (Table 5.1) due to the presence of SH group left a bitter taste following its oral administration. In order to dissociate this side effect from the molecule, the thiol group was replaced with several other polar groups. The effort led to the finding of a lead molecule with carboxy functionality instead of thiol group (Fig. 5.16). Next, in an attempt to further improve the activity of the lead molecule, the structure was extended by adding $-CH_2CH_2-Ph$ moiety, to facilitate binding by introducing a new hydrophobic interaction site. This resulted in the identification of captopril analogue enalaprilat as 'me too' drug.

CCR5 (GPCR) blockers are considered to be the best option for treating HIV, as they prevent the entry of virus in the host cells (Fig. 9.8) Several groups started screening of GPCR-focused library of small compounds against CCR5 receptor. One such study led to the identification of a lead compound (Fig. 5.16) but needed improvement in terms of activity, selectivity, and PK properties. The lead optimization studies following extension of structure led to the identification of aplaviroc (Fig. 5.16) as a preclinical candidate. However, it failed to complete clinical trials as it was associated with severe hepatotoxicity. Subsequently, maraviroc (Fig. 9.8) with no structural resemblance to aplaviroc was discovered by Pfizer as a CCR5 blocker and has been approved by FDA.

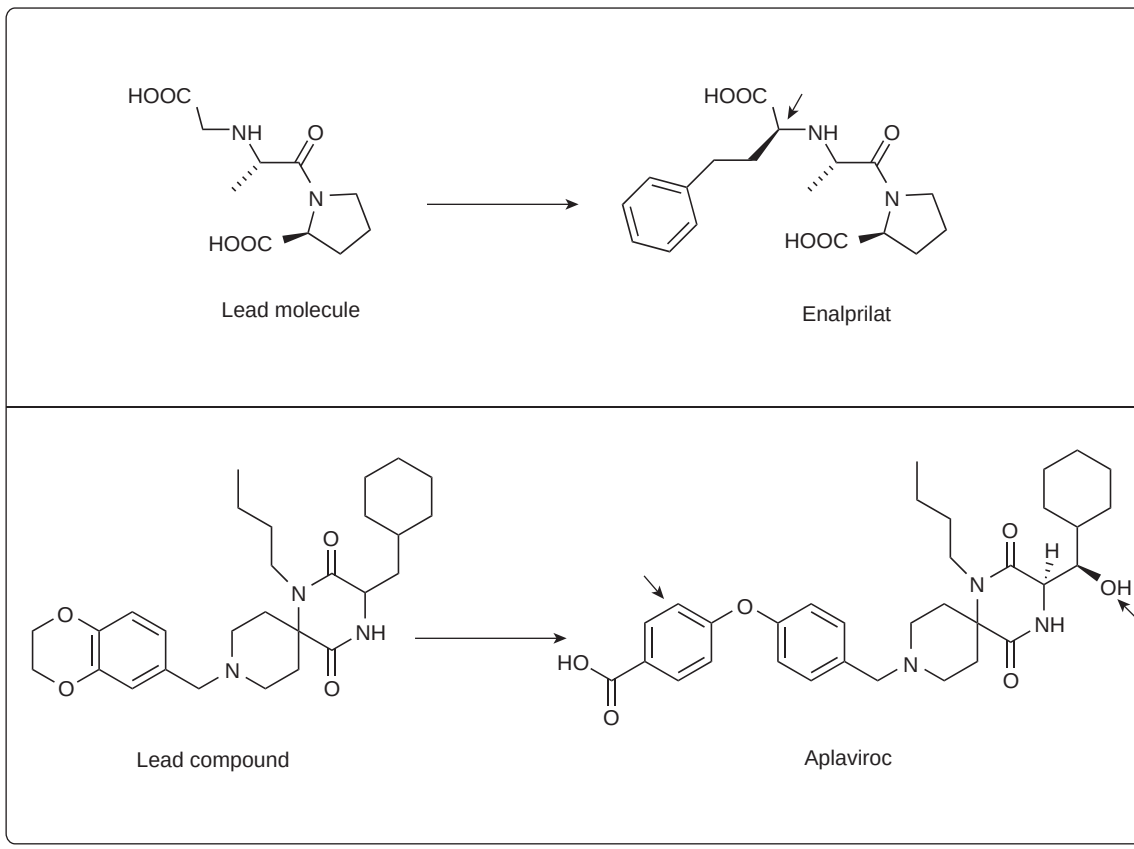
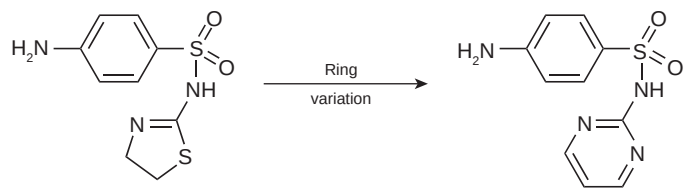


Figure 5.16 Extension of structure in the lead molecules led to clinically useful drugs.

5.4.4.3 Ring Variation

Receptor/enzyme occupancy by a test compound can also be improved by varying the size of the ring that holds the binding groups. Expansion or contraction of ring may facilitate better or full occupancy of space in the binding site by the binding groups attached to the ring or influence the physicochemical properties. Replacement of thiazole ring (5-memberd) in sulfathiazole with a pyrimidine ring (6-membered) afforded sulfadiazine with reduced toxicity (Fig. 5.17). The introduction of electron-withdrawing pyrimidine ring resulted in analogues with improved water solubility, improved acidity, and ionizing ability of the NH proton. The resulting compound silver sulfadiazine is used topically to prevent infection resulting from burns. In another example, refinement of chlorpromazine structure via ring expansion led to the development of antipsychotic compound imipramine (Fig. 5.15). The latter was initially used as antipsychotic for patients suffering with schizophrenia; however, later its antidepressant activity was discovered serendipitously.

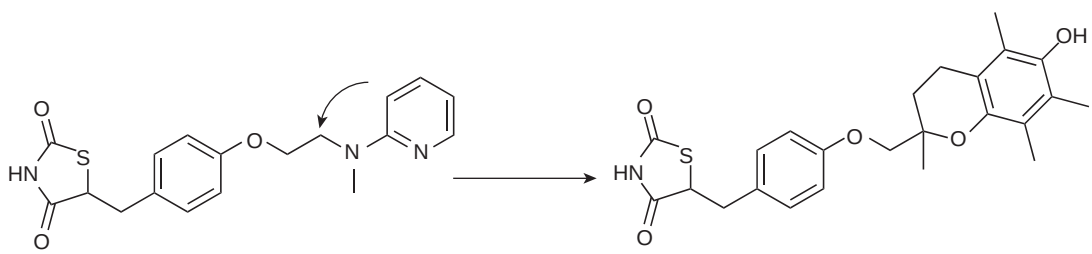


Sulfathiazole Sulfadiazine

Figure 5.17 Ring variation resulted in reduced toxicity.

5.4.4.4 Ring Fusion

To improve drug–target interaction, new rings can be generated and fused with the existing ring in the test compounds. This may orient the binding groups in a manner that will not only improve occupancy of binding site but may also reduce conformational flexibility of the test compounds (Fig. 5.18).



Rosiglitazone Troglitazone
(Withdrawn from market)

Figure 5.18 Example of ring fusion in drug development.

5.4.4.5 Simplification of the Structure

Biologically active lead molecules often derived from natural sources have large and complex molecular structure framework. During lead optimization studies, it has been found that the removal of unnecessary parts, functional groups, carbon skeleton, or molecular fragments that are not part of the pharmacophore furnishes simplified structures with full retention of biological activity. Thus, phenomenon of simplification not only furnishes active compounds but also reduces the cost of the drug as synthesis of simplified structures is commercially viable.

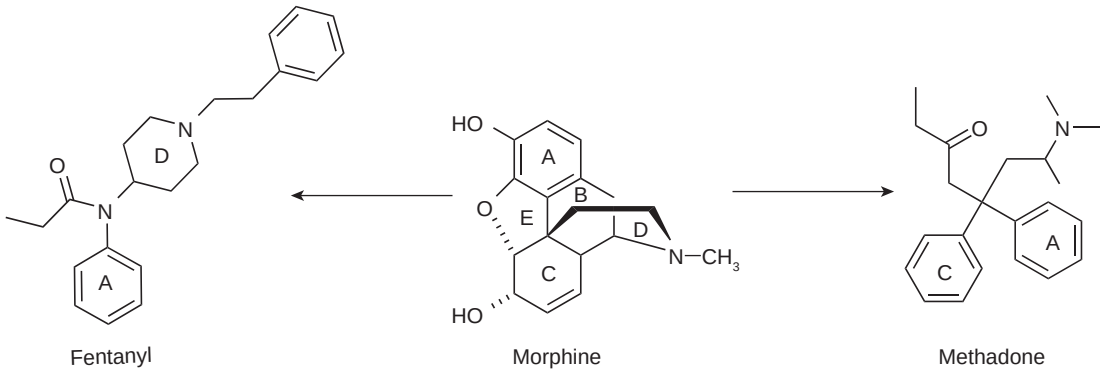


Figure 5.19 Simplification of the morphine furnished clinically useful drugs.

For example, parathyroid hormone (PTH), an 84-amino acid polypeptide, is secreted from parathyroid gland and plays an important role in osteoporosis. Sequential deletion of N-terminal fragments led to the identification of the N-terminal region 1–34 peptide that retained the full activity of natural PTH and is used clinically for treating osteoporosis.

Similarly, removal of rings in morphine led to both active and inactive compounds. Morphinan, without ether linkage ring E, was more active than morphine (Fig. 5.7). Methadone was although less active but was found to be clinically useful for countering addiction (Fig. 5.19). Fentanyl[6] based on 4-phenylpiperidine (clogP = 3.62) was 100-folds more potent than morphine (clogP = 0.57) with comparatively rapid and short duration of action and with fewer side effects. Besides, it also readily crossed the blood–brain barrier (BBB) in both directions and its diffusion through BBB can be attributed to improved hydrophobicity. Fentanyl is clinically used as an anaesthetic agent (Fig. 5.19).

5.4.4.6 Rigidification of the Structure/Conformational Blockers

Molecules in solution are ensemble of conformers and depending on the flexibility of the structures, the population of biologically active conformer may be less, moderate, or high. Thus, in molecules with flexible structures arising due to free rotation, introduction of rigidity or conformational blockers may increase or decrease the population of biologically active conformer. A classic example of introducing conformational blockers is imatinib, an anticancer drug. Initially, *N*-phenylpyrimidin-2-amine (lead molecule) was identified as tyrosine kinase inhibitor which was then subjected to lead optimization studies. Interestingly, introduction of conformational blockers at the *ortho* position of aromatic rings blocked free rotation around aryl-N bond and furnished clinically useful drug imatinib (Fig. 5.20).

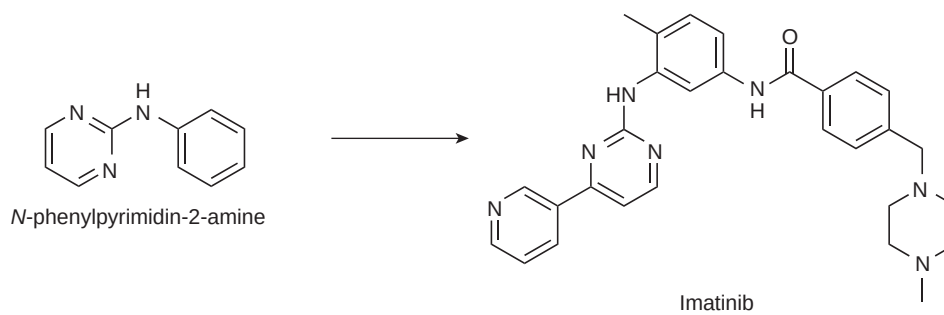


Figure 5.20 Conformational blocker in the drug imatinib.

CASE STUDY 4

Development of Oxaminiquine Using Several Structural Variations

Oxaminiquine is an important clinical drug used for the treatment of schistosomiasis. It has its origin in lucanthone, a drug associated with several toxic side effects and requires 3 to 5 administrations a day to get the desired effect. Attempts to improve its activity commenced with simplification of lucanthone, which resulted in a biologically active compound comprising only a *para* disubstituted aromatic ring. The latter was further optimized by introducing rigidifications that furnished anthelmintic drug (anti-worm) oxamniquine (Fig. 5.21).

Next, introducing substituent in the disubstituted aromatic ring afforded mirasan bearing an electronegative Cl group in place of S in lucanthone with improved biological activity. SAR studies indicated that β-aminoethyl amino side chain may be playing an important role in its binding to the target. In order to facilitate binding, it is important that the linear chain adopts a particular conformation. Indeed, the side chain being linear and flexible group may adopt several conformations, thereby reducing the population of biologically active conformation. This led to the rigidification of the structure in order to reduce the flexibility with the view to enhance the population of biologically active conformation. The resulting bicyclic structure with side chain bonds fused in the ring preventing free rotation and exhibited a dramatic improvement in the biological activity. Further, introduction of rigidification and varying substituent and their positions furnished a clinically useful drug oxaminiquine used as anthelmintic.

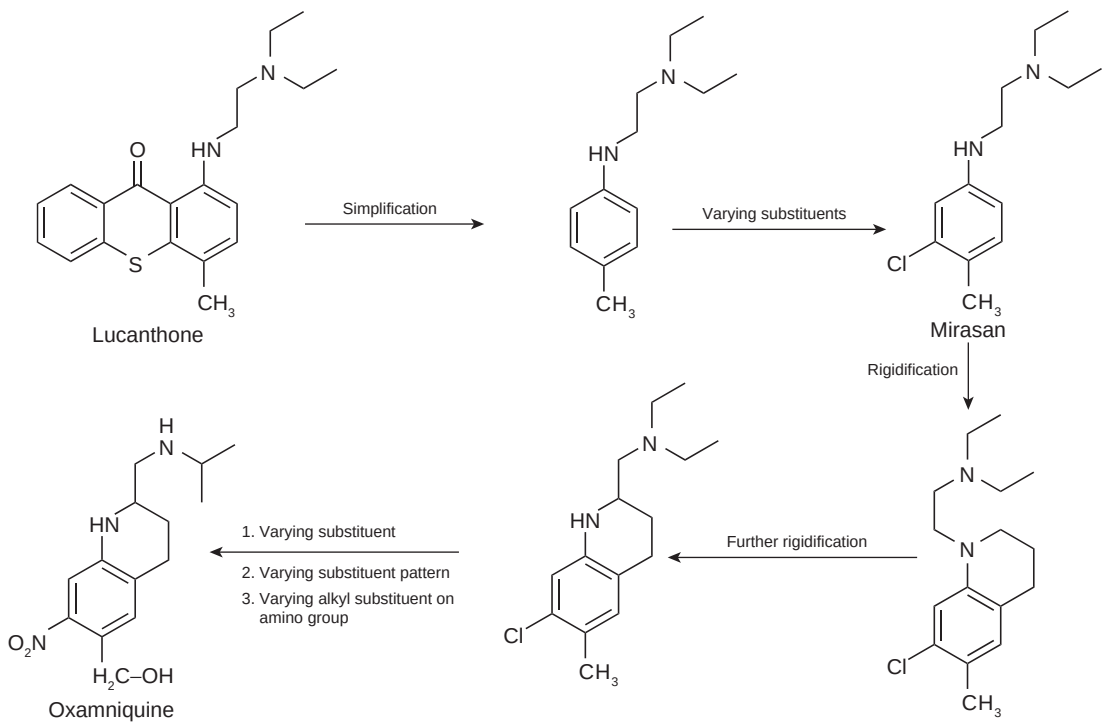


Figure 5.21 Development of oxamniquine.

5.4.5 Structure-Based Drug Design

Structure-based drug design[7] approach involves exploring 3D topographies of the complementary surfaces of ligands/substrates to binding sites in macromolecular protein targets. The strategy[8] involves use of several state-of-the-art technologies for the development of preclinical candidates. It commences with the identification and validation of a druggable disease target using transgenic model followed by cloning, production, and purification of the protein target. Once the protein target is obtained in sufficient quantity, the next step is to determine its 3D structure using either X-ray of the protein crystal or NMR spectroscopy in solution. Besides this, *in vitro* assays using the protein target are also developed for the purpose of screening compounds against the target. In case it is not possible to obtain protein crystals, homology modelling is performed to identify proteins not only with high structural resemblance in terms of amino acid sequence but also where three dimensional (3D) structures are available. The homology modelling is generally carried out using BLAST search available as online servers where amino acid sequence of proteins with unknown 3D structure is matched with known 3D structures stored in the protein data bank (PDB).[9]

Next step is to identify small molecule(s) that can bind with the binding site in the target protein with high affinity in order to inhibit or activate the biological function of the target. This can be carried out using either *in silico* computational screening followed by *in vitro* screening or by directly screening focused libraries against the protein target in the *in vitro* biochemical assays. The best compound identified from this assay with IC_{50} values of <1–10 μM is then co-crystallized with the protein and subjected to X-ray with the view to delineate 3D information about the binding site. This is then followed by several cycles of design and synthesis of analogues (lead optimization studies) around the lead molecule with the view to get compounds with good ADMET parameters, improved affinity, efficacy, and potency. Alternatively, fraction-based drug discovery approach using NMR spectroscopy can be also applied to develop preclinical candidate with optimal ADMET and activity profiles.

CASE STUDY 5

Discovery and Rise of Captopril[10]

In 1960s, a Brazilian researcher while working on the venom of the Brazilian viper *Bothrops jararaca* identified bradykinin potentiating factor (BPF) that potentiated the actions of bradykinin. The potentiation of bradykinin occurred probably by inhibiting the enzyme that inactivated it. The prevention of inactivation of bradykinin in turn increased its effect on vasodilation (blood vessels to dilate) and resulted in the fall of blood pressure.

Next, BPF was tested on angiotensin-converting enzyme (ACE) and was found to be a potent inhibitor thereof (Fig. 5.22). This led to the hypothesis that inhibitors of ACE can be used as a means for treating hypertension. Interestingly, this finding led to award of Nobel Prize to John Vane in 1968, demonstrating that oligopeptides from the Brazilian viper's venom inhibited the activity of ACE from dog lungs. Thus, inhibitors of intracellular ACE not only prevented conversion of Ang I to Ang II but also prevented hydrolysis of bradykinin, thereby lowering blood pressure.

In subsequent years, characterization of oligopeptide(s) from the Brazilian viper's venom led to the identification of a nonapeptide teprotide as hit molecule that inhibited ACE *in vitro* (Fig. 5.23). However, lack of oral activity limited therapeutic utility of the nonapeptide, as peptides are known to get hydrolysed in gut under acidic conditions.

(*Continued*)

Study 5 (*Continued*)

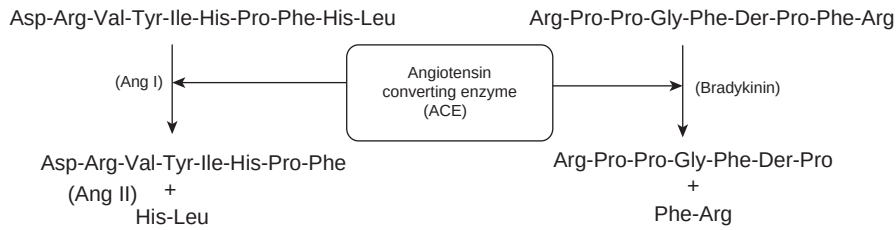


Figure 5.22 Hydrolysis of Ang I and bradykinin by ACE.

Glu–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro……(Hit molecule)

Figure 5.23 Nonapeptide teprotide from the Brazilian viper's venom that inhibited ACE *in vitro*.

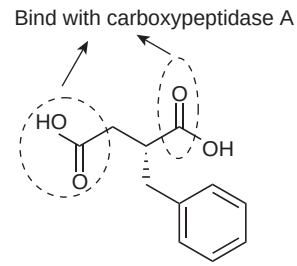


Figure 5.24 L-Benzylsuccinic acid (2(R)-benzyl-3-carboxypropionic acid) as potent inhibitor of model enzyme carboxypeptidase A.

Next, in order to demonstrate the direct role of Ang II in the increase of blood pressure, angiotensin I or II were injected into an animal model and both led to the rise of blood pressure. Next, nonapeptide teprotide from snake venom was injected prior to the administration of Ang I and II. As hypothesized, the rise in blood pressure was observed following administration of angiotensin II but not after injecting angiotensin I. These *in vivo* findings suggest that:

1. Ang II is directly involved in the increase of blood pressure.
2. Nonapeptide teprotide inhibited enzyme ACE, thereby preventing generation of vasoconstrictor substance Ang II.

Till 1970s, there was no report on converting a therapeutic peptide into a non-peptidic therapeutic small molecule that could be administered orally as drug. Squibb's applied a new approach to find small molecules as ACE inhibitors based on structure-based drug design approach. The strategy involved generating information about the active/binding site in the enzyme ACE since the nonapeptide may be inhibiting ACE following binding to its active site. In the absence of crystal structure of ACE (a zinc metalloproteinase), a model exopeptidase enzyme carboxypeptidase A was selected for structure-based drug design, as it was assumed to have the active/binding site (pocket) similar to ACE.

The most potent inhibitor of carboxypeptidase A was found to be L-benzylsuccinic acid (2(R)-benzyl-3-carboxypropionic acid), and it exhibited its inhibitory action probably by interacting with the binding site via succinyl carboxyl group and a carbonyl group (Fig. 5.24).

(*Continued*)

Study 5 (*Continued*)

Next, more than 2000 compounds were tested randomly against ACE that led to the identification of lead molecule succinyl-L-proline with significant improvement in the inhibitory properties (Fig. 5.25) and also preventing the conversion of Ang I and bradykinin.

The identification of prolyl derivative as inhibitor was a significant finding as the teprotide nonapeptide (snake venom peptide) too had proline amino acid as the c-terminal amino acid moiety and the succinyl moiety was thought to mimic the octapeptide region penultimate to Pro residue in the nonapeptide. Further, lead optimization studies using succinyl proline led to a series of active compounds and ultimately furnished captopril as a potent inhibitor of ACE (Table 5.1).

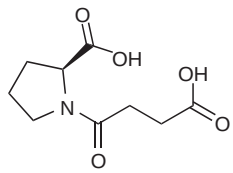
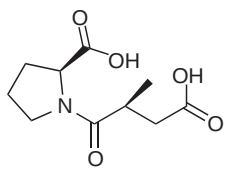
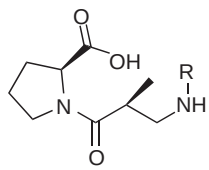
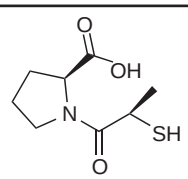
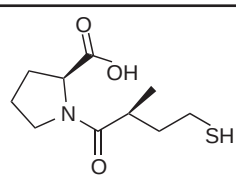
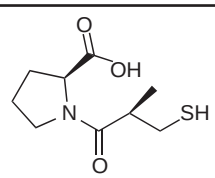


Figure 5.25 Succinyl-L-proline as lead molecule with ACE inhibitory properties.

Table 5.1 Lead optimization studies with L-succinyl proline

	A 15-fold improvement in inhibitory potency was obtained for this compound following introduction of 2-methyl substituent with D configuration.
	Introduction of basicity by replacing –COOH functionality with amide, amine, and guanidine had no effect on the inhibitory potency.
	However, 5-fold increase in activity was obtained with the replacement of –COOH function with –SH.
	A two-carbon extension of –SH group from the molecule furnished compound 50 times more potent than succinyl-L-proline.
	A one-carbon extension of –SH group from the molecule furnished captopril, 1000 times more potent than lead molecule.

5.4.6 Bioisosterism and Drug Design

Bioisosterism is often part of drug design strategies used during transformation of a lead compound into preclinical drug candidates. It generally involves replacement of a group/moiety with other group/moiety having identical physical and chemical properties in a manner that will furnish new analogues with either retention or enhancement of biological activity. For example, let's assume a hypothetical situation where a lead molecule is exhibiting response due to the presence of a group 'X' (or 'R') within the molecule but is having poor PK/PD compliance. For this, we need to change the physicochemical characteristics of the lead compound without affecting its biological activity. One way to achieve this is to replace 'X' (or 'R') within the molecule with analogous moiety 'Y' or ('P'). If the activity is retained in the new analogue following the replacement, then the new analogue is likely to have physical and chemical properties identical to the lead molecule. Under these circumstances, 'X' (or 'R') and 'Y' or ('P') can be called bioisosteres. Thus, bioisosterism in medicinal chemistry plays a major role in the development compound with acceptable PK/PD compliance. However, identifying isosteres remains a challenging task. Introduction of bioisosteres in a lead molecule during its optimization may affect several parameters associated with PK and PD features of test compounds/drugs.[11] The objectives of applying bioisosteres in drug design include:

1. Structural (size, shape, H-bonding)
2. Regulating PK parameters (lipophilicity, hydrophilicity, pK_a, H-bonding)
3. Improving potency by facilitating target interaction
4. Enhancing selectivity
5. Regulating metabolism
6. Substituting toxicophores
7. Creating novel molecules for intellectual property rights

Thus, during lead optimization/drug design approaches, bioisosteres is one approach that can be applied to replace unstable/problematic chemical groups without deviating from the structure of pharmacophore. The technique along with prodrug strategy is commonly applied to improve PK/PD properties of drug candidates. Bioisosteric replacements can be classified into two types: classical and nonclassical bioisosteres.

5.4.6.1 Classical Bioisosteres

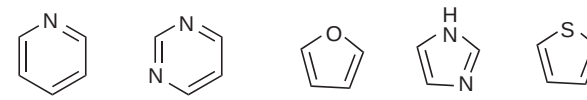
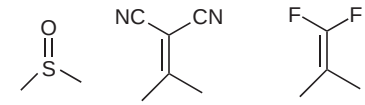
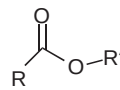
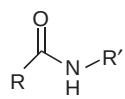
When two groups or molecules or ions bear similar reactive electron shell and shape, same number of atoms, and/or same number of valence electrons, they are called classical bioisosteres. Hydrogen ion and fluoride are examples of bioisosteres as they have same electronic configurations (Table 5.2). Replacement of bioisosteres in test compounds is expected to furnish compounds with retention or improvement in the biological activities.

Table 5.2 List of bioisosteres

Element/Functional Group/Moieties/Rings	Corresponding Isosteres/Bioisosteres
H	D, F
OH	SH, NH_2, CH_3
C	Si
CH_2	–NH–, –O–, –S–
Cl	F, CN, Br, CF_3
O	S, CH_2, NH
–C=C–	–C=N–, –C=O, –C=S

(*Continued*)

Table 5.2 (*Continued*)

Element/Functional Group/Moieties/Rings	Corresponding Isosteres/Bioisosteres
	
	
	
	

F as an Isostere of H (Application of Classical Bioisosteres in Drug Design)

The F has been widely applied in drug design as an isosteric replacement for H in order to modulate a range of medicinal properties of drugs irrespective of diseases for which they have been used. Primarily this has been attributed to approximately close Van der Waals radius (size) between F (1.20) and H (1.35) and also to the extreme electronegativity of F over H.

Fluorination of arenes, for example, can increase a drug's metabolic stability by making it less susceptible to oxidation. Oxidation during metabolism of arenes leading to arenols via arene oxide by far is the most common route to transformation (metabolism) of drugs for the purpose of excretion (Fig. 5.26). Replacing aromatic H with F can prevent oxidation of arenes.

Besides, appropriately positioned fluorine can make a drug more electron deficient by pulling electron density away (due to its extreme electronegativity) from the rest of the molecule. This in turn prevents the molecule from getting oxidized (a phenomenon during metabolism to convert drug into hydrophilic compounds for excretion) since oxidation requires losing an electron. Thus, the electron-deficient drug will be harder to oxidize and will be metabolically more stable.

For example, ezetimibe ($pK_a = 9.48$; acidic) is a drug used for lowering plasma cholesterol levels and it acts by blocking absorption of cholesterol in the gastrointestinal region. F atoms were introduced in the both arene rings (Fig. 5.27) at position 4 with the view to enhance its metabolic stability to oxidation (metabolic dealkylation to generate hydroxyl at *p*-position of arene rings is a common that favors excretion).

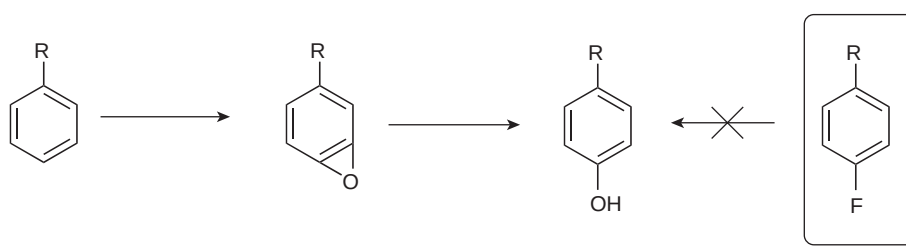


Figure 5.26 Metabolism (oxidation) of arenes and its prevention by introducing F group.

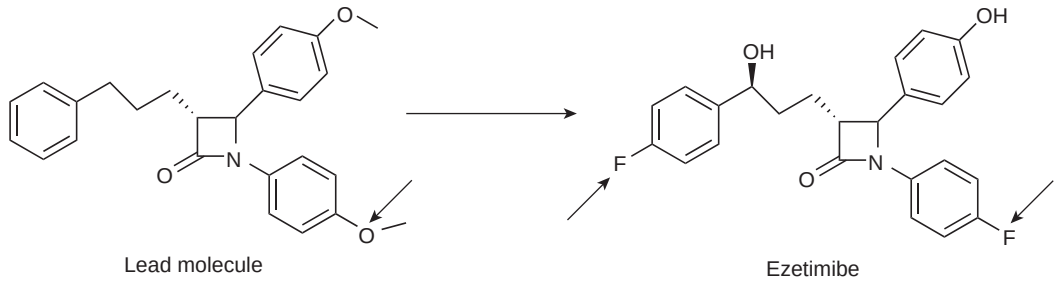


Figure 5.27 Introduction of F atoms to enhance metabolic stability to oxidation.

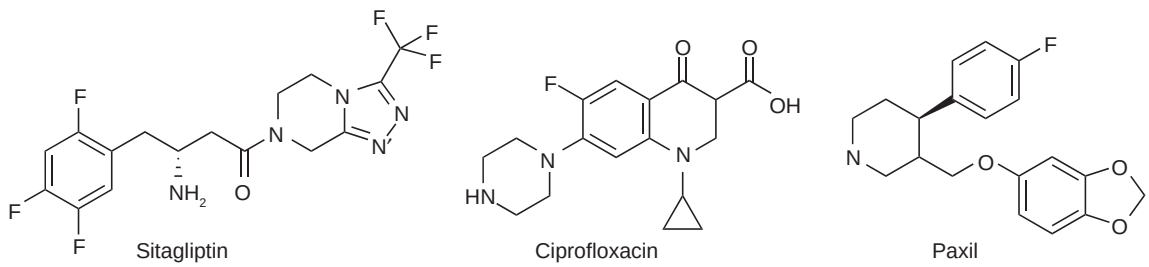


Figure 5.28 20% of all clinically used drugs are fluorinated compounds.

A careful survey of literature revealed approximately 20% of all drugs in used clinical is fluorinated compounds (Fig. 5.28).

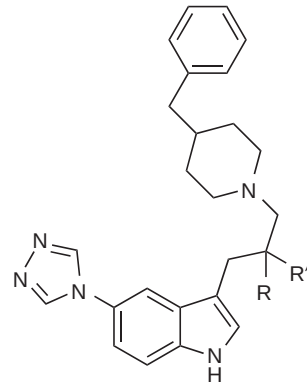
Role of F in Protein–Drug Interaction

Fluorine owing to its extreme electronegativity can have profound effect on the binding affinity of drug towards biological protein target. It has been observed that replacement of C–H bonds with C–F bonds in drug candidates allows fluorine atoms to act as hydrogen bond acceptors. Thus, the presence of fluorine can lead to tighter binding of a drug in the binding pocket of its target protein by increasing the number of potential hydrogen-bonding interactions between the drug and the drug target.

For example, the drug sitagliptin (Fig. 5.28) used for the treatment of type II diabetes contains six C–F bonds, including three on aromatic rings. It has been argued that the presence of three fluorines increased the potency due to increased interaction via hydrogen bonding in the active site (improved binding).

Effect of F on the pK_a

pK_a of a molecule plays a very important role in regulating PK/PD parameters. The extremely electronegative F has been often used to regulate the pK_a of test compounds/drugs. For example, pK_a of CH_3COOH is 2.59, whereas for CH_2FCOOH with an F atom pK_a is reduced to 1.24 and further introduction of F atoms will accordingly introduce further reduction in the pK_a. Knowledge of the pK_a allows the percentage of ionized vs. unionized concentration of drug to be calculated at any given pH, which is essential, for both absorption and diffusion of drugs. Niel *et al.*[12] observed that incorporation of one or two F atoms (Fig. 5.29) in the $5HT_{1D}$ receptor ligands significantly reduced pK_a and in turn had a strong beneficial effect on oral absorption (bioavailability). As can be seen in Fig. 5.29, compound with pK_a value of 6.7 has 50% unionized drug at intestinal pH that led to its increase in bioavailability.



R = R′ = H; pK_a 9.7
R = F; R′ = H; pK_a 8.7
R = R′ = F; pK_a 6.7

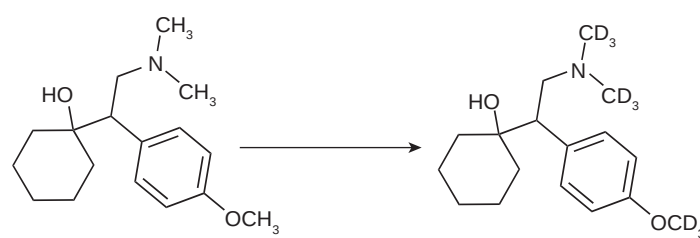
pK_a	At intestinal pH 4 to 7* Unionized			Bioavailability
	4.8	**5.7**	**6.7**	↓ Increases with decrease in pK_a
9.7	0%	0%	0.1%	
8.7	0%	0.1%	1.0%	
6.7	1.2%	9.1%	50%	

**Calculated using software available at https://www.manuelsweb.com/pka.htm (dated 10. 05. 2017)*

Figure 5.29 Effect of pK_a value on the bioavailability.

Replacement of H with D to Reduce Drug Toxicity

Deuterium has been used as bioisostere of H to reduce toxicity of compounds. For example, venlafaxine, a dual serotonin/norepinephrine reuptake inhibitor with antidepression activity was found to be toxic due to formation of O-demthylated and N-demethylated metabolites. Replacement of CH_3 group attached to nitrogen and oxygen with bioisostere CD_3 group furnished deuterated analogue of the drug with significantly reduced metabolism by cytochrome enzyme and with enhanced exposure of the parent drug (Fig. 5.30).



Venlafaxine Deuterated version

Figure 5.30 Replacement of H with deuterium reduced toxicity.

Ring Bioisosteres

The application of ring equivalents is one of the most widely used strategies to design drugs not only with novel intellectual property right but also with enhanced metabolic stability. In a classical example, three COX-2 inhibitors used clinically are from 'me too' class of drugs with bioisosteric variation involving replacement of pyrazole ring as in celecoxib with pyridine as in etoricoxib or isooxazole as in valdecoxib (Fig. 5.31). Thus, pyrazole, pyridine, and isooxazole are ring bioisosteres.

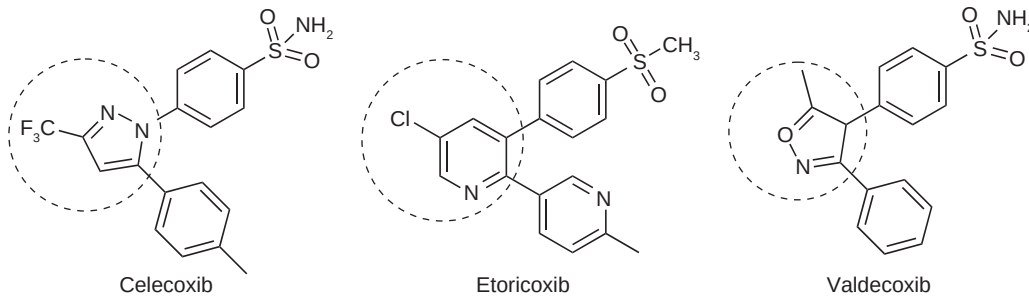
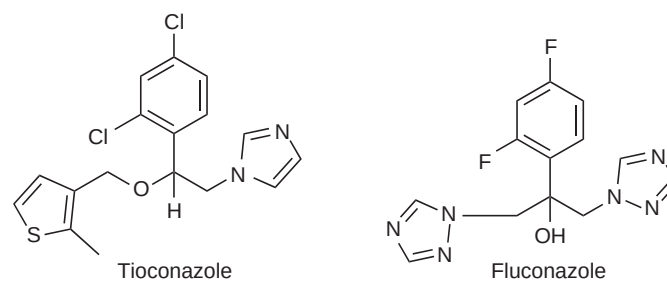


Figure 5.31 COX-2 inhibitors with bioisosteric variation.

Modifying Lipophilicity with Bioisosteres

Using bioisosterism strategies, lipophilicity (which plays an important role in the permeation of drug across membranes) of test molecules can be controlled by introducing appropriate bioisosteres. Tioconazole (Fig. 5.32), an antifungal drug widely used for skin infection, has restricted use (cannot be used for systemic infections) due to its hydrophobicity and poor aqueous solubility (clog = 4.79). Replacing the less polar heterocycles in the molecule with relatively polar heterocycles along with introduction of hydrophilic hydroxyl group furnished orally active drug fluconazole with significantly reduced logP value (clogP = −0.44).



Drug	logP	pK_a	Solubility
Fluconazole	0.5/2.27	1.76	1.39 mg/mL
Tioconazole	4.4/5.3	6.77	0.0165 mg/mL

Figure 5.32 Modifying lipophilicity furnished orally active drug fluconazole.

Application of Bioisosteres in Reducing Toxicity

Aminopyrine remained in clinical use as analgesic and anti-inflammatory drug since its introduction in 1896. However, in 1922, it was found to be carcinogenic and in 1951, Roche replaced dimethylamino group with a bioisostere isopropyl group and were able to remove the carcinogenic activity (Fig. 5.33).

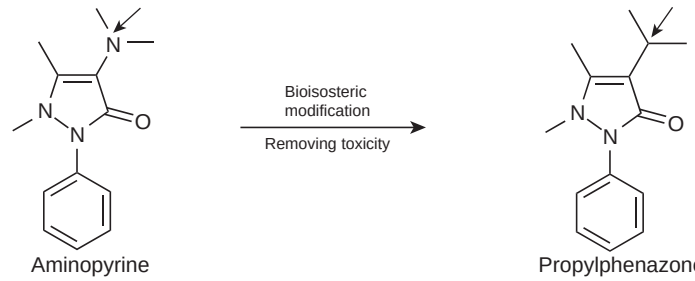


Figure 5.33 Replacement of dimethylamino group with a bioisostere isopropyl group.

5.4.6.2 Nonclassical Bioisosteres

These bioisosteres are structurally different compounds as far as their atomic constitution is concerned but still display similar physicochemical properties. They are generally introduced as replacement for functional groups required for target interaction but are associated with PK issues.

Nonclassical Bioisosteres of Polar Groups

Polar groups besides playing an important role in drug-target interactions also contribute towards pK_a, lipophilicity, and solubility of test compounds/drugs. Thus, on one hand these polar groups facilitate binding with biological targets, on the other hand they may reduce lipophilicity and pose problems by affecting absorption (owing to the ability of polar groups to ionize). If such a situation arises in any potent test compound(s), the polar groups can either be masked (prodrug strategy) to facilitate absorption or can be replaced with nonclassical bioisosteres with similar physicochemical properties.

For example, among several bioisosteres[13] used for –COOH group, replacement with 5-substituted tetrazole ring remains one of the most classical strategies for improving activity. This can be attributed to the presence of acidic proton in tetrazoles like –COOH group, coupled with significantly enhanced lipophilicity (enhancing permeation and bioavailability) of tetrazole anion in comparison to caboxylate anion (Fig. 5.34).

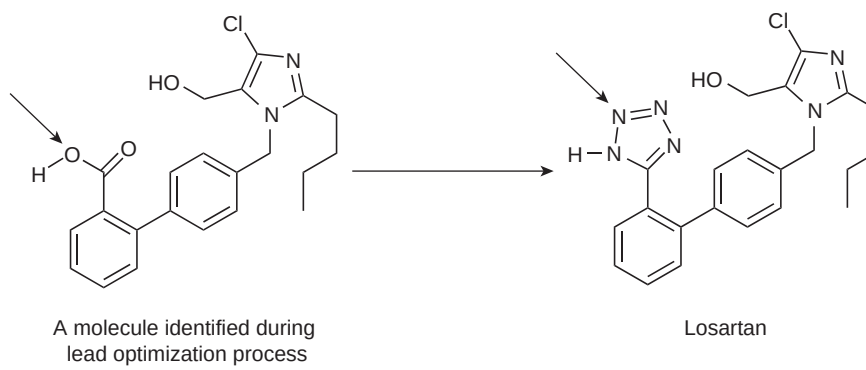


Figure 5.34 Substituted tetrazole ring as bioisostere of –COOH group.

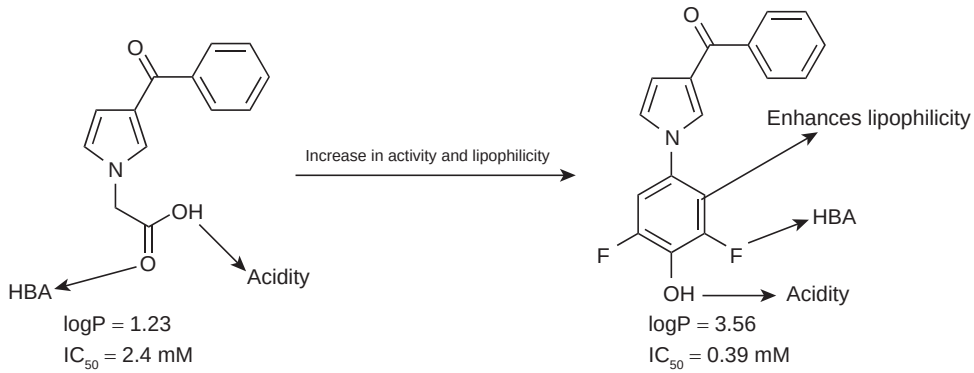


Figure 5.35 2,5 difluorophenol as bioisostere for –COOH functionality.

In yet another interesting example, 2,5 difluorophenol has been used as bioisostere of –COOH functionality for test compounds with aldose reductase inhibitory activity. The replacement with 2,5 difluorophenol enhanced lipophilicity (bioavailability) and activity by maintaining HBA (hydrogen bond acceptor) group and acidity similar to that present in the carboxyl group (Fig. 5.35).

Cyclic and Noncyclic Isostere

Estradiol and dimethylstilbestrol represents a classical example of ring versus noncyclic bioisosteres. The central bond in diethylstilbestrol led to the orientation (*trans*) of the phenol and ethyl groups in a manner that favored its binding to the estrogenic receptors and was found to be equipotent to estradiol (Fig. 5.36).

Sultopride is an antipsychotic drug used for the treatment of schizophrenia. Replacement of amide bond in sultopride with a nonclassical cyclic bioisostere led to selectivity for D3 receptor over D2 receptor (Fig. 5.37).

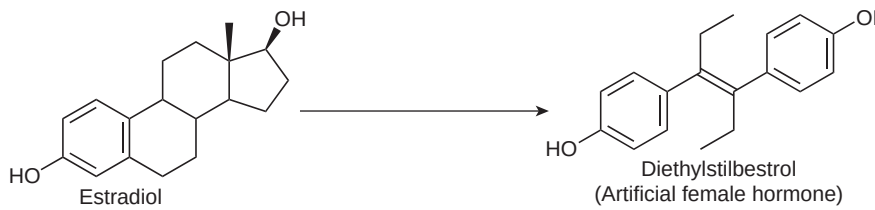


Figure 5.36 Introduction of noncyclic isostere.

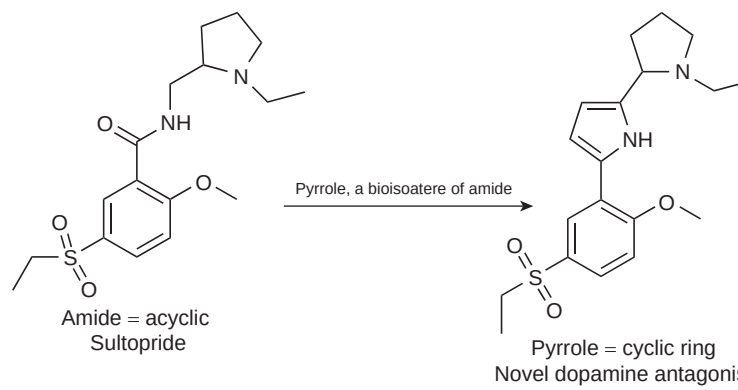


Figure 5.37 Introduction of pyrrole as cyclic isostere.

5.4.7 Stereoisomerism in Drug Design

Isomerism deals with compounds having identical chemical compositions but have different orientations of their atoms in space. Three types of isomerism are possible – constitutional, configurational, and conformational. Stereochemistry involving configurational (geometrical isomers and optical isomers) and conformational isomerism in drugs plays an important role in the pharmacological, PK, and PD properties of drugs (ADMET).

Important Note

The isomeric pairs due to change in stereospecificity have different physical properties (partition coefficient, pK_a, etc.) and thus will differ in their pharmacological activity (PD) and ADMET properties (PK).

5.4.7.1 Optical Isomerism in Drugs

Optical isomerism in drugs can occur either due to enantiomerism or diasteromerism depending on the presence of number of chiral centers. Notably, enantiomers although display similar physical and chemical properties, their physiological activities vary owing to their difference in their binding with protein macromolecules. These bindings in turn regulate interaction with target, metabolism, and distribution of test compounds. The difference in the pharmacological action in a drug with optical isomers is due to the difference in the 3D orientation of functionalities (point of attachment) involved in the binding of the two isomers with protein targets (enzyme or receptor).[14] For example, among the two enantiomers of epinephrine, R-isomer has three-point attachments oriented towards its adrenergic receptor while in S-isomer due to change in orientation, only two-point attachments are possible with the adrenergic receptor. Thus, the formation of H-bond is absent due to changed orientation of the hydroxyl group (Fig. 5.38).

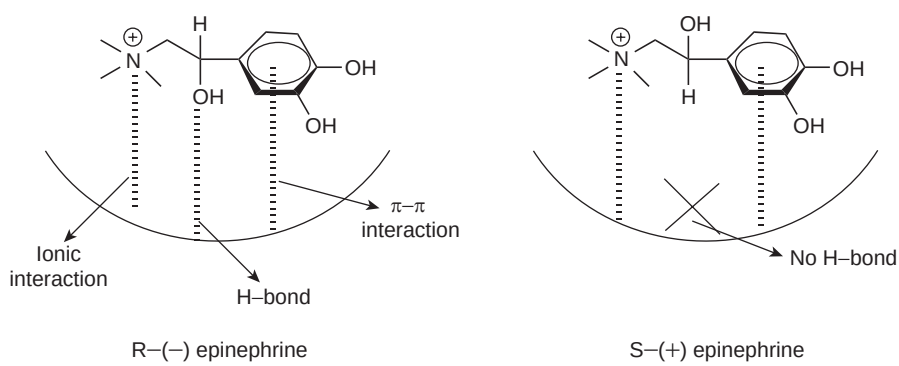
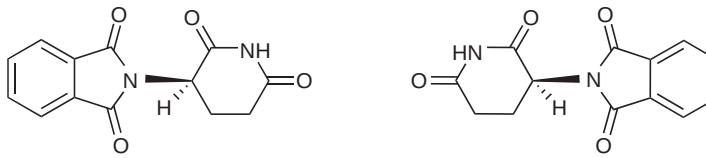


Figure 5.38 R-isomer has 3-point attachments whereas S-isomer has two-point attachments.

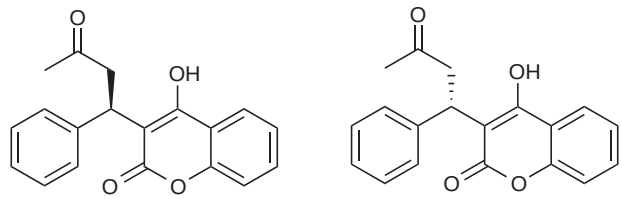
A Horror Drug from the 1950s

Thalidomide was originally launched in 1955 as a hypnotic, sedative, and anxiolytic. Use of this drug by pregnant mothers caused severe birth defects among over 10,000 babies. This was attributed to the presence of a chiral center in the molecule due to which drug existed as racemic mixture of (*R*)- and (*S*)-enantiomers. Both isomers were generated during the manufacturing process of the drug. The (*R*)-enantiomer had sedative effects, whereas the (*S*)-isomer led to birth defects (Fig. 5.39). Besides, it was also found that the isomers interconverted under physiological conditions, thereby restricting its clinical use even as pure isomer. The thalidomide tragedy led many countries to lay new guidelines for enantiomeric drugs.



R – (+)-Thalidomide S – (–)-Thalidomide

Figure 5.39 Thalidomide, a horror drug.



(R) – (+) Warfarin (S) – (–) Warfarin

Figure 5.40 S-isomer is more active than R-isomer



Important Note

"To evaluate the pharmacokinetics of a single enantiomer or mixture of enantiomers, researchers need to develop quantitative assays for individual enantiomers *in vivo* during the early stages of drug development. This will allow assessment of their inter conversion abilites and the ADMET profile of the individual isomers. Unless it is particularly difficult, the main pharmacologic activities of the isomers need to be compared in the *in vitro* systems, in animals and/or in humans."

Clinically, many drugs are used either as racemic mixture since both the enantiomers are active or as a single isomer. Ibuprofen, a nonsteroidal anti-inflammatory drug is used clinically in the form of racemic mixture. Interestingly, in humans, R(–) ibuprofen undergoes unidirectional inversion in its configuration under physiological conditions to furnish S(+) ibuprofen. The latter is 100 times more potent than its corresponding R-enantiomer in inhibiting COX-1.

Verapamil, a calcium-channel blocker, is used as racemic mixture although S-isomer has been found to be 20 times more potent than its corresponding R-enantiomer.

Warfarin used as anticoagulant is also administered as racemic mixture with S-isomer being more active than R-isomer. Although both the isomers elicit same biological response, their potencies differ due to their difference in metabolism and excretion. The active S-isomer is more readily eliminated than R-warfarin (Fig. 5.40).

Benzomorphans

Therapeutic analgesic activity in benzomorphans series was found to be regulated by their absolute configurations. In racemic pentazocine, one of the enantiomer S(–)-pentazocine was found to be 20 times more active than R-enantiomer. Thus, out of the two, S(–) enantiomer is used clinically for the management of pain (Fig. 5.41).

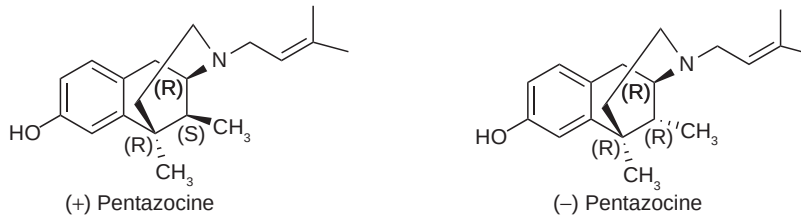


Figure 5.41 (–)-Pentazocine is 20 times more potent than its (+) enantiomer.

5.4.7.2 Conformational Isomerism

Conformational isomerism generally occurs in compounds due to free rotation between two atoms resulting in *syn, anti* (*trans*), and *gauche* conformations. Among these three conformations, *trans* is the most preferred, as it has lowest energy followed by *gauche*. The least stable conformation is the *syn* due to steric and electronic hindrance between the overlapping substituent. Interestingly, many achiral compounds exhibit enantiomeric conformers arising due to dissymmetry in the molecule. Thus, any drug due to free rotation between two atoms too can exist in more than one conformer and hence can bind to more than one protein target (receptor/enzyme). However, in contrast a protein target owing to its specificity will bind to only one conformer. The neurotransmitter acetylcholine (ligand/first messenger) due to the presence of *trans* and *gauche* conformation binds selectively to muscarinic and nicotinic receptors, respectively (Fig. 5.42).

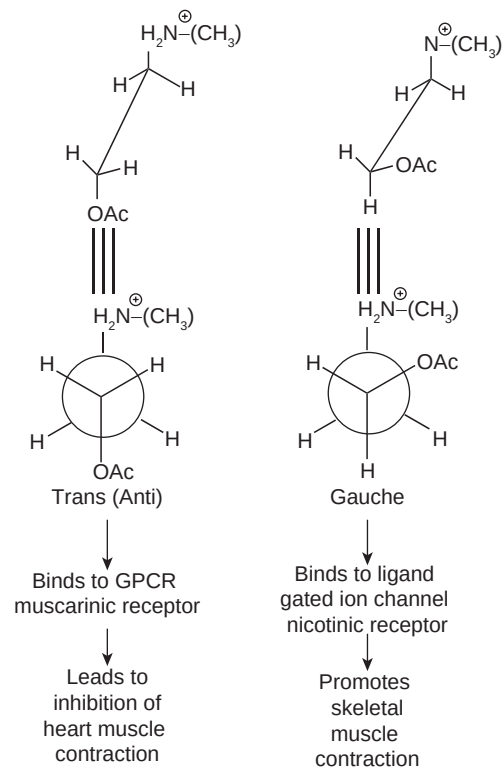


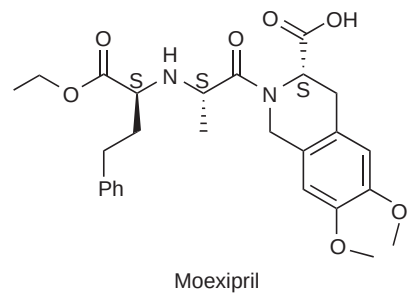
Figure 5.42 Biologically active conformations of acetylcholine and their selectivity for receptors.

5.4.7.3 Diastereomeric Drugs

In general, diastereomeric compounds have two or more chiral centers and accordingly can exist as four or more stereoisomers. The diastereomers differ in their relative stereochemistry and are nonsuperimposable mirror images with different physical properties. Thus, for drugs with two more chiral centers, safety and efficacy of all diastereomers needs to be established with a view to identify a single diastereomeric compound with optimal therapeutic response. Difference in physical properties of diastereomers will affect their ADMET properties.

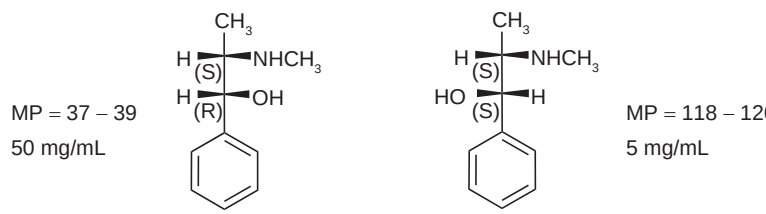
Captopril (Table 5.1), an antihypertensive compound, was the first drug to be released in the market as angiotensin converting enzyme (ACE) inhibitor. This was followed by development of a series of drugs named enalapril, lisnopril, benazepril, moexipril, idapril, etc. One thing that was common in these ACE inhibitors is that they carry two or more chiral centers, thereby resulting in four or more diastereomeric forms; however, only single isomer is used clinically (Fig. 5.43).

Ephedrine and pseudoephedrine, due to the presence of two chiral centers, exist as four stereoisomers. They differ in their chemical structure and in their solubility; ephedrine is 'erythro', whereas pseudoephedrine is 'threo' (Fig. 5.44). However, when it comes to treating orthostatic hypotension, ephedrine is more active than pseudoephedrine. On the contrary, for the treatment of nasal decongestion, pseudoephedrine is preferred over ephedrine.



Moexipril

Figure 5.43 ACE inhibitor with more than two chiral centers.



Ephedrine (Erythro)

Pseudoephedrine (Threo)

Figure 5.44 Diastereomers with differential physical properties.

5.4.8 Drug Design Strategies to Improve Absorption via Modulating Polarity

Hydrophobicity of drugs/test compounds plays an important role in the absorption via diffusion through the GIT lipid bilayer cell membranes. Molecules with moderate hydrophobicity tend to diffuse into the cell while molecules with excessive hydrophobicity enter the lipid bilayer membrane but cannot exit the membrane and enter into cytoplasm. Weakly hydrophobic compounds fail to enter the lipid bilayer.

One approach to regulate hydrophobicity as evident by logP values in the test compounds is to modulate the polarity of compounds. The hydrophobicity in test compounds can be reduced by increasing the polarity either by increasing ionization or by introducing polar groups of different strengths ($COOH > OH > NH_2$). Alternatively, hydrophobicity in test compounds can be increased either by diminishing ionization or by introducing alkyl/aryl moieties with different hydrophobicity (aryl > butyl > methyl).

5.4.8.1 Modulating Hydrophobicity of Compounds by Shifting of Alkyl or Acyl Substituent

Hydrophobicity of drugs plays a very important role in the absorption and distribution of drugs. One approach for regulating hydrophobicity in test compounds is to shift the position of alkyl group within the molecule (Fig. 5.45).

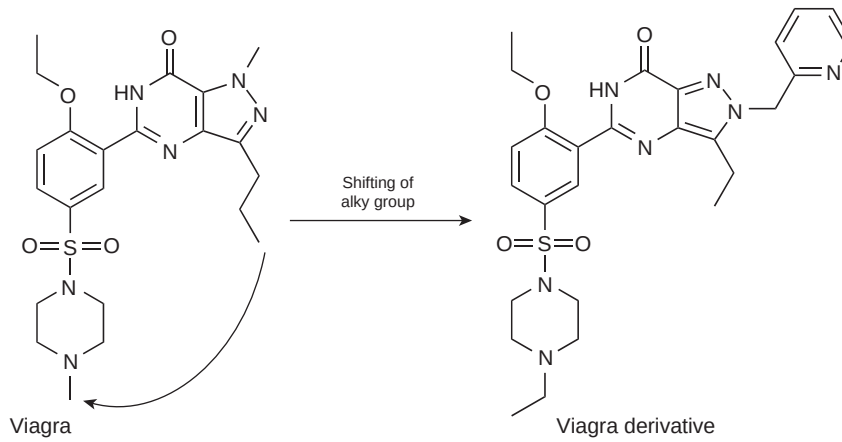


Figure 5.45 Shifting of methyl group furnished viagra derivative with enhanced activity.

5.4.8.2 Modulating Hydrophobicity by Introducing Polar Functional Groups

Among many strategies available to reduce the hydrophobicity of the test compounds, a simple strategy involves introduction of polar groups without compromising with its biological activity. Antifungal drug tioconazole due to its excessive hydrophobicity is restricted to topical use only; however, introduction of hydroxyl group (as polar group) as in tioconazole reduced hydrophobicity and enhanced bioavailability by oral route (Fig. 5.32).

5.4.8.3 Modulating Hydrophobicity by Masking Polar Groups

In yet another strategy to modulate hydrophobicity, polar groups present in the molecule can be masked. However, this may or may not interfere with protein binding. The strategy is extensively used during the design of prodrugs.

$$R - OH \xrightarrow{CH_3I} R - OMe$$

$$R - NHR \xrightarrow{CH_3COCl} \begin{matrix} R \\ \diagdown \\ N - COCH_3 \\ \diagup \\ R' \end{matrix}$$

$$R - COOH \xrightarrow[R'-OH]{H^+} RCOOR'$$

For example, antibiotic ampicillin is administered as inactive prodrug, bacampicillin, by masking the carboxyl function with an acyl group. The latter undergoes enzymatic degradation inside the body to release the active drug ampicillin (Fig. 5.46).

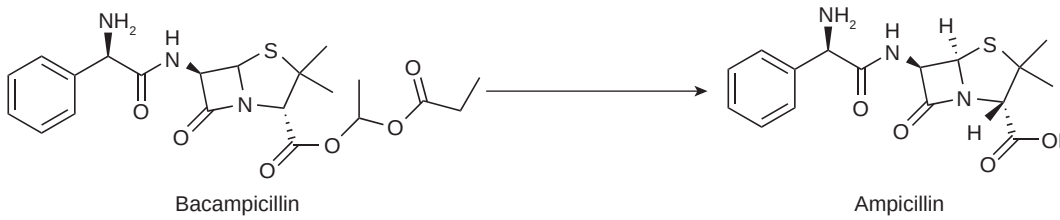


Figure 5.46 Masking of polar group in bacampicillin.

A sedative drug nordazepam, due to the presence of free NH group undergoes rapid metabolism and excretion. This issue was addressed by replacing NH with N-Me, which resulted in diazepam with improved duration of action due to decreased metabolism and excretion (Fig. 5.47).

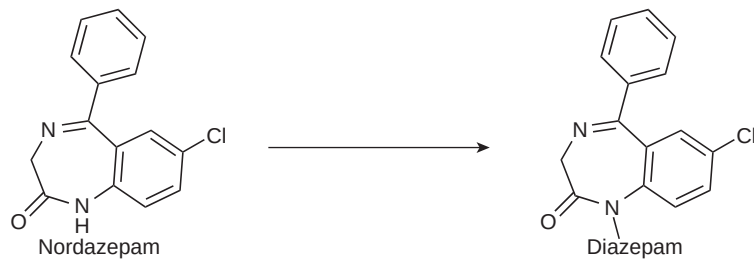


Figure 5.47 Masking polar NH group by NH–CH_3 group.

5.4.9 Preventing Degradation of Drugs (Drug Stability)

Drug design strategies have also been applied to increase the stability of drugs against acid hydrolysis in gut and against CYP 450-mediated metabolic degradation in gut and liver. Improving metabolic degradation and stability of test compounds/candidate drugs in turn may prolong their half-life and duration of action.

5.4.9.1 Steric Shields to Block Hydrolysis

A widely used strategy to prevent enzymatic hydrolysis of acids, esters, or amides is to introduce a bulky group at the site susceptible to hydrolysis (Fig. 5.48).

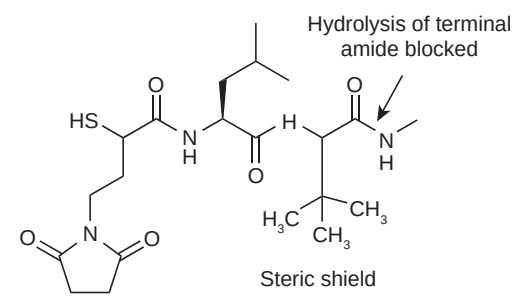


Figure 5.48 Blocking hydrolysis by introducing bulky group.

5.4.9.2 Stereoelectronic Modification

Introduction of steric group coupled with modification in the electronic properties of groups has been affected with the view to prevent degradation of drugs. For example, procaine with an ester group is hydrolyzed quickly; onset of local anesthetic effect occurs between 14 and 18 minutes, and duration of action remains between 60 and 90 minutes. However, replacing ester group with NH–CO group (changed electronic properties) and introducing two ortho-methyl groups in the phenyl ring as conformational blockers furnished lidocaine with enhanced duration of anesthetic effect up to 200 minutes. Thus, stereoelectronic changes in local anesthetic procaine resulted in lidocaine with longer duration of action (Fig. 5.49).

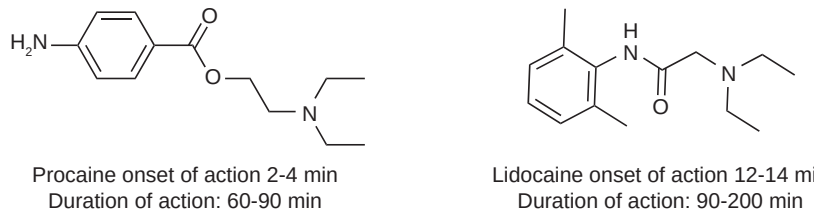


Figure 5.49 Introducing stereoelectronic modification by replacing ester with an amide group.

5.4.9.3 Metabolic Blockers

Metabolism involves enzymatic breakdown of a compound into an active or inactive metabolite. Most drug metabolism primarily occurs in the liver; however, small intestine remains the most important extrahepatic site of drug biotransformation where metabolizing enzymes cytochrome P450 (CYPs) present in the proximal regions (of the small intestine) are responsible for drug metabolism reactions. Cytochrome (CYP) P450 is a family of heme (cofactor)-containing monooxygenase enzymes. It carries out metabolic oxidative transformation of xenobiotics including drugs and endogenous substances.

In general, CYP transform or derivatize the drug into water-soluble (hydrophilic) compounds so as to facilitate its excretion through urine. One of the transformations involves hydroxylation via oxidation by CYP enzymes. For example, during the metabolism of megestrol acetate (oral contraceptive), the position 6 becomes hydroxylated by metabolic enzymes as the position is prone to oxidation. This in turn facilitates its excretion, thereby reducing biological efficacy of the drug. The metabolic oxidation was blocked by introducing a methyl group at position 6, which prolonged the duration of action of the drug (Fig. 5.50).

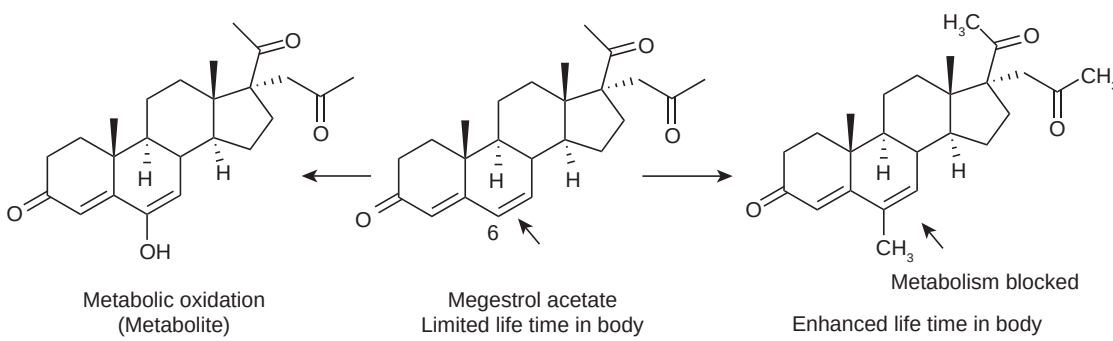


Figure 5.50 Preventing metabolic oxidation.

5.4.9.4 Reducing Metabolic Degradation

Poor efficacy and short duration of action exhibited by a variety of test compound/drugs can be attributed to CYP 450-mediated metabolic degradation. Some of the common metabolic degradation involves transformation of methyl group to –COOH group, hydroxylation of aliphatic/aryl carbons, oxidations, and dealkylation and deamination reactions. Thus, preventing degradation of these susceptible groups can improve the efficacy and duration of action of the drug. Tolubutamide, an antidiabetic drug, undergoes metabolic degradation due to the oxidation of methyl group in the benzene ring into the –COOH group. Replacement of methyl group with chloro group furnished chlorpropamide with prolonged duration of action (Fig. 5.51).

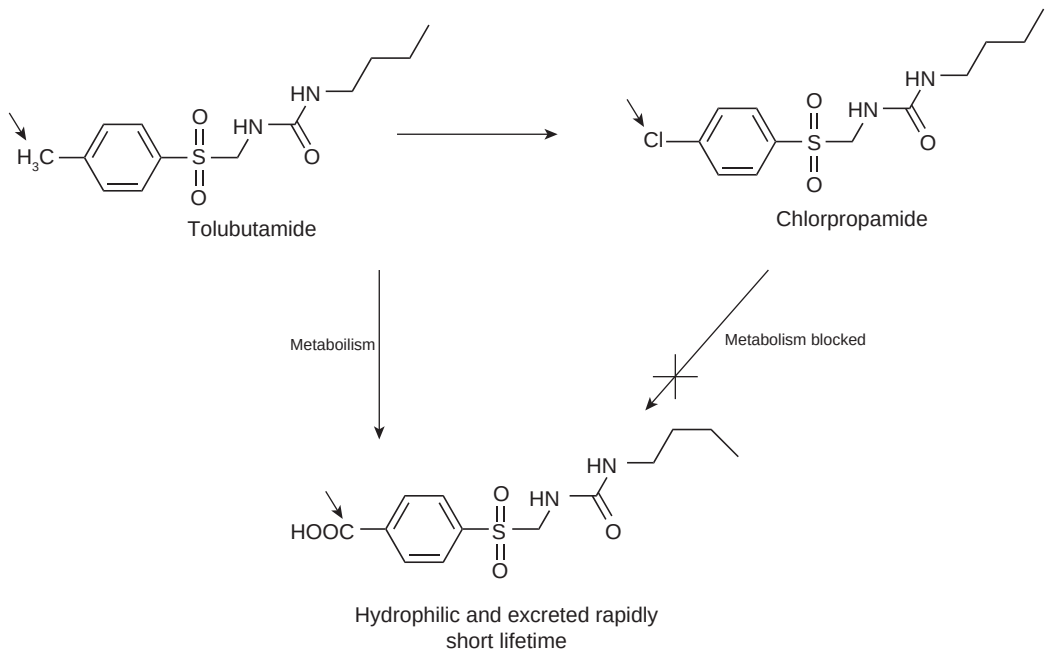


Figure 5.51 Replacing susceptible CH_3 group with chloro furnished chlorpropamide with long duration of action.

5.4.9.5 Dealing with Metabolically Susceptible Groups

Some metabolic reactions during phase II (non-CYP-mediated) may attenuate or terminate biological activity of test compounds/drugs via conjugation reactions involving methylation/acetylation/sulfation/glutathionylation. The resulting conjugated products formed in cytosol are generally water soluble and are easily excreted. For example, in salbutamol (antiasthmatic drug), one of the hydroxyl groups present on the aromatic ring undergoes selective methylation by catechol-O-methyl transferase resulting in the termination of the activity (Fig. 5.52). This can be attributed to loss of binding of drug with target proteins in the absence of the hydroxy group. Thus, in order to prevent methylation of the −OH group, the latter is shifted in a manner that its ability to bind with the target is retained without compromising with activity.

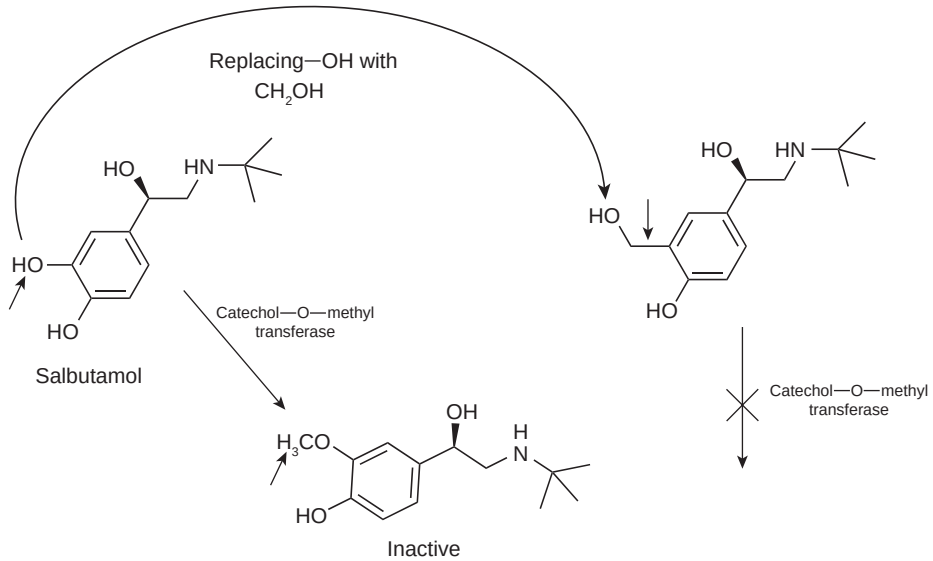


Figure 5.52 Shifting susceptible group to protect its biotransformation (metabolism).

Introducing Susceptible Metabolic Groups

At times during drug development programme, one can come across test compounds/drugs with very long half-life in the human body. This in turn may lead to toxicity and other undesirable side effects by binding non-specifically with other proteins. In such a situation, one can introduce metabolically susceptible groups in a manner that would not only affect its biological activity but will reduce the half-life of drug/test compounds.

For example, during the development of antiarthritic agent etoricoxib (Fig. 5.53), the lead molecule 5-chloro-3-(4-(methylsulfonyl) phenyl)-2,3'-bipyridine had a very long half-life, thereby causing toxicity.

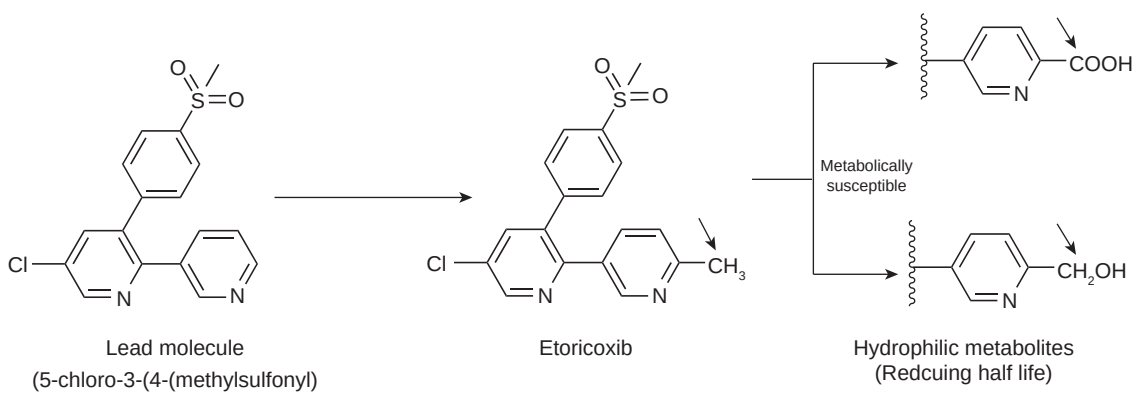


Figure 5.53 Replacing methyl with metabolically susceptible groups.

The half-life of the lead molecule was reduced by strategically introducing methyl moiety as a metabolically susceptible group. The methylated compound etoricoxib following administration was rapidly metabolized into hydrophilic metabolite bearing –COOH or CH_2OH group, thereby facilitating its rapid excretion from the body (reducing half-life of drugs).

Ring Variation to Improve Metabolic Stability

Sometimes the presence of a ring system can make the molecule vulnerable to metabolism, thus introducing variation in the ring may resist metabolism and improve biological activity. For example, during the development of the antihypertensive agent cilazapril as an ACE inhibitor, initially a bicyclic moiety was identified as a lead molecule with moderate antihypertensive activity as it was prone to metabolism. However, introducing ring expansion led to the identification of the clinically useful cilazapril with high affinity for the enzyme (Fig. 5.54).



Figure 5.54 Example of ring variation in the lead molecule led to clinically useful drug.

5.4.10 Drug Design Strategies Used for Targeting Drugs

The major problems associated with administration of systemic drugs/test compounds are as follows:

1. They are evenly biodistributed throughout the body.
2. They lack specific affinity for tissues bearing a pathological site.
3. They require administration in large doses in order to achieve high concentration at the desired site (due to even biodistribution).
4. They exhibit toxicity due to nonspecific binding of drugs to undesired proteins (off-target).

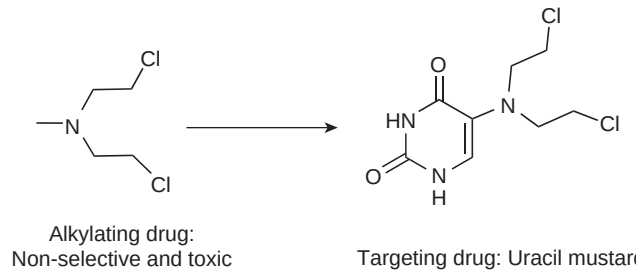
In order to overcome these problems, drug design strategies need to be adopted that would allow enhanced drug concentration at the pathological site (desired site of action), irrespective of route of administration. Thus, access to the drug target requires a targeted approach[15] where the test compound/drug is selectively and specifically targeted or delivered (deposited) only to its binding site by avoiding its exposure to the undesirable target organs or tissues or cells. If a drug can be targeted in such a way, it will minimize even biodistribution and subsequent toxic effects and in turn will maximize therapeutic index.

Historically, Paul Ehrlich for the first time introduced the concept of targeting drugs based on his observation that antibacterial drugs were selectively toxic to microbial cells over human cells. Some of the classical examples depicting drug targeting have been summarized below:

5.4.10.1 Targeting Drug Selectively for Tumor Cells

The major drawback associated with the therapy of cancer is that the drugs are unable to distinguish between cancer cells and normal human cells. In order to overcome this problem, drugs can selectively target

cancer cells. Among many strategies used for this purpose, one strategy involves covalent linking of the drug/therapeutic test compound with an intravital building block (nucleic acid or amino acids) that is required by cancer cells for their proliferation and survival. For example, transformation of nitrogen mustard, an anticancer drug, into targeted drug uracil mustard (Fig. 5.55).



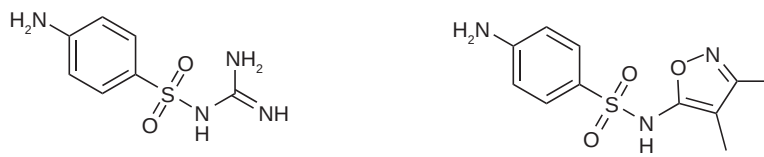
Alkylating drug:
Non-selective and toxic

Targeting drug: Uracil mustard

Figure 5.55 Targeting drug for the treatment of cancer.

5.4.10.2 Targeting Drug for Gastrointestinal Tract Infection

For the treatment of infections in gut, the drugs (antibacterial) need to remain concentrated in the gut and should not under any circumstances get absorbed by gut wall tract. To achieve this, the antibacterial drug should be highly polar (ionized) so that it does not cross the gut wall and urinary tract (only lipophilic compounds are absorbed). One of the examples is antibacterial drug sulfaguanidine (water soluble) that is highly ionized in acidic pH in gut and is incapable of crossing the gut wall. Yet another water-soluble hydrophilic derivative sulfisoxazole is used for treating urinary tract infections (Fig. 5.56).



Sulfaguanidine, are so soluble in water that they are not absorbed from the GI tract (<5%).

A few very water-soluble sulfisoxazole are rapidly excreted via the urinary tract (>90% in 24 hr) mostly in an unchanged form; because of this, they are primarily used to treat urinary tract infections.

Figure 5.56 Targeting drug for infectious diseases.

5.4.10.3 Targeting Drug for Peripheral Regions Instead Central Nervous System

Treating neurological disorders using systemic administration of drug remains a daunting task owing to the presence of unique and protective BBB. Under normal physiological conditions, substances circulating in the blood capillary (made up of endothelial cells) can enter brain by either passive or active transport. For example, lipid-soluble substances such as alcohol, caffeine, nicotine, and anesthetics readily diffuse through (pass through their intercellular clefts) the membranes. Uptake of other essential nutrients (glucose, amino acids, and nucleosides) occurs via carrier-mediated transport proteins. The majority of drugs circulating in blood capillary in brain penetrate BBB through passive diffusion.

Several potent preclinical CNS therapeutics have failed to reach clinics due to their incompatible physicochemical properties, peripheral metabolism (drug enters CNS via the systemic blood circulation) and poor diffusion abilities through BBB.[16] To overcome these hurdles, CNS drugs during conventional administration are given either at higher or in multiple doses so that the desired concentration of CNS drugs can reach the site of action. Nonetheless, this may significantly enhance the risk of systemic toxicity as well.

An alternate strategy for targeting drugs for CNS disorders involves lipidization in order to facilitate passive diffusion of CNS drugs into the BBB. Lipidization is introduced via chemical modification (primarily by adding lipid groups to the polar ends) of the therapeutic candidate/test compound itself into a more lipophilic form. Several studies have shown that for an acceptable CNS permeability, lipophilic analogues with logP value in the range of 1.5–2.5 are preferred. Indeed, drawback of lipidization may lead to poor selectivity and poor distribution of drugs despite enhancement in the volume of distribution. For example, morphine with polar groups has clogP of 0.57; on the other hand, its lipophilic analogue heroin with good permeability across BBB has clogP of 1.48 (enhanced logP).

5.4.11 Prodrug Strategy

During lead optimization studies, the general perception is to design drugs that are neither too lipophilic nor too hydrophilic with pK_a values ranging between 6 and 8 (not a thumb rule). These physicochemical properties of drugs together play an important role in solubility, bioavailability, distribution, metabolism, excretion, and interaction with biological targets to elicit desired biological response. However, often during lead optimization studies/drug design process, we come across preclinical candidates that fail to deliver the desired biological results in the human due to poor bioavailability/access to biological targets or stability issues (PK/PD issues).

This occurs because human system generally treats every drug administered as xenobiotics and tries to either expel them in the gut after absorption by efflux phenomenon or extensively metabolizes them into hydrophilic substances for the purpose of excretion. To overcome these drawbacks associated with a compound of therapeutic value, the concept of prodrug was introduced. The concept of prodrug relies on the transformation of an active molecule[17] into a derivative that itself is inactive but once administered, is converted into the active drug by metabolic biotransformation.[18] In general, prodrug strategies can either address a variety of PK (ADMET)/PD (improving physicochemical, biopharmaceutical, or PK properties) issues or pharmaceutical issues in order to transform a therapeutic drug candidate into a clinically successful drug. Transforming a drug into prodrug assists in good absorption, access to targets in tissue through facile distribution, resistance to metabolism, and with moderate excretion properties.

Design of prodrug can address PK/PD issues by

1. Improving solubility (enhance bioavailability)
2. Improving membrane permeability (distribution)
3. Preventing pre-systemic metabolism (before absorption)
4. Preventing systemic metabolism (during absorption)
5. Improving distribution of drugs from blood plasma into tissues
6. Prolonging of duration of action so as to access biological targets
7. Altering toxicity
8. Altering elimination

Design of prodrug can address pharmaceutical issues by

1. Improvement of taste
2. Improvement of odour
3. Reduction of irritation
4. Reduction of pain on injection
5. Enhancement of drug solubility and dissolution rate
6. Enhancement of chemical stability of drug
7. Drug delivery: By preparing parenteral or injectable formulation

5.4.11.1 Definition of Prodrug

The prodrug is referred to as a pharmacologically inactive compound which when administered inside the body undergoes biotransformation and releases the active drug. The active moiety of the prodrug in the physiological conditions is released either by nonenzymatic or enzymatic process. The biotransformation of prodrug may occur either before or after absorption in the blood stream or at the site of action. Thus, prodrugs are activated (biotransformed) either intracellularly (within cells) or extracellularly (in digestive fluids or in the systemic circulation). Examples of drugs activated intracellularly include lipid-lowering statins, whereas prodrugs activated extracellularly include antibody-prodrug, gene-prodrug, or virus-directed enzyme-prodrugs, used in chemotherapy or immunotherapy.

Another term that is analogous to prodrug and is often used in drug discovery is called latentiation. The latter is defined as chemical/pharmacological modification of an active compound, which when administered in the human body reverts to the original active compound and in turn prolongs its biological action.

Thus, the objective of prodrug is to improve permeability to be absorbed into the blood circulation (absorption), to access target efficiently (distribution), to be stable to survive the physiological journey (metabolism), and to be eliminated in a satisfactory time (excretion). In other words, designing a drug with optimum PK properties can be achieved by implementing one or more of the following prodrug strategies.

5.4.11.2 Classification of Prodrugs

This can be classified into two major categories (Fig. 5.57), based on the method they are transformed into the active drug by human body.

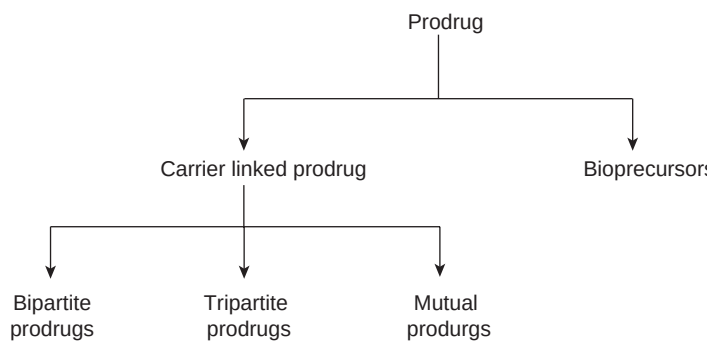


Figure 5.57 Classification of prodrugs.

Carrier-Linked Prodrugs

1. An active drug is covalently attached to a carrier group to furnish prodrug, which itself is inactive.
2. Once a prodrug is administered in the body, the active drug is liberated in the body following enzymatic or nonenzymatic release of the carrier.
3. The carrier groups generally used for the carrier-linked prodrugs are inert, biologically inactive, and devoid of toxicity.

Bipartite prodrugs

It constitutes a linking/carrier moiety directly linked (covalently) to the drug. Indeed, the lipophilicity of the drug is significantly modified due to the presence of carrier moiety which may itself be hydrophobic or hydrophilic. Once this bipartite prodrug is administered in the body, it may undergo enzymatic hydrolytic cleavage to release the active drug.

1. **Bipartite Prodrug for Slow and Prolonged Release (Sustained Drug Action):** Sometimes depending on the pathogenesis of disease, or biological efficacy of the preclinical candidates, need arises to enhance the duration of action of the drug. A common strategy used for this purpose is to link (acylate) the drugs with long-chain aliphatic acids. The resulting prodrug undergoes slow hydrolysis following their administration, thereby causing the slow release of drug and enhances the duration of action.

   Fluphenazine has a shorter duration of action (6−8 h), but prodrug fluphenazine deconaten (derivatized with decanoic acid) has longer duration of action, for about 30 days (Fig. 5.58).

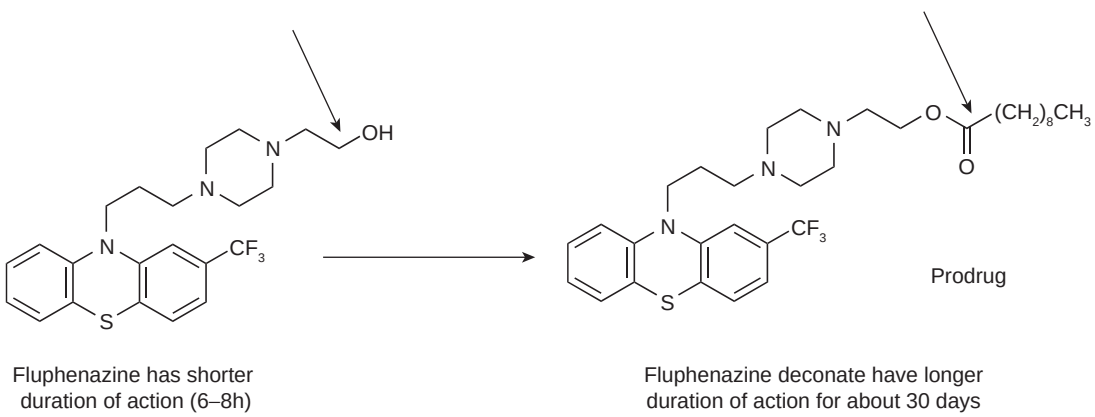


Figure 5.58 Bipartite prodrug for slow and prolonged release.

2. **Design of Bipartite Prodrugs to Improve Transport across BBB:** The treatment of diseases related to CNS remains a challenging task. The presence of protective barrier of the CNS not only protects the brain from unwanted molecules (xenobiotics) but also reduces the efficacy of CNS-active drugs.

   L-DOPA (levodopa) is used as dopamine precursor for the treatment of Parkinson's disease. Since dopamine is too hydrophilic (water solubility 7.43 mg/mL) and cannot penetrate BBB, its precursor L-DOPA (water solubility 3.3 mg/mL) is administered as a prodrug. It is designed to mimic the dopamine and is transported across cell membranes by active transport (not by simple diffusion). Dopamine is produced in the CNS via enzymatic decarboxylation of the prodrug L-DOPA (Fig. 5.59).

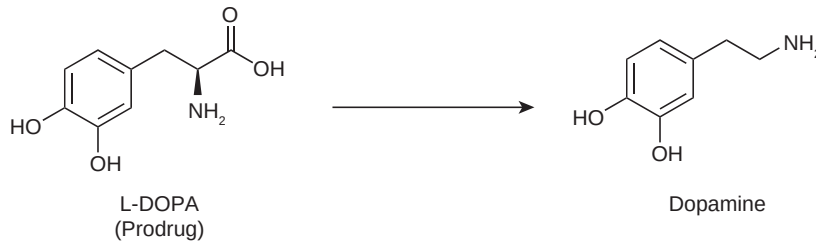


Figure 5.59 Bipartite prodrugs to improve transport across BBB.

In yet another example, morphine cannot diffuse BBB by itself due to the presence of two hydroxyl groups. In an attempt to enhance the lipophilicity, chemical modification via simple derivatization was carried out to design a prodrug that can penetrate BBB (Fig. 5.60). Thus, protecting both the hydroxyl groups with an acetyl group improved lipophilicity of morphine, thereby facilitating its diffusion through BBB. However, improved bioavailability in BBB led to addiction than analgesic.

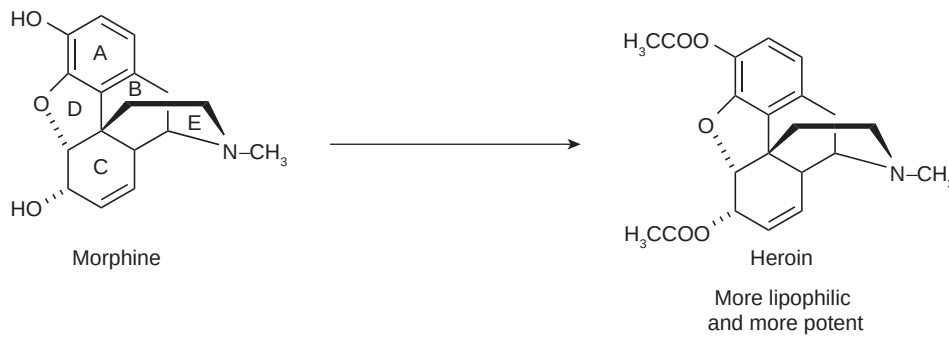


Figure 5.60 Simple acylation furnished compound with improved bioavailability.

Hexobarbitone, a prodrug, was used (1940s) as a rapid-acting agent for inducing anesthesia with short duration of action. The duration of action was enhanced by selectively methylating one of the nitrogens that resulted in a prodrug with reduced polarity and enhanced permeability across the membrane. Following administration of prodrug, enzymatic demethylation occurs in the liver to furnish the active drug (Fig. 5.61).

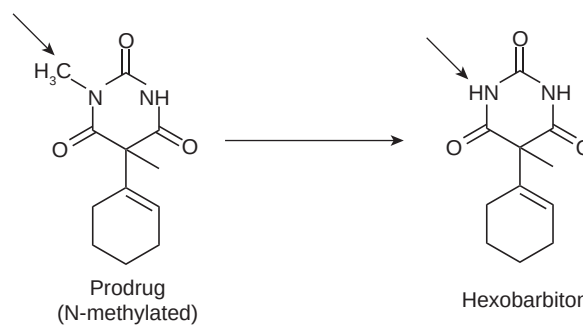
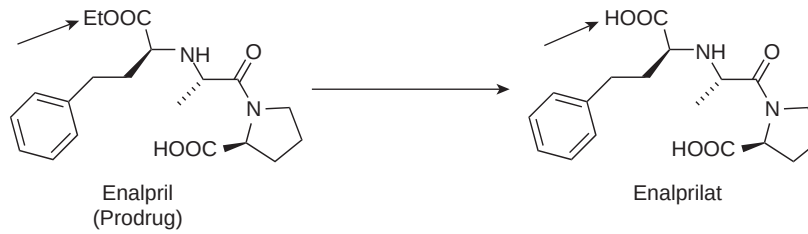


Figure 5.61 Methylating one of the nitrogens furnished prodrug.

3. **Oral Administration Using Bipartite Prodrug Strategy:** Enalaprilat was first discovered as a potent inhibitor of ACE and was found to be suitable for the treatment of hypertension. However, the presence of two –COOH groups makes the molecule highly polar and prevents its accessibility by oral route, thus requiring administration by injection. Esterification of one of the –COOH groups with ethyl alcohol furnished monoethyl esters of enalaprilat generally referred to as enalapril (Fig. 5.62). Following oral administration, the enalapril was bioconverted by esterases into enalaprilat with enhanced bioavailability in the range of 35–44%. Barring captopril and lisinopril, remaining 'me too' ACE inhibitor series (5–6 compounds) are prodrugs.



Enalpril (Prodrug) Enalprilat

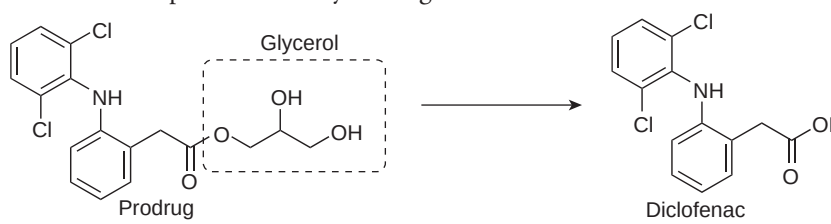
Figure 5.62 Esterification facilitated oral administration of enalapril.

4. **Enhancing Solubility of Drugs via Bipartite Prodrug Strategy:** During drug discovery programme, we often come across therapeutic preclinical candidates whose application in clinics becomes restricted due to poor aqueous solubility and absorption. Approximately >30% of test compounds used for the purpose of drug discovery programme have poor aqueous solubility (<10 μM), and achieving optimal solubility remains one of the challenging tasks for medicinal chemists. Therefore, to improve aqueous solubility, some of the most widely used methods involve derivatization of the lead candidates with acid or sugar moieties.

CASE STUDY 6

Diclofenac

It is a nonsteroidal anti-inflammatory drug widely used for the treatment of mild pain (antipyretic and analgesic) with different routes of administration. It can be administered as tablet by oral route, as injectable, suppository, and as a cream for topical application. For oral administration, the drug is administered either as K or Na salt (tablet form) of the –COOH functionality that makes it water soluble and raises its bioavailability. As injectable, diclofenac sodium is available in aqueous solution. However, for its topical use, the drug is transformed into a diclofenac ester as prodrug. This was achieved by synthesizing and evaluating a series of diclofenac esters[19] as prodrug (Fig. 5.63) and one of them, glycerol diclofenac ester, demonstrated improved solubility and higher transdermal flux across the skin than diclofenac.



Prodrug Diclofenac

Solubility 0.551 μM (aqueous) Solubility 0.00341 μM (aqueous)

Figure 5.63 Glycerol diclofenac ester demonstrated improved solubility.

CASE STUDY 7

Prednisolone

Prednisolone is a steroid used clinically to treat numerous disease conditions such as allergic/breathing disorders, skin conditions, psoriasis, etc. However, prednisolone alone is practically insoluble in water, and this issue of poor bioavailability was addressed by transforming it into a phosphate ester as prodrug (Fig. 5.64). The latter following administration by oral route undergoes hydrolysis by enterocyte (in gut on brush border/apical surface) alkaline phosphatases to release the parent drug.

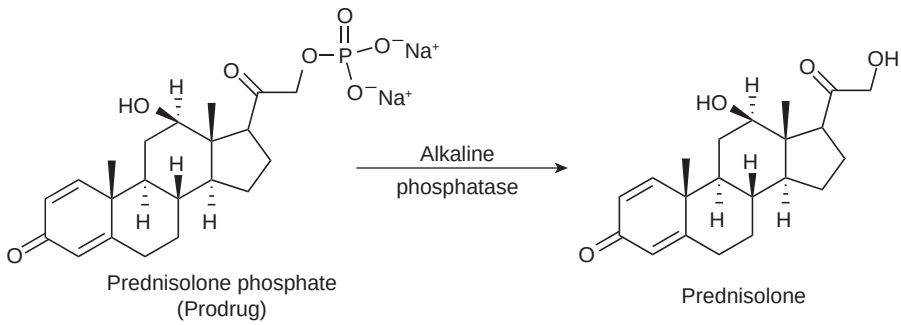


Figure 5.64 Phosphate ester as prodrug.

Tripartite prodrugs

This constitutes three components and differs from bipartite prodrug by the presence of a third component: a spacer or linking moiety between the drug and the carrier (Fig. 5.65).

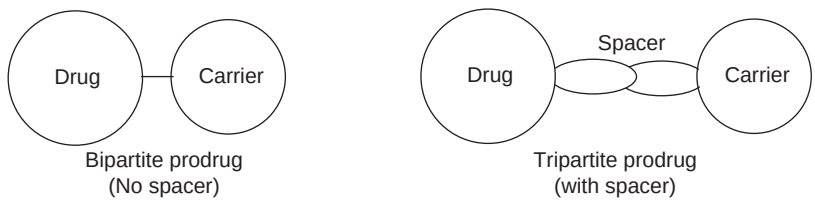


Figure 5.65 Tripartite prodrugs.

Tripartite Prodrug Strategy to Improve Absorption: The β-lactam-derived ampicillin with antibiotic properties has a poor oral bioavailability. However, exterification of carboxyl group in the form of pivaloyloxymethyl ester resulted in a prodrug pivampicillin with two-fold enhancement in the oral bioavailability (Fig. 5.66).

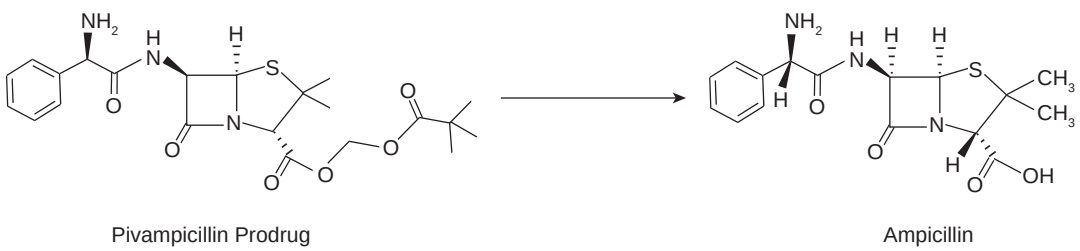


Figure 5.66 Pivampicillin as prodrug.

It is an example of tripartite strategy where a $-CH_2-$ group has been introduced as linker between pivaloyl group and ampicillin. The derivatization of carboxyl function with pivaloyl group enhances the oral bioavailability by increasing the lipophilicity of pivampicillin (clogP = 2.52) compared to that of ampicillin (clogP = −1.20). Upon absorption, pivampicillin is hydrolyzed into its active form ampicillin by esterases. Besides, using esters as carrier in the design of prodrug, other most commonly used (biologically labile) carriers involve amides, carbamates, ethers, phosphates, oximes, imines, etc.[20] Drugs attached to the carriers via these moieties are easily hydrolyzed by various enzymes to release the active drug inside the body.

Mutual prodrugs

As the name suggests, in this approach, two drugs are covalently linked to each other. Each drug acts as a carrier or prodrug for the other and vice versa (Fig. 5.67). The mutual prodrug concept may have synergistic effect, if both the drugs are for same activity or may be additive if pharmacological actions are different. The concept has found application in the treatment of diseases such as cardiovascular, viral, and cancer.

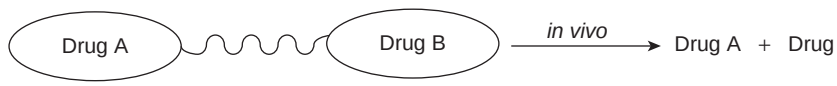


Figure 5.67 Mutual prodrugs.

CASE STUDY 8

Benorylate as a Mutual Prodrug

Benorilate (INN), or **benorylate**, is an ester-linked prodrug of aspirin with paracetamol. It is used as an anti-inflammatory and antipyretic medication (Fig. 5.68).

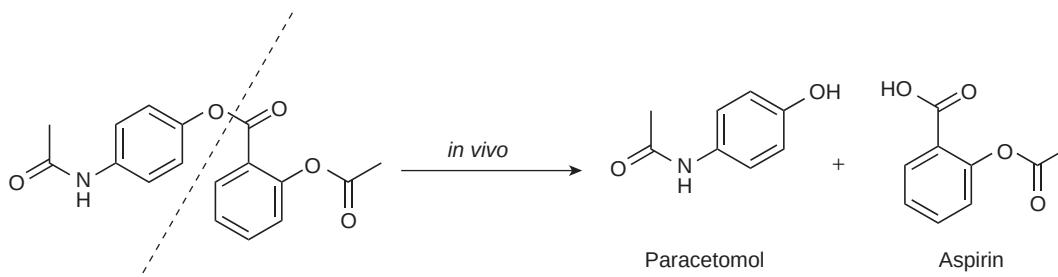


Figure 5.68 Benorylate as a mutual prodrug.

Prodrug to improve patient acceptability

Some drugs when taken by oral route can leave bad taste in mouth or kill the taste for food. The issue can be addressed by introducing acceptable changes in the molecule in a manner that would either remove the bad taste of the molecule or reduce the solubility of the drugs in saliva. Chloramphenicol, an antibiotic, leaves a bitter taste in the mouth following its consumption. The issue was addressed by derivatizing chloramphenicol with palmitate ester as it successfully masked its bitter taste by reducing its solubility in saliva (Fig. 5.69).

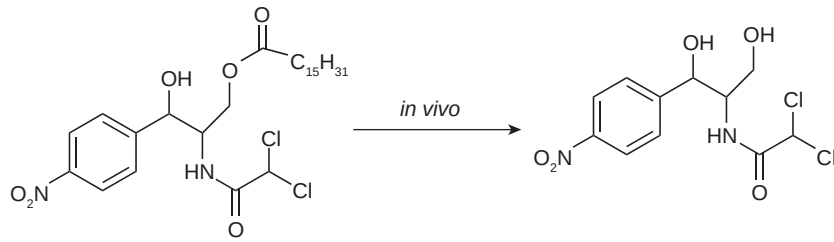


Figure 5.69 The palmitate ester of chloramphenicol masks the bitter taste.

CASE STUDY 9

Azathioprine as Prodrug

Azathioprine (Fig. 5.70) is used as adjunct therapy for preventing rejection of organ in kidney transplant patient and for autoimmune disorders. Azathioprine belongs to the family of prodrug as it is an inactive compound by itself. Once the drug is administered in the body, it is readily absorbed and undergoes non-enzymatic transformation to active 6-MP in the bloodstream.

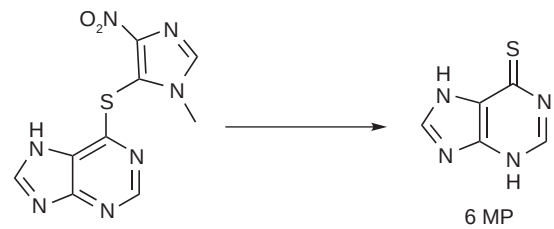


Figure 5.70 Azathioprine as a prodrug.

Prodrug of a Metabolite (Bioprecursors)

Prodrugs are generally considered to be the inactive moieties and once they have been administered in the body, they release the active drug. However, sometimes drugs that are pharmacologically active undergo metabolism in the body and produce a metabolite that may be more active than the parent drug. These classes of drugs are referred to as prodrug of an active metabolite or bioprecursor. Clinically used drugs such as sartans and statins are classical examples of bioprecursors.

CASE STUDY 10

Losartan as a Bioprecursor (But Not a Typical Prodrug)

Losartan, an antihypertensive agent elicits pharmacological response by binding with angiotensin II receptor (AT_1) as an antagonist. Following the oral administration of the drug, losartan undergoes the first-pass metabolism, and approximately 14% gets metabolized by one of the cytochrome P450 enzymes into a metabolite with a –COOH functionality obtained by the oxidation of $–CH_2OH$ present in losartan. The resulting metabolite is 10 to 40 times more active than parent drug and has 10 times

(*Continued*)

Study 10 (*Continued*)

higher affinity for AT_1 receptor than losartan.[21] Thus, losartan can be categorized under bioprecursor but cannot be categorized under prodrug as the pharmacological actions of losartan are mediated not only by losartan itself but also by its metabolite and possibly by other metabolites as well (Fig. 5.71).

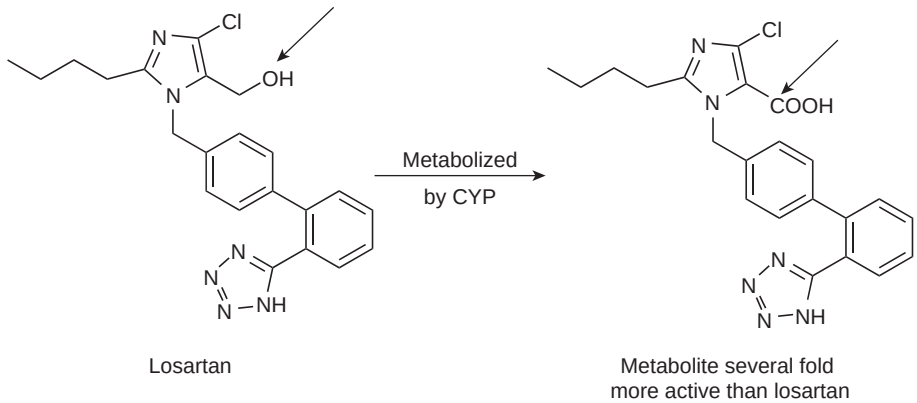


Figure 5.71 An example of bioprecursor.

CASE STUDY 11

Lactone Statins as Prodrug

Statins competitively bind with the enzyme HMG-CoA (5-hydroxy-3-methylglutaryl-coenzyme A) reductase, and in turn blocks synthesis of cholesterol. Among series of statins used clinically (as hypolipedemic), lovastatin, compactin (mevastatin), and simvastatin (Fig. 5.72) are administered as lactone prodrugs.[22] This is in contrast to other statins, which are generally administered as pharmacologically active hydroxyl acid form.

The lactone prodrugs following oral administration are hydrolyzed (metabolized) to their active open-chain dihydroxy acid form (active metabolite) in the liver (Fig. 5.73).

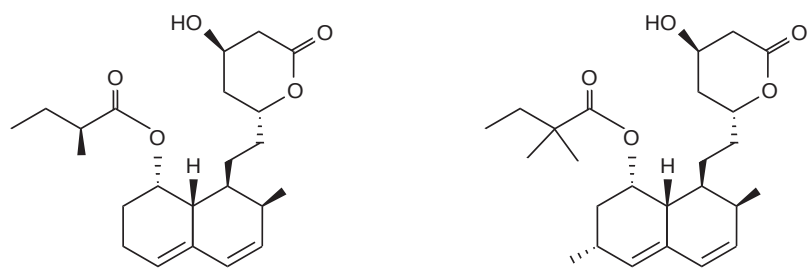


Figure 5.72 Lactone prodrugs.

(*Continued*)

Study 11 (*Continued*)

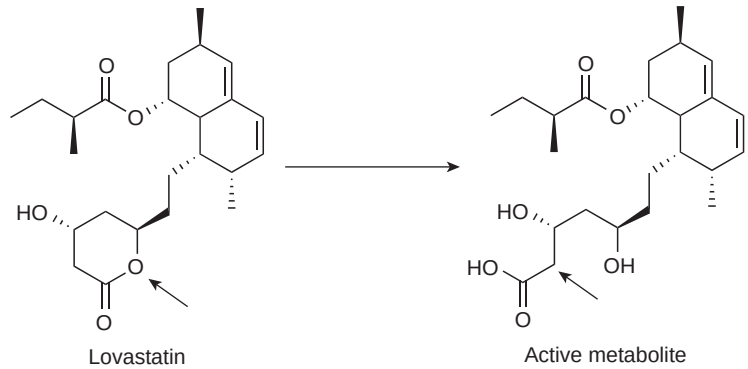


Figure 5.73 Biotransformation of lovastatin to active metabolite.

Naturally Occurring Prodrug

Poppy seeds collected from different sources display wide variations in the concentration of opiates morphine (2–25.1 micro g/g) and codeine (0.4–57.1 micro g/g). Codeine (O-methylmorphine) itself has extremely low affinity for μ opioid receptors and is biotransformed into analgesic morphine (O-demthylation) in the presence of a metabolic enzyme cytochrome P450. Thus, codeine with no analgesic activity[23] can be grouped under naturally occurring prodrugs for morphine (Fig. 5.74).

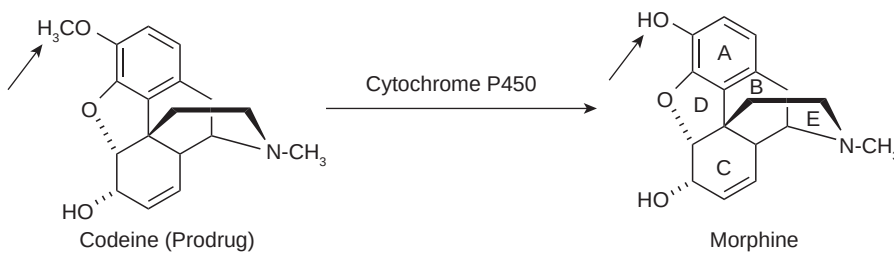


Figure 5.74 Codeine with no analgesic activity acts as prodrug.

5.4.12 Structure–Property Relationship

The SAR studies identify part(s)/functional groups of the molecule that interacts with the target proteins to elicit biological response *in vitro* or *in vivo*. However, often the *in vitro* biological data is not translated in the *in vivo* models due to unacceptable or poor PK profile (ADMET) of test compounds. The *in vivo* ADMET profile is generally governed by the physicochemical properties of test compounds. The chemical structure of test compounds regulates their physicochemical properties which in turn affect ADMET properties and ultimately the therapeutic response. Unacceptable physicochemical properties of test compounds can lead to poor performances exhibited during *in vivo* absorption, distribution, metabolism, and excretion. Thus, besides SAR studies, structure-property relationship (SPR) studies addressing relationship between chemical structure and physicochemical parameters needs to be also carried out under lead optimization studies. Although numerous approaches are available to improve ADMET profile, optimizing

physicochemical properties of compounds remains one of the best options. However, this should be carried out with a caution since changes in physicochemical properties may also have profound effect on therapeutic response. Some of the most common physicochemical properties primarily optimized during SPR studies involve solubility, permeability, lipophilicity (logP, logD), pK_a, polar surface area (PSA), etc. (The role of physicochemical properties in drug design has been discussed separately in Chapter 6). The optimization of SPR will facilitate movement of compounds through the physiological barriers in the body by affecting their ADMET[24] parameters and ultimately allowing their access to the desired protein target. Notably, during lead optimization studies, a balance is required between the efficacy/potency (PD) and good PK profile (integration of SAR and SPR studies) while chemical modifications (analoging) are introduced in lead compounds.

Thus, although during drug design, identification of initial lead molecules is a critical step and is generally based on pharmacologic properties alone, further lead optimization requires integration of SAR and SPR studies for the identification of preclinical candidates. For SAR studies, analogues are assessed in pharmacological assays whereas for SPR studies (ADMET profile), analogues are assessed in the *in vitro* assays for solubility, lipophilicity, permeability, hepatic microsomal stability, plasma stability, plasma protein binding, CYP450 inhibition, induction/inhibition of P-gp (efflux protein transporters), etc (Table 3.3). Physicochemical properties of compounds such as solubility, lipophilicity etc serves as a basis for these *in vitro* ADME assays and providing information about SPR. The structure property relationship studies together with structure-activity relationship studies form basis for a successful translation of the *in vitro* activity data into *in vivo* experiments. *In vivo* ADME profiling is also carried out during lead optimization as well as during selection of preclinical candidates.

Integration of PK (concentration vs. time) with PD (effect vs. time) parameters in the early stages of drug discovery, particularly during transformation of hit-to-lead, lead optimization, and candidate selection processes[25] leads to a quantitative description of the dose–exposure–response relationship.[26] This reflects complete time course of the desired and/or undesired therapeutic effect corresponding to a certain dose regimen (Fig. 5.75). Additionally, this relationship can also assist in increasing the probability of successful translation biological efficacy from preclinical species into clinical settings.[27]

It is pertinent to add that although optimal PK parameters must be accompanied by optimal biological response (PD as end result) *in vivo*, an optimal biological response may or may not be always accompanied by optimal PK properties. This is evident in numerous clinically used drugs such as lipitor (Atorvastatin) that, despite having a low bioavailability of only 14% due to rapid first-pass metabolism in humans (poor PK profiles), it remains a top-selling drug for lipid lowering.

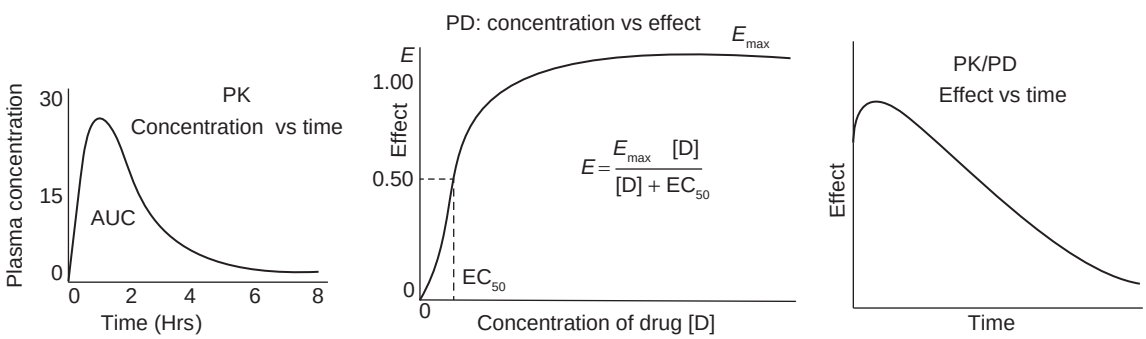


Figure 5.75 Quantitative description of the dose–exposure–response relationship.

5.4.12.1 *Optimizing pK_a as one of the Important Physicochemical Parameters under SPR*

In general, drugs do not undergo complete ionization in aqueous medium. In fact, only a fraction of drug molecules ionizes at a given physiological pH unless pH is equal to or close to pK_a. Since most drugs are weak acids or bases, their degree of ionization helps the drugs to move from lipid and aqueous environment. Thus, the concentration of ionized and unionized fractions is regulated by the pK_a of the drug and local pH. Acidic drugs with pK_a values greater than 6 are weak acids, and basic drugs with pK_a less than 8 are considered to be weak base. During lead optimization studies, one can determine the percentage of ionized and unionized test compound using its pK_a and pH of the physiological medium. The percentage can be calculated using Henderson Hasselbach equation or several online softwares. Higher is the concentration of unionized drugs at the site of absorption, more is the permeation (diffusion) of drugs through the plasma membrane. Thus, changes in pK_a of test compounds can affect the percentage of ionized vs. unionized concentrations of test compound in the physiological pH.

Variation in the pK_a values of the lead compounds can be introduced either by varying the alkyl groups on the amine nitrogen (enhancing basicity) or by introducing substituent in the arene rings with the view to affect (increase/decrease) acidity or basicity of aromatic acids/bases. An increase in activity/potency following change in pK_a can be attributed to an increase in the concentration of the unionized form at the site of absorption. The ionized form being water soluble is required during administration and movement of drugs through aqueous environment such as gut lumen, blood plasma, interstitial fluid, and cytoplasm (Fig. 5.76)

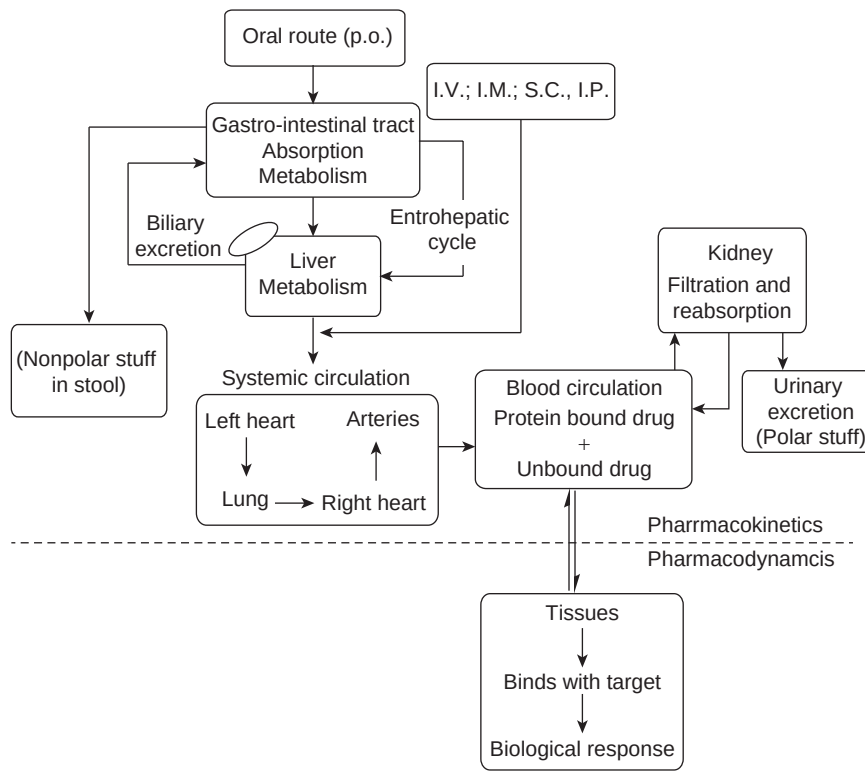


Figure 5.76 Pharmacokinetics/pharmacodynamics involving absorption, distribution, metabolism, excretion, and biological response.

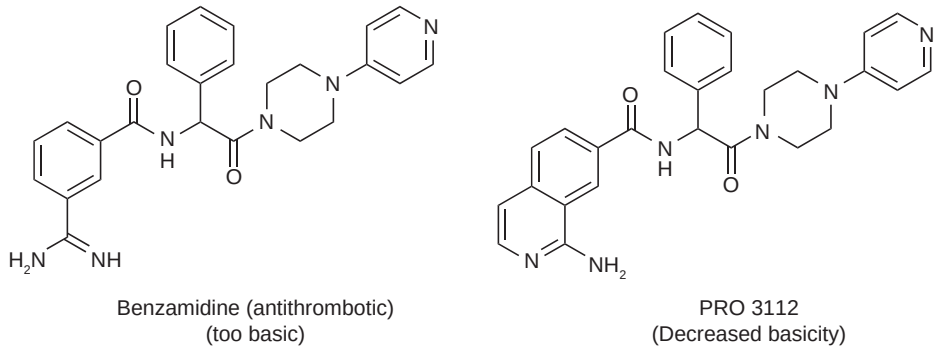


Figure 5.77 Reduction in basicity furnished compound with improved absorption.

while the unionized form is required for diffusion through lipid bilayer membrane. However, while varying pK_a of test compounds, there is a possibility that modification in polar groups may also interfere with binding interactions of compounds with the desired enzyme/receptor (biological targets).

Benzamidine, an antithrombotic drug due to the presence of basic amidine group, gets protonated in gut, thereby reducing the absorption of the drug in its ionic form. In an attempt to reduce the basicity of the drug, the amidine group was incorporated into an arene ring, which afforded compound PRO 3112 with decreased basicity and improved absorption (Fig. 5.77).

5.4.13 Allosteric Modulators as an Emerging Concept in Drug Design

Most of the clinically used drugs exert their effect by directly binding to the primary active/functional (orthosteric) site on the therapeutic proteins (receptor/enzyme) leading to agonistic/antagonistic actions.[28] The disadvantage often encountered with these drugs is decreased selectivity, and this has been attributed to similarity of orthosteric sites between the target and other proteins (isoforms) performing related functions. The sequence of amino acids in the orthosteric (binding) sites in a family of proteins with related functions is a highly conserved region. For example, all serine proteases have Ser195, His57, Asp102 as the conserved region in their binding sites. Consequently, drugs that bind to orthosteric sites often exhibit poor selectivity.

In contrast, allosteric sites present on target proteins display highly variable amino acid sequence and generally are less conserved. Allosteric binding sites are pockets to which effector molecules (signaling molecules discussed under Chapter 1) bind and regulate enzymatic activity by inducing changes in conformation leading to positive or negative effect on orthosteric-bound substance and are termed allosteric regulation. Thus, in a family of isoform proteins, their orthosteric sites are although highly conserved providing poor selectivity for drugs, allosteric sites are less conserved (highly variable) and provide good selectivity.

This topographic difference between these two sites led to the emergence of a novel approach to overcome selectivity issue where more emphasis is being laid to identify drugs that can bind to allosteric site instead of the active (orthosteric) site on the same protein.[29] For example, functional sites (orthosteric sites) in a family of five muscarinic acetylcholine receptors (mAChRs) were found to be highly conserved while allosteric sites in these isoforms were found to be highly variable. Virtual screening of the allosteric site by docking a library of small molecules led to the identification of a selective antagonist for M2 type mAChR as a positive allosteric modulator since it potentiated the antagonistic binding at the orthosteric site.[30]

In yet another example, allosteric modulation approach has been applied to the enzyme phosphoinositide-dependent protein kinase-1 (PDK1). The latter is a signaling molecule that has emerged as an important

therapeutic target for eosinophilic inflammation and has been found to be involved in downstream signaling events (phosphorylation) via PI3K-AKT pathway. The orthosteric site in this protein kinase is known as ATP-binding site, whereas allosteric site is known as PDK1-interacting fragment. Since the catalytic activity of this enzyme is predominantly governed by allosteric modulation, several small molecule allosteric modulators have been identified using structure-based drug design approach.[31]

Thus, an allosteric modulator (drug) approach for drug discovery can be successfully applied to both receptors and enzymes. The allosteric modulator would selectively bind a protein target among its isoforms and induce conformational change in the target protein in a manner that would either facilitate or block the binding of the substrate/ligand to the orthosteric site. Substances that facilitate binding of the substrate/ligand to the orthosteric site are referred to as allosteric positive modulator and the ones that inhibit are referred to as allosteric negative modulator.

5.4.14 'Me Too' Strategies for Drug Design

They are also called 'follow-on drugs' as they are structurally very similar to the originally discovered clinically used drugs (prototypes) with negligible structural difference and have identical mechanism of action.[32] A 'Me too' drug is used for the same clinical purpose as the original prototype drug and could be better than the prototype drug or it could be less active. However, different compounds in a particular prototype class may be approved at different dosages or dosing schedules depending on their PK properties. Yet another major benefit of 'Me too' drugs involves use of 'Me too' drugs from different classes in combination under polytherapy to create more effective therapeutic regimens. For example, HIV is treated with a 'triple cocktail' of three different classes of 'Me too' drugs; similarly, hepatitis C is now cured with combinations of two or three 'Me too' prototypes of antivirals.

For decades, it has been common practice by pharmaceutical companies to market drugs discovered by them as a single player (monopoly) in the market. However, over the period, companies showed interest in this approach of 'Me too' class of drugs. Using this approach of drug design, as soon as the original drug prototype becomes generic following expiry of its patent, numerous 'Me too' drugs are launched in the market by other players. For example, Genzyme enjoyed a monopoly for the treatment of Gaucher for 19 years before Shire released its enzyme in the market,[33] and four years later, Pfizer launched a third such enzyme in this 'Me too' series.[34] In another example, Merck remained the only company to market a DPP4 inhibitor Januvia for type-2 diabetes for three years in the US before other companies joined the fray. Classical examples of 'Me too' drugs have been summarized below.

5.4.14.1 'Me Too' Drugs Based on Serotonin-Specific Reuptake Inhibitors

Serotonin is considered to be happy neurotransmitter and its deficiency in synapse has been associated with depression and related disorders. One approach to treat this deficiency has been to elevate the concentration of serotonin in synapse by inhibiting its reuptake by membranous transporter proteins back into presynapse. The first prototype drug Prozac as SSRI was launched by EliLilly in 1987, and it went off patent in August 2001. Later, the prototype was followed by a series of 'Me too' drugs Paxil, Celexa, Lexapro, and Zolofit (Fig. 5.78).

5.4.14.2 'Me Too' Drugs Based on Thiazolidinones

They are also called glitazones and were developed for the treatment of type 2 diabetes. They act by binding to peroxisome proliferator-activated receptor-gamma (PPRγ; endogenous ligand FFA) and induces uptake of fat into peripheral tissues. This in turn leads to the decrease of circulatory fat concentration, thereby enhancing insulin sensitivity and glucose metabolism (Fig. 5.78). The first prototypical drug in the series was ciglitazone. Subsequently, although some 9–10 compounds in this 'Me too' class of drugs were developed, but only few of them could find use in clinics; rest were withdrawn or discontinued due to severe toxicity.

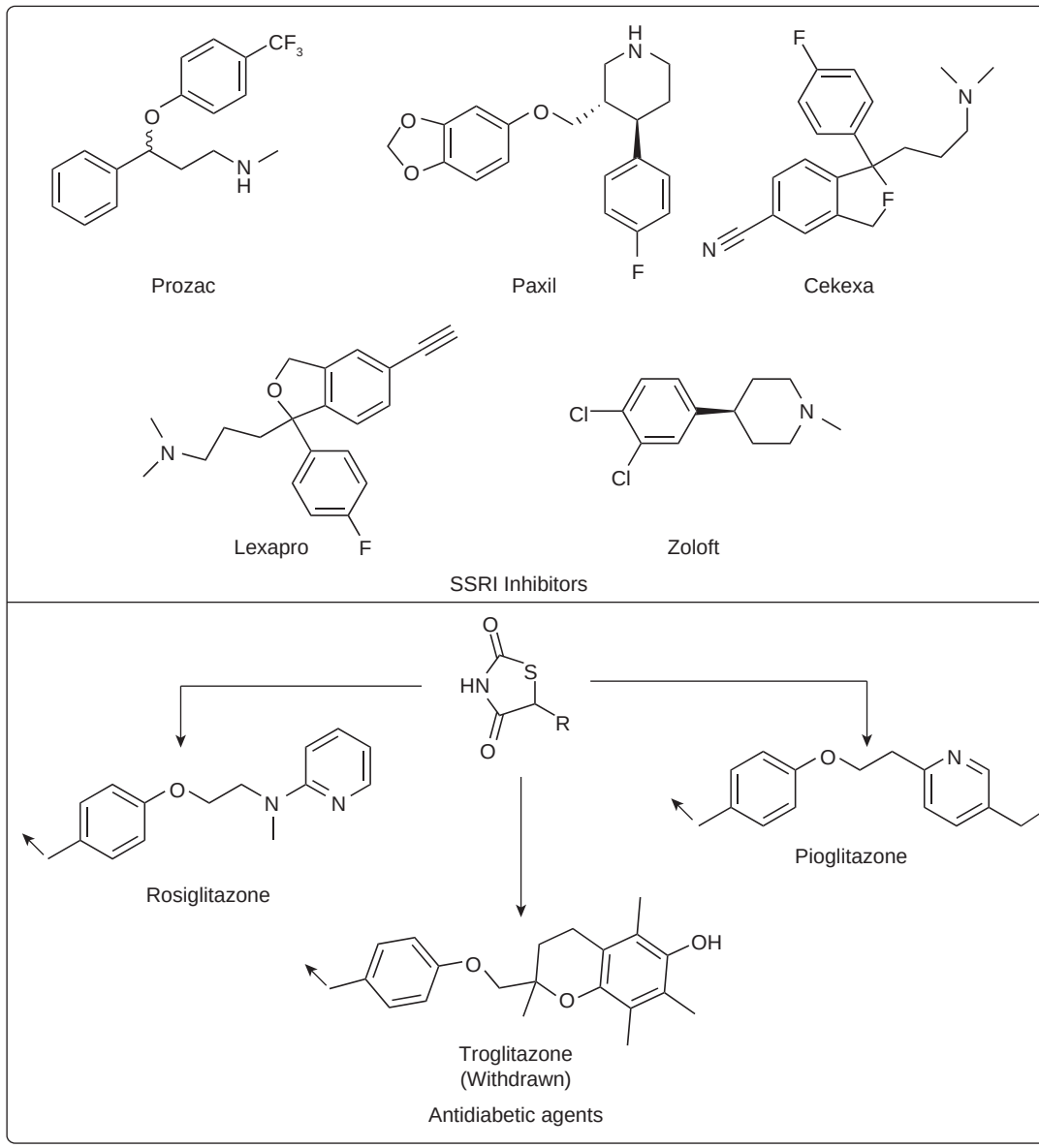


Figure 5.78 Examples of 'Me too' drugs.

5.4.14.3 'Me Too' Drugs Based on Captopril

Captopril is an ACE inhibitor used clinically for the treatment of hypertension. It was the first drug developed using structure-based drug design approach and was released in the market in 1980s. Within six years of its launch, a series of 'Me too' drugs (approximately 10 compounds) using captopril as prototype flooded the market (Fig. 5.79). Interestingly, barring captopril and lisinopril, remaining 'Me too' ACE inhibitors (5–6 compounds) based on captopril (Table 5.1) were designed as prodrugs.

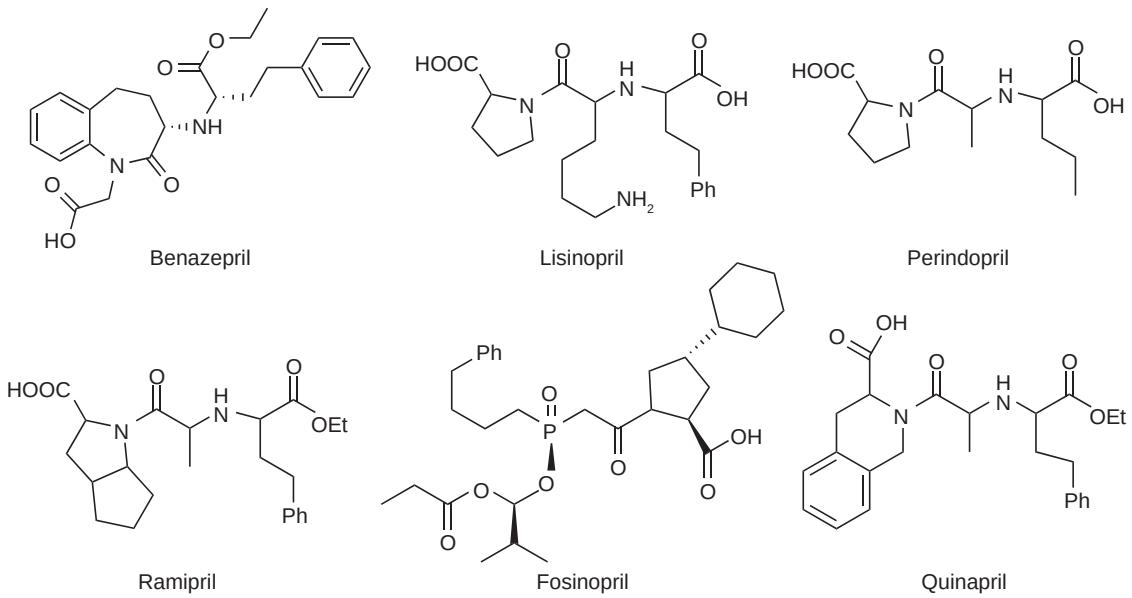


Figure 5.79 Me too drugs derived from captopril.

5.4.14.4 'Me Too' Drugs Based on Statins

Statins, used clinically for the treatment of hypercholesterolemia, display a similar structural pattern to each other, and hence can be classified under 'Me too' classes of drugs. Being a 'Me too' class of drugs, they act by same mechanism of action involving inhibition of the enzyme HMG-CoA reductase that plays a role in cholesterol synthesis. The first prototype drug in statin series was Mevacor (lovastatin), which was derived from natural source and developed by Merck. This was followed by a series of stains as 'Me too' compounds. Out of the 8 statins used clinically, two compounds are of natural origins, two are semisynthetic compounds and remaining four statins are of synthetic origin (For structures see: Table 4.2). However, three of the eight statins with lactone rings are prodrugs.

5.4.15 Antimetabolite Strategy for Drug Design

Antimetabolites[35] can be defined as drugs that inhibit normal metabolic process essential for the survival of cells (division and function). One of the most essential metabolic routes is synthesis of DNA and RNA, and cell division. The antimetabolite concept uses drugs that are structurally analogous to a naturally (intravital) occurring compound that is needed to carry primary anabolic reactions (metabolism). They work through a variety of mechanisms including competition for binding sites on enzymes and incorporation into nucleic acids, while also inhibiting the growth of the most rapidly proliferating cells. This in turn interferes with the normal metabolic processes, thus preventing the synthesis of DNA. The strategy has been used for the development of antibacterial and anticancer agents.

5.4.15.1 Antimetabolites as Antibacterial Agent

Folate is a vitamin B that is naturally present in many foods. Since human body cannot make folic acid, they need folate through dietary source for their cellular functions. Interestingly, although mammals get folate from their diet, gram-positive and -negative bacteria must synthesize their own B-vitamin. The folate synthesis in bacteria involves a chemical reaction between *p*-aminobenzoic acid (PABA) and dihydropterin pyrophosphate (DHPP) in

the presence of an enzyme dihydropteroate synthase (DHPS). This results in the formation of 7,8-dihydropteroate (dihydrofolic acid) from PABA and DHPP. Another enzyme DHFR then catalyzes conversion of dihydrofolic acid into tetrahydrofolic acid required for the synthesis of purines utilized for the generation of DNA, a phenomenon essential for growing cells. Sulfonamides compete with PABA and bind with DHPS, thus preventing bacteria from making folic acid. Due to structural similarity of PABA (natural metabolite) with sulphonamides (antimetabolite drug), the latter mimics (Fig. 5.80) the natural substrate PABA and bind with the enzyme. The sulfa drugs are competitive reversible inhibitors and act as bacteriostatic agents (Fig. 5.81).

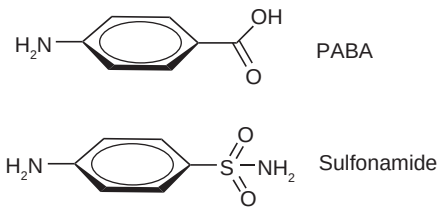


Figure 5.80 Structural analogy (similarity) between PABA (natural metabolite) and sulphonamides (antimetabolite drug).

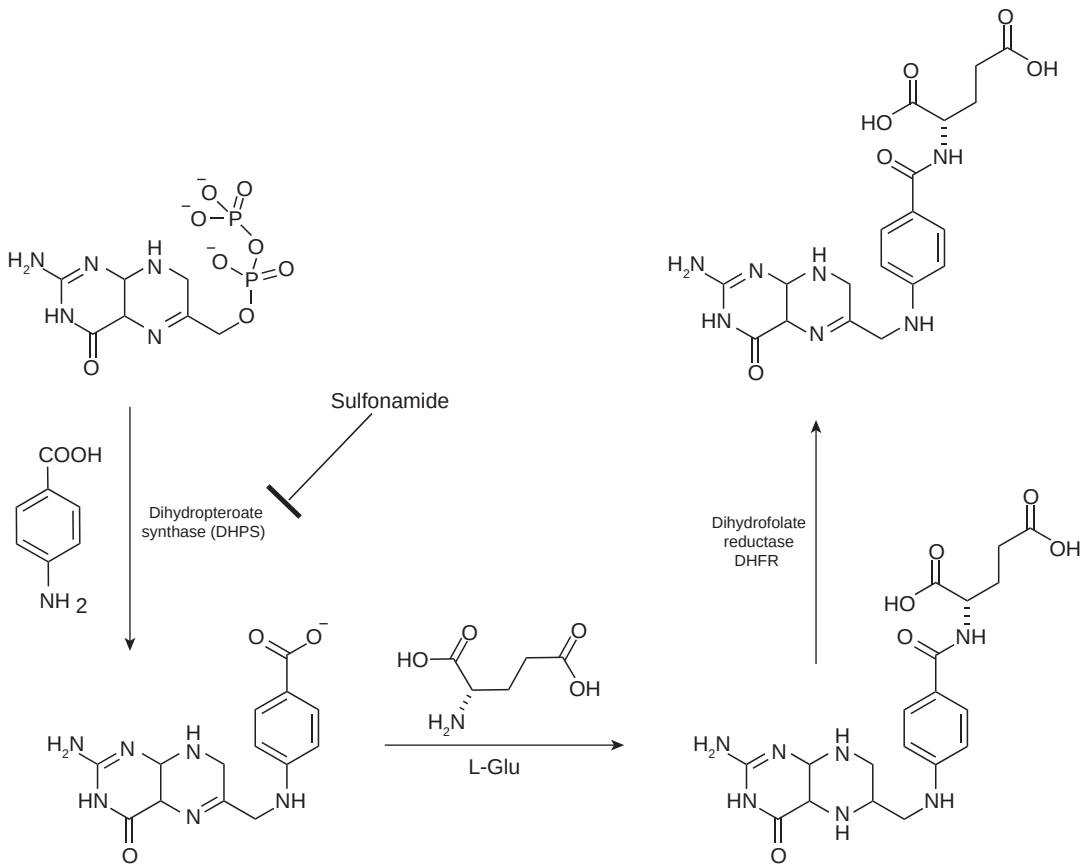


Figure 5.81 Sulfonamides (antimetabolite) inhibiting DHPS enzyme.

5.4.15.2 Antimetabolites as Anticancer Agent

In a multicellular organism, cell division is the route for tissue growth and maintenance. However, for the survival of the multicellular organisms, a balanced growth and division of different cell populations (tissues) with defined/specific functions is regulated by the process of cell proliferation. Interestingly, the basic mechanism involving regulation of growth and division of different cell populations throughout multicellular organisms remains same. The cell division is always accompanied by complete duplication of genetic trait encoded in DNA (called duplication of DNA or DNA replication).

In adult multicellular organisms, while some cells such as skin, liver, and bone marrow continue to proliferate or regenerate following an injury, other types such as bone and muscle cells, the proliferation ceases and tissue renewal (replacement) remains the only option. Cancer is caused by uncontrolled and abnormal proliferation arising from a damaged cell owing to mutated genes and ultimately grows into a tumor.

Among variety of chemotherapy available for treating cancer, antimetabolites are used to interfere with synthesis of DNA and RNA responsible for cell growth. The mechanism of action of antimetabolite (drug) involves competing for the binding sites of enzymes involved in metabolic (anabolic) process and subsequent replacement of natural building blocks (metabolites) in DNA and RNA with antimetabolite. Thus, antimetabolites prevent natural substances (metabolites) from becoming incorporated into DNA during the S phase (of the cell cycle), thereby causing cell cycle arrest (unable to divide) and apoptosis. Cancers of ovary, breast, and leukemia, etc., have been successfully treated using antimetabolites strategy. Examples of antimetabolite drugs have been listed below in Table 5.3.

Table 5.3 List of antimetabolite drugs

Antimetabolite Drug	Developed against Natural Metabolite
5-Fluorouracil and foxuridine	Pyrimidine antagonist
Methotrexate	Folic acid antagonist
6-Mercaptopurine and 6-thioguanine	Purine antagonist

CASE STUDY 12

Antimetabolite drug 6-MP

The antimetabolite drug 6-mercaptopurine (6-MP; brand name Purinethol), a purine analogue, inhibits *de novo* purine synthesis and promotes apoptosis by interfering with protein, DNA and RNA synthesis. The drug is used for the treatment of malignant diseases, autoimmune diseases, and immunosuppression (as azathioprine).

Initially, 6-MP binds competitively with the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and itself is transformed into the nucleotide analogue 6-thioinosinic acid (TIMP) also called 6-MP ribose phosphate. This, in turn, blocks the intravital purine salvage pathway involving formation of inosinic acid (IMP) followed by its conversion to adenine and guanine nucleotide building blocks for DNA and RNA. Additionally, TIMP undergoes methylation to form 6-methylthioinosinate (MTIMP) and both TIMP and MTIMP are inhibitors of glutamine-5-phosphoribosylpyrophosphate amidotransferase, an enzyme specifically involved in *de novo* pathway for purine ribonucleotide biosynthesis.

(*Continued*)

Study 12 (*Continued*)

Next, incorporation of antimetabolite drug in RNA and DNA commences following conversion of TIMP into thioguanine monophosphate (TGMP). The latter, after phosphorylation to di- and triphosphates gets substituted (incorporated) into RNA. Along with this, the other derivative deoxy-ribonulceotide is also formed, that gets incorporated into DNA and eventually furnishes nonfunctional DNA and RNA (Fig. 5.82).

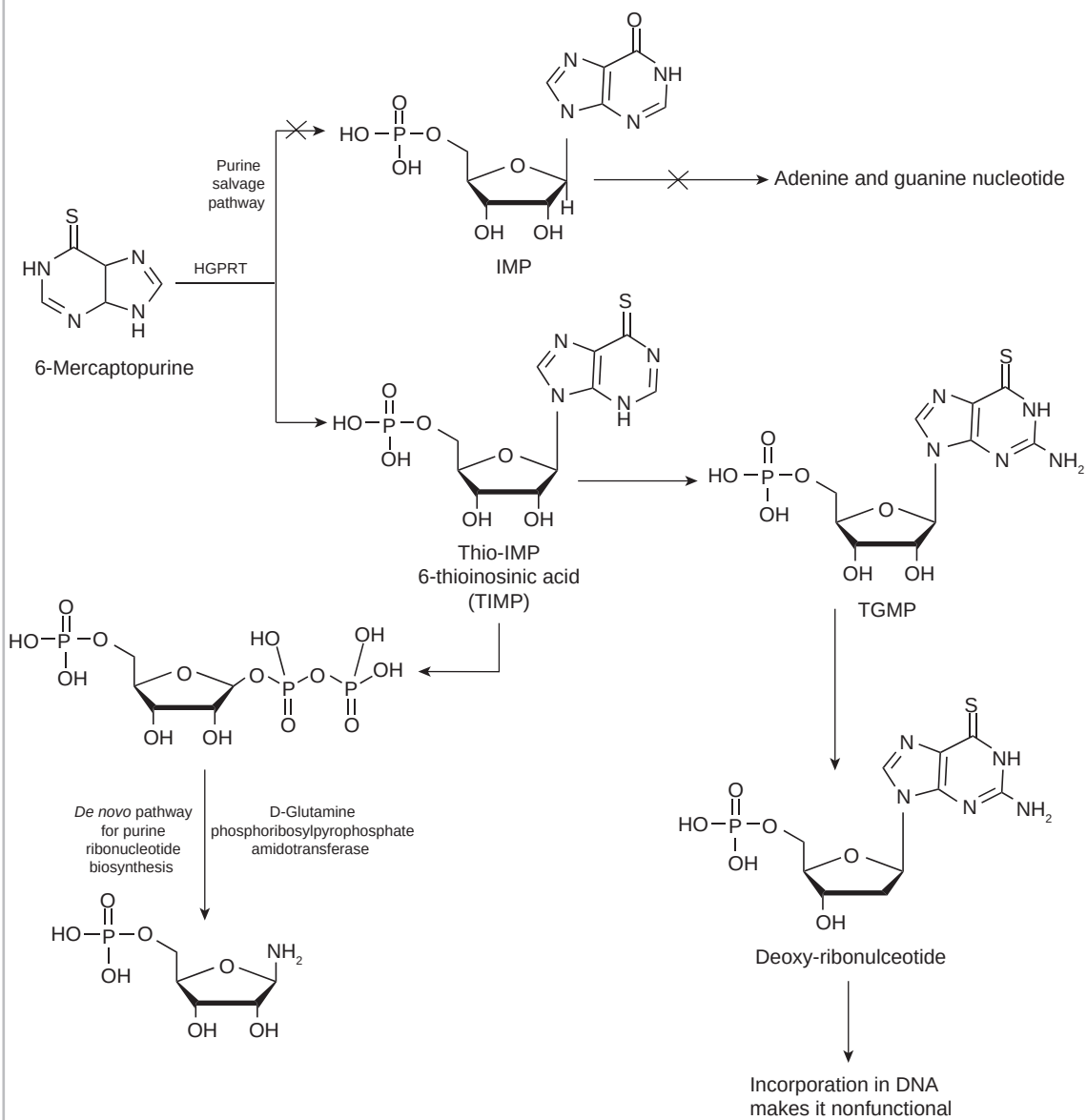


Figure 5.82 Antimetabolite 6-MP and its action involving inhibition of both *de novo* purine and purine salvage pathways.

5.4.16 Role of DMPK for the Selection of Preclinical Candidate

Although the drug development process is time consuming and expensive, approximately 90% of drugs undergoing clinical trials fail to reach clinics. The major cause of failure has been attributed to poor metabolism and PK profile (DMPK) of drug candidates under development. DMPK, or drug metabolism and pharmacokinetics, is an important component of drug discovery programme and is also referred to as ADMET (absorption, distribution, metabolism, excretion, and toxicity).[36] A comprehensive set of high-quality *in vitro* DMPK studies is mandatory for filing application for investigational new drug (IND) to FDA. DMPK provides tools and assays to evaluate test compounds in terms of metabolism, PK properties, and preclinical toxicity. The five basic features for DMPK studies are:

1. **Potency:** The ability of a compound to produce desirable and optimal pharmacological response.
2. **Oral Bioavailability:** Compound with desired physicochemical properties so that it can cross multiple barriers in body following administration such as overcoming first-pass metabolism, efflux, etc., in gut and liver metabolism.
3. **Duration of Action:** The ability of compound to remain in blood circulation with minimal metabolism for sufficient time after it has crossed the barriers through GIT and liver.
4. **Pharmaceutical Properties/Acceptability:** Commercially viable synthetic route, adequate aqueous solubility, and good chemical stability
5. **Toxicity/Safety:** Ability to selectively bind with the desired therapeutic target depicting good therapeutic index (TI).

This can be carried out by *in vitro* ADMET screening and *in vivo* PK profiling that can provide a basis for identifying preclinical candidate(s) with optimal metabolism (both *in vitro* and *in vivo*), PK profile and safety parameters for successful translation during preclinical and clinical studies. *In vitro* DMPK screening tools for test compound in multiple species including humans have been summarized in Table 5.4.These *in vitro* assays for DMPK (ADMET) parameters form basis for establishing dosing of drugs in experimental animal models for the generation of DMPK parameters *in vivo*.

Table 5.4 Checklist for some important *in vitro* DMPK assays for preclinical candidates

Aqueous solubility
logP/logD 7.4
pK_a
Caco-2 permeability
pK_a, neutral or basic with
PSA
Hep G2 hepatotoxicity
MDR1-MDCK/permeability P_{app}
PAMPA assay
Microsomal assay

(*Continued*)



Table 5.4 (*Continued*)

hERG assay
P450 enzyme CYP inhibition
CYP2D6 metabolism
CYP3A4 inducer assay
Plasma protein binding
Effective permeability
Metabolism route
PK/PD
P-glycoprotein assay
Plasma protein binding
MTD (maximum tolerated dose) rodent
Cytotoxicity in suitable cell line

The *in vivo* experiments for DMPK parameters involve:

1. Single dose PK in rat, dog, monkey
2. Single rising dose PK in rat, dog, monkey
3. Multiple rising doses in rats
4. Metabolite profiling and characterization in preclinical species: rat and dog or monkey

In vivo ADME profiling also known as PK studies are carried out by administering compound at a single dose followed by collection and analysis of blood samples to get a plasma concentration versus time curve. From this information about the area under the curve (AUC) and related parameters (Details discussed under Section 11.2.1.2**)** are calculated that provide information about the preclinical candidate from a series with highest estimated exposure.

Currently, DMPK screens[37] involving multiple *in vitro* and *in vivo* techniques are being used routinely as part of lead optimization under the modern drug discovery programme.

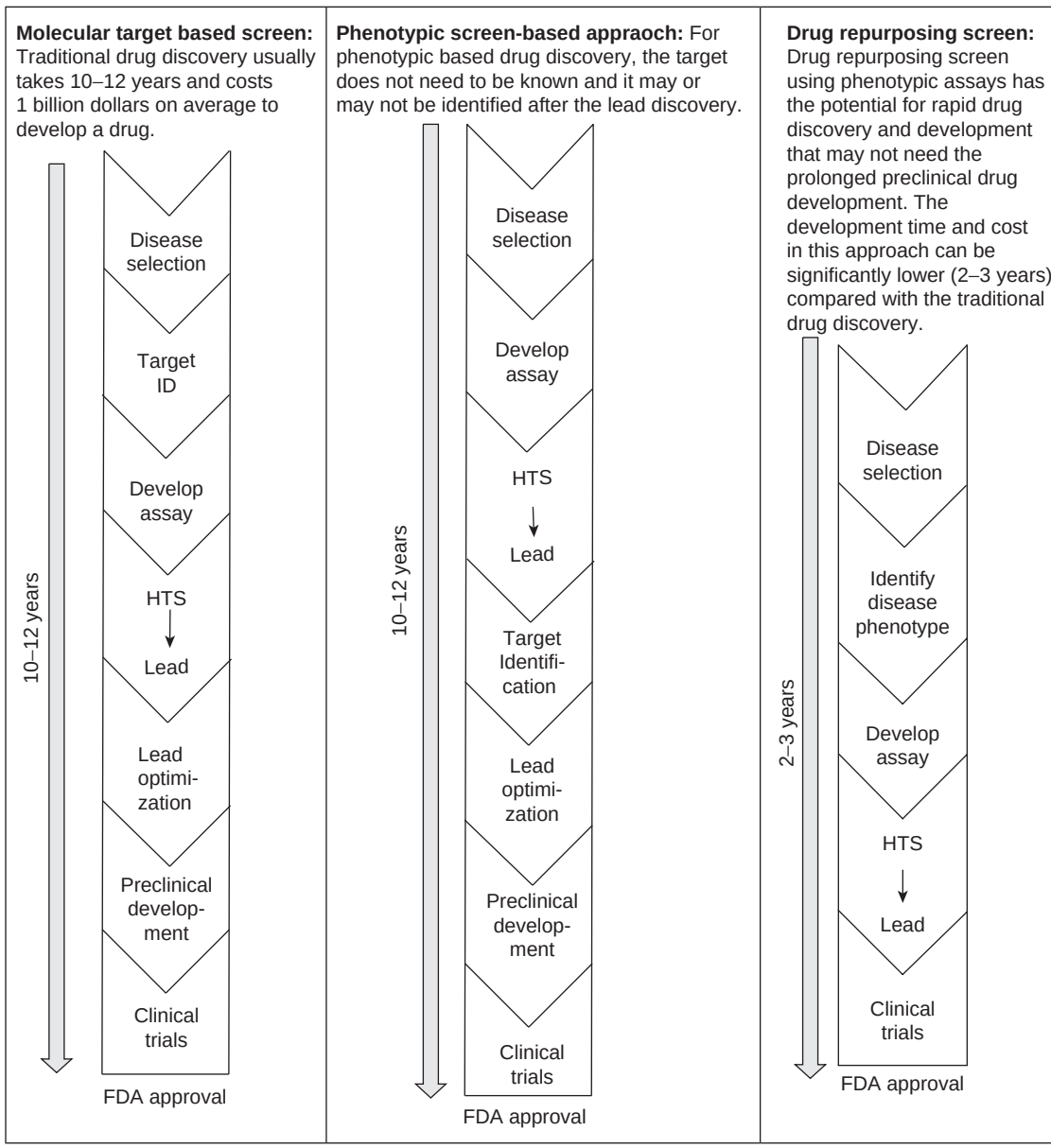
5.5 Timeline of a Pharmaceutical Drug Development from Laboratory to Clinics

The main objective of drug discovery is to identify novel drugs (therapeutic agents) with minimal side effects and effective pharmacological response. But developing therapeutic agents is not only a long and complex process but remains a high-risk adventure. Although researchers continue to strive hard to identify safe drugs, continuous unravelling in the understanding of etiology of the disease at molecular level keeps throwing new challenges. *In the last two decades with the discovery of G-protein-coupled receptors (rhodopsin), diseases are now being redefined from cell-signaling point of view.*



On an average, although it takes around 10–15 years for an idea to reach clinics, drug discovery using repurposing approach *(drug repositioning is the application of available drugs for treating conditions different from the original treatment purposes)* can significantly lower this time frame to 2–3 years (Table 5.5). A scientific report in 2017 pegged the research cost for developing a new drug around $2.6 billion.[38] Similarly, in 2018 a scientific report attributed the success rate for new drug to be less than 2.01%.[39] On the contrary, the cost for developing drug using repurposing strategy has been reported to be $1.6 billion.[29]

Table 5.5 Time frame in different formats of drug discovery



Exercises

1. Discuss role of F as an isostere of H in drug design.
2. Define lead molecule and its characteristics.
3. Describe development of captopril using structure-based drug design approach.
4. Describe characteristics of preclinical candidates.
5. Discuss structure-activity relationship with an example.
6. Discuss role of bioisosterism in drug design.
7. Name advanced in vitro assays for pharmacokinetic properties.
8. Role of optical isomerism in drug design.
9. Write in brief:
   - **(a)** Masking of polar groups
   - **(b)** Conformational isomerism in the activity of drugs
   - **(c)** Allosteric modulators
   - **(d)** 'Me too' drugs
   - **(e)** Antimetabolite strategy for drug design
   - **(f)** DMPK
   - **(g)** Bipartite and tripartite prodrugs with two examples each
   - **(h)** Dual role of estrogen in shutting down testosterone concentration in males
10. Discuss drug design strategies used for targeting drugs.

Competitive Exam Questions

1. What is the primary mechanism of action of local anesthetics?
   - **(a)** Activation of ligand-gated potassium channels.
   - **(b)** Blockade of voltage-gated sodium channels.
   - **(c)** Stimulation of voltage-gated *N*-type calcium channels.
   - **(d)** Blockade of GABA-gated chloride channels. [***GPAT 2012***]
2. All of the following ACE inhibitors are prodrugs except
   - **(a)** ramipril.
   - **(b)** Lisinopril.
   - **(c)** enalapril.
   - **(d)** perindopril. [***GPAT 2014***]

References

1. Ashutosh Kumar Patidar, G. Selvam, M. Jeyakandan, Ashok Kumar Mobiya, Ajay Bagherwal, Gaurav Sanadya and Ramraj Mehta, *International Journal of Drug Design and Discovery*, **2011**, *2*, 458.
2. Jeurgen Bajorath, Encyclopedia of Cancer, DOI 10.1007/978-3-642-27841-9_4502-2, *Springer-Verlag Berlin Heidelberg*, **2014**.
3. Connie L. Smith and Keith R. Powell, *Pediatr Rev*., **2000**, *21*, 368.
4. Palmer, D. C., and Strauss M. J., *Chem. Rev*., **1977**, *77*, 1.
5. (a) Kametani T., Kigasawa K., Hiiragi M., and Wagatsuma N., *Heterocycles,* **1974**, *2*, 79; (b) Clarke, E. G. C., *Nature,* **1959**, *184*, 451.



6. Ross J. Fleischman, David G. Frazer, Mohamud Daya, M.S. Jonathan Jui, and Craig D. Newgard, *Prehosp Emerg Care*, **2010**, *14*, 167.
7. Tom L. Blundel, *Nature*, **1996**, 384SUPP, *7*, 23.
8. Amy C. Anderson, *Chemistry and Biology*, **2003**, *10*, 787.
9. V. K. Vyas, R. D. Ukawala, M. Ghate and C. Chintha, *Indian J. Pharm. Sci.*, **2012**, *74*, 1.
10. Nawazish Naqvi, Ke Liu, Robert M. Graham and Ahsan Husain, *The Journal of Biological Chemistry*, **2005**, *280*, 6669.
11. George A. Patani and Edmond J. LaVoie, *Chem. Rev*., **1996**, *96*, 3147.
12. M. B. van Niel, M. S. Beer, H. B. Broughton, S. K. F. Cheng, S.C. Goodacre, A. Heald, K. L. Locker, A.M. MacLeod, D. Morrison, C.R. Moyes, D. O'Connor, A. Pike, M. Rowley, M. G. N. Russel, B. Sohal, J. A. Stanton and S. Thomas, H., *J. Med. Chem*., **1999**, *42*, 2087.
13. Ballatore C., Huryn D. M., and Smith A. B., *Chem. Med. Chem*., **2013**, *8*, 385.
14. Lien Ai Nguyen, Hua He and Chuong Pham-Huy, *Int. J. Biomed. Sci.*, **2006**, *2*, 85.
15. Torchilin V. P., *Eur. J. Pharm. Sci*., **2000**, *11*, S81.
16. Menelas N. Pangalos, Lee E. Schechter and Orest Hurko, *Nature Reviews Drug Discovery*, **2007**, *6*, 521.
17. Jarkko Rautio, Hanna Kumpulainen, Tycho Heimbach, Reza Oliy, Dooman Oh, Tomi Järvinen and Jouko Savolainen, *Nature Reviews Drug Discovery*, **2008**, *7*, 255–270.
18. Kristiina M., Huttunen, Hannu Raunio and Jarkko Rautio, *Pharmacological Reviews*, **2011**, *63*, 750.
19. Lobo S., Li H., Farhan, N., and Yan G., *Drug Dev. Ind. Pharm*., **2014**, *40*, 425.
20. Daniela Hartmann Jornada, Guilherme Felipe dos Santos Fernandes, Diego Eidy Chiba, Thais Regina Ferreira de Melo, Jean Leandro dos Santos and Man Chin Chung, *Molecules*, **2016**, *21*, 42.
21. Sadoshima J., *Circ. Res.*, **2002**, *90*, 754.
22. Reinoso R. F., Sánchez Navarro A., García M. J., and Prous J. R., *Methods Find Exp. Clin. Pharmacol.*, **2002**, *24*, 593.
23. Paul R. Ortiz de Montellano, *Future Med. Chem*., **2013**, *5*, 213.
24. Han van de Waterbeemd, Dennis A. Smith, Kevin Beaumont and Don K. Walker, *J. Med. Chem.*, **2001**, *44*, 1313.
25. Tove Tuntland, Brian Ethell, Takatoshi Kosaka, Francesca Blasco, Richard Xu Zang, Monish Jain, Ty Gould, and Keith Hoffmaster, *Front. Pharmacol.*, **2014**, *5*, 174.
26. Susan M. Abdel-Rahman and Ralph E. Kauffman, *Annual Review of Pharmacology and Toxicology*, **2004**, *44*, 111.
27. Piet H. Van Der Graaf and Johan Gabrielsson, *Future Med. Chem*., **2009**, *1*, 1371.
28. Ruth Nussinov and Chung-Jung Tsai, *Annual Review of Pharmacology and Toxicology*, **2015**, *55*, 249.
29. Abdel-Magid, A. F., *ACS., Med. Chem. Lett*., **2015**, *12*, 104.
30. Magdalena Korczynska, Mary J. Clark, Celine Valant, Jun Xu, Ee Von Moo, Sabine Albold, Dahlia R. Weiss, Hayarpi Torosyan, Weijiao Huang, Andrew C. Kruse, Brent R. Lyda, Lauren T. May, Jo-Anne Baltos, Patrick M. Sexton, Brian K. Kobilka, Arthur Christopoulos, Brian K. Shoichet, Roger K. Sunahara, *PNAS*, **2018**, *115*, E2419
31. Xu X., Chen Y., Fu Q., Ni D., Zhang J., Li X., and Lu S., *Journal of Enzyme Inhibition and Medicinal Chemistry*, **2019**, *34*, 361.



32. Anupam B. Jena, John E. Calfee,† Edward C. Mansley,‡ and Tomas J. Philipson, *Forum Health Econ. Policy*. **2009**, *12*, 5.

33. http://www.evaluategroup.com/Universal/View.aspx?type=Story&id=207449

34. https://www.pfizer.com/news/press-release/press-release-detail/pfizer_and_protalix_biotherapeutics_announce_fda_approval_of_pediatric_indication_for_elelyso_taliglucerase_alfa_for_injection_for_intravenous_use_for_the_treatment_of_type_1_gaucher_disease

35. Peters G. J., *Nucleosides Nucleotides Nucleic Acids*, **2014**, *33*(4-6), 358–74.

36. Hong Wan, *ADMET and DMPK*, ***2013***, *1*,19.

37. K. C. Cheng, Walter Korfmacher, Ronald E. White and George Njoroge, *Perspectives in Medicinal Chemistry*, **2007**, *1*, 1.

38. Nigel H. Greig, , *Research and Clinical Interventions*, **2017**, *3*, 651.

39. Hanqing Xue, Jie Li, Haozhe Xie, and Yadong Wang, *Int. J. Biol. Sci.*, **2018**, *14*, 1232.



CHAPTER

6

Role of Physicochemical Properties of Chemical Structures in Drug Design

KEY THINGS TO LEARN FROM THIS CHAPTER

- Role of physicochemical properties of test compounds in drug discovery
  - Lipinski rule of five and rule of three
  - Ionization constant/pK_a and its role in ADMET and target binding interactions, Calculation of ionized/unionized content from pK_a of drug and pH of environment, Affect of pK_a on bioavailability
  - Solubility of drugs
  - Lipophilicity (logP), and its relationship with pK_a of drugs, Lipophilic efficiency (LiPE), Effect of lipophilicity PK/PD parameters *in vitro*, Distribution coefficient (logD)
  - HBD/HBA
  - Surface activity
  - Number of rotatable bonds (ROTB)
  - Number of aromatic rings (AROM)
  - Undesirable functional groups (ALERT)
  - Steric factor
  - Molecular size
- Desirable physicoschemical parameters for CNS drugs and preclinical drug candidates.

6.1 Introduction

The physicochemical properties of drugs fundamentally affect both pharmacokinetic (PK) and pharmacodynamic (PD) parameters of drugs once they are administered in our body. While pharmacokinetic deals with the effects caused by the organism on the life span of the drug, pharmacodynamic deals with effects of drug in terms of biological response on the organism (Fig. 6.1).

Once a drug (ligand or substrate) is administered in the body, it ultimately accesses the specific target (receptor/enzyme) to produce the desired biological response. The success largely depends on the following physiological factors:

1. Good bioavailability and distribution
2. Gastric conditions
3. Reduced first pass metabolism

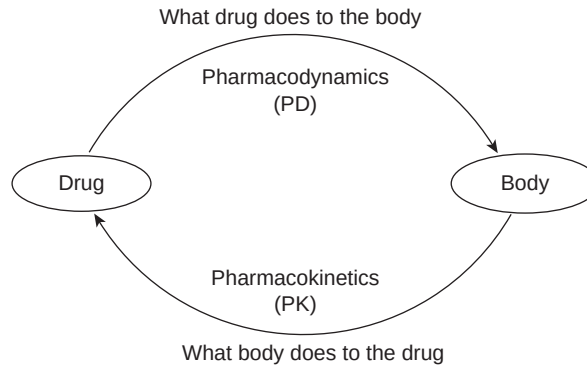


Figure 6.1 Relation between pharmacodynamics and pharmacokinetics.

4. Avoidance of drugs getting effluxed back (P-glycoprotein functions) in the intestine
5. Survival of drugs against intestinal bacteria
6. Minimizing binding of drugs with blood plasma proteins and blood cells
7. Survival of drugs in the range of physiological pH (1.5–8.0) across the body
8. Ability of drugs to diffuse into tissue carrying therapeutic targets
9. Reduced/delayed metabolism: Phase I and II
10. Reduced/delayed elimination of drugs by kidney
11. Avoidance of partition into undesired places (off protein targets/brain)

A successful drug, among many attributes, must have chemical features in terms of functional groups and balanced hydrophobic-hydrophilic components (expressed as lipophilicity) so that it can pass in sufficient concentration through multiple physiological barriers before accessing the site of action to elicit the desired biological response. The presence of functional groups primarily affects solubility and ionizing and binding abilities of the molecule. Ionization (expressed as pK_a) of the drug/test compound plays an important role during absorption through physiological barriers due to the presence of both aqueous and lipid environments. Plasma membranes generally present lipid environment and the drug has to encounter them during absorption as it passes through gastrointestinal region via epithelial cells, diffusion from blood capillaries into interstitial fluids (or extracellular fluids [ECF]) via endothelial cells, and finally diffusion into tissues containing active sites. Aqueous environment is encountered in the gut, in the blood, in the interstitial fluid (part of ECF), and in the cytoplasm within tissues, albeit with variations in their pH values. Thus, for a drug administered by oral route, its journey in the body involves three lipid zones and four aqueous zones before accessing the target. Accordingly, a drug must have the optimal pK_a value in order to facilitate its movement both in the ionized or unionized state in sufficient concentrations while passing through seven aqueous and lipid environments. Besides pK_a, another important aspect of functional groups in the molecule involves its binding to protein targets via weak non-covalent interactions. Thus, while optimizing pK_a value in a molecule, its binding properties also need to be taken into account.

The second important chemical feature, lipophilicity, plays a role in solubility, absorption, uptake of drug into the brain, plasma protein binding, distribution, and metabolism. Highly hydrophobic compounds exhibit poor pharmacokinetic (ADMET) profile as they promote non-specific binding with plasma proteins, bind to off-target protein causing toxicity, get deposited in adipose tissues, fail to diffuse through aqueous zones, etc.

On the contrary, highly hydrophilic compounds fail to get absorbed through lipid bilayer membranes. Thus, lipophilicity, solubility, and pK_a in test compounds must be balanced to achieve optimal physicochemical profile. The poor solubility may lead to decreased bioavailability and distribution. However, increasing aqueous solubility may decrease lipophilicity, thereby decreasing permeability with difficulty in penetrating physiological barriers.

Besides optimizing physicochemical properties of test compounds during lead optimization, the physicochemical properties are also applied as filter to a large database of chemical libraries (unbiased libraries) before they are submitted for *in vitro* screening against a target. A set of physicochemical parameters are generally applied as filter that separates test compounds with drug-like properties or drug-likeness from the huge collections of compounds (library).[1] Drug-likeness or drug-like properties takes into account the properties that are common among clinically used drugs. It has thus become a common practice to analyze physicochemical properties of test compounds (chemical library) in the early stages of drug discovery and only compounds with drug-like properties are subjected to screening. This exercise is predominantly carried out using Lipinski's rule. Computational tools and software are available for filtering out molecules with drug-like properties from a chemical library.

Once the libraries of compounds are screened for their biological activity *in vitro* and *in vivo*, chemical modifications in the hit/lead molecules are carried out with the view to further optimize the physicochemical properties and its effect on PK/PD profile. This is an important exercise during medicinal chemistry, and initial compound selection must be based on *in vitro* binding affinities in nanomolar to picomolar range. Further *in vitro* functional assays are then carried out to establish agonist and antagonist properties along with efficacy and selectivity. These parameters assist not only in getting desired therapeutic response during *in vivo* studies in animal models with human diseases but also in compound selection process.

It is pertinent to add that binding between drugs and therapeutic targets involves both polar (ionic, H-bond, dipole) and hydrophobic interactions yet again justifying requirement for an optimal balance between polar and non-polar chemical components (lipophilicity) in the test compounds during lead optimization. Thus, molecular properties (chemical features) of a molecule can affect its physicochemical properties which, in turn, can affect PK and PD profiles of the test compounds in the organism (Fig. 6.2).

There are few molecular properties of molecules such as size, shape, flexibility, polarity, lipophilicity, and ionization that regulate the following physicochemical properties and are analyzed routinely during drug discovery or lead optimization programme:

1. Lipinski's rule of five (RO5)
2. Ionization constant (pK_a)
3. Solubility (aqueous and fat solubility)
4. Partition coefficient (logP and D)
5. Hydrogen bond donor (HBD)/Hydrogen bond acceptor (HBA)
6. Surface activity
7. Number of rotatable bonds (ROTB)
8. Number of aromatic rings (AROM)
9. Undesirable functional groups (ALERT)
10. Steric factor
11. Molecular weight
12. Polar surface area
13. Electron density

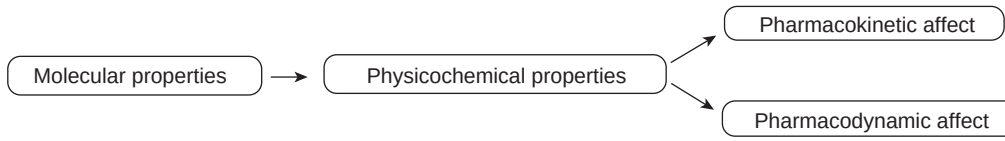


Figure 6.2 Physicochemical properties of drugs fundamentally affect both PK and PD parameters of drugs.

6.2 Lipinski's Rule of Five

The foundation of any drug discovery programme lies in the quality of chemical library submitted for screening against a particular disease target(s). The selection process may involve either prior art information or 'filtration' of drug-like compounds from a library or a focused library depending on the structure and function of therapeutic targets. The process of 'filtration' involves selection of compounds with certain physicochemical properties that are common among drugs. Criteria for these properties (physicochemical/drug-like) are related to a range of properties for successful clinically used drugs in terms of their oral bioavailability, safety/toxicity, and clinical success. Although predictive performances *in vivo* with compounds having drug-likeness have several exceptions, Lipinski's rule[2] and Veber's criteria[3] remain two of the most widely used methods for the selection of compounds with drug-like properties. It is pertinent to add that both the rules have been developed as filtering criteria for the selection of compounds for drug-likeness only for the purpose of orally administered compounds (not applicable to routes other than oral) and are based on parameters such as solubility and permeability. Although both the rules for drug-likeness are easy to use, application to drugs involving routes of administration other than oral is often debatable. This gets support from the comparative applicability of both the rules to approximately 1100 clinically used drugs categorized under oral and non-oral routes of administration (Table 6.1). Thus, although either of the rules are not a guarantee for establishing drug-likeness with high degree of confidence, the physicochemical parameters laid down in these rules have become integral part of drug discovery programme and are used routinely irrespective of routes of administration or disease areas.

Lipinski's rule, also called as *Rule of Five* (RO5) plays an important role in drug design as it addresses ADME issues of drug candidates. There should not be more than one violation out of the four parameters for oral drugs. It is considered to be a thumb rule and is associated with the following physicochemical properties:

1. Molecular weight should be less than 500 (<500).
2. LogP, a parameter for lipophilicity should be less than 5 (<5).
3. Hydrogen bond donor (HBD) should be less than 5 (<5).
4. Hydrogen bond acceptor HBA should be less than 10 (<10).

However, currently more physicochemical parameters are being added that involve pK_a, solubility, logD_{pH}, and PSA.

Table 6.1 Comparison of Lipinski's rule and Veber's rule as reported in the literature[4]

Route of Administration	Lipinski's Rule			Veber's Rule		
	Pass	Fail	% Pass	Pass	Fail	% Pass
Oral	709	59	92	639	130	83
Non-oral	333	90	78	267	156	62

In 2006, Hopkins et al.[5] published an article on number of therapeutic drugs used clinically and their relationship with RO5.

Total number of small molecule drugs:	1204
Orally administered drugs:	803
Parenterally administered drugs:	421
Topically administered drugs:	275
Prodrugs:	192
Compliance of drugs to RO5:	885
Out of this actually administered by oral route:	619
Number of drugs failed at least in one of the RO5:	159

6.2.1 The Rule of Three (RO3)

The rule of three is generally applied during fragment-based drug discovery approach.[6] The approach (Discussed in chapter 4) involves use of small fragments as starting point for filling up the pockets in the binding site of therapeutic target followed by combination of fragments to get a therapeutic candidate. The definition of rule is as follows:

1. Molecular weight is greater than 300 g.
2. The number of hydrogen bond donors is less than or equal to 3.
3. The number of hydrogen bond acceptors is less than or equal to 3.
4. clogP is 3.
5. Other parameters found useful fragment selections are number of rotatable bonds is less than or equal to 3 and polar surface area is less than or equal to 60 m^2.

6.3 Ionization Constant (pK_a)

The pK_a (acid-base dissociation constant) plays central role in drug discovery and is considered to be one of the most crucial physicochemical parameters in drug design. It is also fundamental to five other important physicochemical parameters: solubility, lipophilicity, permeability, plasma protein binding, and binding with therapeutic targets that in turn affect PK and PD profiles of test compounds/drugs. The pK_a is defined as the pH where a drug exists in 50% ionized and 50% unionized (equal concentration) form. Knowledge of the pK_a allows calculation of percentage of ionized vs unionized concentration of a drug at a physiological pH. The ionization of compounds at a particular pH regulates solubility, $logD_{pH}$, absorption via diffusion of drugs through plasma membrane (bioavailability), distribution and binding, etc. Notably, only unionized form diffuses through the lipid bilayer membrane. Approximately two-thirds of drugs on the market contain at least one group capable of ionization in a pH range of 2–12, and a majority of them are either weak acids or weak bases. Since ionization of the drug depends on its pK_a and pH of the physiological medium (Fig. 6.3),[7] modulating pK_a value of test compounds during lead optimization can have profound effect on bioavailability including ADMET. For example, all local anesthetics (weak bases), with their pK_a values slightly higher than physiologic pH (7.4), have more molecules in the lipid soluble (lipophilic) unionized form. However, at the physiologic pH, ionization equilibrium shifts towards ionized form, and concentration of unionized form of drug becomes less than 50%. Thus, while the unionized hydrophobic form diffuses through the axonal membrane to initiate neural blockade, the ionized form which cannot cross membranes gets eliminated by urine in acidic pH. However, the unionized form that enters cytoplasm following diffusion gets ionized (cationic form) in the presence of H^+ and binds to the ion channel from cystosolic side to reduce or block firing of neurons (Fig. 6.11).

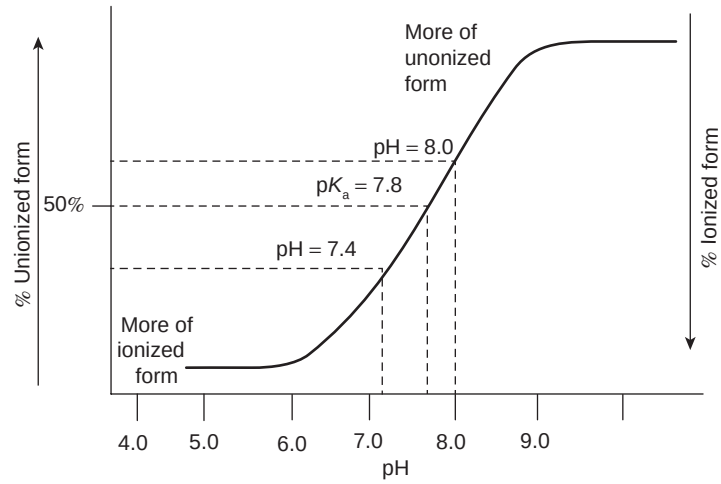


Figure 6.3 Relationship between pK_a, pH, and ionization of a basic compound.

The following points indicate the significance of modulating the pK_a values of test compounds during drug design/lead optimization strategies (for administration by oral route):

1. In general, drugs do not undergo complete dissociation in aqueous medium. In fact, only a fraction of drug molecules ionizes at a given physiological pH unless pH is equal to or close to pK_a.
2. Acidic drugs with pK_a values greater than 6 are weak acids and basic drugs with pK_a less than 8 are considered to be weak base.
3. The ionization of drugs will depend on the pK_a of the drug and pH of the surrounding medium in the gastrointestinal region and, in turn, affects their site of absorption varying from saliva to large intestine.
4. Only the unionized form of a drug can partition across biological membranes.
5. The ionization of drugs may significantly reduce their ability to permeate membranes and in turn affect their bioavailability.
6. Highly hydrophilic drugs cannot cross membranes.
7. The ionized form tends to be hydrophilic (more water soluble), and this feature is required during administration of drug, distribution of drug in plasma, binding of drug at target, and elimination through urine.
8. Knowledge of the pK_a allows calculation of the percentage of the ionized form at any given physiological pH using Henderson-Hasselbalch equation on online software.
9. Weakly acidic drugs are preferably absorbed in stomach, as under acidic environment (pH 1.4–2.0) the drug molecules will mostly remain in the unionized form and diffuse readily through lipid bilayer membrane.
10. Weakly basic drugs will preferably get absorbed in the intestine (pH 7.5–8.0) as under basic environment, the drug molecules will mostly remain in the unionized form and diffuse readily through lipid bilayer membrane.

11. The pK_a influences not only the key physical properties of a drug-like molecule: solubility, lipophilicity, permeability, and protein binding but also affects PK parameters. The pH values along gastrointestinal tract (GIT) ranges from 1.5 to 8.0.

12. As a general rule, when $pH < pK_a$ by 2 units, for acids 99% is unionized, while for bases 99% is ionized. Similarly, when $pH > pK_a$ by 2 units, for acids 99% ionized while for bases 99% unionized.

6.3.1 Ionization of Base

$$\ddot{B} + H_2O \rightleftharpoons BH^+ + OH^-$$

Application of Henderson-Hasselbalch equation to calculate the ratio of $[BH^+]$ to [B]:

$$pOH = pK_b + \log_{10}\left[\frac{\text{conjugate acid}}{\text{weak base}}\right]$$

$$pOH = pK_b + \log_{10}\left[\frac{BH^+}{B}\right]$$

6.3.2 Ionization of Acid

$$AH \rightleftharpoons A^- + H^+$$

Application of Henderson-Hasselbalch equation to calculate the ratio of $[A^-]$ to [HA]:

$$pH = pK_a + \log_{10}\left[\frac{\text{conjugate base}}{\text{weak acid}}\right]$$

$$pH = pK_a + \log_{10}\left[\frac{A^-}{AH}\right]$$

$$pH - pK_a = \log_{10}\left[\frac{A^-}{AH}\right]$$

Relationship between pK_a and strength of acidic and basic compounds is shown in Fig. 6.4.

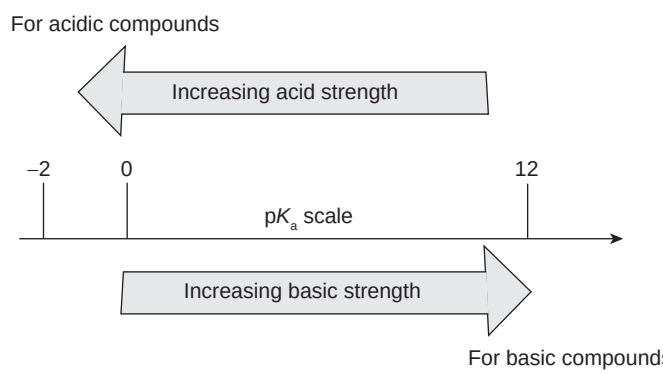


Figure 6.4 Relationship between pK_a and strength of acid/base.

6.3.3 Role of pK_a in ADMET and Target-Binding

Medicinal chemists in general prefer to work on hydrophobic compounds in the early stages of drug discovery owing to their solubility in organic solvents. However, in the later stages, hydrophobic compounds were found to be associated with poor ADMET profile and required introduction of polar groups in last stages of drug discovery. Introduction of polar groups on the one hand had effect on pK_a and on the other hand played a role in binding with targets. Thus, taking pK_a as an additional physicochemical parameter into account during early stages of drug discovery, during structure-property relationship along with other parameters molecular weight, logP, $logD_{7.4}$, HBA/HBD and polar surface area may furnish compounds with good ADMET profile. The pK_a value of test compounds in isolation has no significance and needs to be optimized with other physicochemical properties.

Presence of ionizable groups in the test compounds facilitates binding (electrostatic interaction) with the hydrophilic region of the binding site in the target. Most of the electrostatic drug-target interactions involve H-bonding, ionic interaction, dipole-dipole interaction, and cationic-π interaction. Since target protein receptors or enzymes are present in aqueous environment, water molecules in the active site may reduce the strength of electrostatic interactions. This issue can be addressed by introducing hydrophobicity in the molecule, as desolvation of hydrophobic groups requires significantly less energy. Thus, enhancing hydrophobicity may improve biological activity, but too much of hydrophobicity may ultimately furnish 'fatty molecules' with poor ADMET profiles. A careful survey of oral drugs in market revealed that more than 78% have ionizable groups in their molecular framework.[8] For example, CNS drugs bind to G-protein coupled receptor proteins (GPCR) which carry aspartic acid residue as the key residue that binds with drug/endogenous ligands. Thus, most of the CNS ligands are amine-containing small molecules such as endogenous catecholamines or amphetamine drugs and consequently they have pK_a (basic compounds).[9]

If the physiological pH of test compounds shifts the equilibrium towards the unionized form, the drug would be absorbed. However, if the pH shifts the balance towards the ionized form, the drug would not be absorbed. The relationship between pK_a of acidic and basic drugs and their bioavailability/receptor binding has been summarized in Table 6.2.

As a general rule, when pH < pK_a by 2 units, for acids 99% is unionized while for bases 99% ionized. Similarly, when pH > pK_a by 2 units, for acids 99% ionized while for bases 99% unionized.

Table 6.2 Relationship between pK_a of acidic and basic drugs and their bioavailability/receptor binding

pK_a	Acidic Drug, at pH 2.5, in Stomach			Basic Drug, at pH 5.0, in Small Intestine		
8.0 ↓ 2.0	Concentration of unionized drugs	Bioavailability	Binding with protein target	Concentration of unionized drugs	Bioavailability	Binding with protein target
	↓ Increases	↓ Increases	↓ Decreases	Increases ↑	Increases ↑	Decreases ↑

6.3.4 Strength of Acidic/Basic Compounds in Periodic Table

Across the periodic table, acidity increases with electronegativity, and downwards the periodic table, acidity increases with size. For acidic compounds, an increase in pK_a values decreases acidity, whereas for basic compounds, increase in pK_a values increases basicity (Fig. 6.5).

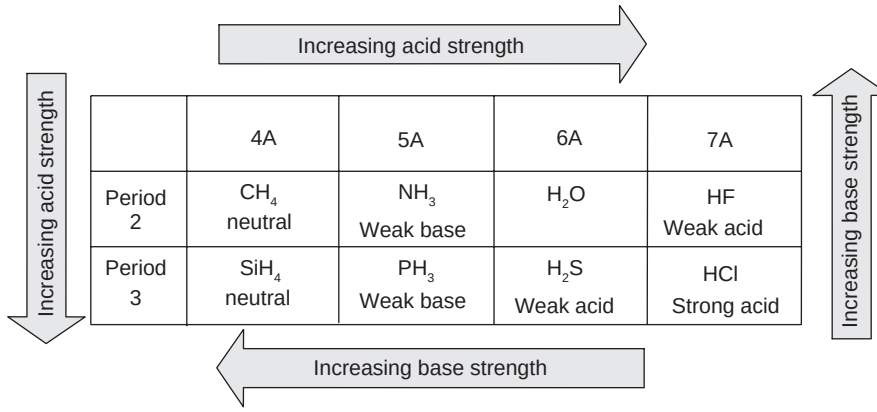


Figure 6.5 Acid/base strength in periodic table.

CASE STUDY 1

Relationship between pK_a/pH/Gut Absorption

Let us analyze absorption of following hypothetical drugs with varying pK_a values depicted in Table 6.3. For establishing a relationship between the pK_a values of a drug with its absorption in gastrointestinal region at different pH, initially percent population of ionized form at different pH is calculated.

Using online software at https://www.manuelsweb.com/pka.htm, the approximate populations of ionized form in terms of percentage at a particular physiological pH (in gastrointestinal region) can be determined and has been summarized in Table 6.3. Next, based on the respective ionization percentage at each physiological pH, one can derive information regarding the absorption of drugs in the gastrointestinal region.

Table 6.3 Concentration of ionized form present at different pH for drugs A to E

Drug	pK_a	% Ionized at pH = 2.2	% Ionized at pH = 4.4	% Ionized at pH = 6.9
A (Acid)	−1.8	100	100	100
B (Acid)	4.8	0.3	28.5	99.2
C (Acid)	7.2	0	0.2	33.4
D (Base)	8.3	100	100	96.2
E (Base)	9.7	100	100	99.8

(*Continued*)

Study 1 (*Continued*)

1. Which one will be the best absorbed drug from the stomach (pH = 2.2)?
Ans: As is evident from Table 6.3 at pH 2.2 drugs A, D, and E will be in the ionized form, whereas drugs B and C will be in an unionized form. Therefore, drugs B and C will be best absorbed at pH 2.2.

2. Which one will be best absorbed from the small intestine (pH = 4.4)?
Ans: As is evident from Table 6.3, at pH 4.4 only drug *cis* in an unionized form (100%), and so it will be the best absorbed followed by drug B having 50% concentration in an unionized form.

3. Which one of them will pass most readily from the plasma into the brain (pH of plasma = 6.9)?
Ans: As is evident from Table 6.3, at pH 6.9 drug C is 67% unionized, and therefore will be the absorbed drug. Remaining drugs are highly ionized and will not penetrate.

4. Which one will be eliminated least readily from the kidney (urine pH of 4.4)?
Ans: Drugs readily eliminated from kidney via urine should be hydrophilic, so only ionized form will be eliminated. As can be seen from Table 6.3, at pH 4.4 drug C will be least eliminated (ionized form 33.4%) and will be re-adsorbed by the tubules in the kidney.

CASE STUDY 2

Calculation of Ionized Content from pK_a Value

Query: An acid has a pK_a of 4.8. What percentage of acid will be ionized at pH 7.4?

Solution: The % ionization of an acid is given by the equation:

$$\text{Percent ionized} = \frac{100}{1+10^{(pK_a - \text{pH})}}$$

$$= \frac{100}{1+10^{(4.8-7.4)}} = 99.74\%$$

6.4 Solubility of Drugs

Drug solubility is an important parameter among physicochemical properties of test compounds/drugs as it requires compounds to be soluble in both water and lipid media. This is because, following administration, the drug has to pass through both aqueous and lipid media inside the body. The aqueous media in the body is present in GIT lumen, blood plasma, cytoplasm, and extracellular/interstitial fluids through which drugs have to pass after exiting blood capillaries and bind with the target proteins in tissues. Aqueous solubility helps to achieve the desired concentration of drug in systemic circulation required for distribution in sites of action and for eliciting desired pharmacological response following binding to the target. On the contrary, lipid solubility of drugs plays a major role in drug absorption (bioavailability), distribution in tissues, and while exiting from blood capillaries through the biological lipid bilayer membranes. Thus, during lead optimization studies, it is important to introduce ionizing and non-ionizing properties in the molecule in a manner that will balance the act of solubility in both the media (Fig. 6.6). Notably, more than 80% drugs used clinically are administered orally.

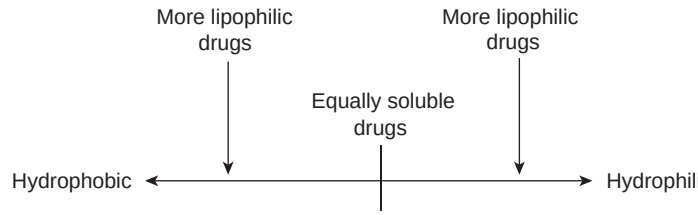


Figure 6.6 Solubility/high lipophilicity/hydrophilicity.

For the dissolution of a test compound in either physiological aqueous or lipid environment, it is a prerequisite that attractive forces are in effect between the solute and the solvent. In the drug discovery programme, the kinetic solubility of test compounds needs to be measured before being submitted for biological screening. This can be done routinely in high throughput format using nephelometric or turbidimetric assays.

CASE STUDY 3

Solubility Prediction in Chloramphenicol

The presence of functional groups containing oxygen and nitrogen usually enhance aqueous solubility, the presence of non-ionizable hydrocarbon chains and ring systems contributes to enhanced lipid solubility (Fig. 6.7). The pK_a values of drugs can be used as a tool to establish aqueous solubility of drugs. For example, diclofenac with pK_a 4.08 (acidic) will have poor aqueous solubility at lower pH due to lower population of ionized drug, whereas at higher pH, the aqueous solubility is enhanced due to higher concentration (population) of ionized drug (Table 6.3).

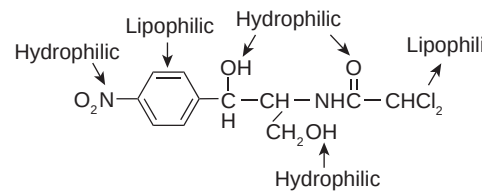


Figure 6.7 Chloramphenicol with balanced water- and lipid-soluble moieties.

Similarly, the aqueous solubility of 4, 5-diphenylimidazole (pK_a 5.88; basic) can also be established with its ability to ionize at a particular pH. Thus, for acidic drugs, a lower pH than the pK_a of the drug decreases aqueous solubility, whereas for basic drugs, a lower pH than pK_a of the drug enhances aqueous solubility (Table 6.4). Besides solubility, pK_a can also affect several additional parameters, as summarized in Figure 6.8.

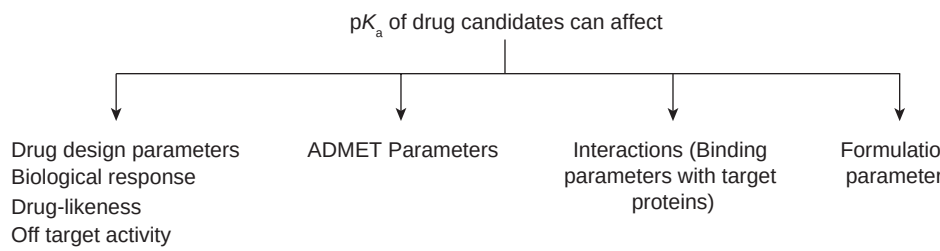


Figure 6.8 Effect of pK_a on various parameters related to drug discovery process.

Table 6.4 Effect of pK_a on solubility of drugs at different pH

Diclofenac pK_a 4.08 (acidic)	**pH 3.0**		**pH 5.0**	
	Solubility	Concentration of ionized drug*	Solubility	Concentration of ionized drug*
	17 μM	7.7%	>350 μM	89.3%
4,5 diphenylimidazole pK_a 5.88 (basic)	**pH 3.0**		**pH 9.0**	
	Solubility	Concentration of ionized drug*	Solubility	Concentration of ionized drug*
	>350 μM	99.9%	84 μM	0.1%

**Calculated using software available at https://www.manuelsweb.com/pka.htm (dated 10. 05. 2017).*

6.5 Lipophilicity

After pK_a, lipophilicity is the second most important physicochemical parameter that plays an important role in drug design addressing both PK and PD issues. Using this parameter one can derive information about drug transport through lipid structures, hydrophobic interactions of drugs with binding sites in target protein, solubility, hepatic clearance, lack of selectivity, and non-specific toxicity (ADMET). Thus, lipophilicity of test compounds regulates absorption in GIT and diffusion in tissues through lipid bilayer membranes, distribution in tissues, and blood-brain barrier (BBB) permeation, binding to target proteins including off-target binding (toxicity) and greater metabolic clearance by cytochrome P450 (CYPs).

Lipophilicity is represented by logP, a measurement of partition coefficient value (ratio of concentration in two liquids) when a test compound is exposed between two immiscible solvents octanol and water. Octanol has both a polar and a non-polar region and resembles phospholipid in cell membranes. Thus, the experimentally generated logP value for a test compound can be a good indication for its ability to get absorbed through lipid bilayer membrane *in vivo*. Higher logP value suggests higher lipophilicity for the test compound. Notably, logP considers the ratio of distribution of unionized compounds between phases.

Experimentally, lipophilicity of a molecule can be calculated using octanol and water (human body is made up of water and lipids) as two immiscible solvents. The concentration of a molecule in each solvent is determined by measuring the relative solubility (in the absence of ionization) of an organic compound in water (at physiological pH 7.4 buffer) and in a lipid media (usually n-octanol).In the equation given below involving ratio of concentrations, it is evident that the partition coefficient P is directly proportional to concentration of compound in octanol. The findings suggest that higher the partition coefficient (P) value, higher is the solubility of the compound through the bilayer cell membrane *in vivo*.

$$\text{Partition coefficient}\left(P_{o/w}\right) = \frac{\text{Conc. of compound (o)}}{\text{Conc. of compound (w)}}$$

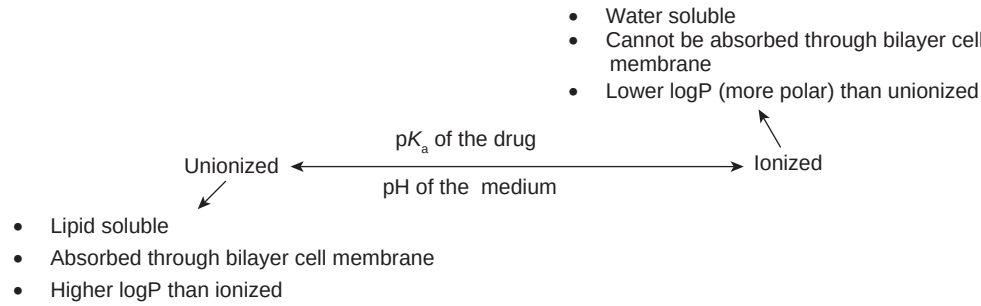


Figure 6.9 Effect of pK_a of drugs on solubility/lipophilicity.

Partition coefficient $P_{o/w}$ determined experimentally is generally expressed as $\log_{10}P$ or logP. However, calculated logP values are referred to as clogP.

In the context of PD (what a drug does to the body), the lipophilicity is the driving force in addition to electrostatic interactions for the binding of drugs to their receptor targets. However, drugs with high lipophilicity are known to be more toxic as they are generally retained longer (e.g., in adipose tissues) and are in turn widely distributed within the body. Besides, they non-selectively bind with proteins other than the desired ones (off-target) and are often extensively metabolized. Hence, it is advisable to balance the hydrophilicity and lipophilic properties in the drug so that it cannot only enter cells through diffusion but retains adequate binding affinity to the therapeutic protein target.

Although logP deals with lipophilicity, the ionizing abilities (pK_a) of drugs have not been taken into consideration in the equation. Besides, since partitioning of the drug in the octanol and water phase depends on the ionization of the drug, logP alone would not reflect actual distribution. Thus, ionizing abilities of drugs at different physiological pH should have effect on the logP values. The logP of non-polar/unionized form should be higher than polar/ionized form (Fig. 6.9).

6.5.1 *Effect of pK_a on Lipophilicity*

1. When the drugs become ionized, the polar form will dissolve in water more readily due to its facile ion-dipole interaction with water molecules.
2. The ionized form of the drug due to its polar nature has lower logP than the unionized from.
3. The ability of the drugs to ionize at different body pH coupled with lipophilicity needs to be taken into consideration during drug development programme.
4. The ionization and lipophilicity value of the drug can give useful information about its absorption and distribution in tissues.
5. For ketoconazole with pK_a values of 6.43 and 3.64, different logP values were obtained at different pH values. This can be attributed to its difference in ionization abilities into ionic and neutral form (Table 6.5).

Table 6.5 Effect of pH on logP values of ketoconazole

pH	3.0	5.0	7.4	9.0
logP	0.35	2.37	3.46	3.56

6.5.2 Effect of Lipophilicity on Some of the PK/PD Parameters in vitro

Although enhancing lipophilicity often furnishes active compounds *in vitro*, overall effects on other PK/PD parameters are not encouraging. Increasing lipophilicity although may have few favorable *in vitro* effects such as increase in response, increase in binding to target protein, and increase in bioavailability. It is also accompanied by unfavorable effects such as increase in degradation of test compounds by metabolic enzymes (short duration of action), increase in binding to plasma proteins (reducing distribution), increase in binding to hERG (resulting in cardiotoxicity), and decrease in aqueous solubility (Fig. 6.10). Thus, increasing lipophilicity may result in compounds with poor ADMET profile *in vivo*.

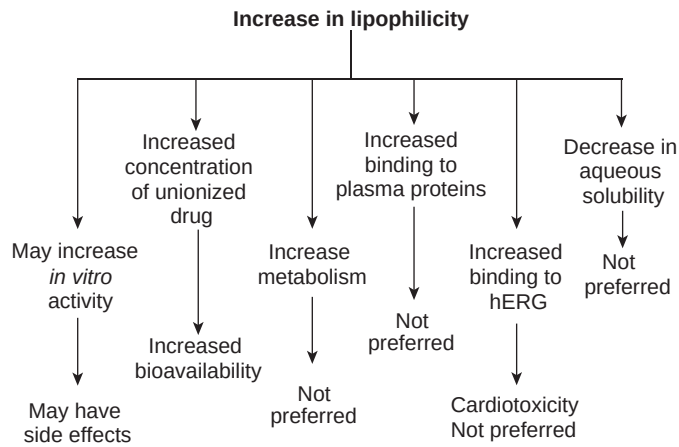


Figure 6.10 *In vitro* effects of increasing lipophilicity of test compounds on other PK/PD parameters.

6.5.3 Apparent Permeation Coefficient (P_{app})

Mathematical equation involving logP and pK_a:
P_{app} has been used to predict the behavior of a compound at all pH values, as logP value is known.

For acids:

$$P_{app} = \frac{P}{1+10^{\mathrm{pH}-\mathrm{p}K_a}}$$

For bases:

$$P_{app} = \frac{P}{1+10^{\mathrm{p}K_a-\mathrm{pH}}}$$

The permeability of drug molecules in Caco-2 cell lines is expressed as apparent permeation coefficient P_{app}, and this is routinely used in *in vitro* or *ex vivo* experiments as part of a general screening process to study drug absorption.

6.5.4 Distribution Coefficient (logD)

The logP deals with partition coefficient in the absence of ionization for a particular compound in two immiscible solvents. But since drugs used in clinics are weak acids or bases, ionization of compounds

depending on pH needs to be considered while determining lipophilicity. Thus, logD has been used to refer to lipophilicity at a particular pH. It deals with distribution of compound under ionized condition at a given pH in two immiscible solvents and is represented by $\log D_{pH}$. Thus, it takes both lipophilicity and degree of ionization into account at a given pH. It can be calculated with the ratio (partition coefficient) between concentration of unionized compound and concentration of ionized species, if any, in octanol versus concentrations of ionized plus unionized species in water. Both logP and $\log D_{pH}$ can be calculated as well as measured experimentally and are routinely used to correlate with lipophilicity in drug discovery.

$$\begin{matrix}\text{Partition coefficient}\left(D_{o/w}\right)\\ \text{(Lipophilicity at a particular pH)}\end{matrix} = \frac{[\text{Conc of unionzed compound}] + [\text{Conc of ionzed compound}]_{(o)}}{[\text{Conc of unionzed compound}] + [\text{Conc of ionzed compound}]_{(w)}}$$

For acidic compounds:

$$HA \rightleftharpoons A^- + H^+$$

$$D = \frac{[HA]_o + [A^-]_o}{[HA]_w + [A^-]_w}$$

$$\log D = \log P - \log[1 + 10^{(pH - pK_a)}]$$

For basic compounds:

$$BH^+ \rightleftharpoons B + H^+$$

$$D = \frac{[BH^+]_o + [B]_o}{[BH^+]_w + [B]_w}$$

$$\log D = \log P - \log[1 + 10^{(pK_a - pH)}]$$

In view of the above, drugs need to be designed with moderate lipophilicity without compromising with activity, diffusion, and binding affinity. Lipophilicity can be regulated by modulating pK_a/polarity of the test compounds.

Thus, the three most crucial physicochemical properties logP, $\log D_{7.4}$, and pK_a together regulate ADMET properties, life span of drugs in the body, and binding with targets; they thus play an important role in drug discovery. Automated techniques are available to determine these parameters with very small quantity of samples. Together these three parameters correlate well with the *in vivo* behavior of drugs in terms of PK/PD.

6.5.5 Lipophilic Efficiency

Drug-likeness and lipophilicity are two important properties of molecules in a chemical library used for the purpose of drug discovery programme. In order to estimate the drug-likeness of active compounds following screening, a mathematical equation has been developed linking potency with lipophilicity. This has been referred to as lipophilic efficiency (LiPE)[10] and can be calculated subtracting logP (lipophilicity) values from pIC_{50} values.

$$\text{LiPE} = pIC_{50} - \log P$$

The LiPE plays an important role during lead optimization studies.

CASE STUDY 4

Role of Ionization in Biological Activity with Reference to Local Anesthetics

Local anesthetic drugs deal with a localized loss of nociception. LA drugs bind more readily to sodium channels in the activated state and interfere with passage of Na^+ ions across cell membrane.

LA diffuses (enters) nerve fiber as neutral unionized free base and then is transformed into the cationic form in cytoplasm which binds at the inner surface (accessible from cytoplasmic side) of the Na channel (Fig. 6.11) and in turn blocks conduction (firing of neurons).

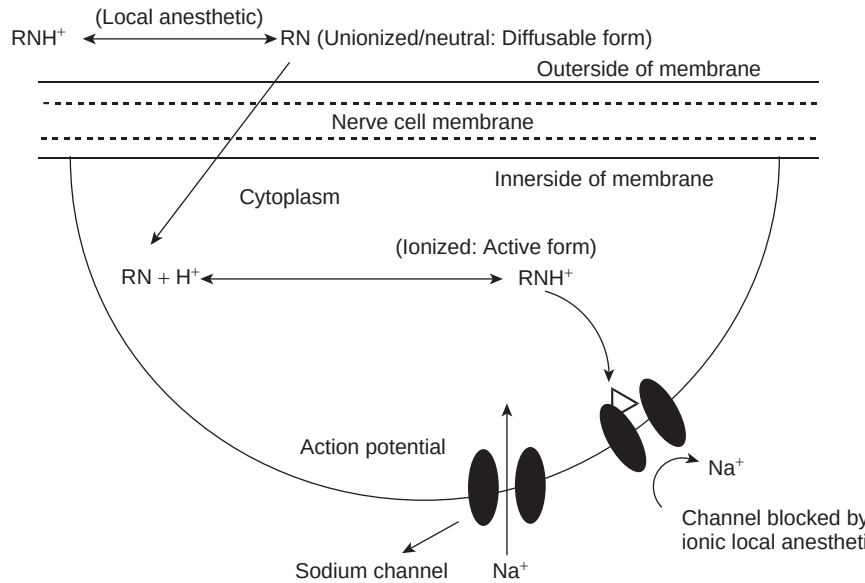


Figure 6.11 Role of ionization in the biological activity of local anesthetic.

6.5.6 Effect of Lipophilicity on Local Anesthetics

Comparing physicochemical properties and biological activities among a series of LAs, the lipid solubility (logP) plays a major role in the potency, plasma protein binding, and duration of action of LAs. Among the series, tetracaine with highest lipid solubility (logP = 3.51) had a longest duration of action (Table 6.6).

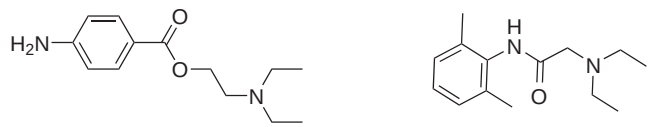
Table 6.6 Physicochemical properties and biological activities of a series of LAs

Drug Name	logP	Relative Potency	% Plasma Protein Binding	Duration of Action (min)
Procaine	0.88	1	6	60–90
Lidocaine	2.26	2	65	90–200
Bupivacaine	3.31	–	95	240–480
Tetracaine	3.51	8	80	180–600

6.5.7 Effect of pK_a on Local Anesthetics

The pK_a (7.7–9.0) of most LA agents is higher than physiologic pH which plays an important role in the onset of anesthesia by LAs. Rapid onset of action can be expected by LA with relatively lower pK_a values (but higher than 7.4) as the concentration of unionized (hydrophobic) form will be more and, accordingly, higher population of drug will diffuse through neuron membrane. Once inside the cytoplasm, it will exert its effect by binding to the Na^+ channel from cytoplasmic side (Fig. 6.12). For anesthetics with higher pK_a, the concentration of unionized drug is decreased, thereby slowing down the onset of anesthesia. For example, at pH 7.4, lidocaine with pK_a of 7.7 has a unionized concentration of 24%, whereas bupivacaine (pK_a 8.4) has 16% unionized drug, which is less than lidocaine. This explains the faster onset of action by lidocaine at the same dose. Thus, delay in the onset of anesthesia for bupivacaine can be directly correlated with relatively less concentration of unionized drug (Table 6.7), which is indeed regulated by pK_a.

In general, lipophilicity (logP) affects many important PK and PD parameters (Tables 6.8 and 6.9).



Procaine Lidocaine

Figure 6.12 Structures of clinically used LAs.

Table 6.7 Effect of pK_a on the action of LAs

Drug Name	pK_a	% Free Base at pH 7.4	Onset of Anesthesia (min)
Lidocaine	7.9	24	2–4
Bupivacaine	8.1	16.6	5–8
Tetracaine	8.42	8.7	5–10
Procaine	9.1	2	14–18

Table 6.8 Effect of lipophilicity on PK parameters

Increases ↑	Increases ↑	Decreases ↓	Increases ↑
logP	Concentration of unionized species	Aqueous solubility	Absorption through membrane

Table 6.9 Effect of lipophilicity on binding and metabolism

Increases				
↑	↑	↑	↑	↑
logP	Binding to target protein	Binding to CYT P450 metabolizing enzyme	Non-specific binding to blood/tissue protein	Binding to hERG channel

Thus, optimization of logP of test compounds during lead optimization is an integral part of drug discovery programme. Although one can envision a direct link between solubility and lipophilicity, role of ionization of drug and its solubility cannot be ignored. The solubility of an ionizable compound increases with increase in difference between pH of environment and pK_a of drug. Gleeson et al.[11] has demonstrated that for >44000 GSK compounds, solubility increased with logP <3. Michael J. Waring[12] based on physicochemical data for clinically used drugs in the literature proposed optimal logP/D (lipophilicity values) values for solubility, permeability, clearance and metabolism, bioavailability, and toxicity (comprising ADMET properties) that in turn might enhance the chances of identifying drugs during lead optimization studies or while applying drug design strategies.

6.5.7.1 Solubility of drugs

1. Compounds with logP >3 have solubility of only 1% (500 μM for drug with molecular weight 500 Da).
2. Compounds with logP <3 have solubility of 50%.

Permeability of Drugs across Intestinal Membranes

To achieve optimal permeability, lipophilicity varies with molecular weights of drugs:

MW 350–400	logP >1.7
MW 400–450	logP >3.1
MW 450–500	logP >3.4
MW >500	logP >4.5

Clearance and Metabolism

1. For metabolic stability against cytochromes, a logD values of <3 is recommended.
2. For renal clearance, the metabolite or unchanged drug should be hydrophilic so that they are easily excreted by urine (aqueous).
3. 50% of compounds with reduced logD value of <0 exhibit net renal clearance.
4. 50% of compounds with logD value >1.2 exhibited net renal reabsorption.

6.6 Hydrogen Bond Acceptor (HBA)/Hydrogen Bond Donor (HBD)

Presence of functional groups in the structural skeleton of test compounds that can form H-bonds within the molecule or with protein targets is a key parameter in drug design. On one hand the presence of

intramolecular H-bonds in test compounds impacts solubility, permeability and efficacy of drug candidates. On the other hand, formation of intermolecular H-bonds between the drug and the target enhances efficacy and potency of drug candidates.

Hydrogen bonds are the strongest of all intermolecular forces among reversible interactions. The formation of H-bonds results either from intermolecular attraction between slightly positive hydrogen on one molecule (test compound/amino acid) and a slightly negative atom (nucleophile/electronegative atom) on another molecule or via intramolecular attraction within the test compound. In living organism, presence of multiple H-bonds contributes to the stability, structure and function of numerous macromolecules involving proteins and DNA. They also play a critical role in the assembly of the three-dimensional structure of numerous intravital folded proteins including enzymes, receptors and antibodies. Since the formation of H-bond occurs between two atoms, they are referred as: hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD). The hydrogen bond donor is the electronegative atom (e.g., N, O, F) of a functional group to which hydrogen(s) is covalently bonded and can readily donate the hydrogen(s) for H-bond formation. For example, hydrogen attached to strongly electronegative atom such N, O or F acts as a HBD. The hydrogen bond acceptor is the electronegative ion or functional group in vicinity that carries a lone electron pair in order to form an H-bond. In fact, partial positive charge on hydrogen attracts lone pair of electrons located on the neighboring atom to form an H-bond. Examples of electronegative atoms as H-bond acceptors are F, N or O. Although H-bonds involve HBD and HBA functionalities, they can be either part of the same molecule in proximity leading to intramolecular H-bond or part of two molecules resulting in an intermolecular H-bonding (For details please refer to Chapter 10).

In medicinal chemistry, formation and disruption of intramolecular H-bonds have been used as one of the design parameters to enhance biological activity of test compounds. The presence of intramolecular H-bonds may have following favorable effect and assist in the success of drug design:

1. Stabilize and enhance the population of the bioactive conformation in test compounds.
2. Improves lipophilicity and other physicochemical properties of drug candidates.
3. Provide protection to polar groups in test compounds thereby facilitating passage of test compounds through environment with low dielectric constant (membrane permeability).

6.7 Surface Activity

The striking property of the stomach wall is its hydrophobic texture. Interestingly, this hydrophobicity (as quantified by the contact angle made by the droplet on the surface) can be eliminated by the addition of 'barrier breakers' that is bile, salts, ethanol, and aspirin.

Water does not form droplets on some surfaces due to surface tension that involves tendency of water molecules to attract one another (called *cohesion*). When cohesion factor crosses threshold value, the water forms spherical droplets or may even form sheets of water. However, the surface tension can be reduced by adding surface-active agents or surfactants. In other words, surfactant is a wetting agent when it comes in contact with a hydrophobic surface. It works via deposition (absorption) of a surface-active agent on greasy (mucus) surfaces, adhering with its hydrophobic part. The hydrophilic part of the surface-active agent remains outside and allows wetting of the mucus surface with water.

Biophysical characteristics of mucus relate to its hydrophobic character and serve as a protective layer or barrier. Thus, a decrease in mucosal hydrophobicity in the presence of surfactant even in traces can enhance the rate of absorption through gut walls.

There are four different types of surface-active agents:

1. Anionic compounds, for example salt of bile acids, salts of sulfate or phosphate esters of alcohols, and salts of sulfonic acids.
2. Cationic compounds, for example high-molecular-weight aliphatic amines and quaternary ammonium derivatives.
3. Non-ionic compounds, for example polyoxyethylene ethers and glycol esters of fatty acids.
4. Amphoteric surfactants

6.7.1 Applications

Antibacterials are frequently used as quaternary ammonium salts. This is because the ammonium cations interact with the anionic regions located in the outer layer of bacterial cell walls, thereby disrupting the cell membranes. Thus, these drugs deposit (absorb) on greasy (mucus) surfaces, adhering with its hydrophobic part. The hydrophilic part of the surface-active agent remains outside and allows wetting of the mucus surface with water.

6.8 Number of Rotatable Bonds

A rotatable bond is referred to as a *single bond* (non-ring bond), covalently linked to a non-terminal and non-hydrogen atom (bound to a non-terminal heavy atom excluded). The number of rotatable bonds (ROTBs) is the number of single bonds which allow free rotation around themselves. C–N bond in $CONH_2$ is excluded because of restricted rotation. The number of ROTBs can also be correlated with molecular flexibility (Fig. 6.13). Veber *et al.*[3] demonstrated a direct relationship between oral prediction and number of ROTBs/polar surface area/total number of hydrogen bonds in test compounds. Test compounds with equal to or more than 10 ROTBs may have poor bioavailability. For example, atenolol with ROTB of 8 has bioavailability of 50%, whereas propranol with ROTB of 6 has bioavailability of 90%.

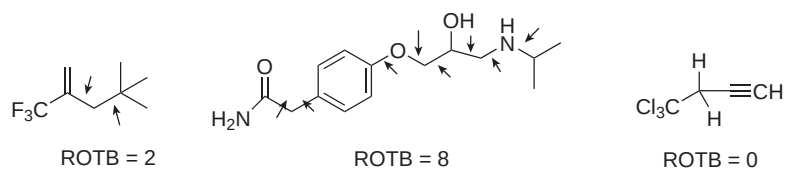


Figure 6.13 Calculation of number of ROTBs.

6.9 Number of Aromatic Rings

The ubiquitous existence of aromatic ring in endogenous ligands such as hydrophilic catecholamines (chemical messengers/neurotransmitters) strengthens significance of aromatic ring in drug discovery. A careful analysis of these catecholamines reveals presence of aromatic ring to enhance lipophilicity and amino group to introduce hydrophilicity in the molecules. Thus, catecholamines (discussed in Chapter 7) are classical design by nature to balance lipophilicity, pK_a, and solubility in their endogenous substances. Medicinal

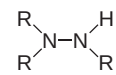
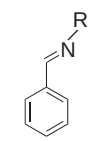
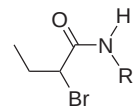
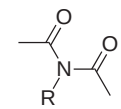
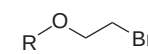
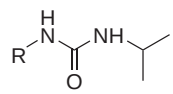
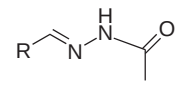
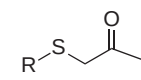
chemists often introduce aromatic rings in the library of test compounds before embarking with screening of compounds during drug discovery programme. This gets support from the fact that more than 74% clinical drugs atleast have one or more aromatic rings and only less than 5% drugs have no aromatic rings in their skeletons.[13] However, the presence of higher Number of aromatic rings (AROM) in a molecular framework can increase hydrophobicity and may be accompanied by decrease in solubility that could act as negative factor for drug development. Thus, increase in aromatic ring count may affect many physicochemical and physiological parameters such as solubility, lipophilicity, serum protein binding, cytochrome P450 inhibition, and hERG binding.

Timothy J. Ritchie et al.[14] studied effect of AROM in 280 in-house drugs in pipeline that were under different development stages of clinical trials. They found that on an average, presence of 2–3 aromatic rings in a given molecular architecture may be a good indicator for further developability of the test compound. However, earlier studies pegged this number for aromatic rings to 1.6.[15]

6.10 Undesirable Functional Groups (ALERT)

While applying physicochemical parameters to separate compounds with drug-like properties in a chemical space comprising millions of compounds, it is advisable to remove compounds with harmful/toxic functional groups.[16] Some of the groups not recommended in drugs have been summarized in Table 6.10.

Table 6.10 List of undesirable functional groups

6.11 Steric Factor

Although PK/PD plays an important role in the movement of drug through the physiological barriers so as to access the target, ultimately the tight binding with target protein is all that is needed for therapeutic response. Given that protein target is made up of chiral amino acids, it poses steric (3D spatial) hindrance[17] for the incoming drug candidate and regulates the strength of drug-protein binding. The resulting steric hindrance due to chiral amino acids has been quantified using Taft equation.[18] The equation was developed using hydrolysis of ester as a model reaction under the influence of steric factors introduced by substituents (Fig. 6.14).



Figure 6.14 A model reaction used by Taft.

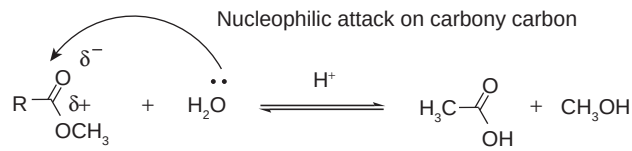


Figure 6.15 Increase in size of R will decrease the rate of reaction.

Table 6.11 Values reflecting resistance

H	1.24 Positive value is reflection of weak steric resistance on hydrolysis
Me	0.00 Internal standard in the Taft equation
t-Bu	−2.78 Negative value is reflection of a huge steric effect on hydrolysis

In this reaction, the size of R has been varied to study its effect on the rate of hydrolysis.[19] Increasing the size of R will create steric hindrance for the nucleophilic attack by water (Fig. 6.15).

The steric effect can be quantified (Table 6.11) using Taft parameter E_S.

$$E_s = \log k_{RCOOCH_3} - \log k_{CH_3COOCH_3}$$

$$= \log \frac{k_{RCOOCH_3}}{k_{CH_3COOCH_3}}$$

Where k is the rate constant for ester hydrolysis.

6.12 Molecular Size

Molecular size is probably one of the most crucial structural parameters affecting therapeutic response. There are various factors that affect molecular size.

6.12.1 Molecular Weight

Molecular weight bears a direct relation with the size of the molecule. Molecular size is an important descriptor as most physicochemical properties and many biological properties are strongly size related. Increase in size interferes with the dissolution of compounds in both the physiological environments: aqueous and lipid. This is in accordance with Lipinski's rule restricting use of compounds with <500 molecular weight as molecular weight >500 can have issues with PK parameters.

6.12.2 Electron Density

By looking at the structure of a molecule on a paper, it is difficult to assume the 3D space the molecule can occupy. This is a very important physicochemical characteristic of test compound because filling up 3D space can not only provide volume, size, shape, and orientation of pharmacophores/functional groups

but may also provide valuable information about steric hindrance faced during target binding. One of the methods used for this purpose is space-filling models, which use spheres representing the size of the atom in the 3D space. Thus, a 3D structure built using space-filling models can mark the boundaries of the space occupied by the molecule and also establish interatomic distances, orientations, and possible flexibility in the molecule. However, now computational tools are available that can be used to draw the information about the electron density in any molecule.

6.12.3 Polar Surface Activity

Continuing with physicochemical properties for drug-like properties, Polar surface activity (PSA)[20] deals with determining abilities of compounds for their diffusion properties, particularly during gastrointestinal absorption (bioavailability) and BBB permeation. Although Lipinski's rule dealing with logP and logD are popular techniques for the purpose of predicting absorption, an additional parameter PSA deals directly with absorption/bioavailability through transport. It is defined as the sum of polar atoms exposed onto the surface (primarily oxygen and nitrogen, also including their attached hydrogens) within the molecule. It has been found to correlate well with the *in vitro* experimental transport assays for determining passive molecular transport (permeability) such as Caco-2 monolayer, BBB transport, etc. The conventional methodologies to calculate PSA are relatively time consuming, as it requires generation of 3D molecular geometry followed by calculation of the PSA (Fig. 6.16). The data generated using this method is referred to as 3D PSA and is expressed as Å^2.

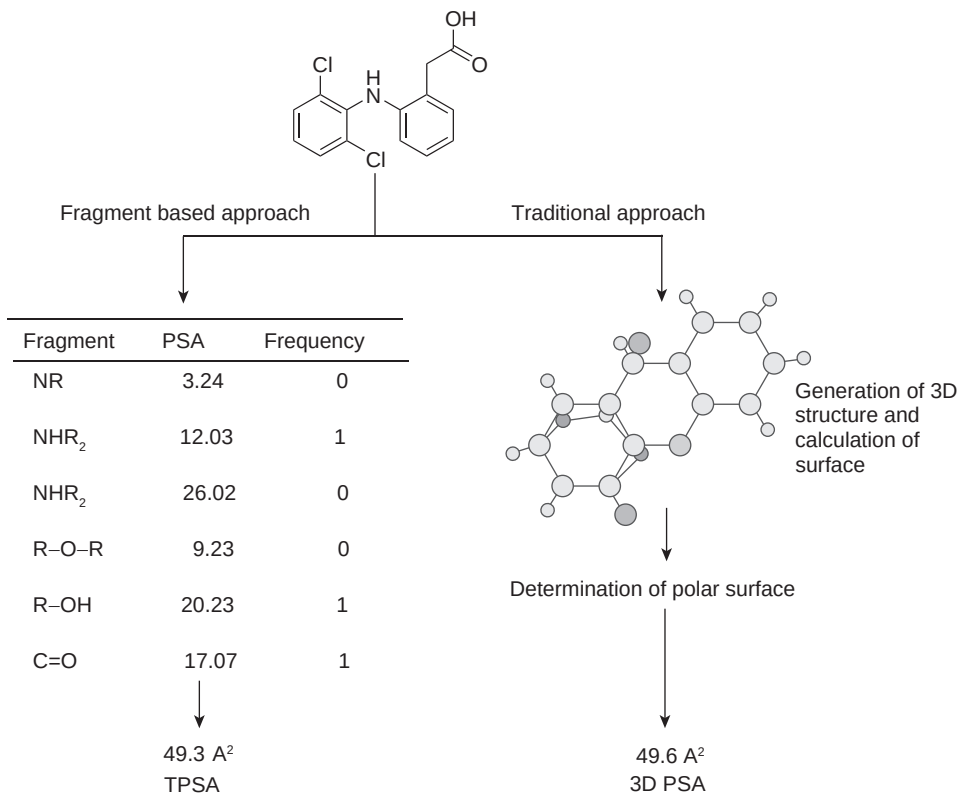


Figure 6.16 Two approaches for generating PSA of compounds.
(*Reprinted with permission*[20])

However, a fast methodology to calculate the PSA involves summation of PSA contributions from each polar group available on the surface. This is designated as topological polar surface area (TPSA) and can be calculated for large number of molecules using computational tools. Thus, PSA can be used as rider for filtering a large database of chemical compounds separating compounds with good (virtual) bioavailability, before libraries are submitted for *in vitro* screening (lead identification). Veber et al.[3] evaluated bioavailability of 1100 compounds in rat and came up with drug-likeness values by taking some of the physicochemical properties into account. They assigned ROTB > 10, PSA < 140, or HBA/HBD < 12 as good bioavailability parameters for test compounds albeit in rats. The authors argued that if any of the two parameters are within limit, the compound is likely to exhibit acceptable bioavailability. Similarly, Chico et al.[21] have proposed values for some of the physicochemical parameters for CNS candidates such as MW < 400, logP < 4, and PSA < 80 Å^2 that can have good penetration through BBB.

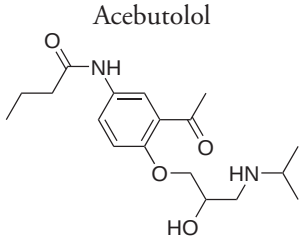
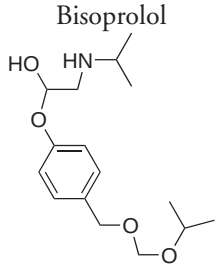
These findings get support from the studies carried out by Kelder et al.[22] who calculated PSA for more than 2000 drugs undergoing phase-II clinical trials by *p.o.* route. They concluded that for oral non-CNS drugs transported passively, the PSA should not be > 120 Å^2. For oral CNS drugs PSA with < 60 – 70 Å^2 favored BBB penetration, thereby suggesting that a relatively smaller PSA value is desirable for drugs to permeate BBB.

CASE STUDY 5

Physicochemical Parameters and Bioavailability of α1-Receptor Antagonists

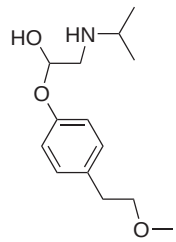
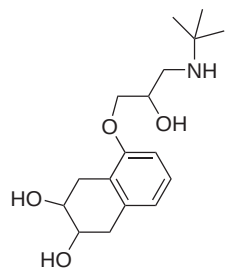
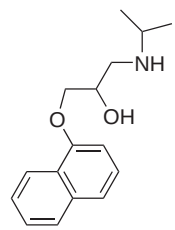
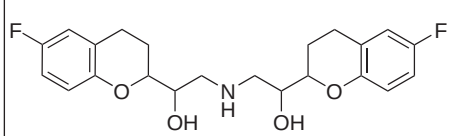
Activation of β1-receptors by endogenous ligand epinephrine (catecholamines) is known to increase the heart rate and the blood pressure. The β1-receptor antagonists are used clinically to block activation of β1-receptors, thereby lowering the heart rate and blood pressure. In an attempt to correlate bioavailability and physicochemical properties such as clog P, PSA, AROM, RTOB, serum protein binding, and aqueous solubility of β1-receptor antagonists, we compared the values and analyzed the data as depicted in Table 6.12.

Table 6.12 Physicochemical properties of clinically used β1-receptor antagonist drugs

Drug	AROM/ clogP	ROTB/ TPSA	Bioavailability	Protein Binding	Solubility
Acebutolol 	1 1.43	9 50.72	Approximately 40%	26%	0.172 mg/mL
Bisoprolol 	1 2.3	12 59.95	Appoximately 80%	30%	0.0707 mg/mL

(*Continued*)

Table 6.12 (*Continued*)

Drug	AROM/ clogP	ROTB/ TPSA	Bioavailability	Protein Binding	Solubility
Metoprolol 	1 1.8	9 50.72	Approximately 50%	12%	0.402 mg/mL
Nadolol 	1 1.23	6 81.95	Approximately 30%	30%	2.25 mg/mL
Propranolol 	2 3.03	6 41.49	Approximately 25% (10 mg tablet)	90%	0.0794 mg/mL
Nebivolol 	2 2.44	6 70.95	Approximately 12–96%	98%	0.0403 mg/mL

As is evident, we find that although all drugs are used clinically, there remains tremendous variation among physicochemical parameters: ROTB varied from 6 to 12, and TPSA varied from 40 to 80 Å^2. However, two parameters c logP (1.2–3.0) and AROM (1–2) are within permissible limits justifying drug-likeness.

CASE STUDY 6

Physicochemical Parameters and Bioavailability of Sartans as Angiotensin-Receptor Blockers

Among sartans, yet another class of antihypertensive drugs, in general compounds with ROTB values of < 10 exhibited good bioavailability whereas compounds with ROTB values > 10 exhibited relatively poor bioavailability (Table 6.13). However, along with ROTB in this series, an additional property—TPSA also exhibited drug-likeness with values ranging between 70 to 140 Å^2 that were within permissible limits (< 120 Å^2).

Table 6.13 Physicochemical properties of clinically used antihypertensive drugs sartans (angiotensin-receptor blockers)

Drug	AROM/ c logP	ROTB/ TPSA	Bioavailability	Protein Binding	Solubility
Azilsartan	3 4.24	10 139.57	60%	99%	0.00703 mg/mL
Candesartan	3 7.08	13 143.34	15%	99	0.00771 mg/mL
Eprosartan	3 4.80	13 92.42	13%	98%	0.00866 mg/mL
Irbesartan	3 6.04	8 87.13	60–80%	90%	0.00884 mg/mL
Losartan	4 3.85	11 92.51	33%	99.7%	0.0047 mg/mL
Olmesartan	4 2.66	11 154.34	26%	99%	0.0105 mg/mL
Telmisartan	6 7.29	7 72.94	67	99.5%	0.0035 mg/mL
Valsartan	3 4.86	10 112.07	23%	94–97%	0.0234 mg/mL

Thus, for both classes of drugs (β 1-blockers and sartans), atleast two physicochemical parameters are within drug-likeness limits, which is in accordance with hypothesis by Veber et al.[3], that if any of the two physicochemical parameters are within limits, the compound is likely to exhibit acceptable bioavailability.

6.13 Physicochemical and ADMET Properties of a Successful CNS Drug[13]

1. Potent activity: low to subnanomolar
2. Highly selective
3. Molecular weight < 450
4. Minimal hydrophobicity (clogP < 5)
5. Number of H-bond donor < 3

6. Number of H-bond acceptor < 7
7. ROTBs < 8
8. H-bonds < 8
9. Aqueous solubility > 60 μg/mL.
10. pK_a, neutral or basic with pK_a 7.5–10.5 (avoid acids)
11. Polar surface area < 60 – 70 Å^2
12. > 30-Fold margin between hERG IC50 and effective unbound plasma concentration
13. Metabolic stability with > 80% remaining after 1h
14. P450 enzyme CYP inhibition < 50% at 30 μM
15. No significant CYP2D6 metabolism
16. Not a potent CYP3A4 inducer
17. Effective permeability > 1×10^{-6} cm/sec
18. Not an efficient P-glycoprotein substrate (*in vivo*).
19. Not a high-affinity serum albumin ligand (K_D < 10 μM).
20. Poor hERG inhibition

6.14 Physicochemical and ADMET Parameters Required for a Preclinical Candidate Drug[23]

Activity	Low to subnanomolar
Selectivity	Potency, affinity, therapeutic and adverse effects
Serum stability	To measure the degradation of your compounds
Molecular weight	≤ 450
Hydrophobicity	(clogP < 5)
Number of H-bond donor	< 3
Number of H-bond acceptor	< 7
ROTBs	< 8
H-bonds	< 8
Aqueous solubility	> 60 μg/mL
pK_a, neutral or basic with	pK_a 7.5 – 10.5 (avoid acids)
Polar surface area	< 60 – 70 Å^2
Margin between hERG IC50 and effective unbound plasma concentration	> 30-Fold
Metabolic stability	with > 80% remaining after 1h.
P450 enzyme CYP inhibition	< 50% at 30 μM
No significant CYP2D6 metabolism	A major metabolic pathway for many drugs and a source of interindividual variability in patient response

(*Continued*)

(*Continued*)

Not a potent CYP3A4 inducer	Predicts clinical drug-drug interaction
Effective permeability	$>1 \times 10^{-6}$ cm/sec
Not an efficient substrate for P-glycoprotein (*in vivo*)	Efflux of drugs by P-glycoprotein (P-gp) in the intestine limits bioavailability
Not a high-affinity serum albumin ligand ($K_D < 10\ \mu M$)	High-affinity binding may provide a reservoir for a long duration of action and ultimately seriously impact PK properties (ADME) of therapeutic drugs
Poor hERG inhibition	To prevent cardiotoxicity

Exercises

1. Discuss role of Lipinski rule drug design.

2. Discuss role of lipophilicity and pK_a in drug design.

3. Discuss chemical libraries with drug-like properties in drug discovery.

4. Explain role of physicochemical properties in drug design.

5. Discuss role of pK_a in drug absorption.

6. Discuss role of bioisosterism in drug design

7. Name advanced in vitro assays for pharmacokinetic properties

8. Role of optical isomerism in drug design

9. Write short notes on:

(a) Ionization constant/pK_a

(b) LogP and D

(c) Role of PSA in drug design

(d) Lipophilic efficiency

(e) Number of rotatable bonds in drug design

(f) HBD/HBA

(g) Solubility of drugs

Competitive Exam Questions

1. According to Lipinski's rule of 5, which of the following properties of drug molecules are likely to cause poor oral absorption?

(a) A molecular weight lesser than 500

(b) less than 5 hydrogen bond donors

(c) A logP of less than 5

(d) More than 10 H-bond acceptors

[***GPAT 2013***]

2. What will be the fraction of lidocaine in the ionized form, if its pK_a is 7.9 and pH of the infected tissue is 8.9?

(a) 99% **(b)** 90%

(c) 9% **(d)** 1% [***GPAT 2017***]

3. Indicate which of the following general statements are true?

(a) A weakly basic drug is fully ionized when pH of the solution is at least 2 pH units greater than its pK_a.

(b) A weakly acidic drug is unionized when pH of solution is at least 2 pH units below its pK_a.

(c) The higher the pK_a of a weak acid, the stronger is the acid

(d) Acidic drugs are less soluble in alkaline solution. [***GPAT 2017***]

References

1. Fei Mao, Wei Ni, Xiang Xu, Hui Wang, Jing Wang, Min Ji and Jian Li, *Molecules*, **2016**, *21*, 75.
2. Lipinski C.A., Lombardo F., Dominy B.W., Feeney P.J., *Adv. Drug Deliv. Rev.*, **2001**, *46*, 3.
3. Daniel F. Veber, Stephen R. Johnson, Hung-Yuan Cheng, Brian R. Smith, Keith W. Ward, Kenneth D. Kopple, *J. Med. Chem.*, **2002**, *45*, 12, 2615.
4. http://www.ddw-online.com/drug-discovery/p217618-rules-for-drug discovery:-can-simple-property-criteria-help-you-to-find-a-drugspring-14.html.
5. John P. Overington, Bissan Al-Lazikani and Andrew L. Hopkins, *Nature Reviews Drug Discovery*, **2006**, *5*, 993.
6. Harren Jhoti, Glyn Williams, David C. Rees and Christopher W. Murray, *Nature Reviews Drug Discovery*, **2013**, *12*, 644.
7. Manallack, D. T., Prankerd, R. J., Yuriev, E., Oprea, T. I., and Chalmers, D. K., *Chem. Soc. Rev.*, **2013**, *42*, 485.
8. David T. Manallack, Richard J. Prankerd, Elizabeth Yuriev, Tudor I. Oprea and David K. Chalmers, *Chem. Soc. Rev.*, **2013**, *42*, 485.
9. David T. Manallack, *Perspect. Med. Chem.*, **2007**, *1*, 25.
10. Kevin D. Freeman-Cook, Robert L. Hoffman, Ted W. Johnson, *Future Med. Chem.*, **2013**, *5*, 113.
11. Gleeson M.P., *J. Med. Chem.*, **2008**, *51*, 817.
12. Michael J Warning, *Expert Opin. Drug Discov.*, **2010**, 235.
13. Aldeghi, M., Malhotra, S., Selwood, D. L., and Chan, A. W., *Chemical Biology and Drug Design*, **2014**, *83*, 450.
14. Timothy J. Ritchie and Simon J.F. Macdonald, *Drug Discovery Today*, **2009**, *14*, 1011.
15. Hann, M.M. et al., *J. Chem. Inf. Comput. Sci.*, **2001**, *41*, 856.
16. Robert F. Bruns and Ian A. Watson, *J. Med. Chem.*, **2012**, *55*, 9763.
17. Sakloth F., Kolanos R., Mosier P. D., Bonano J. S., Banks M. L., Partilla J. S., Baumann M. H., Negus S., and Glennon R. A., *Br. J. Pharmacol.*, **2015**, *172*, 2210.
18. Taft, R. W., *J. Am. Chem. Soc.*, **1953**, *75*, 4538.
19. McClelland, R. A. and Steenken, S. *J. Am. Chem. Soc.*, **1988**, *110*, 5860.
20. Caron G., Ermondi G., Gariboldi M.B., Monti E., Gabano E., Ravera M., Osella D., *Chem, Med, Chem.*, **2009**, *4*, 1677.
21. Chico L.K., Van Eldik L.J., Watterson D.M., *Nat. Rev. Drug Discov.*, **2009**, *8*, 892.
22. Kelder J., Grootenhuis P.D., Bayada D.M., Delbressine L.P., Ploemen J.P., *Pharm. Res.*, **1999**, *16*, 1514.
23. Hassan Pajouhesh and George R. Lenz, *J. Amer. Soc. for Exp. Neuro Therapeutics*, **2005**, *2*, 541.

CHAPTER

7

Receptorology

KEY THINGS TO LEARN FROM THIS CHAPTER

- Receptors as drug target
- Ligands: Exogenous and endogenous ligands
  - Ligands that enter a cell or do not enter a cell
  - Ligand induced signal transduction
- Distribution of receptors on the cell
  - Cell-surface receptors
    - G-protein coupled receptor (GPCR): Mechanism of action downstream signaling and termination of signal transduction
    - An enzyme-linked receptor: Receptor tyrosine kinase, Guanylate cyclase receptor
    - Ionotropic receptors: Ligand gated, voltage gated, second messenger gated, a cyclic nucleotide gated, mechanically gated receptors.
  - Intracellular receptors

7.1 Introduction

Most of the clinically used drugs (specific class of drugs), following their administration, elicit pharmacological response by binding to a therapeutic protein targets, a receptor or an enzyme. Accordingly, drugs can either act as a ligand or a substrate based on the mechanistic pathway for which they have been developed to treat pathophysiological conditions of a disease. As ligands, drugs act as first messengers, bind to receptors, and initiate their pharmacological effect through cell signaling and, in turn, modulate intracellular signal transduction. Substrates on other hand bind to extracellular or intracellular signaling enzymes furnishing products that regulate cellular/physiological functions.

The access of drugs (ligands) to the receptor and their binding (following their administration) is regulated by the physicochemical properties of drugs. Some important features that contribute to the physicochemical properties of drugs arise from their own cyclic/acyclic molecular skeleton, the presence or absence of certain functional groups, spatial relationships between groups (intramolecular distance) and shape of the

molecules, lipophilicity, solubility, pK_a, polar surface area (PSA), etc. The receptors through their orthosteric/allosteric binding site interact with drugs via specific topography of the binding site that is complementary to the chemical structure of drugs with areas of polarity/hydrophobicity for electrostatic and hydrophobic interactions.

7.2 Receptors as Drug Target

Receptors are macromolecular component generally derived from proteins that bind with the ligand (drug or chemical messenger) and elicit its pharmacologic response by regulating (agonistic or antagonistic) cell signaling and subsequent intracellular signal transductions (Fig. 7.1). In general, receptors following their binding with endogenous ligands undergo a conformational change and transduce an intracellular signal that initiates a variety of intracellular post-translational modifications of signaling enzymes responsible for cellular/physiological cell-specific functions. They are one of the most widely used targets in drug discovery and act like an electric switch that can be turned on or off. When turned on, receptors elicit signals via cascade of events or can be turned off to block any undesired biological response through no signaling.

Generally, there are two kinds of receptors: cell-surface receptors and intracellular receptors known as *nuclear receptors* (transcription factors) that participate in numerous fundamental physiological mechanisms (Fig. 7.2) via cell signaling. The cell-surface receptor is designed to detect intravital and extracellular signal chemical molecules (ligands/drugs) such as neurotransmitters, hormones, cytokines, etc. Since these chemicals are hydrophilic molecules, they are unable to cross the phospholipid bilayer membranes, and thus bind to the portion of the membrane-bound receptor protruding outside the cell membrane. Upon binding with ligand, the receptor generally undergoes significant change in its conformation to initiate a cascade of intracellular events involving a number of other mediators, including signaling molecule effectors, second messengers, signaling enzymes, effector proteins (transducers), etc.

On the contrary, nuclear receptors are activated by non-polar (hydrophobic) intravital ligands (steroids)/drugs as signal molecules, as they are readily able to diffuse across the plasma membrane (Fig. 7.2). In general, both the receptors are class of recognized therapeutic protein targets and remain primary targets for many drugs used clinically.

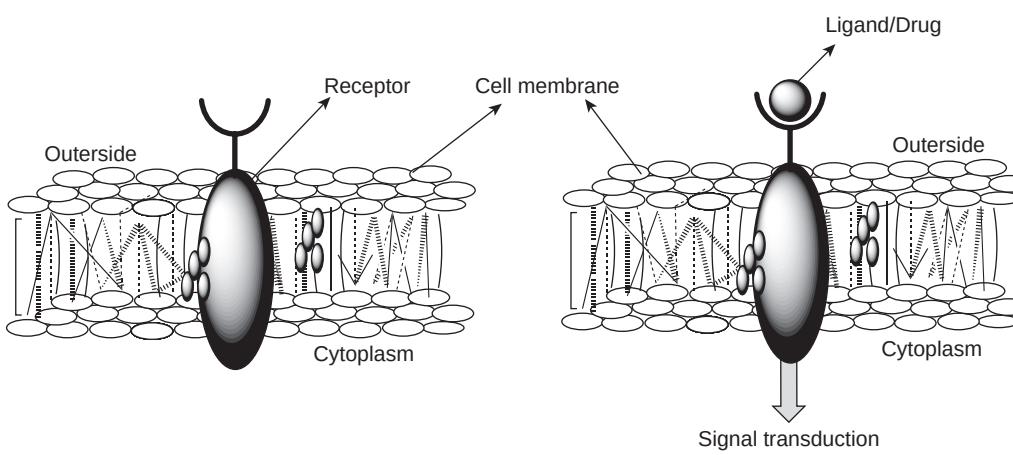


Figure 7.1 A diagrammatic view of signal transduction following binding of ligand/drug to a cell surface receptor.

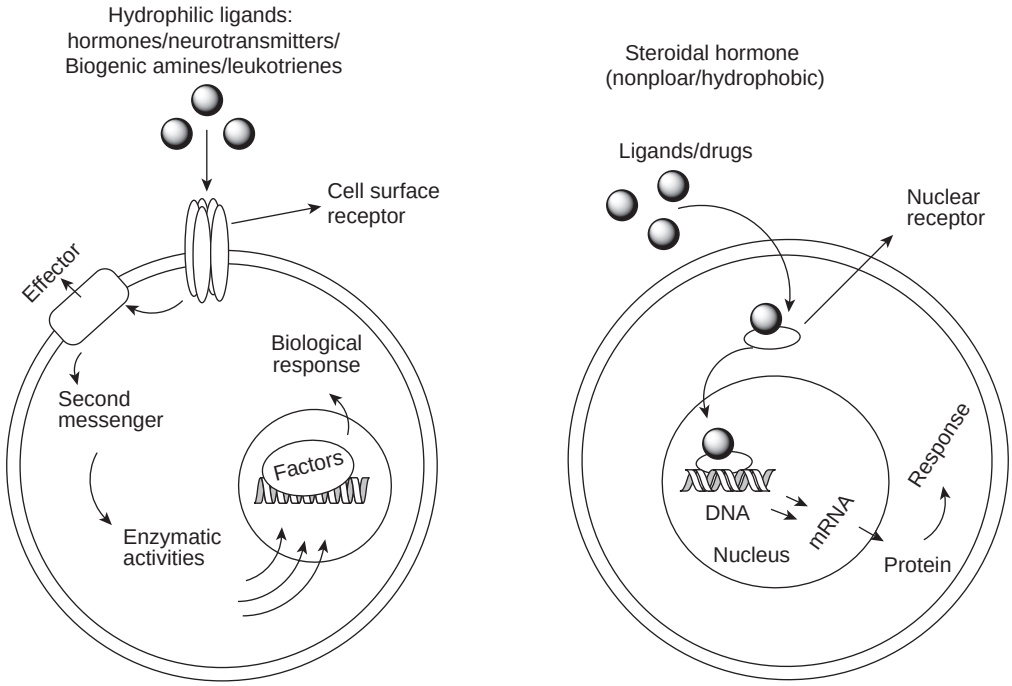


Figure 7.2 Cell-surface and intracellular receptors.

7.2.1 Comparison between Receptors and Enzymes

Difference between receptors and enzymes include:

1. The primary difference between receptors and enzymes lies in the manner they function. While enzymes catalyze a reaction and convert its substrate/drug to a product, receptors after binding with a ligand/drug produce signal.
2. An enzyme undergoes slight (minor) change in its conformation following its binding to its substrate, resulting in an even tighter fit. On the contrary, ligand binds to a receptor and induces changes in protein conformation to initiate a series of events.
3. A ligand is often a soluble molecule such as a hormone or neurotransmitter that binds to a receptor. A substrate is a molecule that in the presence of enzyme undergoes transformation into product (Fig. 7.3).

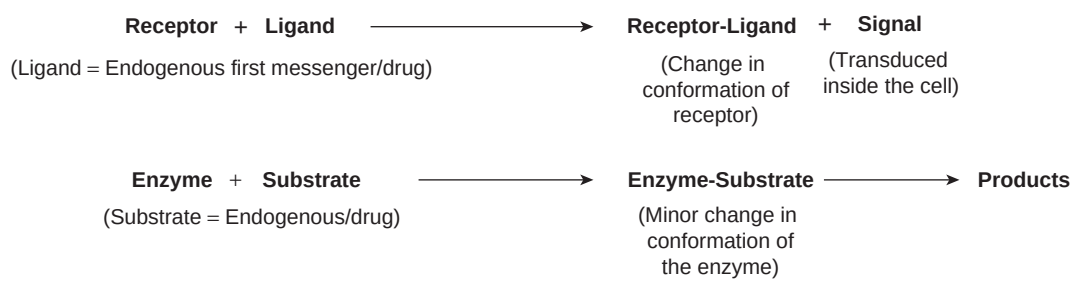


Figure 7.3 Difference between enzymes and receptors (protein targets).

Similarities between enzymes and receptors include:

Both enzymes and receptors are made up of proteins and depict the same primary, secondary, tertiary, and quaternary structure. Thus, the similarities between enzymes and receptors allow both systems to be modeled computationally by applying the same mathematical equations.

7.2.2 Intravital Ligands (Endogenous)

Endogenous ligands that are produced by signaling cells interact with receptors and have structurally diverse chemical structures. They range from proteins (hydrophilic) and catecholamines to hydrophobic molecules like steroids to even gases like nitric oxide. They can be classified into two groups depending on their hydrophobic/hydrophilic nature. The hydrophilic endogenous ligands comprise polypeptides/proteins/factors/amino acid derivatives, whereas lipophilic ligands comprise hydrophobic molecules such as estrogen, thyroid hormones, vitamin A and D, etc.

7.2.3 Ligands that Enter Cells

Endogenous ligands of hydrophobic origin can enter cell via passive diffusion or carrier protein-mediated transport through the lipid bilayer cell membranes. Following their entry in the cytoplasm, they bind with intracellular receptors either in the cytoplasm or nucleus. However, the ligand-receptor complex produces signal once inside the nucleus and, in turn, regulates gene expression, thereby controlling numerous physiological functions of the organism.

7.2.4 Ligands that do not Enter Cells

Endogenous ligands of hydrophilic origins cannot diffuse through lipid bilayer cell membranes due to their polar nature. Instead, these endogenous ligands bind with the extracellular domain of receptors located on the cell surface. Hormones and neurotransmitters comprise one of the largest sets of hydrophilic endogenous ligands. Hormones are molecules released in the blood stream in response to stimulus and are generally used as first messenger (signaling molecules). Examples include insulin, glucagon, LHRH, etc.

Neurotransmitters are chemical messengers used not only for direct signaling between neurons at a synapse but also act as hormones. A classic example of overlap is norepinephrine (noradrenalin) and epinephrine which are released into the bloodstream by the adrenal glands as hormones and by sympathetic nerve endings (neurons) in the brain as neurotransmitters. Release of norepinephrine (noradrenalin) and epinephrine from adrenal glands (adrenal medulla) occurs during adrenalin rush (fight and flight response) part of defense mechanism in response to stress signals. Physiological functions of norepinephrine and epinephrine involve regulation of practically every organ via membrane-bound (cell surface) adrenergic receptors (α and β). Other examples of neurotransmitters are as follows:

1. Small-molecule neurotransmitters: Dopamine (biogenic amines), serotonin, histamine, amino acids, acetylcholine (ACh), purines (ATP).
2. Neuropeptides: Oxytocin, somatostatin, substance P, cocaine and amphetamine-regulated transcript, opioid peptides.

Dopamine, yet another important neurotransmitter that acts as chemical messenger in many physiological functions is synthesized in brain, kidney, and locally in peripheral systems (except blood vessels). In brain, it is released by neurons that send signal to other ells via several distinct dopamine pathways. Outside brain, dopamine functions as a local messenger in physiological activities, for example in blood vessels it acts

as a vasodilator by blocking the release of norepinephrine. Other physiological roles of dopamine include increase of sodium excretion via urine in kidney, modulating insulin production in pancreas, reduction in gastrointestinal motility in gut, etc.

The neurotransmitters adrenaline, noradrenaline, dopamine, and L-DOPA are derived from L-Tyr during catecholamine biosynthesis. Although they are involved in diverse physiological functions, they bear a strong structural resemblance comprising a catechol moiety (arene ring with two hydroxyl groups) and an amine group in the side chain (Fig. 7.4).

Among other endogenous chemical messengers (ligands), ACh is a low-molecular-weight neurotransmitter (organic compound) of immense significance (Fig. 7.5). The physiological functions generally affected by ACh are referred to under cholinergic activities.

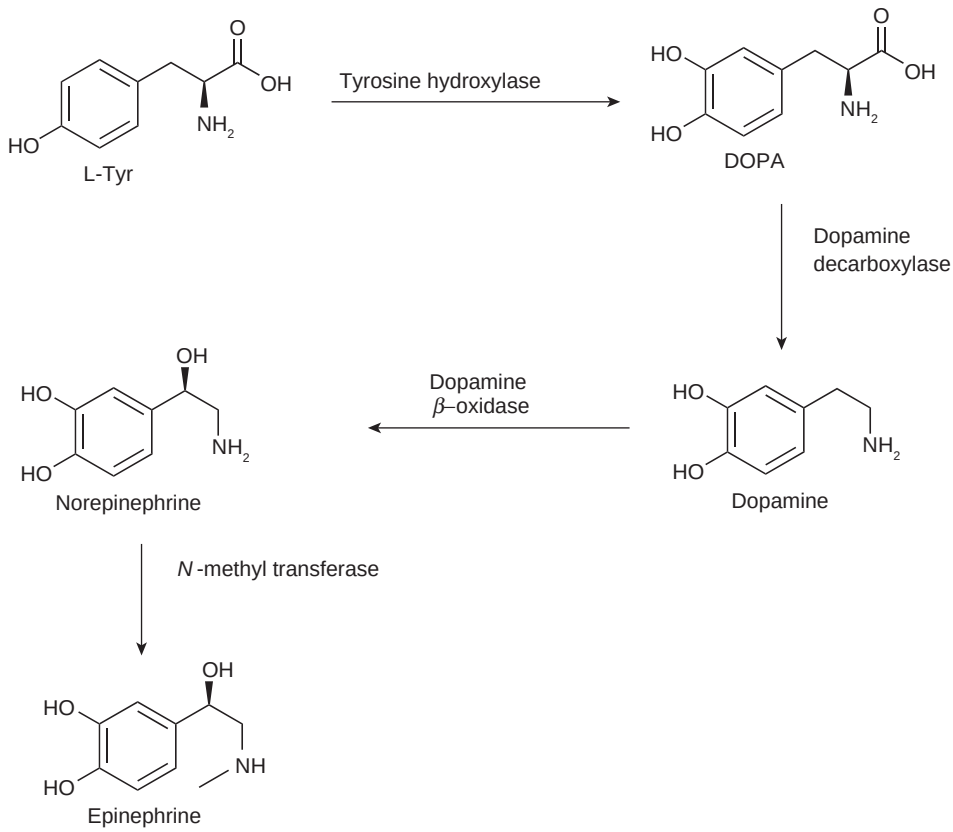


Figure 7.4 Biosynthesis of catecholamines.

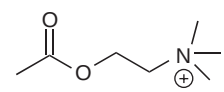


Figure 7.5 Structure of ACh; an important endogenous neurotransmitter in cholinergic functions.

Neuropeptides comprise endogenous opioid peptides (endorphins, enkephalins, dynorphins) that bind with μ-opioid receptors in the CNS (discretely distributed in the human brain) and play a role in the expression of analgesic effects by eliciting inhibitory signal for downstream pain modulation pathway.

7.2.5 Signal Transduction

This involves intracellular transduction of a signal that is generated in response to the binding of a first messenger (exogenous or endogenous cues) to the receptor. In other words, external molecular cues of environmental or endogenous origins are transmitted to the interior of cells for specific cellular response. The first stage of signal transduction commences with the reception of chemical signal (ligands) by protein receptors on cell surface. This triggers activation of the protein receptor, which is generally accompanied by a conformational change, a signal generating event. The second stage of signal transduction involves transmission of signal to the cytosolic part of the receptor that initiates the process of intracellular signal transduction through propagation and amplification inside the cells. Amplification of a signal involves initiating the ability of a cytosolic molecule to carry out catalytic enzymatic reactions multiple times and producing more than one product. In other words, a signal generated from a single ligand leads to specific biological response multiple times. Propagation of signal involves activation of a series of signal enzymes involved in the downstream signaling pathway, leading to the biological response. Thus, activation of receptor in response to ligand is followed by cytosolic initiation and propagation of signal through a chain of molecules called second messengers, effectors, enzymes, etc. The chains of molecules involved in signal transduction are as follows (Fig. 7.6):

1. First messenger (chemical messenger/ligand)
2. Second messenger
3. Effector
4. Effector proteins, etc.

The third stage of signal transduction involves biological response in the form of specific cellular functions. The chain of reaction during propagation finally enters nucleus and modulates the function of target genes (modulating protein synthesis). Other cell-surface receptors like ion channels may regulate cellular functions via modulating conductance of ions. A cell can carry several types of cell-surface receptors and respond to specific signal molecules.

The fourth stage involves termination of signal process, which occurs by two routes. One of the routes involves termination of a signal by the cytosolic Regulator of G-protein (RAG). The latter hydrolyses GTP from the activated Gα protein via GTPase activity. Alternatively, internalization of ligand-bound receptor inside the cell via endocytosis can also terminate a signal. The internalized receptors undergo endolysosomal degradation, and receptors are recycled back in its native form. Termination of signaling process in protein ion channels is caused by rearrangement of its conformation or by interfering with the generation of second messenger such as cAMP.

The understanding of signaling pathways involving communication among cells/tissues has become essential tool in the drug discovery programme for treating diseases.

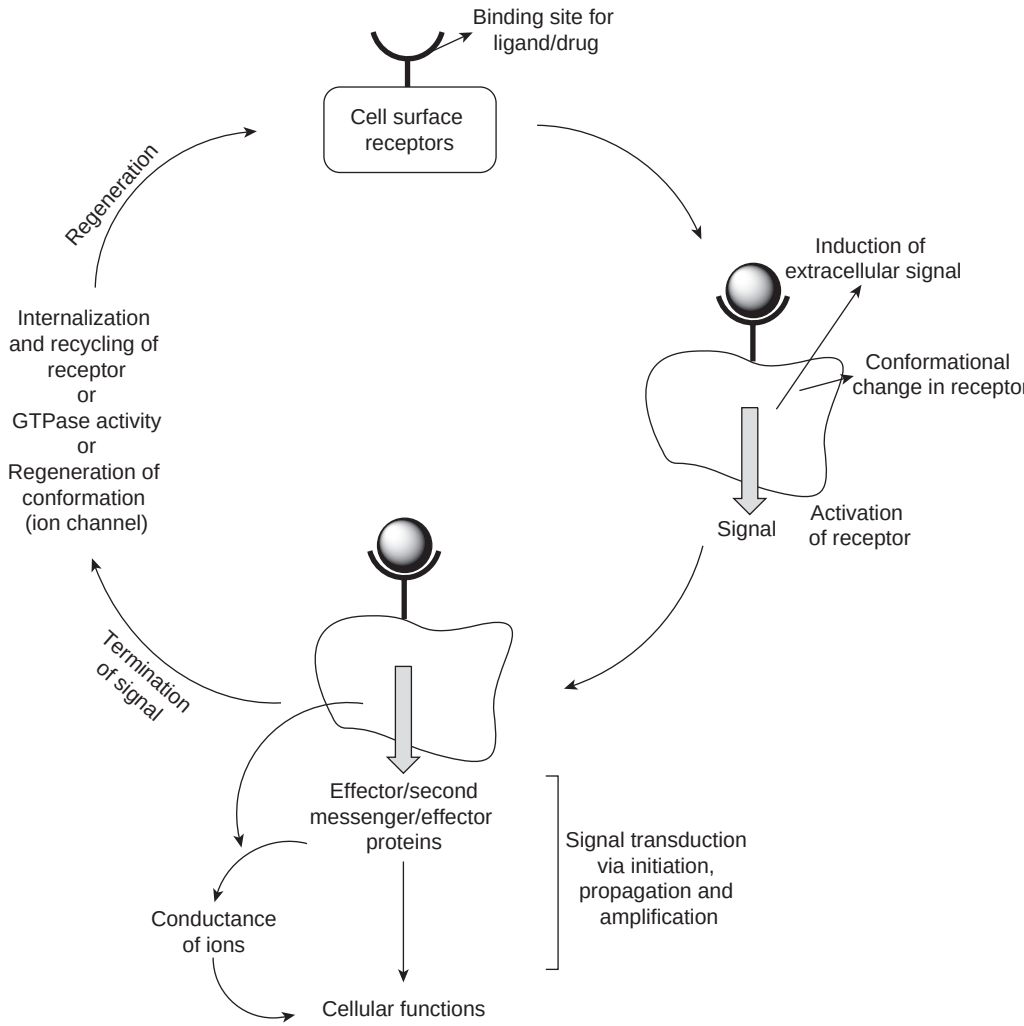


Figure 7.6 A typical signal transduction.

7.3 Distribution of Receptors in the Cell

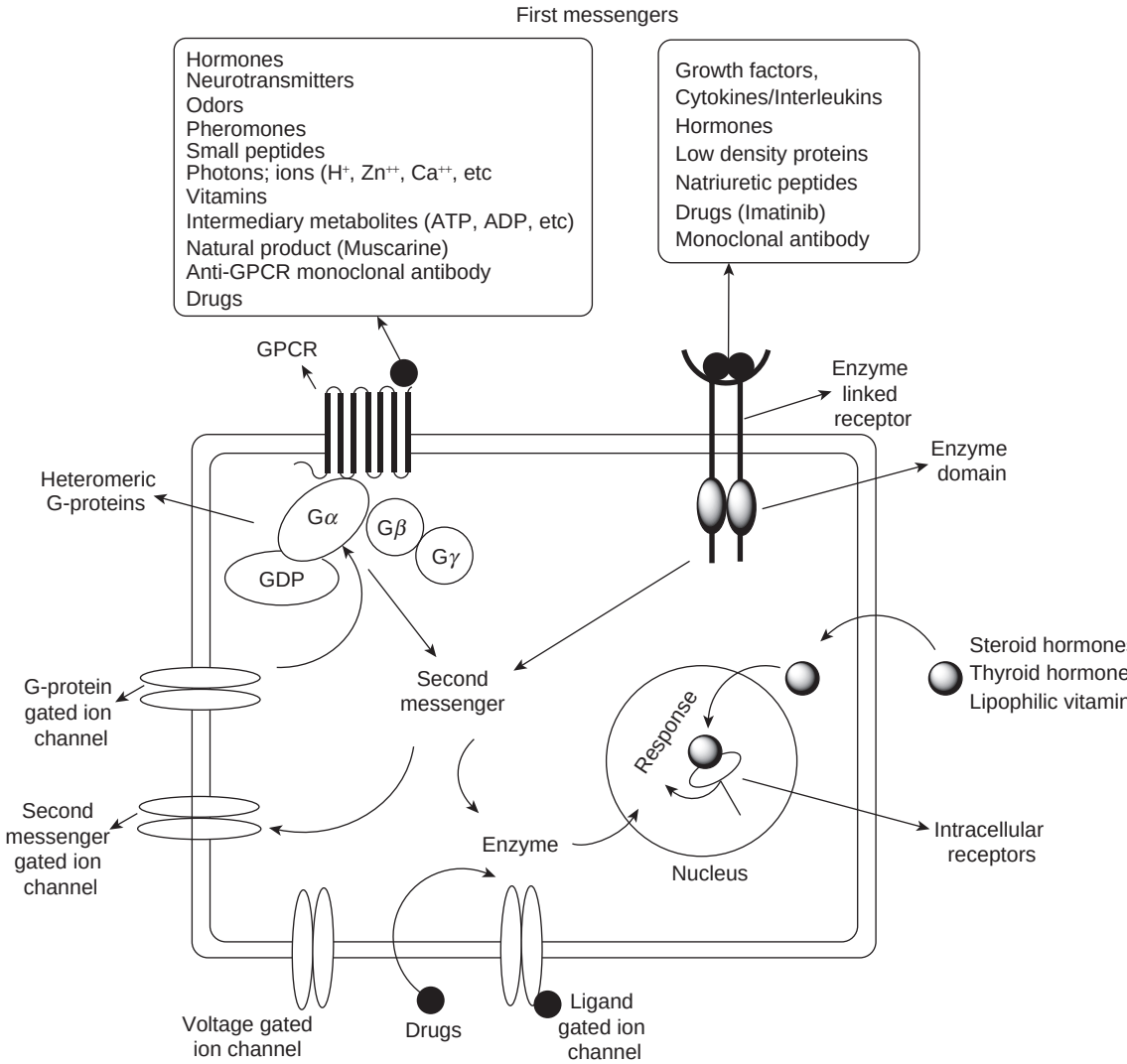
The cells in the body contain thousands of different receptors and can be divided in two categories:

1. Cell-surface receptors
2. Intracellular receptors

7.3.1 Cell-Surface Receptors

They are embedded in the plasma membranes (transmembrane) of cells, part of the region facing extracellular region of the cell and part of it remains hanging in the cytoplasm. The cell-surface receptors contain a binding site that is recognized by the endogenous chemical messengers (signaling molecules) or xenobiotics

including drugs. The binding of ligands with receptors generally follows induced-fit mechanism (lock-key is not prevalent) and involves non-covalent weak interactions. This results in the change of conformation of the protein receptor and triggers intracellular signal transduction or conductance of ions. (More than 60% of clinically used drugs bind with cell-surface receptors).



GPCRs:

1. Intracellular G-protein mediated activation
2. Major signal transduction pathways are cAMP pathway and IP3 pathway

Enzyme linked receptors:

1. Intracellular enzyme (linked to receptor) mediated activation
2. Major signal transduction pathways is cGMP pathway

Ionotropic receptors: Activates conductance of ions that regulates cellular response

Second messengers: cAMP, cGMP, Ca^{2+}, IP3, DAG, NO

Figure 7.7 Extracellular and intracellular receptors.

The main roles of cell-surface receptors include:

1. Endogenous hydrophilic ligands bind to the extracellular domain of cell surface.
2. Binding of ligands with receptors generally follows induced-fit mechanism and involves non-covalent weak interactions.
3. Binding of ligands triggers changes in conformation of receptor and results in a 'domino' effect-involving activation, initiation, propagation, amplification, and biological response.
4. Termination of signal involves GTPase activity or endolysosomal degradation of internalized ligand-bound receptors followed by recycling of native receptors.
5. Endogenous neurotransmitters and hormones are major source of ligands that bind with cell-surface receptors.
6. They remain one of the most preferred therapeutic targets for drug discovery.

Among numerous cell-surface receptors, the three most extensively used receptors as drug targets are as follows:

1. G-protein-coupled receptor systems
2. Enzyme-linked receptor or catalytic receptor
3. Ionotropic receptors

7.3.1.1 G-Protein-Coupled Receptors

G-protein-coupled receptors (GPCRs)[1] are proteins that span the plasma membrane seven times and are also known as seven-membrane domain receptor (Fig. 7.8). The N-terminal domain of the GPCR protrudes outside the cell surface, whereas C-terminal domain is present inside the cytosol. This characteristic orientation of extracellular N- and cytosolic C-terminal regions plays a role:

1. In the synthesis and insertion of GPCR in the plasma membrane following cytosolic endolysosomal processing.
2. Coupling of heterotrimeric G-proteins to the C-terminal side of the GPCR in the cytoplasm for triggering selective downstream signaling.
3. In regulating receptor signaling via N-terminus.[2]

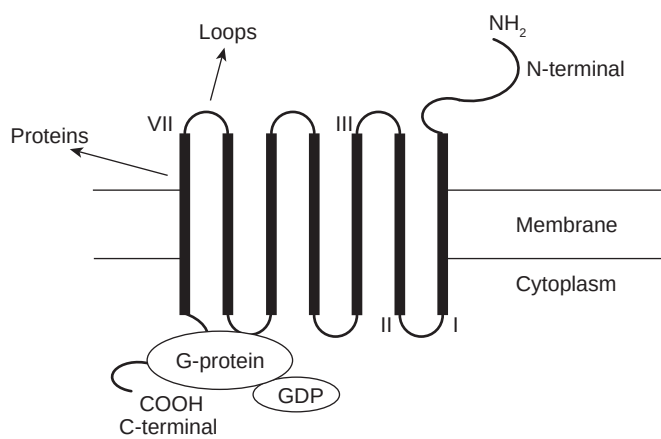


Figure 7.8 A typical GPCR.

Subsequent to the binding of a ligand with the receptor and changes brought about in its conformation, the extracellular domain of GPCR transduces signal through heterotrimeric G proteins present in the cytosolic domain (inner surface of plasma membrane) of the receptor. This intracellular signal transduction triggers a series of cascadic events and thus regulates numerous cell-specific physiological functions. The heterotrimeric G-proteins comprising α-subunits and β, γ dimers provide the key connection between numerous receptors and various effectors, such as adenylyl cyclase, calcium and potassium channels, and phospholipases. Although many receptors can interact with only one type of G-proteins, many receptors interacting with more than one G-proteins have also been found. Thus, a wide variety of G-protein-signaling pathways can occur owing to numerous possible combinations of α, β, and γ subunits.

Following activation of G-proteins, they bind with GTP and trigger production of second messengers such as cAMP, cGMP, NO, etc., in the cytoplasm (Fig. 7.9). The release and decrease of second messengers play a crucial role in many physiological functions, e.g., maintenance of memory in brain, water absorption in kidney, autoimmune and inflammatory disorders, etc. The GPCRs following binding with a therapeutic agent can stimulate or inhibit the production of the second messenger within the cell. In fact, GPCRs comprise the largest family of protein receptors[3] and have been identified as potential therapeutic target for a variety of diseases. A majority of drugs both as agonists and antagonists act via GPCRs. GPCRs comprise one of the largest protein families of therapeutic targets and have been implicated in numerous physiological functions. They recognize a wide range of ligands ranging from low-molecular-weight small molecules to proteins (Fig. 7.7), and intracellular signal transduction generally occurs by two principal pathways: cAMP and IP3/DAC metabolic pathways. Approximately, 34% drugs[4] used in clinics act on more than 100 GPCR therapeutic targets and are used as antihistamines, antihypertensives, antipsychotics, opioid analgesics, etc.

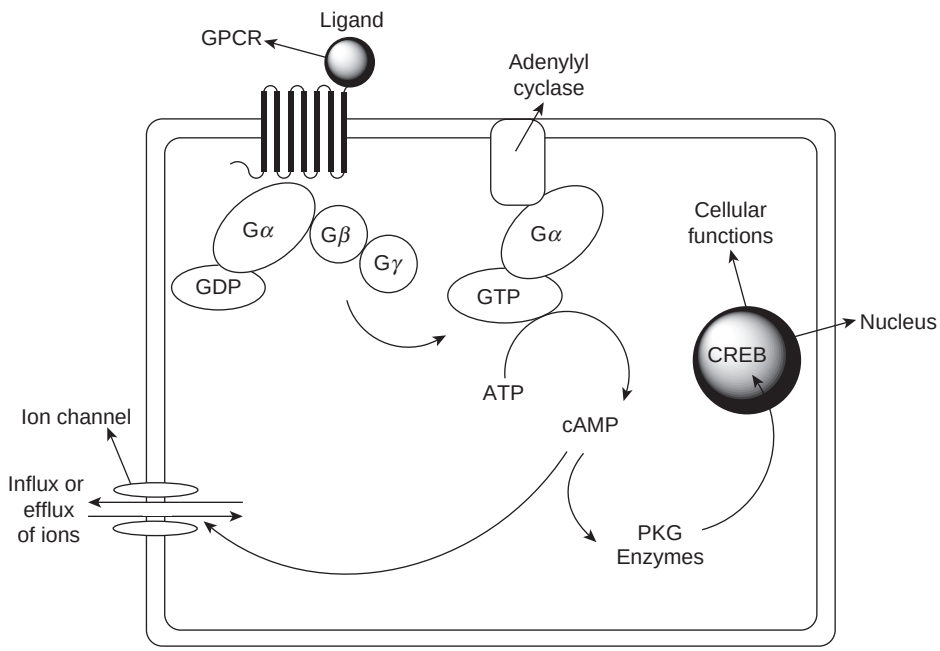


Figure 7.9 A typical GPCR and intracellular signal transduction via effector and second messenger (cAMP).

Mechanism of Action of GPCRs

During the resting stage, the G-proteins (α, β and γ subunits) remain associated with cytosolic domain of the receptor. There are many types of Gα subunit proteins: $G_s\alpha$ (G_s stimulatory), $G_i\alpha$ (G inhibitory), $G_o\alpha$, G_q/α, etc. Following ligand binding and activation of the receptor, the GDP in the Gα subunit is replaced by GTP, which then triggers its disassociation from the Gβ and Gγ subunits. The Gα subunit moves along the inner membrane and allosterically binds with one of the effector Adenylyl cyclase (AC) enzyme that in turn induces production of second messenger cAMP from ATP (Fig. 7.10). The cAMP as a second messenger is responsible for downstream signaling and plays a role in cell proliferation, differentiation, migration, survival, and apoptosis.

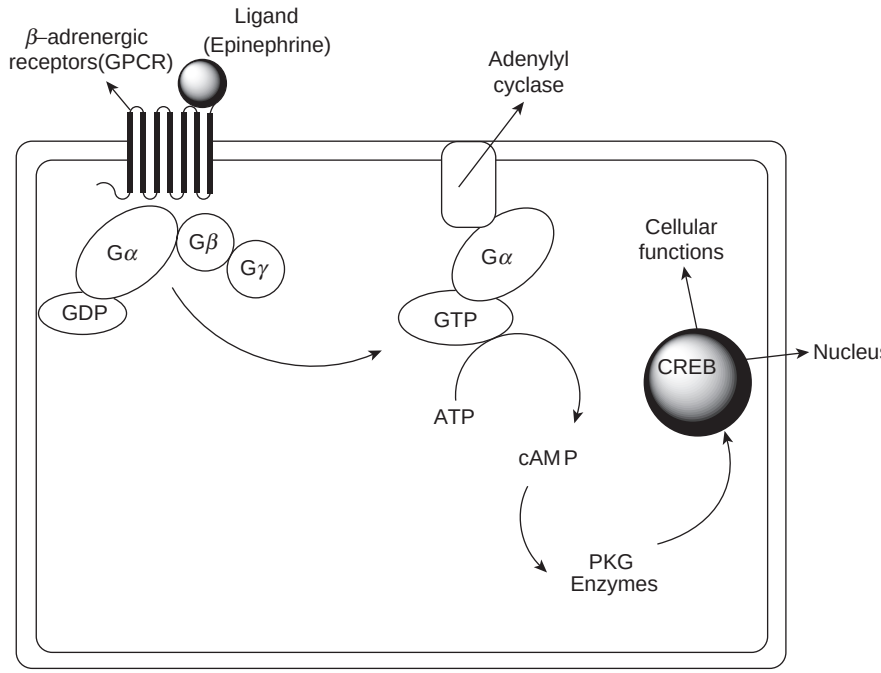


Figure 7.10 Activation of a GPCR-β-adrenergic receptor in a cardiac muscle cell via cAMP pathway.

Protease-Activated Receptor (PAR)

Serine proteases are ubiquitous proteolytic enzymes and generally play a role in protein degradation. They modulate numerous physiological functions either via their proteolytic activity or via intracellular cell signaling. For intracellular cell signaling, proteases just like endogenous hormones and neurotransmitters exert their effect by binding to GPCRs called Protease-activated receptors (PARs). However, the nature of their binding with the receptor is not reversible as observed with hormones and neurotransmitters. In fact, the activation of PAR is very distinct and is accompanied with proteolytic nature of the protease. The activation of PAR involves removal of an N-terminal peptide chain from the GPCR in the extracellular domain by the serine protease. This exposes the remaining peptide chain as a new N-terminal chain linked (tethered) to GPCR in the extracellular region that acts as a tethered ligand and binds (intramolecularly) to its binding own region in GPCR and transduces intracellular signal (Fig. 7.11).

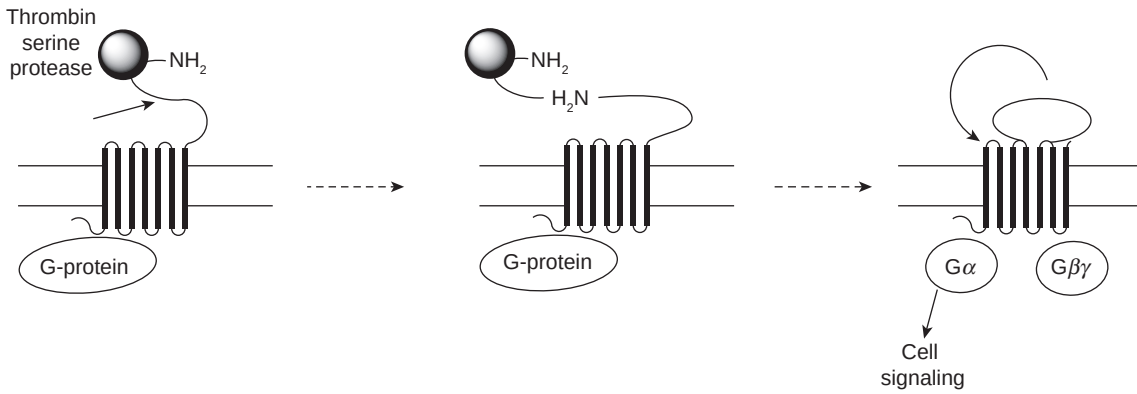


Figure 7.11 Activation of protease-activated receptor (PAR) by serine protease thrombin.

Thus, unlike other endogenous ligands for GPCR, serine proteases do not function like ligands for PAR; they instead help PAR to unravel its own ligands via proteolysis. Thrombin, a serine protease activates subtype PAR1 located on platelets and acts by binding and cleaving the N-terminal peptide from the receptor.[5] The new N-terminal chain then binds intramolecularly through a region involving a hexapeptide Ser-Phe-Leu-Leu-Arg-Asn and triggers activation of intracellular functions through signal transduction. Uncoupling from signaling following activation involves phosphorylation of PAR1, internalization within cytoplasm of PAR1, and disposal via lysosomes. This is unlike neurotransmitter-bound GPCR where receptors after internalization are recycled back to membrane except that ligands are disposed by lysosomes. Thus, most of the effects of thrombin are believed to be regulated by PAR and its intramolecular signal transduction.[6] In an attempt to use PAR-1 as therapeutic target, a monoclonal antibody against PAR-1 located on platelets was found successfully blocking activation of platelets, which paved way for the development of thrombin receptor antagonist (antiplatelet drug) Voraxapar.

CASE STUDY 1

Cardiac Cell GPCRs with Opposite Effects

Acetylcholine (ACh) is released at the junction between nerve and muscle cells, and slows down heart rate, whereas adrenaline (epinephrine) speeds it up. Ach plays a major role in maintaining heart's rhythm when at rest. ACh binds to M2 muscarinic receptors (a subclass of cholinergic receptors) that are found on cardiac cells. The M2 receptors are coupled to Gi protein, and activation of GPCR by endogenous ligand ACh brings decrease in the level of cAMP by inactivating the enzyme AC (also known as adenylate cyclase). In contrast, β1-adrenoceptors (GPCR) present in cardiac and smooth muscle cells is coupled to Gs proteins. Activation of β1-adrenoceptors by endogenous ligand epinephrine (adrenalin) increases the heart rate and frequency of contraction/relaxation by increasing cAMP levels via activation of AC. Thus, β1-adrenoceptors increase cAMP levels; on the contrary, muscarinic receptors (M2) have opposite effect and decreases cAMP levels by inhibiting AC (Fig. 7.12). Besides, G$\beta\gamma$-protein activation linked to M2 receptors leads to the activation of K_{ACh} channels (parasympathetic nervous system) which, in turn, triggers efflux of K^+, thereby causing hyperpolarization (reducing heart rate) of the cells.

(*Continued*)

Study 1 (*Continued*)

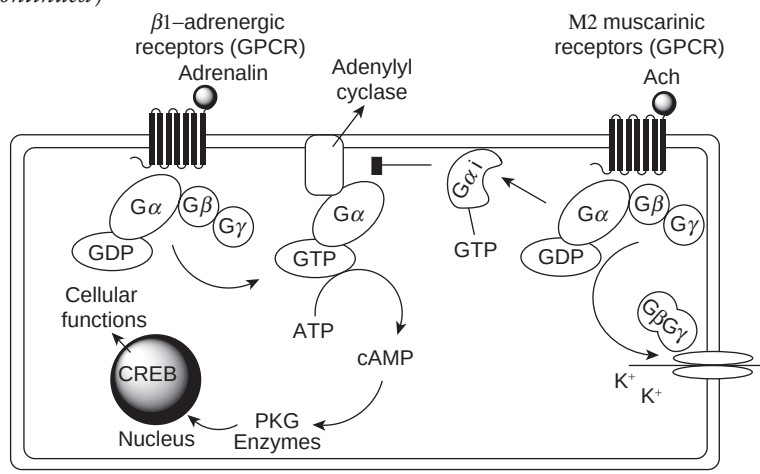


Figure 7.12 β-Adrenoceptors increase cAMP levels; muscarinic receptors (M2) have opposite effect and decrease cAMP levels.

CASE STUDY 2

GPCR Activation by IP3 Pathway

The α_1 (α_1) adrenergic receptor (or adrenoceptors) is a GPCR located predominantly on vascular smooth muscle cells. When endogenous ligand norepinephrine (noradrenaline) binds with the alpha receptor, it results in vasoconstriction. This increases the blood pressure and the direction of blood flow returning to the heart. The receptor binding is followed by activation of smooth muscle contraction through the IP3 signal transduction pathway (Fig. 7.13).

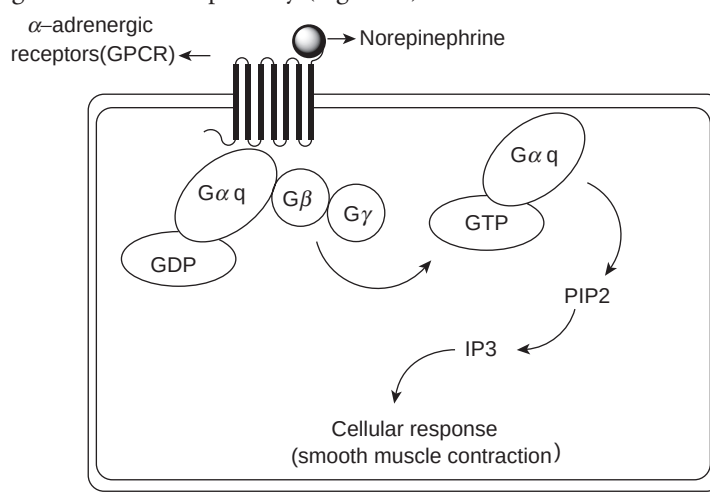


Figure 7.13 Activation of a GPCR α_1 adrenergic receptor in vascular smooth muscle cell via IP3 pathway.

CASE STUDY 3

GPCRs in Vascular Smooth Muscle with Opposite Effects

Depending on the tissue and type of vessel, vascular smooth muscles carry two GPCR-based receptors: alpha-adrenoceptors alpha$_1$ (α_1) and alpha$_2$ (α_2) with distinct functions and pathways. The difference in mechanism of action involves difference in G proteins and second messengers. The $\alpha_1$receptor mediates its signal via Gq protein and leads to the generation of IP3 (second messenger) whereas α_2 receptors mediate their signal via Gi-protein but instead of activating, reduce the concentration of second messenger cAMP. Thus, an increase in the concentration of IP3 and decrease in the concentration of cAMP leads to the activation of smooth muscle contraction (Fig. 7.14).

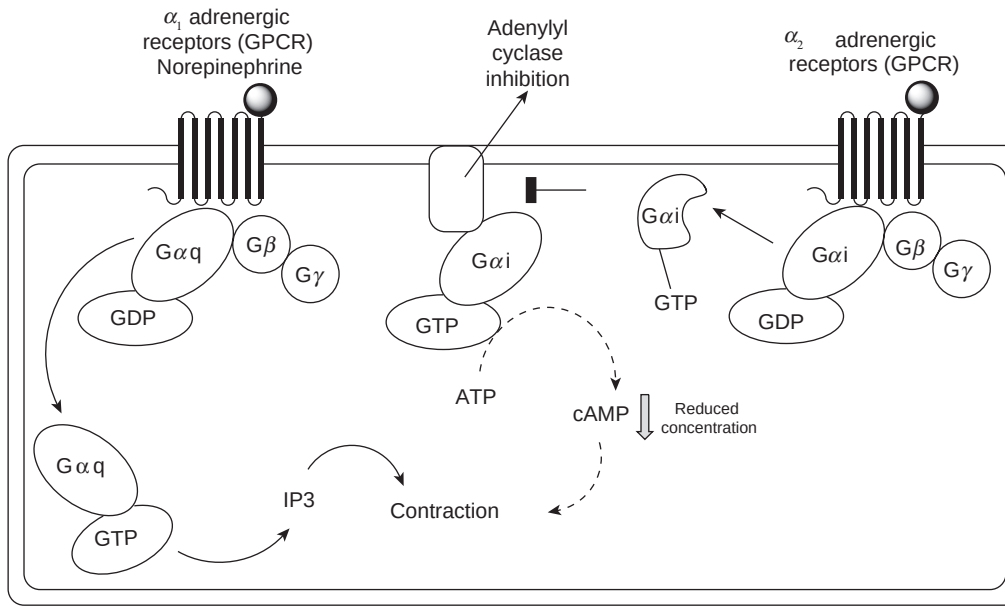


Figure 7.14 Alpha-adrenoceptors: alpha$_1$ (α_1) and alpha$_2$ (α_2).

CASE STUDY 4

Role of Second Messenger cAMP and cGMP in the Relaxation and Contraction of Smooth Muscle Cell

Smooth muscles distributed ubiquitously within organs in the body undergo contraction and relaxation in a regulated manner. Some major physiological functions of the organs involved in contraction and relaxation are inhalation and exhalation in respiration, peristaltic movement in gut, uterine contraction during childbirth, etc. The phenomenon of contraction and relaxation of smooth muscle cell is Ca dependent and is governed by phosphorylation and dephosphorylation of Myosin light chains (MLCs), as shown in Figure 7.15.

(*Continued*)

Study 4 (*Continued*)

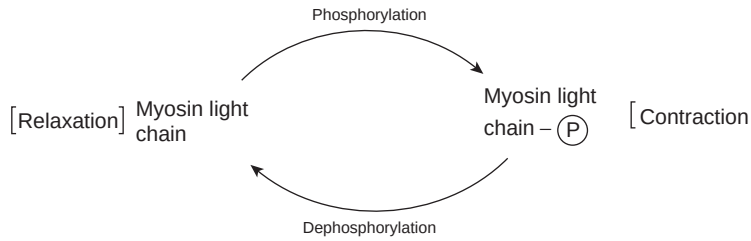


Figure 7.15 Contraction and relaxation of smooth muscle cell is Ca dependent and is governed by phosphorylation and dephosphorylation.

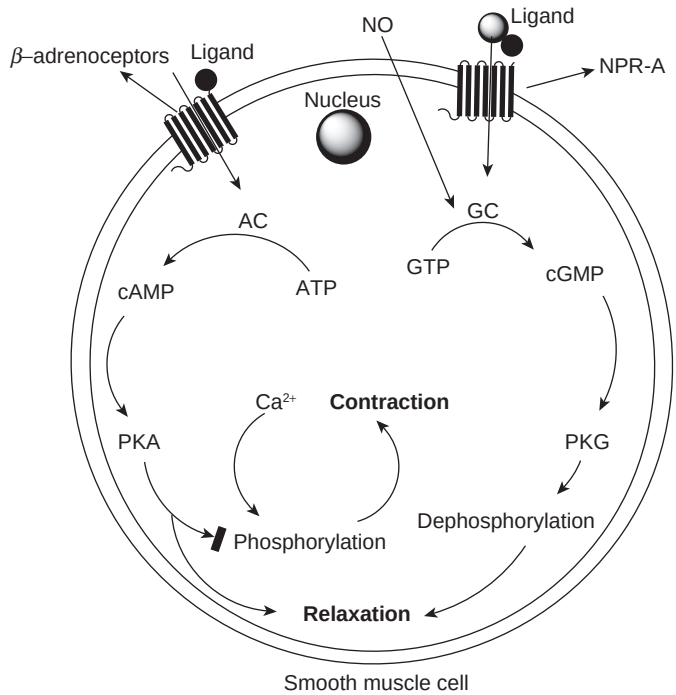


Figure 7.16 Role of second messengers (cGMP/cAMP) in the contraction and relaxation of smooth muscle cell.

The free calcium binds to calmodulin, a calcium-binding protein, and triggers energy-driven phosphorylation of MLCs by activating the enzyme myosin light chain kinase (MLCK), and in turn lead to smooth muscle contraction (Fig. 7.16). An increase in the concentration of cAMP inhibits phosphorylation via PKA, thereby reducing phosphorylation and relaxing the smooth muscle. Relaxation also occurs following increase in the concentration of cGMP that ultimately triggers phosphatase-activated MLC dephosphorylation via PKG.

Thus, a simple phosphorylation reaction triggers cross-bridge cycling of myosin and shortening or contraction of the smooth muscle cell, whereas relaxation occurs when there is reduced phosphorylation.

Inactivation of Second Messengers (cGMP/cAMP) by Phosphodiesterases

A super family of phosphodiesterases (PDEs) that degrades and inactivates intracellular second messengers cAMP and cGMP and provides a feedback loop between cAMP/cGMP levels and PDE activity. Some PDE families are strictly cAMP-specific whereas others are cyclic guanosine monophosphate (cGMP)-specific. Besides, some PDEs hydrolyze both cAMP and cGMP establishing cross-regulation of both pathways with important therapeutic implications (Fig. 7.17). In view of this, design of specific PDE inhibitors as therapeutic strategy has been found useful in numerous pathophysiological conditions. Some examples (Fig. 7.18) of clinically used drugs as PDE inhibitors have been described below:

1. Rolipram is a selective phosphodiesterase-4 inhibitor discovered and developed as a potential antidepressant drug.
2. The oxindole, a PDE2 inhibitor, is used as a therapeutic agent for the treatment of dementia, cancer, and hematologic disorders among other PDE2 mediated by activities.
3. Cilostazol, a selective phosphodiesterase type 3 (PDE3) inhibitor, enhances the production of cAMP and is associated with antiplatelet activity.
4. Sildenafil is used as a PDE5 inhibitor that blocks the degradation of second messenger cGMP into an inactive GMP in the smooth muscles and is used in the treatment of erectile dysfunction (Fig. 1.5; Chapter 1). Papaverine (Fig. 7.18), an opium alkaloid, is also an inhibitor of PDE and exerts its effect by increasing the concentration of second messenger cAMP.

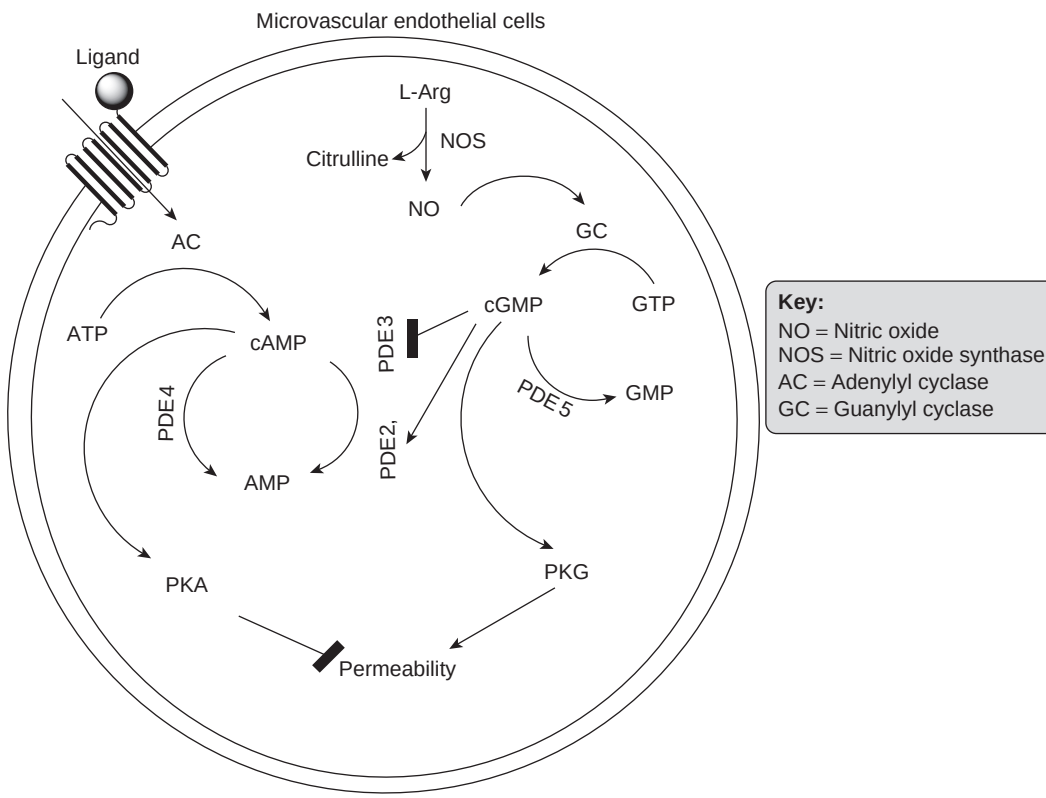


Figure 7.17 Inactivation of second messengers (cGMP/cAMP) by PDEs.

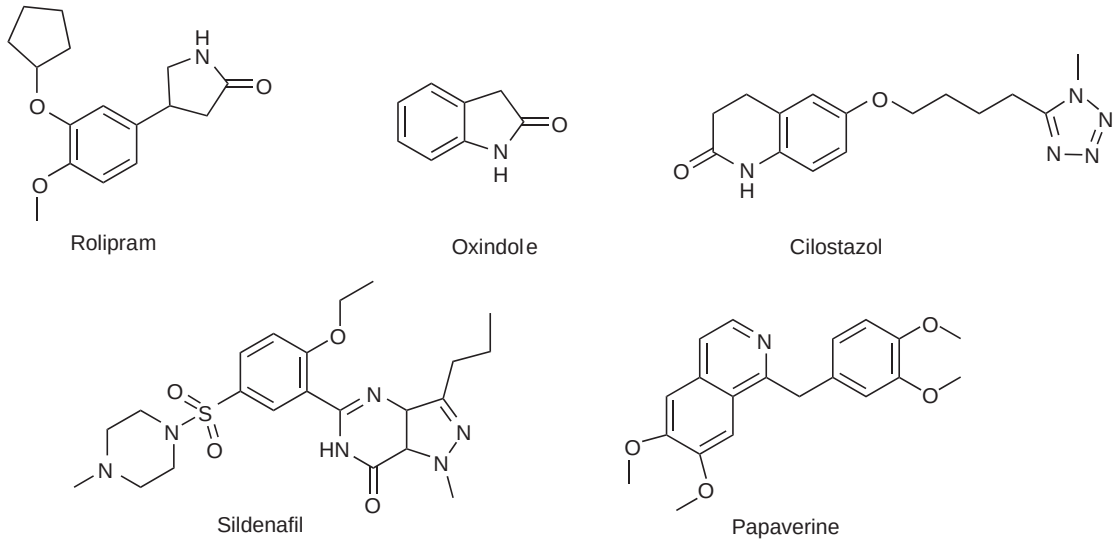


Figure 7.18 Clinically used PDE (II to V) inhibitors.

CASE STUDY 5

Vascular Permeability in Microvascular Endothelial Cells via cAMP/cGMP/PDE Pathway

The vascular endothelial cells play a major role in the smooth functioning of blood vessels. In pathological conditions such as oedema and sepsis, the dysfunction arises due to increased permeability and vascular leakage. Vascular permeability deals with the ability of a blood vessel wall to allow flow of drugs, nutrients, water, ions in and out of the vessel to enter in the tissues. Among many causes attributed to the permeability dysfunction, signaling by second messenger cyclic nucleotides cAMP and cGMP and their degradation by PDEs (2-5) play a critical role in endothelial barrier function. Both PDE2 and PDE3 on one hand hydrolyze cAMP, on the other hand, action of PDE3 is blocked by cGMP; thus, PDE 2 and 3 act as a bridge between the two PKA and PKG-mediated signaling pathways resulting in loosening and tightening of the endothelial barrier (Fig. 7.17).

Role of GPCR in Diabetes

Glucagon-like peptide-1 (GLP-1) (peptide) is a neurotransmitter secreted in intestine in response to the presence of glucose in food. It binds with GPCR family of receptors called GLP1R, which are cell-surface receptor expressed in pancreatic beta cells. Binding of GLP-1 with GLP1R induces change in the conformation of GLP1R and transduces signal within the cell stimulating the adenylyl cyclase (second messenger) pathway which triggers downstream activities and leads to the increase in the production of insulin (synthesis) and its release. During clinical settings such as diabetes, one of the possible causes could be degradation of GLP-1 by an extracellular enzyme DPP-4. The latter is expressed on the surface of most cell types as an intrinsic membrane glycoprotein and is a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides. Thus, both GLP1R (a receptor) and DPP-4 (an enzyme) are good biological targets for the management of diabetes. Exenatide (ligand; a 39-amino-acid peptide), an agonist of GLP-1 and vildagliptin (a substrate), an inhibitor of DPP4 enzyme is used for the management of diabetes (Fig. 7.19).

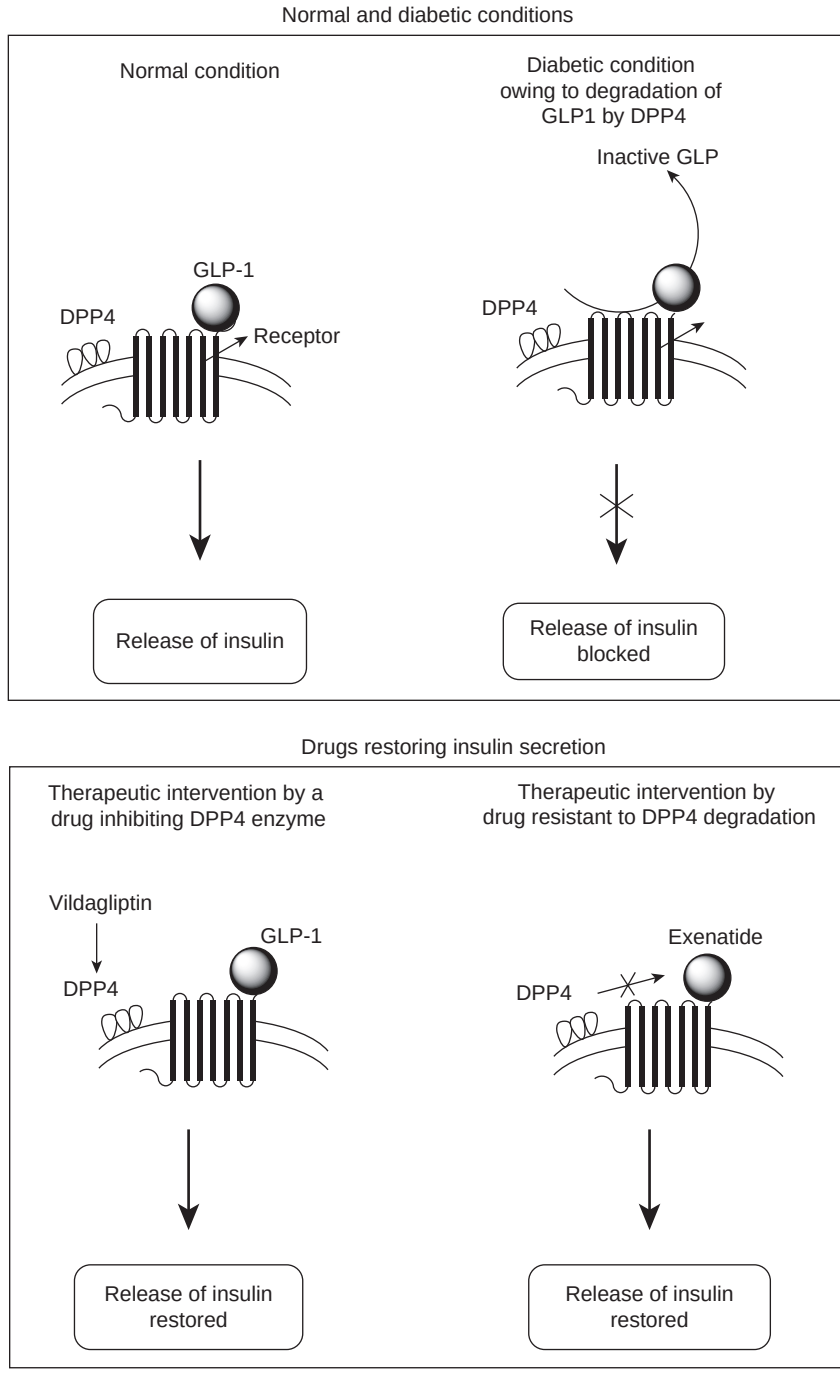


Figure 7.19 Therapeutic intervention in diabetic patients either with an inhibitor of an enzyme DPP4 or by an agonist ligand (first messenger) for the receptor GLP1R.

Some other examples of drugs that act on GPCRs are listed in Table 7.1.

Table 7.1 Drugs and their action on GPCRs

Drug	Major Receptor	Drug Action	Clinical use
Salbutamol	β_2-adrenergic	Partial agonist	Bronchodilation
Fexofenadine	Histamine H_1	Antagonist	Antiallergic
Atropine	Muscarinic	Antagonist	Pupil dilation
Haloperidol	Dopamine	Antagonist	Antipsychotic
Morphine	Opioid	Agonist	Pain killer
Losartan	Angiotensin	Antagonist	Antihypertensive
Clopidogrel	Adenosine	Antagonist	Anticoagulation

Termination of Signal Transduction

This involves resting to activation to again resting stages of GPCRs. Once a GPCR is activated following the binding of the ligand/drug (first messenger), the activated GPCR undergoes conformational change followed by dissociation of G proteins and a chain of events involving effectors and second messengers. This in turn, eventually triggers the ligand-specific cellular response. However, after the activation of the cellular response, the ligand/drug cannot remain attached to the receptor and has to leave. This is affected by a protein called Regulator of G-protein signaling (RGS) that allosterically binds with cytoplasmic G-proteins component and allow reassociation/regeneration of GPCR followed by release of the first messenger/drug (Fig. 7.20).

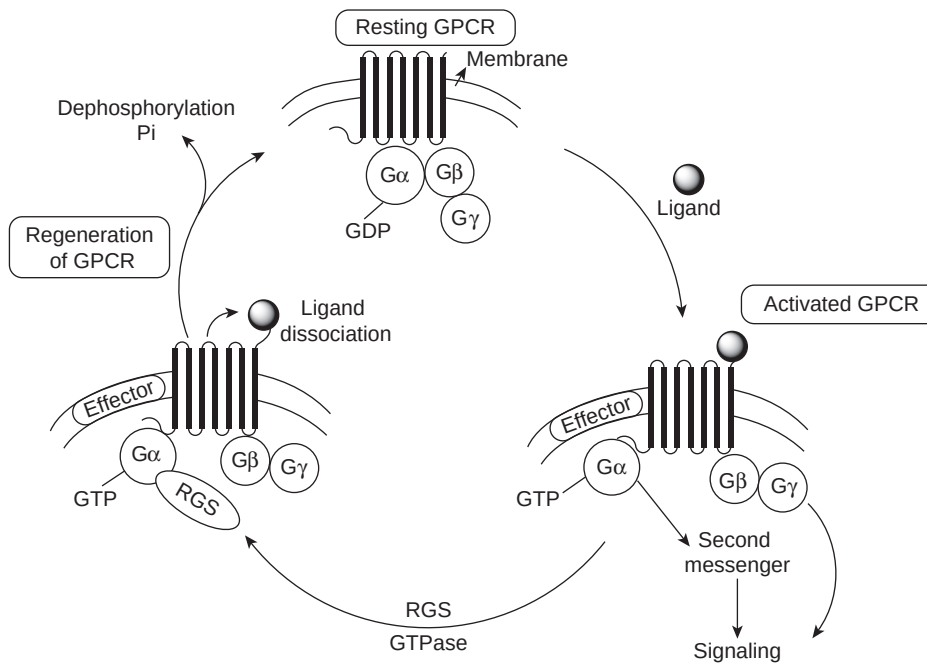


Figure 7.20 Binding and dissociation of ligand/drug/first messenger; (RGS = regulator of G-protein signaling).

The latter, after completing its task, is released in interstitial fluid, taken up by capillaries in bloodstream, and metabolized for the purpose of excretion.

However, the other mechanism of termination of signal transduction involves internalization of receptors via endocytosis into the endosomes from cell membrane. This internalization of ligand-bound receptor may either enhance the duration of signal[7] or in due course of time may be processed via endolysosomes for the purpose of recycling (Fig. 7.21) of the receptors back to the cell surface or degradation.[8]

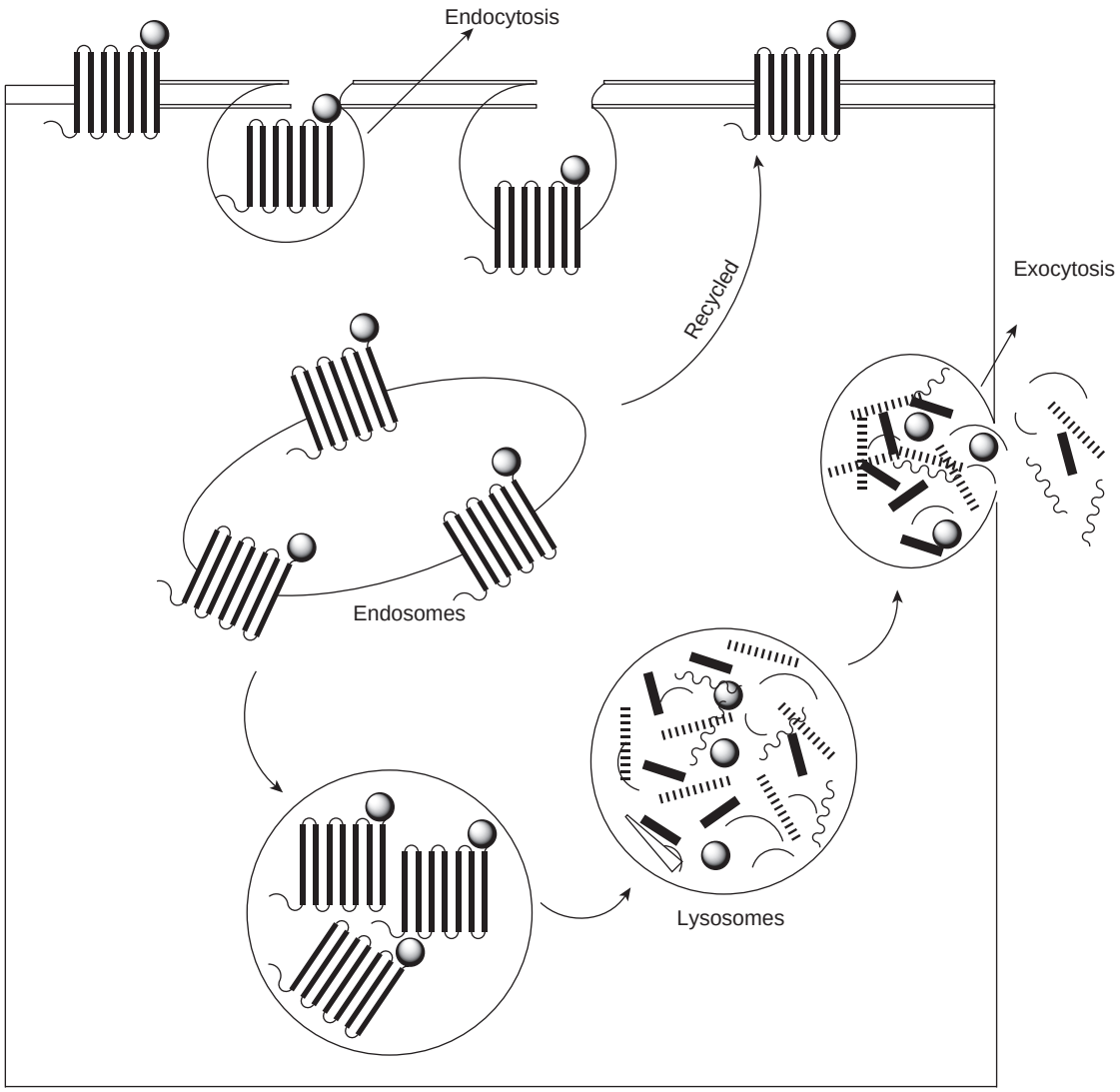


Figure 7.21 Termination of signal transduction involve internalization of GPCR, regeneration and elimination of wastes via exocytosis.

7.3.2 Enzyme-Linked Receptors

It is also referred to as a catalytic receptor and is a transmembrane helical protein. It is made up of three domains: an extracellular domain that acts as receptor for ligand and protrudes outside the cell in interstitial fluid, a lipophilic membrane embedded region in the plasma membrane, and an intracellular enzyme domain on cytoplasmic side associated with catalytic functions. Some receptor-linked enzyme receptors which have both receptor and enzymatic functions have been summarized below:

Examples of the enzyme-linked receptors include the following:

1. Receptor tyrosine kinase (RTK)
2. Guanylate cyclase receptor
3. Serine/threonine kinase receptors
4. Histidine kinase receptor

7.3.2.1 Activation of Enzyme-Linked Receptor

Ligands upon binding to enzyme-linked receptor induce dimerization that brings about conformational change in the cytosolic enzymatic domain required for the expression of enzymatic activity (Fig. 7.22).

Majority of the enzyme activity involves tyrosine kinase (TK) and phosphatase activity, serine or threonine kinase activity, and guanylyl cyclase activity. Thus, the signal generated extracellularly is relayed intracellularly to the nucleus regulating cellular activities. Both domains (receptor and enzyme) have been used as therapeutic target for developing drugs against various diseases. A common pathway for many enzyme-linked receptor-mediated signal transduction and tissue-specific downstream activities have been depicted in Figure 7.23.

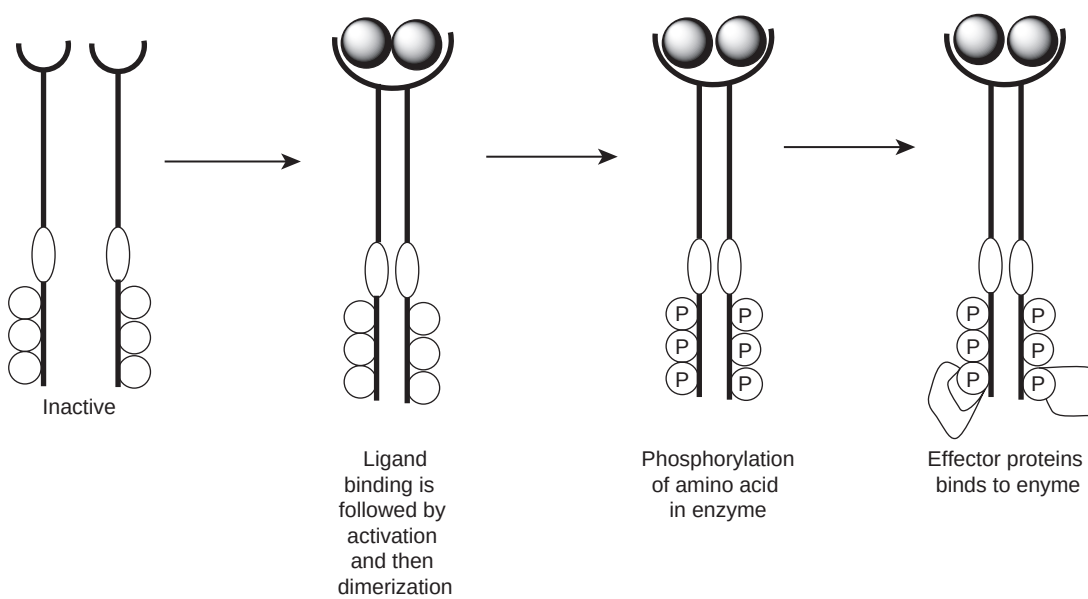


Figure 7.22 A general route to activation of enzyme-linked receptor.

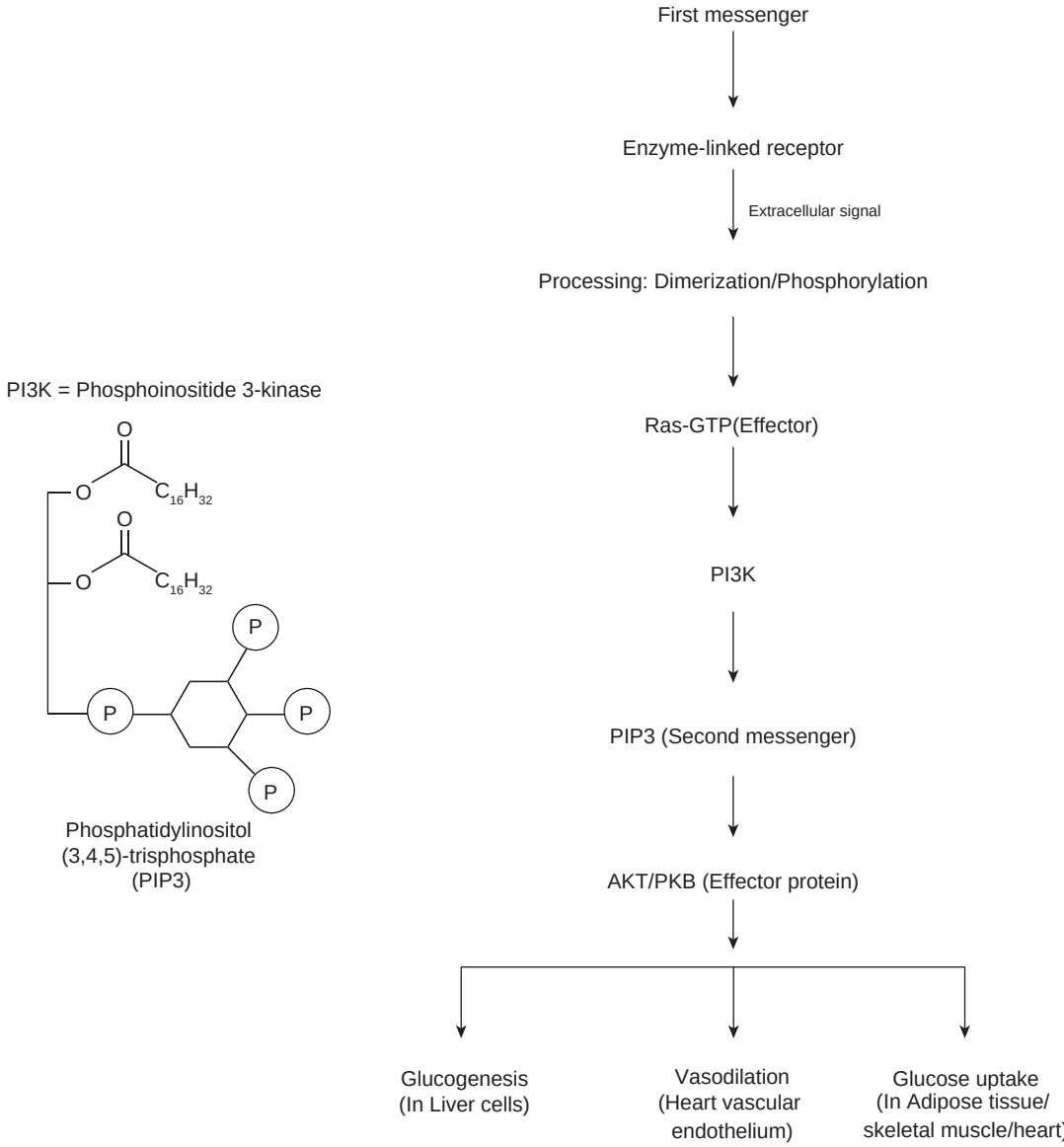


Figure 7.23 The PI3K (kinase)-AKT pathway may be common for many enzyme-linked receptor-mediated signal transduction; however, downstream activities are tissue specific.

Receptor Tyrosine Kinases (Enzyme-Linked Receptor or Catalytic Receptor)

This class of receptors serves dual role—receptor plus enzyme (bifunctional receptor and enzyme).[9] Binding of ligands to receptor occurs in the extracellular domain followed by signal transduction through intracellular domain involving enzymes.

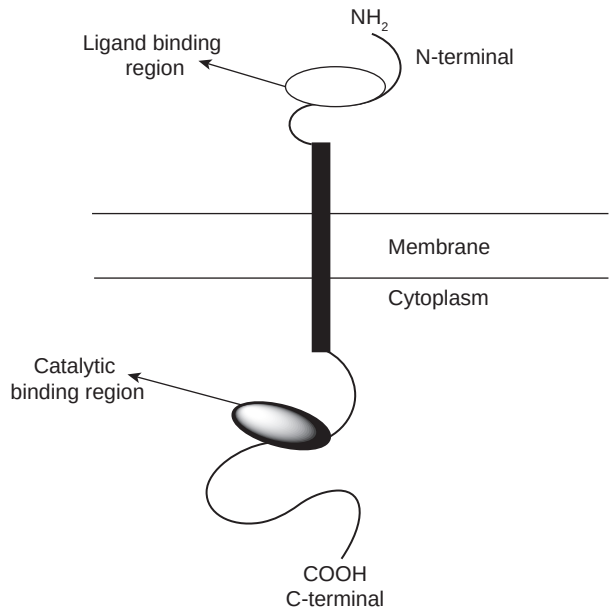


Figure 7.24 A general diagrammatic representation of tyrosine-linked receptor (RTK).

The binding of receptor with the first messenger follows induce-fit model leading to changes in the shape of the protein. This triggers the opening of the active site in the enzyme and catalyzes reaction within cells (Fig. 7.24). The signal transduction in the enzyme domain (kinases) is accomplished by catalyzing phosphorylation of another protein by transfer of a phosphate group from ATP. Some kinases are membrane bound, and others reside in the cytoplasm. Cell-signal-mediated phosphorylation occurs in all the three hydroxyl amino acids Tyr, Ser, and Thr present in proteins. RTKs are generally activated by endogenous hormones or exogenously administered drugs as first messenger and their overexpression has been implicated in cancer.

CASE STUDY 6

Role of Receptor Tyrosine Kinase in Breast Cancer

Drugs can be developed to inhibit TK activity by either targeting extracellular domain of TK with the view to block signal transduction or, in other words, inhibiting initiation of intracellular cascade activity. Inhibition of kinases offers a powerful approach for treating diseases particularly when the pathophysiological conditions are due to signals induced by first messenger. Drugs can be designed in the form of ligands to inhibit signals at the receptor level or can be designed in the form of substrates (inhibitors) to inhibit intracellular kinase activity. Human epidermal growth factor receptor 2 (HER2) is a protein with an intracellular TK domain and an extracellular ligand-binding domain.

(*Continued*)

Study 6 (*Continued*)

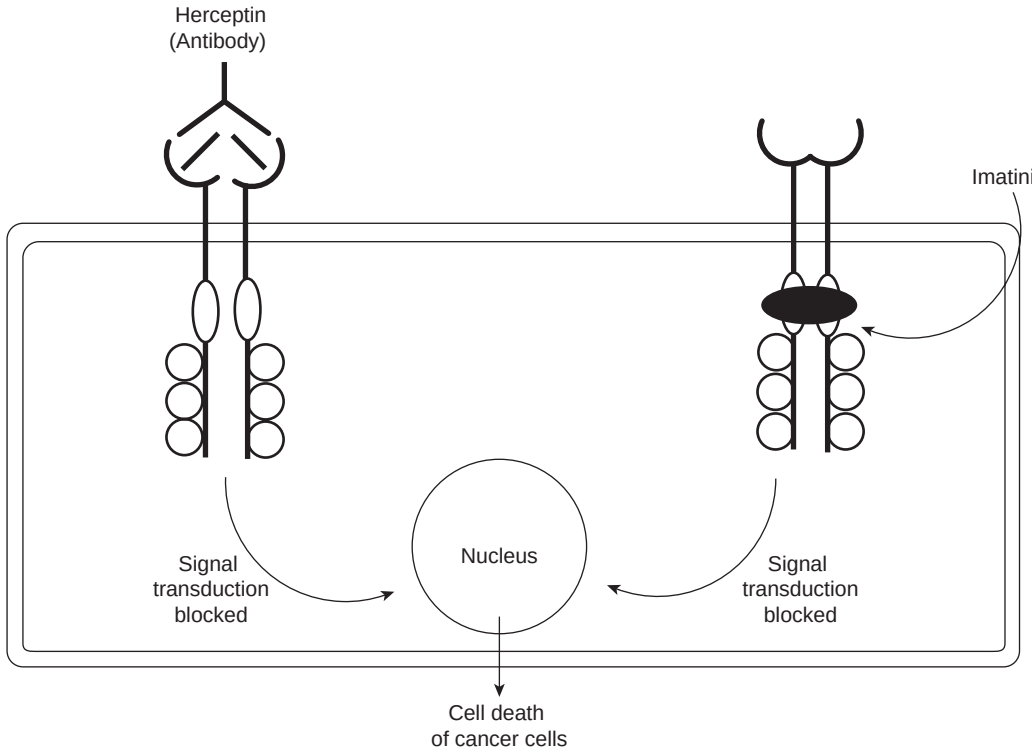


Figure 7.25 Two therapeutic approaches to inhibit activation of receptor TK for treating breast cancer.

HER2 receptors are overexpressed on the surface of all cells in breast cancer cells. Herceptin, a monoclonal antibody (trastuzumab) works as ligand/first messenger and attaches itself to the HER2 receptors on the surface of breast cancer cells. This, in turn, blocks HER2 receptors from receiving endogenous growth signals and slows down or stops the cellular functions of the breast cancer cells (Fig. 7.25). Alternatively, kinase activity can be inhibited by imatinib mesylate drug (a small-molecule substrate) that binds to the intracellular portion of the membrane-bound kinase (Fig. 7.25). Thus, herceptin (a hydrophilic biologics) blocks the receptor extracellularly and imatinib mesylate (a hydrophobic molecule; logP = 3.4/4.3) inhibits membrane-bound enzyme kinase intracellularly.

Role of Kinases and Phosphatases as Target Proteins

There are two major signaling enzyme pathways that work in tandem by opposing each other and control numerous cellular functions specific to the cells. On one hand, protein kinases following activation of the extracellular receptor catalyze the phosphorylation of hydroxy acids in proteins to furnish phosphoproteins and phosphatases; on the other hand, they catalyze removal (dephosphorylation) of phosphate groups from phosphoproteins (Fig. 7.26). Since protein kinases following activation are involved in signal transduction leading to phosphorylation, any aberration in the activation by receptor can cause diseases, e.g. overexpression of HER2 receptors in breast cancer. Thus, both the enzymes serve as important therapeutic target in drug discovery.

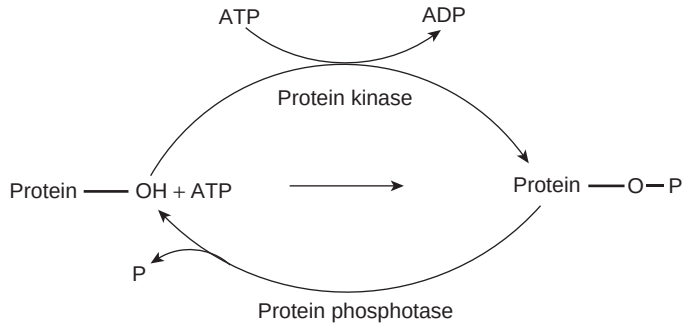


Figure 7.26 Role of kinases and phosphatases in phosphorylation and dephosphorylation.

CASE STUDY 7

Insulin Receptor Tyrosine Kinase Mediated Signal Transduction

Insulin secreted by β-cells in pancreas binds with insulin receptor tyrosine kinase (IR) present on the target cells of skeletal muscle, adipose tissue, and the liver, and exerts its action by promoting uptake of glucose from circulation. The IR comprises one pair of extracellular α-domain and a pair of transmembrane β-domain (Fig. 7.27). Once insulin binds to the insulin receptor in extracellular domain, it transduces intracellular signal that triggers not only autophosphorylation of kinases but also activates other proteins including IR substrate proteins (IRS) via phosphorylation.

This in turn exerts therapeutic effect of insulin; however, this action is reversed by dephosphorylation in the presence of protein tyrosine phosphatases (PTPs). Thus, inhibition of PTP1B can be a good therapeutic target for the treatment of diabetes (Fig. 7.28).

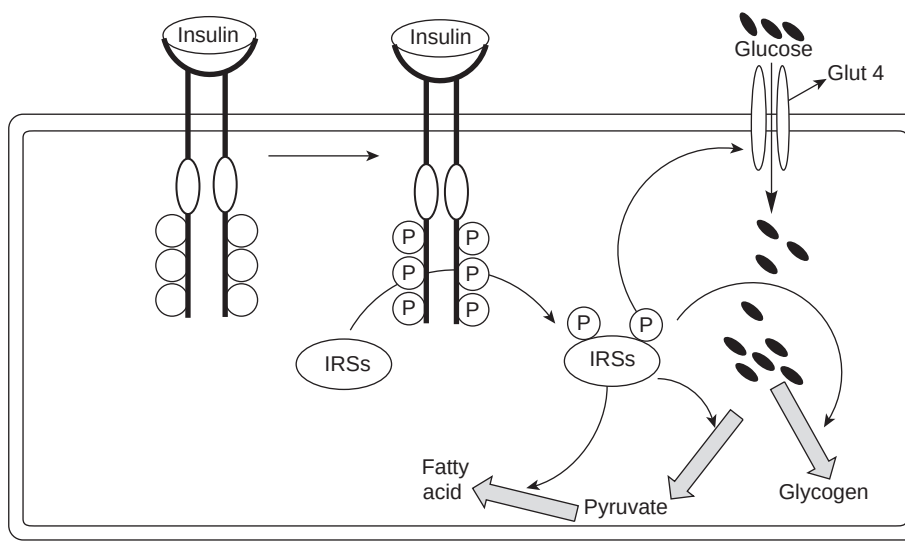


Figure 7.27 The IR-mediated signal transduction.

(*Continued*)

Study 7 (*Continued*)

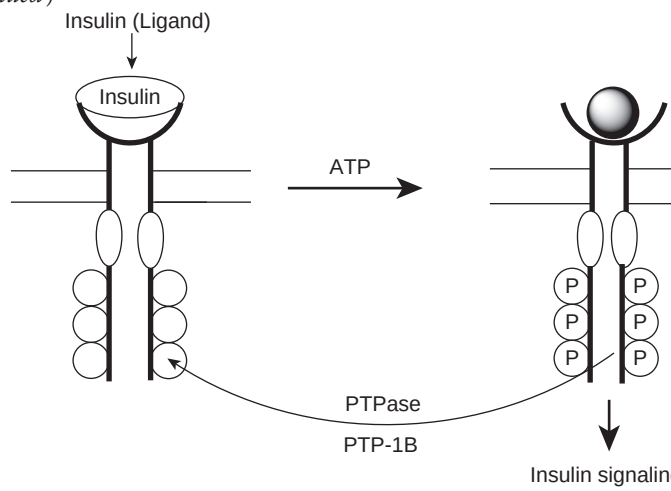


Figure 7.28 PTP1B and IR desensitization.

Trodusquemine (substrate) is a naturally occurring cholestane and non-competitive, allosteric inhibitor of PTP1B, with potent hypoglycaemic, anti-diabetic, and anti-obesity activities (investigational drug candidate). Upon administration, trodusquemine selectively inhibits PTP1B and inhibits the dephosphorylation of the insulin receptor (thereby preventing PTP1B-mediated signaling). This, in turn, improves insulin signaling and insulin sensitivity, and decreases blood glucose levels. However, trodusquemine has not yet been released in the market and still remains an investigational drug candidate.

Receptor Transactivation or Crosstalk between Different Classes of Receptors

Besides stimulation/signaling of GPCRs and RTKs, etc., by their corresponding ligands, cross communication between signaling arising from two different classes of receptors also exists. This in turn allows integration of stimuli received by cells under diverse physiological situations (Fig. 7.29). Typical examples include interconnected communication network via the activation of GPCR followed by epidermal growth

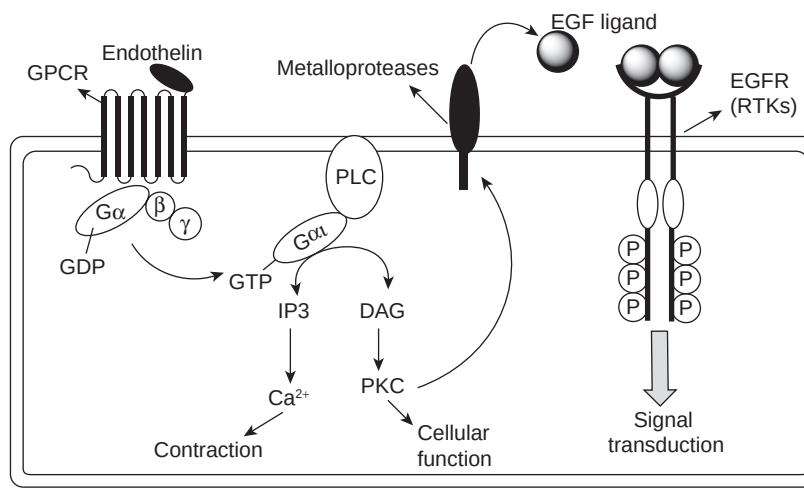


Figure 7.29 Transactivation of EFGR via crosstalk between different signaling systems.

Factor receptor (EGFR; a family of RTKs) in cancers. The interconnected communication network involving GPCR and EGFR works via initial activation of GPCR following binding with agonist (endothelins). The activation eventually leads to the production of PKC (intracellularly) which then leads to the activation of metalloproteases that generates the mature EGFR ligand (extracellularly). The EGFR ligand binds and activates the EFGR, which is followed by subsequent downstream signaling cascades including mitogen-activated protein kinase (MAPK) transduction pathway that controls cell proliferation. A list of drugs targeting TK receptors have been summarized in Table 7.2, and the structures of few RTK inhibitor have been depicted in Figure 7.30.

Table 7.2 List of RTKs and their corresponding inhibitory drugs.

RTKs and their Ligands	Therapeutic Implications	Monoclonal Antibody Therapy	Small Molecule Drugs
VEGFRs bind with VEGF	Stimulate angiogenesis	Bevacizumab (monoclonal antibody) binds to VEGF	Sorafenib and Sunitinib (small organic molecule)
The EGFR binds to EGF	Mutations affecting EGFR expression or activity could result in cancer	Cetuximab (Erbitux) (monoclonal antibody)	Gefitinib (Iressa) and Erlotinib (Tarceva). (small organic molecules)
HER2/erbB-2	Breast cancer	Herceptin (monoclonal antibody)	Imatinib (small organic molecule)
TrkA (nerve growth factor)	Regulates functioning of nervous system	–	Larotrectinib

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HER2, human epiermal growth factor receptor 2; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFRs, vascular endothelial growth factor receptors.

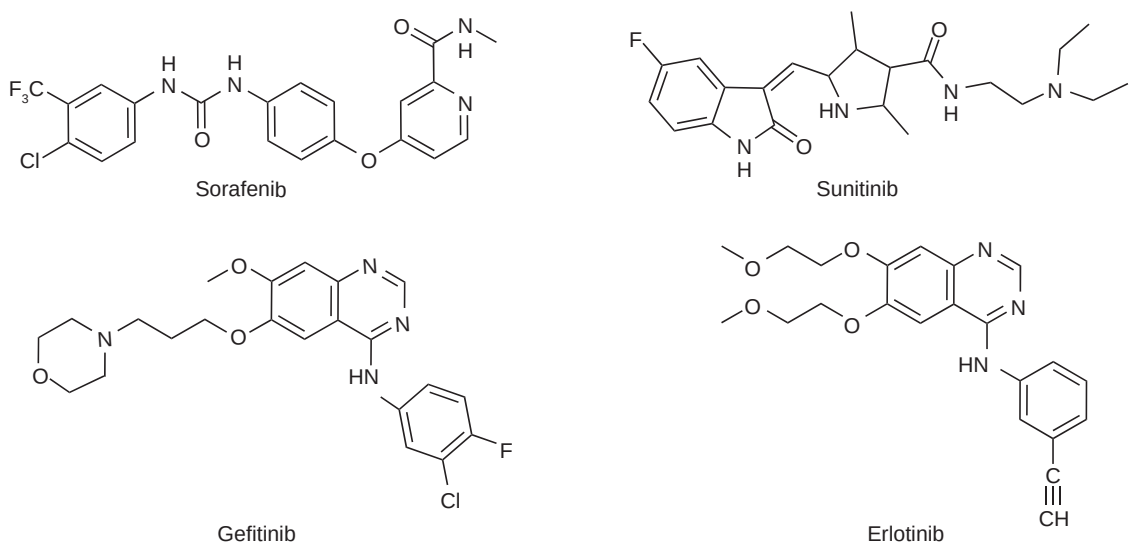


Figure 7.30 Structures of TRK inhibitors as drug.

CASE STUDY 8

Receptor Guanylate Cyclase (rGC)

Membrane-bound guanylate cyclase-coupled receptors belong to the class of TRK family with an extracellular binding site and an intracellular guanylyl cyclase catalytic domain. A number of receptor guanylyl cyclase have been reported, which uses cGMP as an intracellular mediator (second messenger) for modulating specific downstream signaling via kinases, phosphodiesterase and ion channels. The signaling pathway involves activation of the receptor guanylyl cyclase by natriuretic peptides (ANPs) as first messenger/endogenous ligand followed by intracellular activation of receptor-linked GC. This in turn causes transformation of GTP to second messenger cGMP responsible for physiological functions (Fig.7.31).

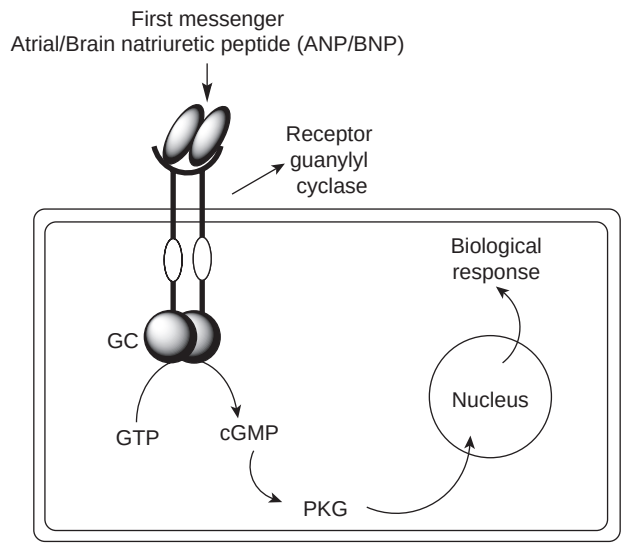


Figure 7.31 cGMP/PKG signaling pathway via receptor guanylyl cyclase.

7.3.3 Ionotropic Receptors

Ions play an important role in regulating physiological functions throughout the body. In the central nervous system, the flow of ions between neurons is essential for the passage of electrical signals through the neurons. The continuous exchange (conductance) of ions across neuron (excitable cells) membranes helps in generating polarized membranes/action potential, resulting in firing of neurons. The key ions that play an important role in membrane/action potential are calcium, potassium, sodium, chloride, and copper ions. The resting stage of membrane potential (−55 mV) requires more of K^+ inside the neuron and more of Na^+ in the ECF outside the neuron in synapse.

Notably, the bilayer cell membrane (hydrophobic) themselves are not permeable to ions; instead membranes are impregnated with several openings in the form of ion channels through which ions are transported in and out of the cells. The ion channels are transmembrane proteins that allow influx and efflux of ions in response to specific stimuli. There are three major kinds of channels:

1. Ligand-gated channels
   - **(a)** Intracellular
   - **(b)** Extracellular
2. Voltage-gated channels
3. Second messenger gated channel

7.3.3.1 Ligand-Gated Ion Channel

These receptors are present in neurons dealing with CNS-related activities and generally respond (open) to neurotransmitters as ligands. They predominantly regulate synaptic transmission either between two neurons or between a neuron and a muscle cell. The excitation of presynaptic neuron triggers secretions of neurotransmitter stored in the vesicles inside the presynapse. Following its release in the synapse, the neurotransmitter binds to the ligand-gated receptors present on the surface of postsynaptic neurons. This in turn triggers change in the conformation of the ion-channel receptor as they open up and allow movement of ions through the channels. This causes change in membrane potential and initiates depolarization, an excitatory response followed by repolarization to hyperpolarization, an inhibitory response restoring back to the resting potential of the postsynaptic neurons (Fig. 1.25; Chapter 1).

The ligand-gated ion channels (transmembrane proteins) can be found both in the covering membrane of cells as well as on cytosolic intracellular organelles. Accordingly, it can be gated with either extracellular ligands or intracellular ligands. Generally, the transmembrane receptor protein ion channels are made up of two different domains: a transmembrane domain structurally arranged in the form of a centrally aqueous channel (ion pore) and an extracellular domain which carries site for ligand binding.

The mechanism of action of ligand-gated receptors involves induced fit binding of a neurotransmitter (ligand) to the ligand-gated receptor followed by conformational change of protein which results in the temporary opening of the ion channel (hydrophilic). The momentary opening of ion channel allows bidirectional movement of ions (Na^+, Ca^{2+}, Cl^-, K^+) across the cell between the interstitial fluid and intracellular cytoplasm. Due to direct link between activation of receptor by a ligand and opening of ion channels, the ligand gated channels remain fast signaling receptors (millisecond latency). Opening is also followed by rapid termination of signaling leading to the closure of channels since neurotransmitters dissociate rapidly. On the contrary, GPCR produces much slower signaling responses and ranges from hundreds of milliseconds to minutes or even longer. Notably, a neurotransmitter may bind and activate both GPCR and ion channel during the transmission of impulse through neurons producing both fast and slow postsynaptic potentials (PSP*)*. Dysfunction of ion channels have been implicated in epilepsy, Alzheimer's, Parkinson's, schizophrenia diseases to name a few.

7.3.3.2 Extracellular Ligand-Gated Ion Channel Activity

As the name reflects**,** the transient opening of ion channel occurs when a signaling molecule (a ligand) binds to the extracellular region of the ion channel. The binding of specific ligands to ion channels allows transient opening of the ion channels (Fig. 7.32), which is generally fast, occurs within milliseconds, and is short-lived (<0.1 s).

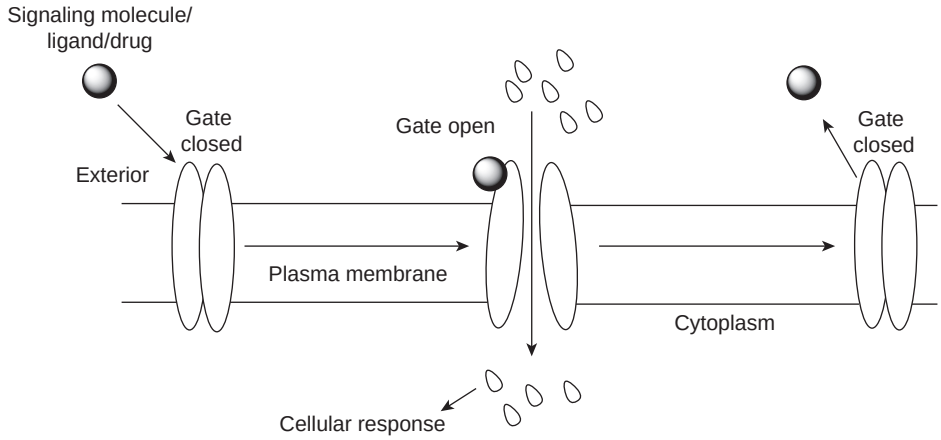


Figure 7.32 Ligand-gated ion channel receptor.

7.3.3.3 Intracellular Ligand-Gated Ion Channel Activity

These ligand-gated channels are activated by GPCR-driven chemical signals generated within the cytoplasm of neurons (from an intracellular side). This involves the extracellular binding of ligands (such as ACh) at GPCR, which triggers G protein-mediated signaling in the cytoplasm and activates the ion channel (Fig. 7.33) from the cytoplasmic side. This class of ion channels carries ligand-binding domains on their intracellular surfaces (cytosolic side) that interact with G-proteins. These G-protein-gated ion channels are widely distributed in CNS neurons and muscular walls of heart and are involved in the regulation of physiological functions through movement of ions.

CASE STUDY 9

Muscarinic ACh Receptors in Heart Muscle Cells (Protein-Gated Channel)

Two of the physiological functions of heart involving propagation of electrical impulses and cardiac muscle contraction are regulated by GPCR-mediated control of ion channels.

The muscarinic (subtype M2/M4) acetylcholine receptors (GPCRs) present in the presynapse neurons or heart muscle cells (non-neuronal effector cells) binds with the acetylcholine (Ach; a neurotransmitter) released in the synaptic cleft. Binding of ACh with GPCR stimulates cardiac muscle cells by activating G-proteins (Fig. 7.33). This involves separation of G-proteins into Giα and G$\beta\gamma$ in the cytoplasm. The G$\beta\gamma$ then binds to K channels (protein-gated channel) present in the presynaptic cell membrane or smooth muscle cell membrane in heart from the cytosolic side, thereby opening the K channels that trigger efflux of K^+. This in turn hyperpolarizes (further increase in the negative charge inside the cell) the membrane which leads to either decrease in the release of acetylcholine in the synapse or reduction of the frequency of heart muscle contraction (produces slower heart rate/slowing of impulse conduction through the atrioventricular node). Besides, Giα protein decreases the level of cAMP by inactivating the enzyme adenylyl cyclase (Fig. 7.12).

(*Continued*)

Study 9 (*Continued*)

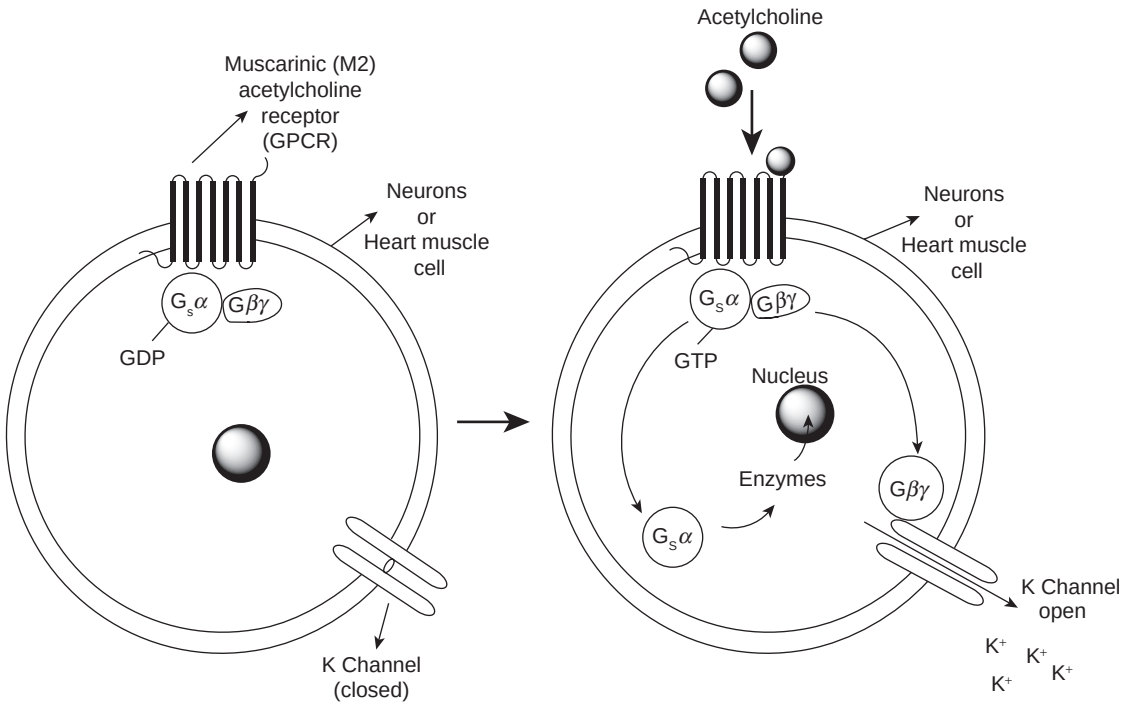


Figure 7.33 G-protein-gated channel-activation of K^+ Channels: Ach activates muscarinic Ach receptors in presynaptic neurons/cardiac muscle cells.

Muscarinic and Nicotinic Receptors

ACh**,** an endogenous ligand (neurotransmitter) exerts its physiological functions by binding to two cell-surface acetylcholine receptors (AChRs): muscarinic and nicotinic generally found on both sides of the synapse (presynaptic and postsynaptic). The muscarinic receptors belong to a family of GPCRs widely distributed in both CNS and peripheral nervous system, and regulate functioning of lungs, heart, upper GI tract, etc. The nicotinic receptors are ligand-gated ion channels and allow movement of Na^+, K^+, and Ca^{2+} ions across cell membranes. Three important physiological functions and location of these ACh receptors are as follows:

1. **CNS Receptors:** They comprise both muscarinic and nicotinic receptors and clinical implications involve regulation of memory, wakefulness, and sleep.
2. **Autonomic Receptors:** They comprise both muscarinic and nicotinic receptors and clinical implications involve a wide range of involuntary physiological functions, such as the parasympathetic component of the autonomic nervous system that produces nerve effects in the heart, smooth muscles, and glands.
3. **Neuromuscular Receptors:** They are nicotinic receptors and clinical implications involve cholinergic activation of skeletal muscles.

7.3.3.4 Voltage-Gated Channel (Postsynaptic Membrane)

This class of channels responds to changes in the electrical properties of the cell membrane in which the ion channel is embedded. These channels have voltage sensor with positive charges which spans the membrane (Fig. 7.34). In fact, the phenomenon of depolarization involving increase of Na^+ concentration inside the cell (positive charges) causes physical movement of sensor. In general, such channels remain closed when the membrane is hyperpolarized, an inhibitory stage (negative voltage inside membrane, −70 mV), but open up upon depolarization, an excitatory stage (when the voltage becomes less negative).

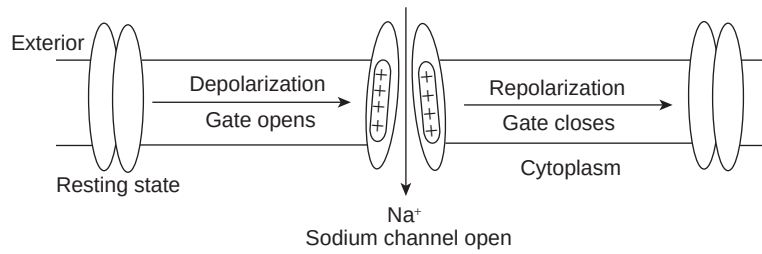


Figure 7.34 Voltage gated ion channel receptors.

CASE STUDY 10

Verapamil as Calcium Channel Blockers

Verapamil, an L-type calcium channel blocker/antagonist (prevents Ca from entering cells mainly in heart and blood vessel walls) gains access to its binding site present in the ion channel via allosteric modulation (an intracellular route; Fig. 7.35). It preferentially binds to ion channels allosterically in an open state (excited state) and blocks the movement of Ca ions. The therapeutic cardiac effects using verapamil involve decrease in contractility, heart rate, and conduction velocity.

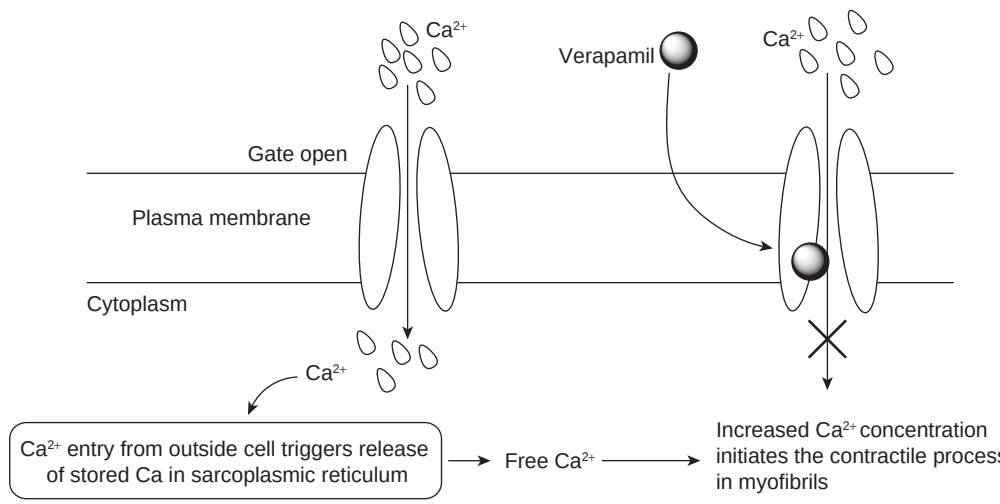


Figure 7.35 Verapamil, an L-type calcium channel blocker (prevents Ca from entering cells mainly in heart and blood vessel walls) gains access to its binding site *via* an intracellular route.

CASE STUDY 11

Local Anesthetics as Ligands for Ion Channel Receptors

Local anesthetic (LA) drugs (lidocaine/procaine) with nerve-blocking abilities are used clinically for inducing local and regional anesthesia as well as for their cardiac antiarrhythmic actions. Their target is the voltage-gated Na^+ channels, and the binding site is located within the channel's inner pore which is accessible from the cytoplasmic side. Na channels are the source of excitatory currents (depolarization stage during action potential) for the nervous system and muscle (Fig. 6.3; Chapter 6).

Thus, during firing of neurons (conduction of nerve impulses) generation of action along the axon occurs due to the inflow of Na^+ from outside cell membrane into the cell as the Na channels are open. The binding of cationic LA drugs to Na channel from the cytosolic side prevents influx of Na^+. Accordingly, when the influx of sodium is interrupted, there is no action potential and thus conduction of pain-causing signal is inhibited lasting the duration of anesthesia induced by the drug.

7.3.3.5 Second Messenger-Gated Ion Channel

The binding of first messenger/ligand to receptors on the cell surface of postsynaptic membrane triggers the intracellular production of second messengers via activation of membranous cytosolic enzyme Phospholipase C (PLC). Next, second messenger IP3 binds with the ligand gated ion channels present on the membranes of endoplasmic reticulum. This then selectively triggers the release of Ca^{2+} (a second messenger) from the lumen of the endoplasmic reticulum into the cytoplasm (Fig. 7.36). The increased concentration of Ca^{2+} in cytoplasm then leads to a variety of cellular/physiological functions and also binds with K^+ ion channel on plasma membrane from the cytoplasmic side and opens the channel for potassium to move out of the cell, thereby causing hyperpolarization (blocking firing of neurons).

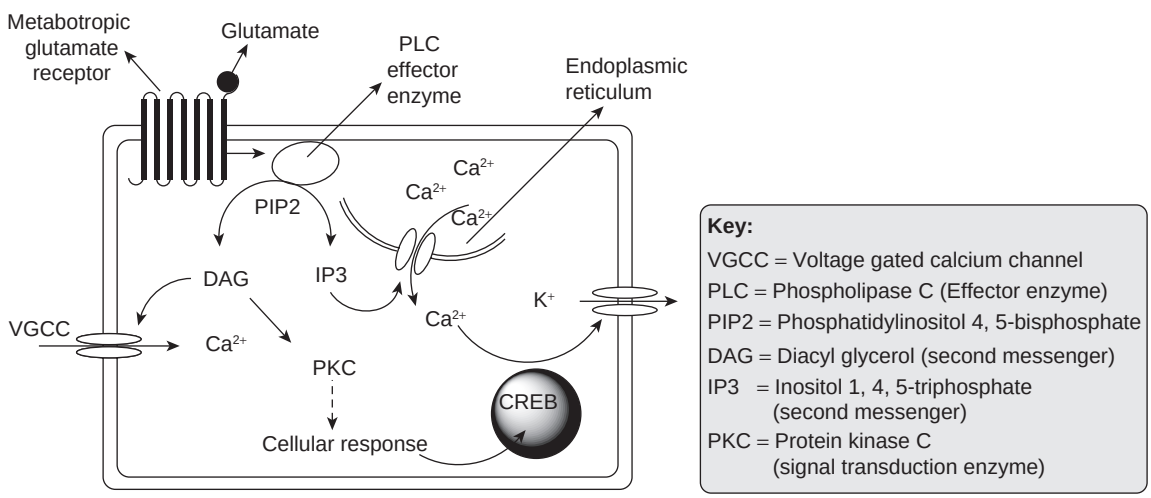


Figure 7.36 Following activation of GPCR, second messenger IP3 is released which binds with ion channel on the endoplasmic reticulum following which Ca^{2+} ion is released.

7.3.3.6 Cyclic Nucleotide–Gated Ion Channels

As the name suggests, these ion channels open up in response to yet another second messenger cyclic nucleotide cGMP/cAMP which is generated intracellularly following binding of a ligand to membrane-bound receptors. For example, receptor guanylyl cyclase (rGC) following its binding with endogenous ligand ANP triggers conformational change in the receptor followed by the intracellular activation of receptor-linked enzyme guanylyl cyclase which in turn catalyses the conversion of GTP to cGMP (Fig. 7.37). The cGMP binds with the ion channels from the cytosolic side and triggers efflux of K^+ and influx of Na^+ and Ca^{2+} ions (hyperpolarization stage).

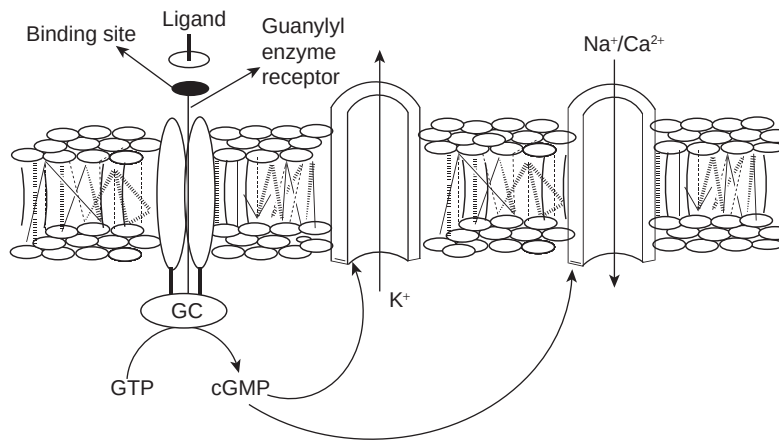


Figure 7.37 Cyclic nucleotide–gated ion channel receptors.

7.3.3.7 Mechanically Gated Channel

As the name reflects, the ion channel opens following a physical distortion of the cell membrane. Many channels that respond to the sense of touch are referred to as mechanically gated (Fig. 7.38).

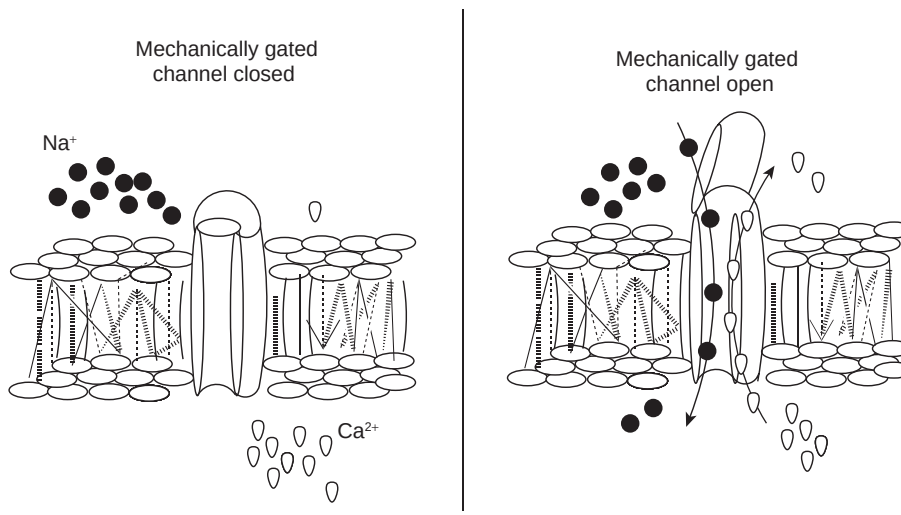


Figure 7.38 Mechanically gated ion channel receptors.

For example, a local change in the temperature of skin or application of pressure on the skin may cause these mechanically gated channels open and allow ions to enter the cell for the flow of pain electrical signals through the neurons.

7.3.4 Intracellular Receptors (Nuclear Receptors)

These classes of receptors are located within the cytoplasm/nucleus of the cell and are generally activated by hydrophobic ligand (first messenger) that can pass through the plasma membrane. The mechanism involves entry of hydrophobic messenger in the cell followed by its binding to receptor which triggers dimerization of receptor and binding to a coactivator protein. The resulting complex next binds to DNA to activate or inhibit transcription/protein synthesis (Fig. 7.39).

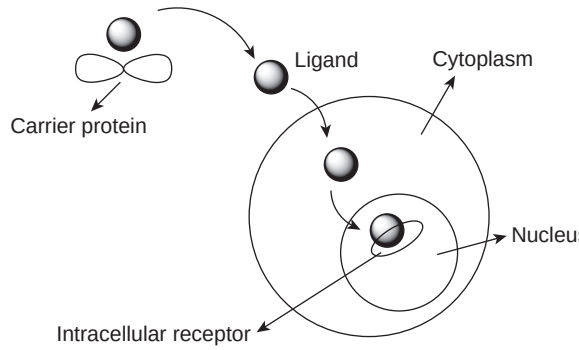


Figure 7.39 Intracellular receptors.

7.3.4.1 Examples of Intracellular Receptors

Examples of ligands for intracellular receptors comprise lipid-soluble hormones (steroids/thyroid), fat-soluble vitamins, fatty acid metabolites, and cholesterol metabolites. They are able to diffuse directly through the lipid bilayer cell membranes of both the endocrine cell where they are produced and that of the target cell where they bind and exhibit pharmacological response.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptor superfamily comprising three subtypes: PPARα, PPARγ, and PPARβ/δ. They are stimulated in response to small lipophilic ligands.

1. Activation of PPAR-α reduces triglyceride level. It is the target for fibrates, a class of drugs used in the treatment of dyslipidaemia.
2. Activation of PPAR-γ causes insulin sensitization and enhances glucose metabolism. It is the target for the antidiabetic drug thiazolidinediones (glitazones; ligand and agonists).
3. Activation of PPAR-β/δ enhances fatty acid metabolism.

7.3.4.2 Thiazolidinediones (Glitazones) as Antidiabetic Drugs Activates Nuclear Receptor PPAR-γ

These nuclear receptors are found in muscle, liver, and fat. Stimulation of PPARγ regulates diabetes type II by upregulating the expression of genes involved in lipid and glucose metabolism, insulin signal transduction, and adipocyte differentiation. The thiazolidinone class of drugs (agonists) are synthetic ligands that

bind to and activate PPARγ. The synthetic ligands (drugs) compete with endogenous ligands: free fatty acids (FFAs) and eicosanoids for PPARγ. Among many mechanisms, one mechanism that contributes to the hypoglycaemic effect of thiazolidinediones involves an increased expression of the glucose transporter GLUT4 (Fig. 7.40). The increased expression of GLUT4 increases the ability of cells (e.g. adipocytes) to take up glucose from extracellular region in the presence of insulin.

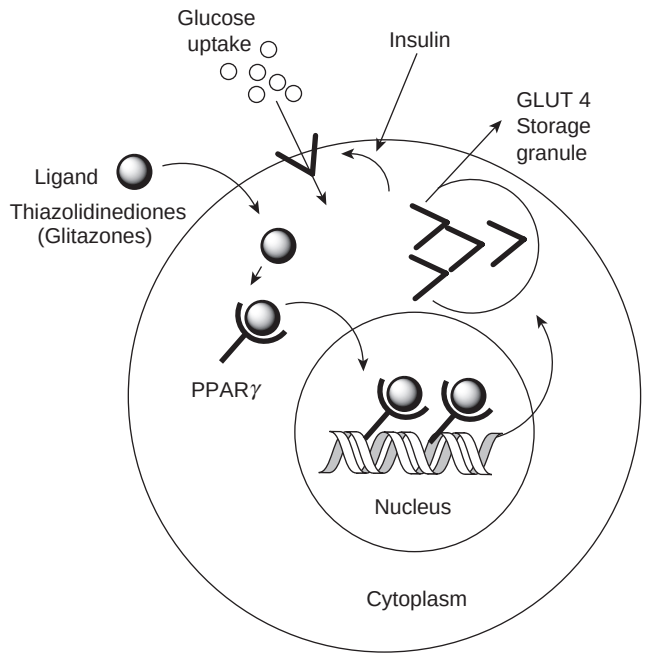


Figure 7.40 Glycaemic mechanism of action of thiazolidinedione 'insulin sensitizers' in adipocytes via intracellular receptor.

Exercises

1. Discuss role of GPCR as therapeutic targets.
2. Explain mechanism of GPCR in intracellular signal transduction.
3. Describe classification and distribution of receptors in cells.
4. Explain role of enzyme linked receptor with an example.
5. Discuss roles of GPCRs in cardiac cells with opposite effects.
6. Explain GPCR activation via second messenger with an example.
7. Write differences between (i) ligands and enzymes; (ii) receptors and enzymes.
8. Describe ion channel receptors with examples.
9. Write short notes on:
   - **(i)** Protease activated receptors
   - **(ii)** Ligands
   - **(iii)** Intracellular receptors with example

10. Discuss roles of second messengers cAMP and cGMP in the relaxation and contraction of smooth muscle cells.

11. Explain resting-activation-resting stages of GPCR.

12. Discuss degradation and inhibition of second messengers by phosphodiesterase.

13. Role of GPCR in diabetes as therapeutic target.

Competitive Exam Questions

1. The gonadal hormones like estrogens, androgens and progestins bind with

(a) receptors located in the cytoplasm.

(b) receptors located in the nucleus.

(c) receptors located in the vacuoles.

(d) none of the above.

[***GATE Pharmacy 1998***]

2. Identify the receptor which demonstrates the fastest onset response, when stimulated.

(a) Nuclear receptors

(b) GPCR

(c) Insulin receptors

(d) Ionotropic receptors

[***GATE Pharmacy 2007***]

References

1. Stephen J. Hil, *Br. J. Pharmacol.*, **2006**, *147*(Suppl 1) S27–S37.

2. Coleman, J. L. J., Ngo, T., and Smith, N. J. *Cellular Signaling*, **2017**, *33*, 1.

3. Christofer S. Tautermann, *Bioorganic and Medicinal Chemistry Letters*, **2014**, *24*, 4073–4079.

4. Hauser A. S., Attwood M. M., Rask-Andersen M., Schiöth H. B. and Gloriam D. E., *Nature Reviews Drug* Discovery, **2017**, *16*, 829.

5. Lutz Heinis, Kenji Ishiinit, Shaun R. Coughlinn, and Brian K. Kobilk, *J. Biol. Chem.*, **2016**, *269*, 27719.

6. Coughlin S. R., *J. Thromb. Haemost*, **2005**, *3*, 1800.

7. Calebiro D. and Godbole A., *Best Pract. Res. Clin. Endocrinol. Metabol.*, **2018**, 32, 83.

8. Alexander Sorkin and Mark von Zastrow, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 609.

9. Aude I. Ségaliny, Marta Tellez-Gabriel, Marie-Françoise Heymann, and Dominique Heymann, *J Bone Oncol.*, **2015**, *4*, 1–12

CHAPTER

8

Drug Receptor Theories

KEY THINGS TO LEARN FROM THIS CHAPTER

- Drug-receptor occupation theories: Hypothesis of Clark, Ariens and Stephenson, Simple Occupancy theory, rate theory, two state theory, macromolecular perturbation theory, activation aggregation theory, receptor cooperativity and mobile receptor models.
- General mechanism of drug-receptor interaction: Lock and Key hypothesis and Induced fit model.
- Drug-receptor complex formation and dissociation: The dose response relationship,
- Binding affinity, K_D, calculation of receptor occupancy.
- Drug Affinity, Drug efficacy/response (Intrinsic Activity) (E_{max}), Drug Potency
- Spare receptors.
- Agonists, partial agonists and inverse agonists.

In general, drugs exhibit their physiological response following their binding to the desired therapeutic protein target; it could be a receptor, an enzyme or a cell membrane impregnated transporter protein. In addition, drugs also bind to plasma proteins affecting their distribution and metabolic enzymes for their metabolism and excretion, thus regulating ADME or life span of drugs inside the body. Finally, drugs may cause toxicity (T) by binding to off-target proteins, such as blocking of hERG channels that leads to cardiotoxicity and sudden death. In this chapter, functional parameters involved during the interaction of drugs with receptors are discussed.

8.1 Drug–Receptor Occupation Theories

Receptor theory provides a relationship between occupancy of receptors by drugs, drug-receptor interaction and quantification of therapeutic response. Several theories have been discussed to describe the basis and model of receptor occupancy by drugs and consequent prediction of biological response. Some of the most common theories are outlined below.

8.1.1 Hypothesis of Clark

'*The Pharmacologic effect of the drug depends on the percentage of the receptors occupied*.' Occupation of receptors can elicit maximal response and the ligand–receptor binding will follow the Law of Mass Action.

8.1.2 Hypothesis of Ariens and Stephenson

'Effectiveness of a drug lasts as long as the receptor remains occupied.' Many substances possess different effects:

1. Some have high affinity for the receptor.
2. Some have low affinity.
3. Some are not effective, and those ineffective substances block or inhibit the receptor.

8.1.3 Simple Occupancy Theory

Intensity of a pharmacological response exhibited by a drug is directly proportional to the number of receptors occupied by that drug. In other words, maximal response may occur when all available receptors have been occupied (Fig. 8.1). However, the occupancy theory fails to provide any explanation for the difference in the activity exhibited by two drugs bound maximally to all receptors for the same disease.

In general, administration of drugs is accompanied by a biological response; however, the observed response following occupancy of receptors by ligands is not a linear function; instead, it follows a hyperbolic path. This suggests that for a full pharmacological response all receptors need not be fully occupied. Thus, maximal biological responses can be observed even when a fraction of available receptors have been occupied (less than maximal receptor occupancy).

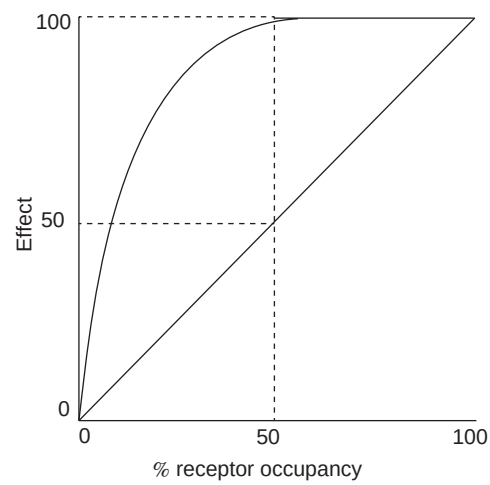


Figure 8.1 Receptor occupancy and biological effect.

8.1.4 Rate Theory

Hypothesis of Paton describes, *'Effectiveness of a drug does not depend on the actual occupation of the receptor but by obtaining proper stimulus.'*

8.1.5 Macromolecular Perturbation Theory

The theory postulates that binding of drugs to receptor causes two kinds of perturbations in the molecular protein comprising the target: a specific conformational perturbation induced by agonist leads to a therapeutic response, while a non-specific conformational perturbation effected by antagonist leads to a no response.

8.1.6 Activation Aggregation Theory

The theory suggests that in the absence of drug, protein receptors always exist in a dynamic equilibrium between an inactivated state (T_o) and activated state (R_o). The presence of agonist drug shifts the equilibrium to the activated state while the presence of an antagonist shifts the equilibrium to the inactivated state. Partial agonists are expected to bind both T_o and R_o.

8.1.7 The Receptor Cooperativity Model

The model suggests that when a ligand binds to a one subunit of a receptor, it can either activate or inactivate rest of the subunits leading to either positive or negative cooperativity. Binding of molecular oxygen to hemoglobin is an example of positive cooperativity. The deoxy-hemoglobin as such has poor affinity for the oxygen but binding of a single oxygen to a single heme triggers significant increase in the affinity of oxygen by the resulting mono-oxygentaed hemoglobin. This in turn triggers binding of oxygen to the remaining three chains in a hemoglobin molecule. In the negative cooperativity, binding of ligand to receptor causes significant drop in the affinity of receptor for the ligand thereby decreasing the ability of the receptor to bind to the ligand.

8.1.8 The Mobile Receptor Hypothesis

This hypothesis is based on the fact that protein receptors float freely within the "sea" of lipid bilayer plasma membrane. The mobile receptor model has been proposed to describe the interaction of hormone (First messenger or ligand) with the transmembrane cell surface receptors (floating) and latter's affinity for cytosolic membrane bound effectors such as adenylyl cyclase. Latter plays a crucial role in the signal transduction (Details discussed in Chapter 1) or cell signaling, transducing external molecular signals from the exterior of a cell inside the cytoplasm thereby regulating cellular/physiological functions. Notably, when the receptor is bound to the hormone, the affinity of the receptor for the cytosolic effector is significantly enhanced (reversible association).

8.1.9 Two-State Receptor Theory

The draw back with above traditional theories is that they overlooked mechanism involved in the activation of receptors following their occupancy by drugs leading to the induction of signal. The current concept of two state model of activation theory states that cell surface receptors such as G-protein coupled and ion channels following binding with drugs undergo conformational change thereby leading to the activation of receptors. Thus, two-state model involves occupancy of receptors by drugs followed by change in the conformation of the receptor thereby inducing change in the state of receptor from inactive to active state (Details discussed in Chapter 7).

8.2 General Mechanism of Drug–Receptor Interaction

The receptor located on the plasma membrane or within cells including neurons bind with endogenous ligand or drug. The binding activates the receptors and modulate cellular/physiological functions. The activation of receptor following binding of receptor to the ligand/drug depends on the nature of interactions between them. The drugs/ligands generally bind to the receptors either by lock and key or induced fit models.

8.2.1 Lock and Key Hypothesis

The primary requirement for this model is that shape of both binding site in the receptor and ligand must be complementary to each other. In other words, during binding, the ligand can simply place itself in the binding site and produce a signal. Since the shapes for both of them are complementary, they are called *lock and key model*. This is an older model and is not prevalent among receptors (Fig. 8.2).

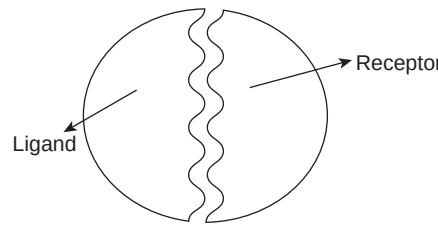
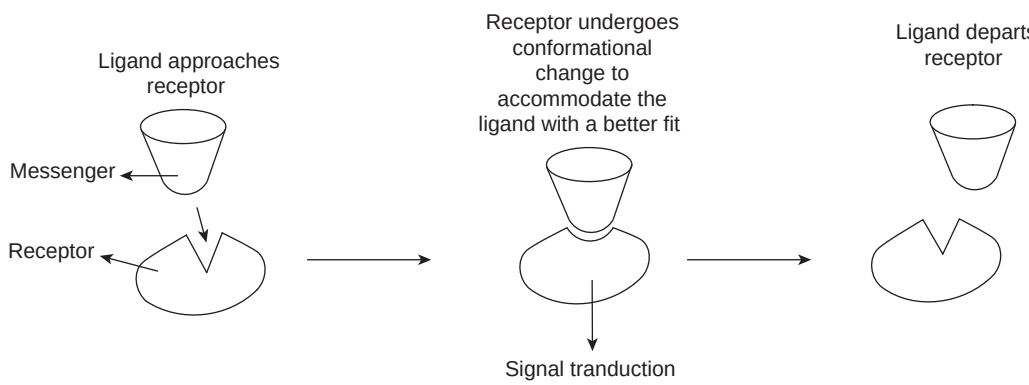


Figure 8.2 Lock and key model.

8.2.2 Induced Fit Model

The induced fit model, proposed by Daniel Koshland in 1958, asserts that when an appropriate ligand makes contact with the binding site on the receptor, the latter undergoes conformational change to accommodate the ligand with a better fit. This adjustment of the receptor to fit the substrate is called *model of induced fit*. A change in the conformation of receptor induces signal transductionand directs the cell to perform its designated physiological/cellular function. After the completion of stimulation by ligand, the conformation reverts back to normal and the ligand is released (Fig. 8.3) and excreted via the following metabolism.



(In a disease situation, drugs can be designed to block or trigger signal transduction).

Figure 8.3 Induced fit model.

8.3 Drug–Receptor Complex Formation and Dissociation

The drug-receptor interaction is governed by the Law of mass action; the rate of formation of drug-receptor complex is proportional to the drug concentration. Nevertheless, the binding of a drug (D) to a receptor (R)

results is a reversible formation of a drug–receptor complex (DR). At equilibrium ($K_{association} = K_{dissociation}$), the rates of drug–receptor complex formation [DR] and its dissociation are equal.

The dynamic equilibrium reaction is governed by rate association constant K_1 for forward reaction ($K_{association}$) and rate dissociation constant K_2 for backward reaction ($K_{dissociation}$).

$$\underset{\text{(Drug)}}{[D]} + \underset{\text{(Receptor)}}{[R]} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \underset{\text{(Drug-Receptor Complex)}}{[DR]} \longrightarrow \text{Response} \qquad \text{(Eq. 1)}$$

Where,
K_1 = Rate of association
K_2 = Rate of dissociation

At equilibrium,

$$\text{Association} = \text{Dissociation}$$

$$K_1[D][R] = K_2[D][R] \qquad \text{(Eq. 2)}$$

Applying law of mass action:
The equilibrium constant:

$$\text{Equilibrium association constant, } K_A = \frac{[DR]}{[D][R]} = \frac{K_1}{K_2} \qquad \text{(Eq. 3)}$$

$$\text{Equilibrium dissociation constant, } K_D = \frac{[D][R]}{[DR]} = \frac{K_2}{K_1} \qquad \text{(Eq. 4)}$$

Three crucial parameters of drugs that are regulated by DR complex formation are affinity, efficacy, and potency. The biological activity (efficacy) exhibited by a drug is related to the stability of the drug–receptor complex, in other words reflecting binding affinity for the receptor. On the contrary, K_D of the drug–receptor complex represents potency of the drug.

8.3.1 The Dose–Response Relationship

It is a relationship between the dose and therapeutic response in an exposed test population. A normal dose–response curve is hyperbolic whereas log of concentration gives sigmoid curve (Fig. 8.4).

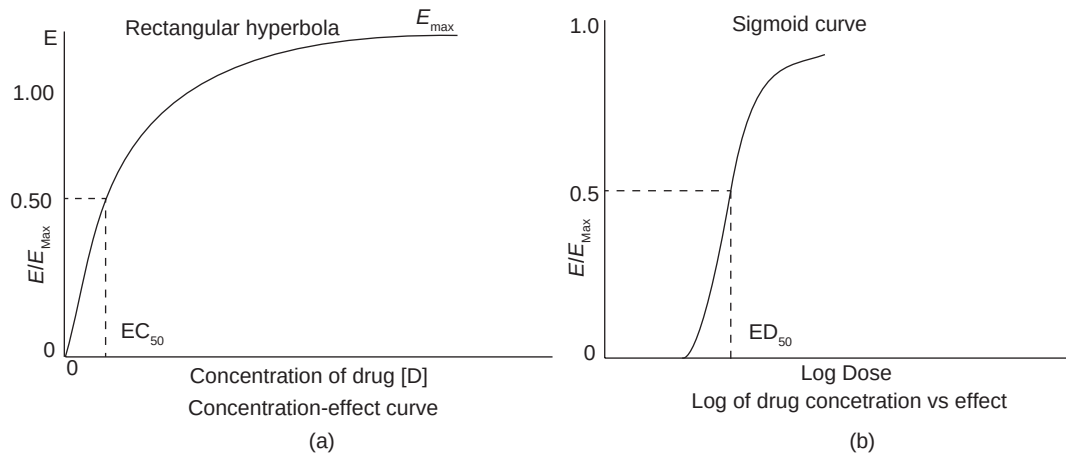


Figure 8.4 (a) Concentration–effect curve and (b) Log of drug concentration–effect curve.

8.3.2 Binding Affinity and K_D

Binding affinity represents the strength of the binding interaction between protein targets to its specific ligand/drug. Binding affinity between the protein and the ligand is affected by non-covalent and reversible intermolecular electrostatic and hydrophobic interactions.

In bimolecular interactions involving drugs and receptors, $K_{association}$ represents binding affinity whereas K_D represents equilibrium dissociation constant. Thus, binding affinity can be assessed by calculating the equilibrium dissociation constant (K_D) that ranks order of strengths of bimolecular interactions. A low K_D value means that the drug has high association and greater affinity for the receptor. Thus, K_D is inversely proportional to K_A and is expressed as moles/L (Eq. 5).

$$K_D \propto \frac{1}{K_A} \quad \text{(Eq. 5)}$$

For example, the K_D value is 6.2×10^{-6} moles/L for warfarin drug, which reflects that when the receptor occupancy by the drug (ligand) is 50%, the number of free/unbound molecules in per liter blood left is 6.2×10^{-6}.

In contrast to the value of K_D which is reflected in moles/L, values for K_A are represented in L/mol. For example, K_A value of 1.6×10^5 L/mole for a drug means that 1 mole of unbound drug is present in 1.6×10^5 L of plasma. A relatively higher value for K_A reflects high affinity of the ligand for the receptor.

8.3.3 Relationship between Formations of Drug–Receptor Complexes with Occupancy of Receptors

A mathematical correlation has been established between formations of drug–receptor complexes with the occupancy of receptors by drug molecules.

In this mathematical equation, P_{DR} (amount of drug bound to the receptor) represents the fraction of receptors occupied by drugs (D), whereas P_R represents fraction of free (unoccupied) receptors.[1] Assuming that sum of free and bound receptors is equal to 1 (when population of receptors remains constant), the fraction of occupied receptors can be calculated using following equations:

$$P_R + P_{DR} = 1 \quad \text{(Eq. 6)}$$

$$K_D \frac{P_{DR}}{[D]} + P_{DR} = 1 \quad \text{(Eq. 7)}$$

$$P_{DR} = \frac{1}{\frac{K_D}{[D]} + 1} \quad \text{(Eq. 8)}$$

$$P_{DR} = \frac{[D]}{K_D + [D]} \quad \text{(Eq. 9)}$$

The equation states that the receptor occupancy of drug relies on K_D and concentration of drugs. Using an alternate mathematical equation, the total population of free or occupied receptors represented by R_T (R_{total})can be correlated with K_D. Accordingly, P_{DR} and P_R can be represented as

$$P_{DR} = \frac{[DR]}{R_T} \text{ and } P_R = \frac{[R]}{R_T}$$

Substituting Eq. 9 in Eq. 5:

$$\frac{[DR]}{R_T} + \frac{[R]}{R_T} = 1 \quad \text{(Eq. 10)}$$

$$[DR] + [R] = R_T \quad \text{(Eq. 11)}$$

$$[R] = R_T - [DR] \quad \text{(Eq. 12)}$$

Substituting [R] in Eq. 11 with by (R_T – [DR]) as in Eq. 12 and rearranging the equation leads to

$$\text{Response} = \frac{[DR]}{R_T} = \frac{[D]}{K_D + [D]} = \frac{\text{Effect}}{\text{Max effect}} = \frac{B}{B_{max}} \quad \text{(Eq. 13)}$$

Where,

B = Observed biological response following binding of drug to receptor

B_{max} = Maximum biological effect following full occupancy of receptors (saturated) by drug or total number of receptors in a particular tissue

[D] = Drug concertation

[R] = Unoccupied receptors

8.3.4 Calculation of Receptor Occupancy

Since K_D deals with receptor occupancy, the percentage of receptor-bound drug (occupancy) at a given drug concentration can be calculated using the following equation (% occupancy of by a drug):

$$P_{DR} = \frac{[D]}{K_D + [D]} \times 100 \quad \text{(Eq. 14)}$$

Let's assume that a drug has a K_D value of 0.50 nM in equilibrium; then the receptor occupation in terms of percentage by the drug at 0.5 and 1.0 nM concentration can be calculated as follows:

$$\%\ \text{Occupancy at } 0.5\,\text{nM} = \frac{0.5}{0.5 + 0.5} \times 100 = 50\%$$

$$\%\ \text{Occupancy at } 1.0\,\text{nM} = \frac{1.0}{0.5 + 1.0} \times 100 = 66\%$$

8.3.5 K_D and EC_{50}

K_D deals with receptor occupancy by drugs while EC_{50} deals with response by drugs at a particular concentration. As depicted in Fig. 8.5, K_D can be represented by the 50% occupancy of the total number of receptors (B_{max}). On the contrary, EC_{50} can be represented by concentration of drug required to give half of the maximal response (E_{max}).

If EC_{50} value for a drug is less than K_D, it reflects that 50% of maximal response for a drug can be obtained even with less than 50% receptor occupancy (Fig. 8.6).

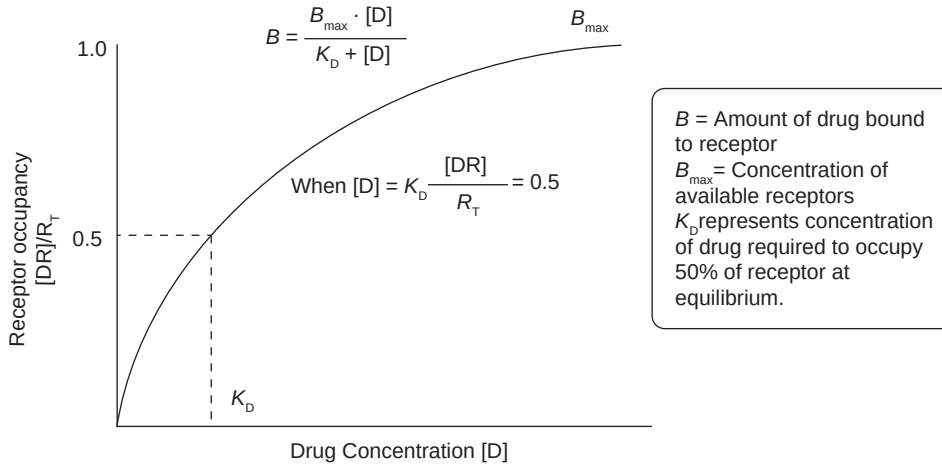


Figure 8.5 A hypothetical curve depicting receptor occupancy and drug concentration.

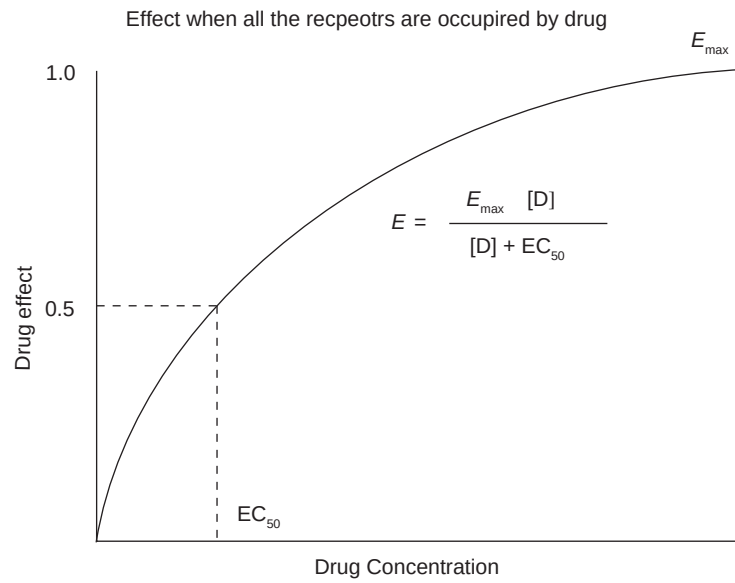


Figure 8.6 A graded dose–response curve.

8.4 Agonists/Partial Agonists/Inverse Agonists

Drugs that activate receptors to produce signal as functional response are called *agonists*. A drug (ligand) following binding with receptors can produce three different biological responses: full, partial, and inverse agonists, and have therapeutic application in drug design (Fig. 8.7). The functional response of an agonist is associated with binding affinity and intrinsic activity as parameters.

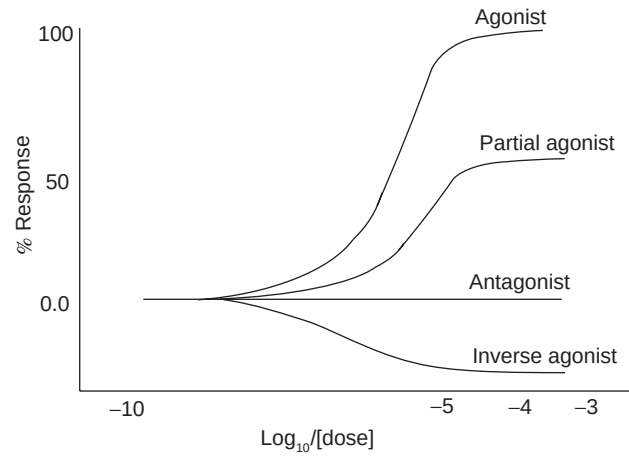


Figure 8.7 Agonists: positive intrinsic activity; antagonist zero intrinsic activity; inverse agonist: negative intrinsic activity.

8.4.1 Drug Agonists

Drugs that produce full pharmacological response following its binding with the receptor are termed *full agonists*. In some cases, they are able to produce maximal response even without occupying the entire population of receptors.

1. They mimic endogenous ligands.
2. They bind and activate the target receptors to produce a signal.
3. They bind to precise molecular regions in the receptor called *binding site*; however, in some cases, drugs bind at a site different from the binding site (allosteric) designated for endogenous ligands.
4. In many drug agonist–receptor interactions, the binding results in a change in the conformation of the receptor. The conformational change may enhance the affinity of the drug for the receptor (induced fit).
5. A single receptor can recognize and bind many dissimilar ligands. This provides an opportunity and rationale to design higher-affinity drugs that need not be derived from the same structure.[2]
6. They have both high affinity and activity.
7. The molecular structure of an agonist dictates the physicochemical properties that contribute to its specific binding to the receptors, e.g. pK_a, hydrophobicity, conformation, stereochemistry, etc.
8. Binding of agonists with receptors involvesnon-covalent interactions such as ionic interaction, Van der Waals forces, hydrophobic interactions, hydrogen bonds, etc.
9. Receptor-binding pockets for agonists are highly specific, and small changes in the agonists can have a large effect on the affinity of the drug–receptor interaction.

8.4.1.1 Drug Affinity to Receptors

Binding affinity[3] describes ability of drugs to bind with a receptor (fast/strong binding = higher affinity). Binding affinity is affected by non-covalent intermolecular interactions involving hydrogen bonding, hydrophobic interactions, ionic interactions and Van der Waals forces between the protein and the ligand. Salient features of affinity are as follows:

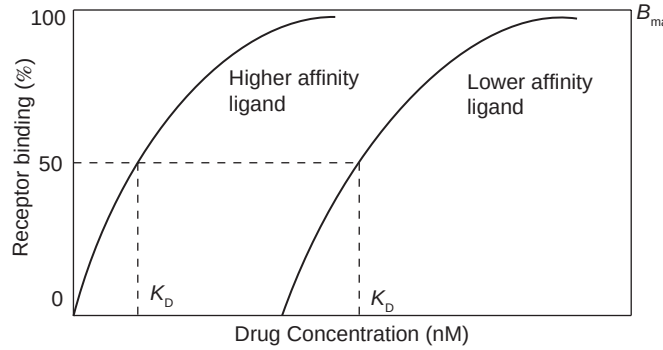


Figure 8.8 A higher-affinity ligand has a lower K_D value.

1. Affinity refers to strength with which ligand is bound to the receptor (Fig. 8.8).
2. Once bound to the receptor, the ligand is activated and elicits signals causing a downstream effect within cells.
3. A low K_D reflects a powerful drug with high receptor affinity.
4. Some drugs may have higher affinity while others may have a lower affinity for the same binding sites in the receptor.
5. A high affinity drug can replace a low affinity drug from the active site.

8.4.1.2 Drug Efficacy (Intrinsic Activity)

Efficacy[4] is the relationship between receptor occupancy and the ability of the drug to elicit a maximal pharmacological response at the physiological/cellular level. It deals with the measurement of the action of a ligand; a drug with low efficacy may elicit a decrease in the maximal response. Efficacy is also referred to as *intrinsic activity* of the ligand/drug and is represented by E_{max}. Efficacy represents the effect of a drug following binding; more the effect, more efficacious is the drug. Some drugs (agonists) have high affinity and high efficacy while some drugs (agonists) may have a high affinity but low efficacy. However, in order to compare difference in efficacy between two drugs, the studies should be carried out at higher doses. Thus, drugs/agonists can differ in terms of their efficacy or maximum response. As depicted in Fig. 8.9, drug A has higher efficacy than drug B.

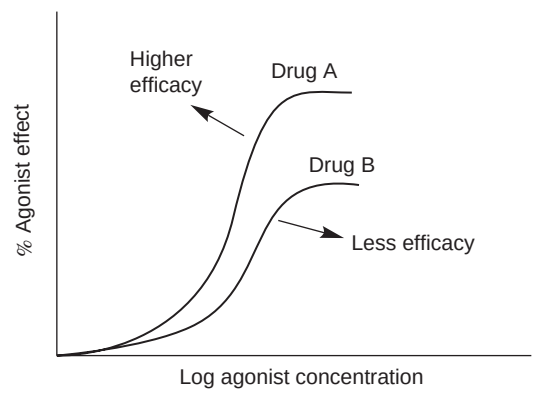


Figure 8.9 Drug A has higher efficacy than drug B.

8.4.1.3 Drug Potency

The potency depends on both the affinity and efficacy, and is a relative term used for the purpose of comparison. Affinity describes ability of drugs/ligands to bind to the receptors while efficacy is an inherent feature associated with drugs and describes its ability to produce therapeutic response. In other words, affinity deals with binding of drug to receptor, and efficacy deals with drug-bound response. Thus, potency can be defined as the amount of drug that is necessary to produce the desired effect. The potency of a drug is commonly represented by EC_{50}, (other parameters used are ED_{50}, LD_{50}, K_D, etc.), the half maximal effective concentration. It can be defined as the molar concentration of an agonist, which produces 50% of the maximum possible response (halfway between the baseline and maximum in the graph) for that agonist.

Differences in drug potency are evaluated by comparing their effective concentrations. A highly potent drug elicits a given response at low concentration while a drug of low potency elicits same response at higher concentration (Fig. 8.10 and 8.11).

Salient features of these comparative studies are as follows:

1. Drugs eliciting same biological response may or may not have same EC_{50} values and may have different efficacy and response (Fig. 8.10).
2. In Figure 8.10, drug A is more potent and has higher affinity for receptor than drug B, as the former elicits same maximal biological response at comparatively lower EC_{50} value.
3. In Figure 8.10, drug B has lower potency because it exhibits higher EC_{50} value.

Similarly, drugs with different EC_{50} values against a given receptor may elicit either maximal response or differential responses (Fig. 8.11). As is evident from this figure, drug C exhibits low efficacy than drug D, but the potency of drug C is more than drug D as drug C shows lower EC_{50} value than drug D.

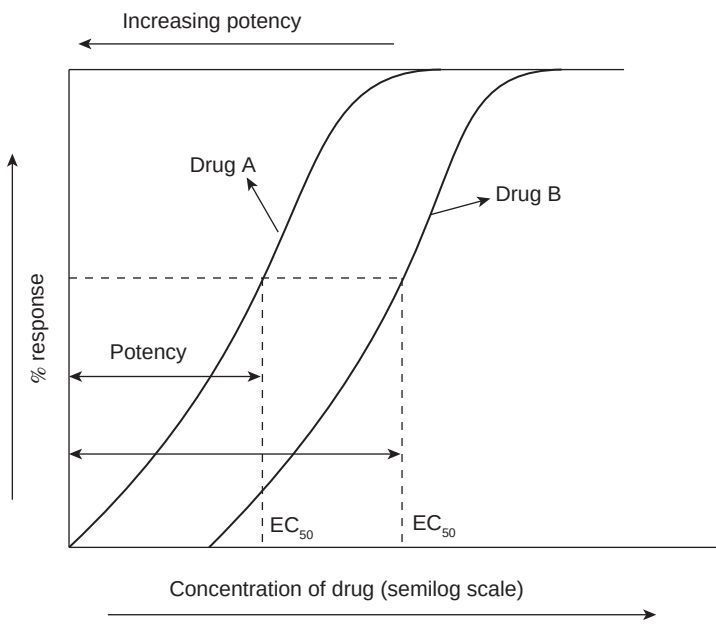


Figure 8.10 Drugs with similar efficacy but differing in terms of their potency.

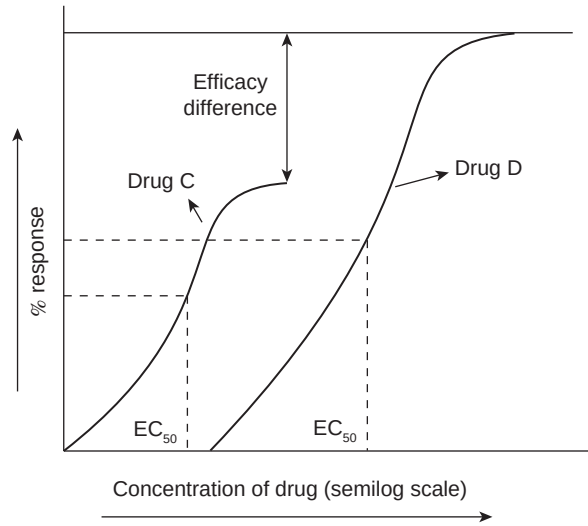


Figure 8.11 Dose–response relationship for two drugs with differential efficacy and potency.

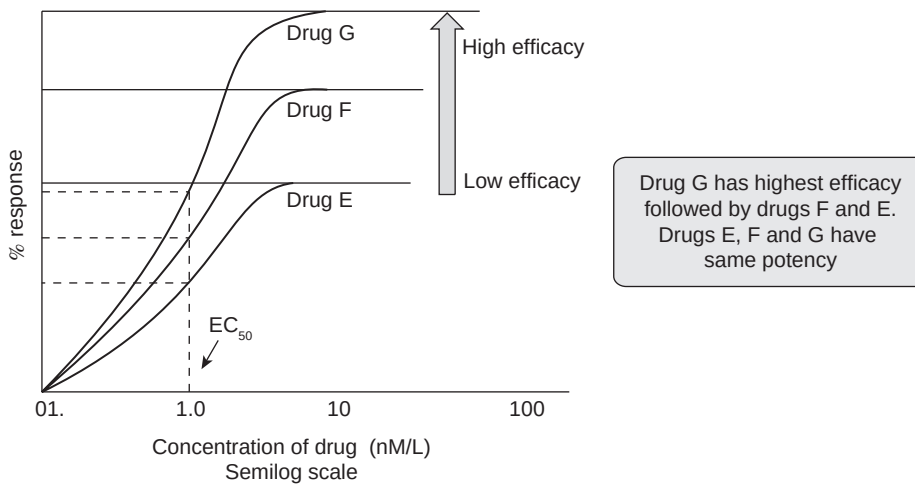


Figure 8.12 Drugs with same EC_{50} (potency) but different efficacy.

A comparison of three drugs E, F, and G with same potency and differential efficacy has been depicted in Fig. 8.12. As is evident, drug G has the highest efficacy, followed by drugs F and E.

8.4.1.4 In vivo *Potency of Drugs*

Potency among different drugs for the same disease can be established by comparing their ED_{50} values. It refers to the dose (ED_{50}) of a drug required to produce 50% of that drug's maximal response. For example, out of the four drugs A, B, C, and D, the biological response for the same disease differs in their ED_{50} values (Fig. 8.13). Drug A is more potent than B, C, and D, since it exhibits lowest ED_{50} value.

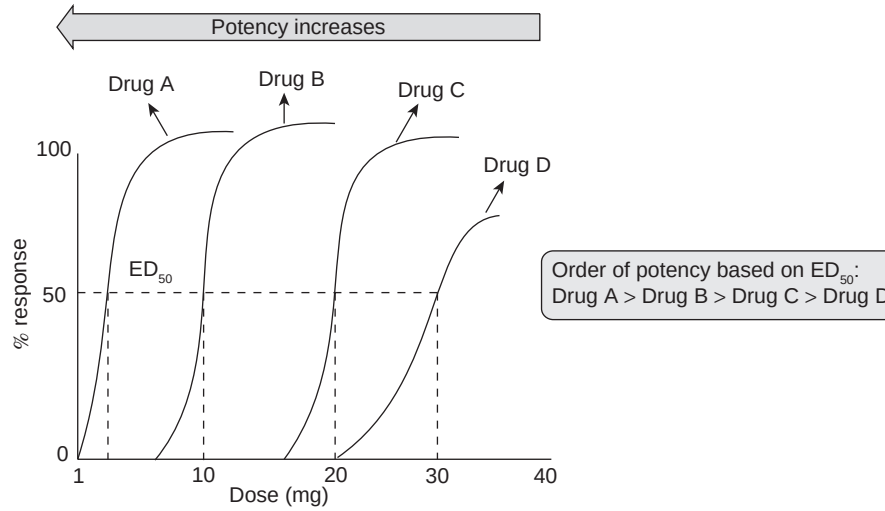
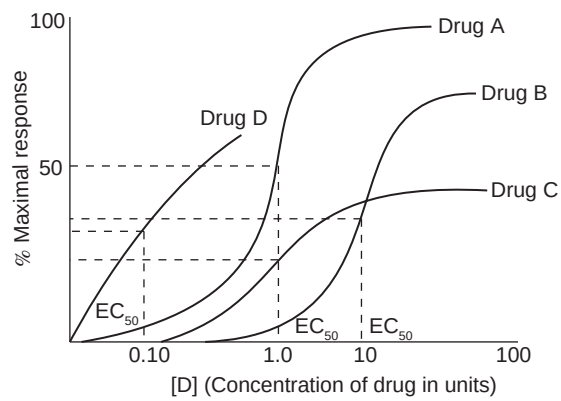


Figure 8.13 *In vivo* potency of drugs is measured by comparing their ED_{50} values.

8.4.2 Partial Agonists

Drugs in general are designed in a manner that they should bind selectively to only one receptor; however, non-biologic drugs can interact with several receptors and elicit diverse pharmacologic/toxic (desired/undesired) responses. The affinity to receptor and therapeutic response of a drug are the two primary factors that control the particular effect manifested by a drug. The magnitude of structurally diverse drugs that binds to the same receptor may differ even if the same number of receptors are occupied in a given disease situation.

Partial agonists are drugs that bind to and activate a given receptor but differ in their efficacy which may be in the form of partial response in comparison to a full agonist. Notably, the decreased response exhibited by partial agonists should not be interpreted in terms of decreased affinity for receptors (Fig. 8.14).



Drug A: Full agonist; Maximal response

Drug B: Partial agonist; low intrinsic activity (efficacy) and potency (higher EC_{50}) than Drug A

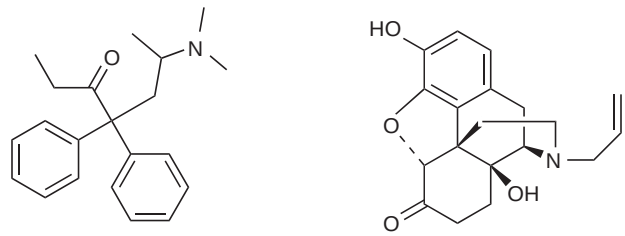
Drug C: Partial agonist; low efficacy than Drugs A and B, potency equivalent to Drug A.

Drug D: Partial agonist; low efficacy, and low EC_{50}(more potent/hihgh affinity) value than Drug A.

Figure 8.14 Characteristics of full and partial agonists.

8.4.2.1 Therapeutic Implication of Partial Agonists

Buprenorphine (analgesic), a partial agonist at the μ opioid receptor, is clinically used for treating patients addicted to opioids (Fig. 8.15). It has a very high affinity with low EC_{50} value (more potent) and low intrinsic activity at the μ receptor in comparison to Methadone (Fig. 8.15), which is a full agonist, at the same receptor. Thus, buprenorphine due to its partial agonistic properties (Fig. 8.16) coupled with high potency is able to readily displace methadone and other opiates with full agonistic properties albeit having less potency. Therapeutic use of buprenorphine has several clinical advantages over full agonists: cannot be displaced by full agonists, better safety profile associated with overdosing, minimal drug addiction, and withdrawal symptoms.



(a) Methadone (b) Buprenorphine

Figure 8.15 Example of full and partial agonist drugs.

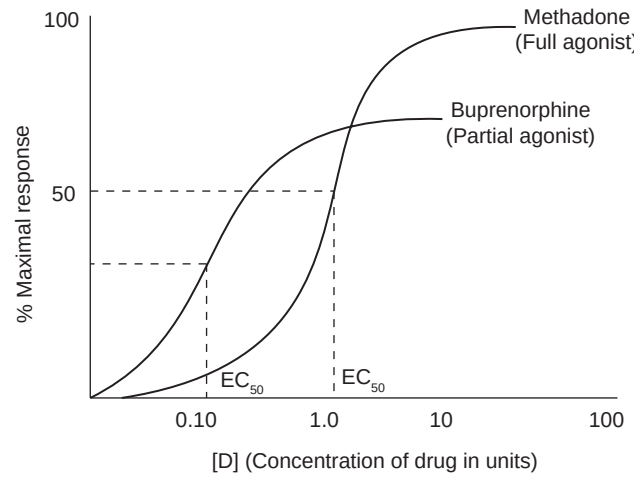


Figure 8.16 Examples of full and partial agonists.

8.4.3 Inverse Agonists

Some receptors such as dopamine, cannabinoids, and benzodiazepines display certain basal level of activation (constitutively active) even in the absence of ligands. Drugs that are administered to reduce the basal level of inherent activation are called *inverse agonists*. For example, in general, antihistamines (antagonists) for H1 and H2 receptors act as inverse agonists. Similarly, the therapeutic responses for numerous anti-psychotics and AT1 blocker drugs are associated with inverse agonism (negative intrinsic efficacy).

8.5 Spare Receptors

The drug receptor binding involves interaction between two chemical entities and, therefore, the concentration of drugs and population of receptors are crucial for biological response. Three scenarios can arise at the site of action:

1. Concentration of drug is more than the population of receptors.
2. Population of receptors is more than drug.
3. A rare situation where concentration and population of both drug and receptor are equal.

Generally, under the receptor theory concept, it is argued that the manifestation of a maximal response by a drug occurs when all receptors are fully occupied. In that case at the half of the maximal effect, EC_{50} is equal to K_D. However, sometimes, full effect is observed at a fractional receptor occupation leading to 'Spare' receptors (unbound or free receptors). In other words, a maximal biologic response could be seen even when receptors are not fully occupied. It is pertinent to add that there is absolutely no difference between biological response of spare receptors and the receptors occupied by the drugs. They are equally functional and can be responsive when occupied by drugs. Their role can be considered as backup receptors, say if in a disease situation some of the receptors are either blocked or become nonfunctional due to side effect caused by other drugs; in that situation, the spare receptors can bind with desired drugs to give the maximal desired reposne (Fig. 8.17).

Experimentally, the existence of unoccupied receptors can be established by co-administering an agonist with an irreversible antagonist and plotting the observed response versus concentration. As is evident from Figure 8.18, curve B obtained following co-administration of agonist with antagonist has higher EC_{50} value than the agonist administered alone.

The findings suggest that in curve B, a higher concentration of agonists ($EC_{50} = 10$) (in comparison to $EC_{50} = 5$ for agonists alone) is required to generate similar response because irreversible antagonists are occupying some of the receptor population. Notably, the height of both the curves remains the same suggesting availability of unoccupied receptors or spare receptors.

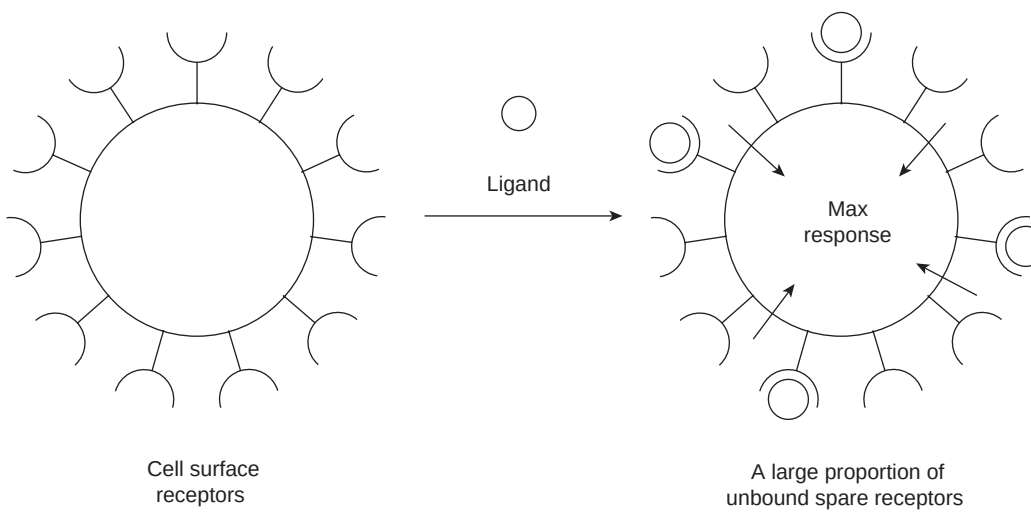


Figure 8.17 Diagrammatic representation of spare receptors.

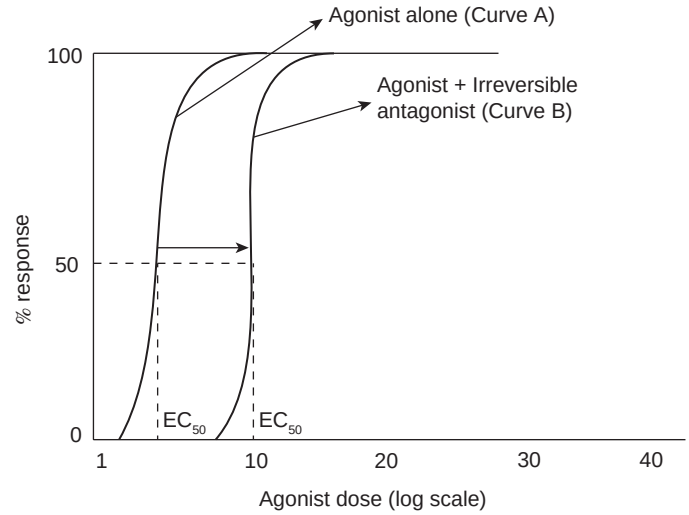


Figure 8.18 A hypothetical experiment demonstrating spare receptors.

8.5.1 Examples of Spare Receptors

Among G-protein-coupled receptors (GPCR), asingle agonist receptor complex can interact with several G-proteins, thereby multiplying the original signal (amplification of signal) by several folds. As we know that the extracellular signal is propagated intracelluarly via 'effector' molecules and second messenger, it is the duration of intracellular activation of this effector that was found to persist even longer than the duration of extracellular drug–receptor complex. As an example β-adrenoceptors (GPCR), an occupation of 90% receptors by ligands is sufficient to give maximal response and approximately 10% are spare receptors. The similar signal amplification phenomenon is also observed for enyzme-linked receptor. Kinetic studies reveal that <1% occupancy of insulin receptors (a class of enzyme-linked receptors) by insulin hormone is sufficient to initiate insulin-response signaling. This implies that 99% of receptors remain unoccupied and are spare receptors.[5] (In healthy adults, daily release of insulin ranges between 30 and 50 units in bloodstream).

Exercises

1. Discuss implications of rate constants in drug-receptor complex formation
2. Explain relationship between binding affinity and K_D (Dissociation constant)
3. Describe receptor occupancy and its consequences.
4. Explain partial agonists with an example.
5. Discuss drug-receptor theories.
6. Write short notes on:
   - **(i)** Induced fit model
   - **(ii)** Drug affinity
   - **(iii)** Drug potency
   - **(iv)** Drug efficiency
   - **(v)** Spare receptors
   - **(vi)** Difference between K_D and EC_{50}
   - **(vii)** Calculation of receptor occupancy
   - **(viii)** Simple Occupancy theory
   - **(ix)** Inverse agonists

References

1. Mark Nickerson, *Nature*, **1956,** *178*, 697.
2. Buyong Ma, Maxim Shatsky, Haim J. Wolfson, and Ruth Nussinov, *Protein Sci.*, **2002**, *11*, 184.
3. Lambert D. G., *Continuing Education in Anaesthesia, Critical Care and Pain*, **2004**, *4*, 181.
4. https://www.princeton.edu/~ota/disk3/1978/7805/780504.PDF
5. Barry G. Saver and Jeremy R. Knowles, *Biochemistry*, **1982**, *21–22*, 5398.

CHAPTER

9

Receptor Antagonists

KEY THINGS TO LEARN FROM THIS CHAPTER

- Orthosteric binding site
- Allosteric site
  - Positive Allosteric modulators (PAMs)
  - Negative allosteric modulation (NAM)
  - Silent allosteric modulators (SAMs)
- Receptor antagonists
  - Antagonists of cell surface receptors
  - Enzyme-linked receptor blockers
  - Ion channel blockers
  - Nuclear receptor antagonists
- Types of Antagonism
  - Reversible Competitive Antagonism
  - Irreversible Competitive receptor antagonism
  - Noncompetitive antagonism

9.1 Introduction

Endogenous ligands generally elicit signals (response) after binding to a precise location present as cavities/pockets on the surface of the receptors. The signal is then transmitted inside the cell or inside the nucleus via signal transduction, which in turn modulates signal specific cellular/physiological functions. These cavities to which ligands bind are referred to as binding sites/orthosteric sites. Often endogenous ligands and drugs may bind the same orthosteric sites. However, sometimes receptors may have additional cavities on the surface very distinct from orthosteric sites called allosteric binding sites. Ligands binding to allosteric sites can modulate the activity of orthosteric sites (Fig. 9.1).

9.2 Orthosteric Binding Site

Orthosteric binding site is defined as the primary binding site (active site) of a receptor to which the endogenous ligands bind to produce its therapeutic effects. Some of the endogenous ligands are hormones, neurotransmitters, steroids, proteins, etc. Similarly, drugs that act as agonists, generally mimic the endogenous ligands and binds to a receptor to produce a signal. On the contrary, there is a type of ligand called *receptor antagonists*

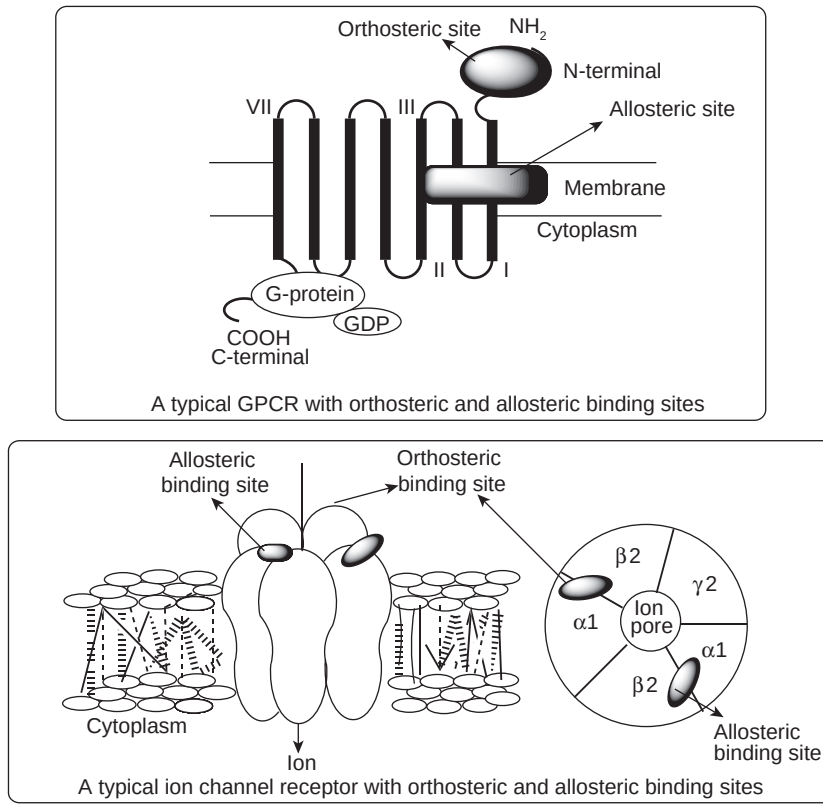


Figure 9.1 A diagrammatic view of receptors with orthosteric and allosteric sites.

that binds to the orthosteric site of the receptor and blocks or diminishes the signal transduction. Endogenous or synthetic ligands, in general, bind to the receptors via noncovalent weak interactions such as hydrogen bonding, hydrophobic binding, ionic interactions, van der Waals interaction, etc. These binding parameters together not only assist in achieving selectivity but also improve efficacy of the compounds. It is pertinent to add that amino acid sequence in orthosteric sites in a family of similar proteins (isoforms) remains highly conserved, thereby causing selectivity issues during the drug development against a particular therapeutic target.

9.3 Allosteric Site

This is a subunit of receptor (secondary binding site, which is topographically distinct from the orthosteric site) that binds with endogenous ligand and induces a change in the receptor's conformation in a manner that regulates the activation/inactivation of the orthosteric sites. A ligand that binds to the allosteric site (Fig. 9.1) in the receptor is known as allosteric ligand. The presence of this druggable allosteric site[1] on a wide range of receptor families offers an alternative approach for therapeutic intervention. Small molecule-based drug can be designed to modulate allosteric receptor function (ligand-based signaling) and bring about desired biological consequences with significantly enhanced selectivity (Fig. 9.2). This may be attributed to the fact that amino acid sequence in the allosteric site is less conserved in comparison to orthosteric sites that remain highly conserved.

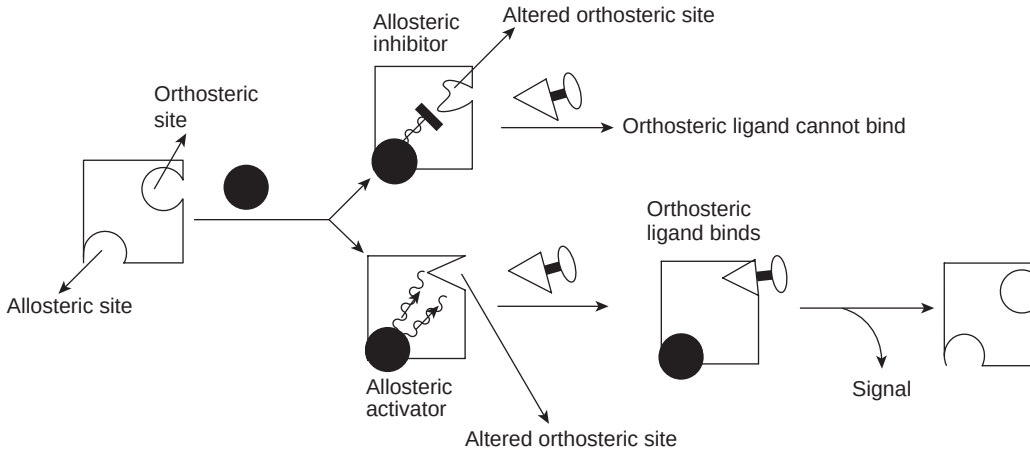


Figure 9.2 Multiple pathways induce conformational change at the active site.

CASE STUDY 1

Orthosteric and Allosteric Binding Sites in $GABA_A$ Receptor

Gamma-amino butyric acid (GABA) receptor ($GABA_A$ is a ligand-gated ion-channel receptor) is a G-protein coupled receptor (GPCR) that primarily binds to endogenous inhibitory neurotransmitter GABA. In central nervous system, GABA remains one of the principal inhibitory neurotransmitters. Most of the sedative and hypnotic drugs bind with either of the three α, β or γ subunits of $GABA_B$ receptor, leading to the activation of the receptors. The activation of receptors results in the opening of chloride channels that causes influx of Cl^- ion followed by hyperpolarization (inhibits firing of neurons) of cells thereby leading to pathological symptoms of depression. Figure 9.3 depicts a simplified GABA receptor with two orthosteric sites for GABA and an allosteric site for benzodiazepines, a class of psychoactive drugs. Latter as allosteric ligands potentiate the action of endogenous ligand GABA.

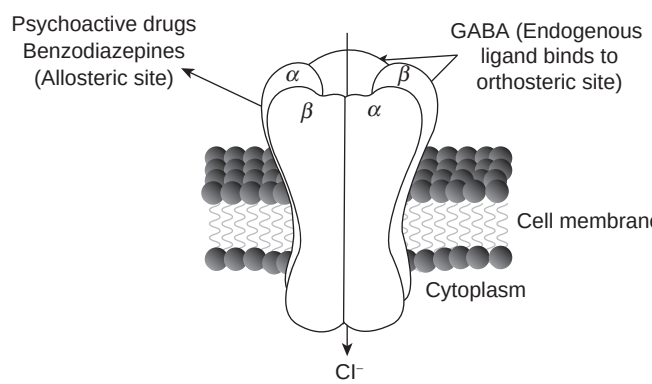


Figure 9.3 Diagrammatic representation of $GABA_A$ receptor with two orthosteric sites and one allosteric site.

9.3.1 Positive Allosteric Modulators (PAMs)

Positive allosteric modulators are also referred to as allosteric enhancers/activators, and drugs under this class are able to increase the activity of a receptor indirectly. The effect of positive allosteric modulators (PAMs) is manifested by their interaction with an allosteric site which in turn amplifies the effect of the orthosteric agonist-mediated receptor response. In fact, their binding to allosteric sites regulates and stabilizes the shape of the orthosteric site to facilitate ligand binding at the orthosteric site with a better fit.

The PAM can either enhance the functional efficacy (Curve b in Fig. 9.4) or enhance the binding affinity/potency (Curve c in Fig. 9.4) of the orthosteric agonist for the target protein.

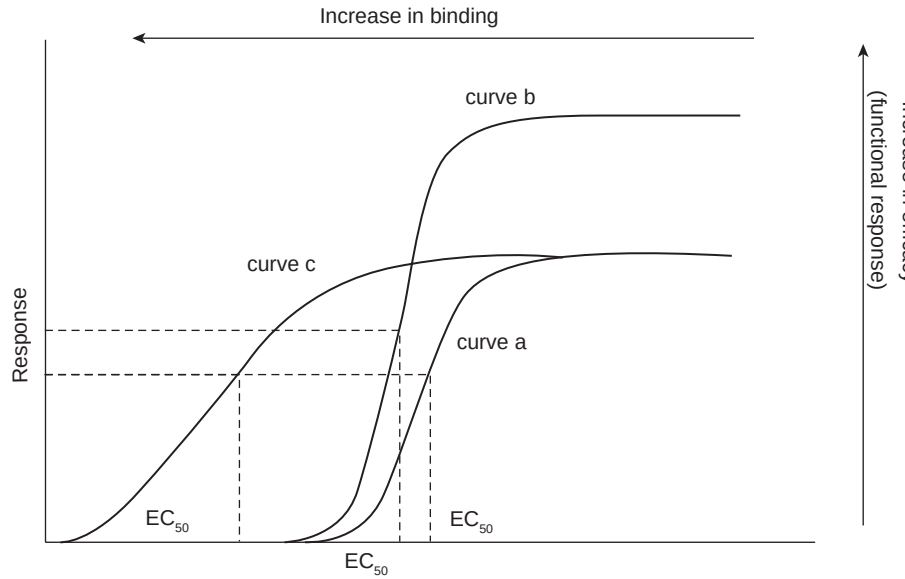


Figure 9.4 An amplification of the orthosteric agonist's effect induced by PAM.

9.3.2 Negative Allosteric Modulation (NAM)

Negative allosteric modulation is also known as allosteric inhibition. This occurs when the binding of an allosteric ligand to allosteric site decreases the potency and efficacy for the orthosteric ligand at the orthosteric site. Following the binding of ligand to the allosteric site, the conformation of the receptor is stabilized in a manner that noncompetitively decreases orthosteric ligand-mediated response (Fig. 9.5). The negative allosteric modulator (NAM) can either reduce the binding affinity/potency (curve b in Fig. 9.5) or reduce the functional efficacy (curve c in Fig. 9.5) of the orthosteric agonist for the target protein.

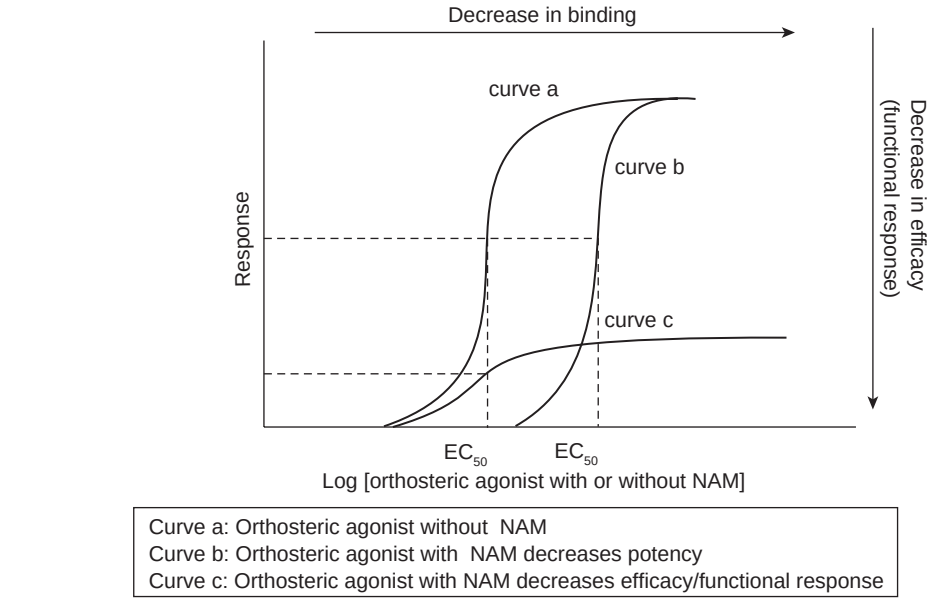


Figure 9.5 NAM induces reduction in the effect of orthosteric agonist either by decreasing the binding affinity (curve 'b') or the functional efficacy (curve 'c').

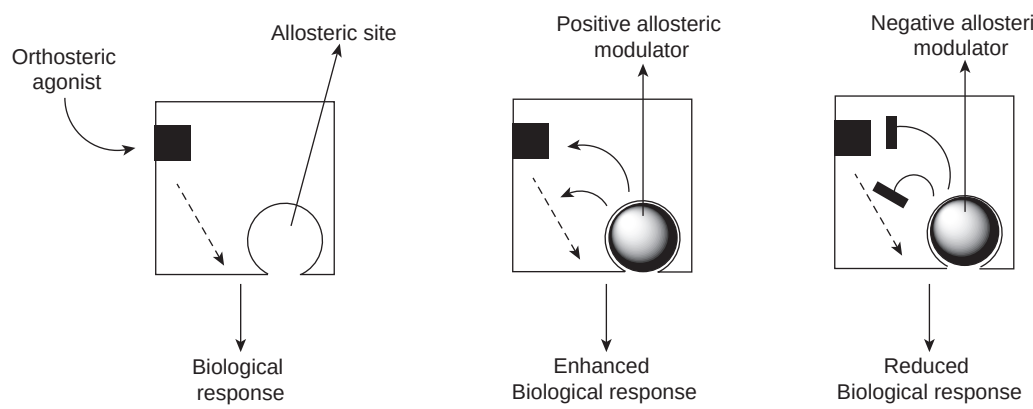


Figure 9.6 Diagrammatic representation of PAM and NAM (↓: activation signal; ⊥: inhibitory signal).

9.3.3 Silent Allosteric Modulators

Silent allosteric modulators are class of neutral allosteric ligands that binds to the allosteric sites without affecting affinity or efficacy of the orthosteric agonist. They are used as competitive antagonists for blocking binding of PAMs or NAMs to the same allosteric site (Fig. 9.7).

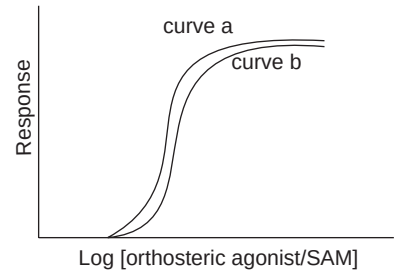


Figure 9.7 Binding of SAM has no effect on the binding and efficacy of orthosteric agonist.

CASE STUDY 2

Drug Maraviroc as PAM for GPCR

CCR5 has been identified as one of the GPCR family coreceptors present on T cells and implicated in HIV infectivity. The mechanism of action for the entry of HIV into the host cell involves initial binding of virus through its gp120 protein to CD4 receptors on T cells. This is then followed by additional binding to CCR5 coreceptors on T cells, which then allows entry of virus in T cell. Maraviroc is a clinically used allosteric drug (NAM) that binds with CCR5 coreceptors and prevents entry of HIV in T host cells. In fact, the allosteric binding of Maraviroc induces conformational change in the CCR5 that prevents attachment of virus through gp120 protein (Fig. 9.8).

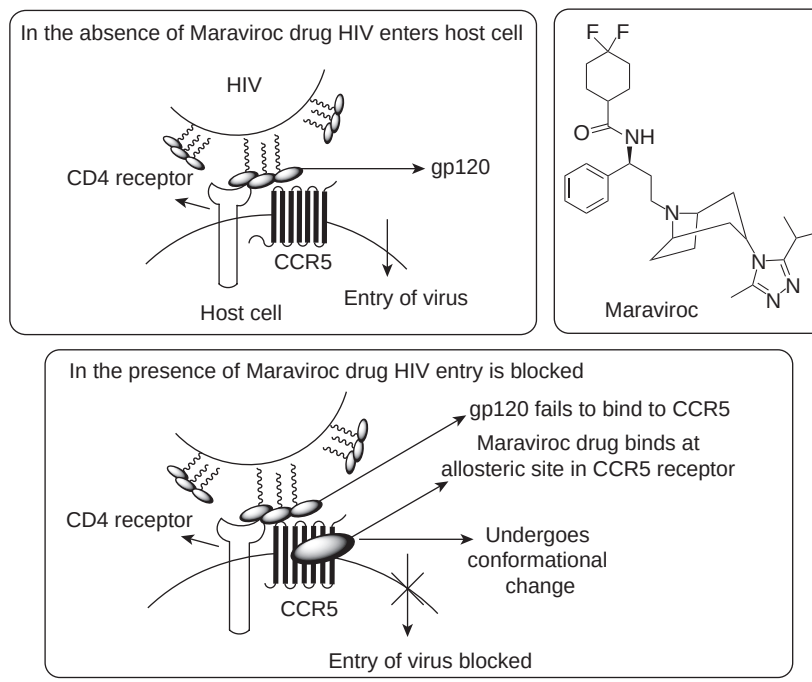


Figure 9.8 Maraviroc as PAM for GPCR.

CASE STUDY 3

Drug Cinacalcet as NAM for GPCR

The calcium-sensing receptors (CaSRs) are family of GPCR and are expressed on the cells in parathyroid glands, thyroid glands, kidneys, and bones. The receptors continuously monitor concentration of extracellular Ca^{2+} and thus play a role in calcium homeostasis (Fig. 9.9). When concentration of Ca^{2+} level drops too low, parathyroid hormone (PTH) is released, which increases the concentration of Ca^{2+} in blood. The secretion of PTH is regulated by CaSR that senses the level of extracellular Ca^{2+}. High level of extracellular Ca^{2+} stimulates CaSR and through number of downstream signaling pathways reduces or blocks the release of PTH.

Cinacalcet, a small organic molecule, is the first GPCR PAM drug to be approved by FDA for treating PTH-related diseases. It allosterically binds to CaSR and potentiates the action of Ca^{2+} at the orthosteric site of the CaSR by reducing the release of PTH (Fig. 9.9).

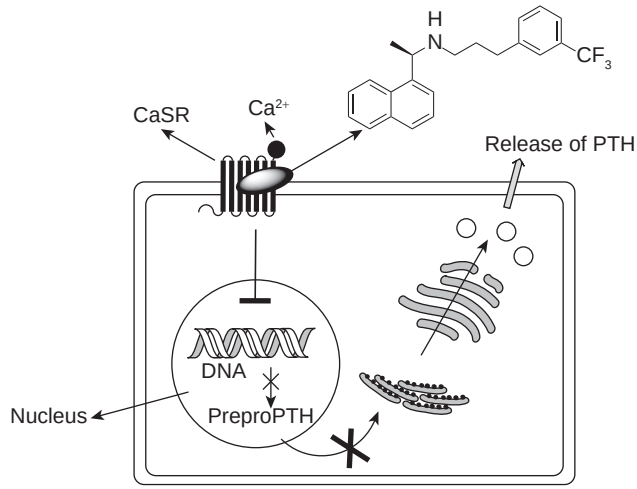


Figure 9.9 Cinacalcet as NAM for GPCR.

CASE STUDY 4

PAM for Ion Channel Receptors

GABA, an endogenous neurotransmitter molecule in brain cells, binds with the extracellular part of $GABA_A$ receptor, a ligand-gated ion channels and signals a decrease in neuronal excitability (Fig. 9.10). Thus, GABA by causing hyperpolarization in the neurons serves as an inhibitory neurotransmitter of central nervous system.

However, in disease situations involving CNS, diseases such as insomnia, anxiety, etc., PAM $GABA_A$ receptor drugs are administered that causes decreased anxiety, sedation, muscle relaxation, and retrograde amnesia. These drugs bind to allosteric site on the $GABA_A$ receptor and potentiate the action of endogenous ligand GABA that is further reducing the neuronal excitability. Indeed, in the absence of endogenous inhibitory neurotransmitter GABA, these PAM $GABA_A$ receptor drugs do not exhibit their activities.

(*Continued*)

Study 4 (*Continued*)

GABA-mediated inhibitory neurotransmission is a major target of modern neuropharmacology, and a variety of structurally diverse CNS drugs with positive and negative allosteric properties have been developed. Examples of $GABA_A$ PAMs include benzodiazepine class of drugs (Fig. 9.11): diazepam (Valium®), alprazolam (Xanax®), zolpidem (Ambien®), and the barbiturate drugs.

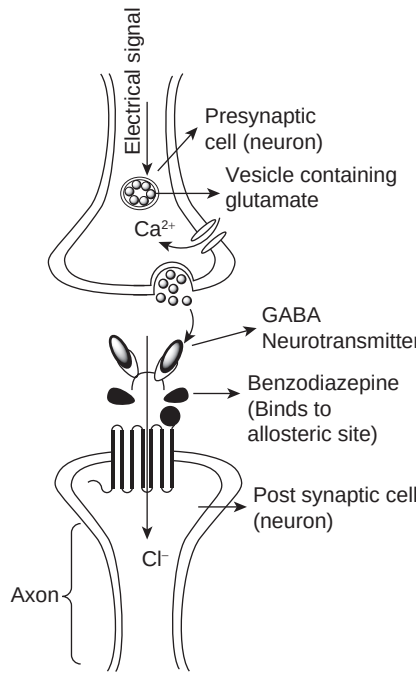


Figure 9.10 Benzodiazepine as PAM binding to allosteric site on $GABA_A$ receptor.

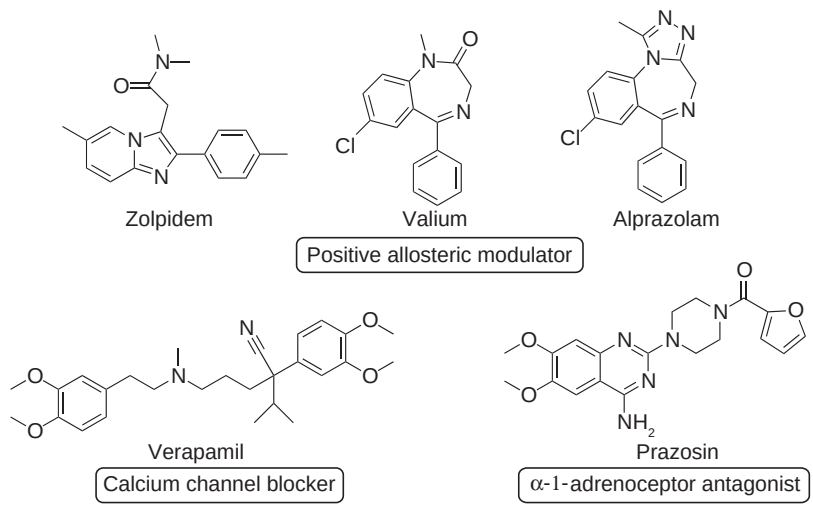


Figure 9.11 Structure of drugs as PAMs binding to $GABA_A$.

9.4 Receptor Antagonists

They belong to the class of molecules (drugs) that have high affinity for the receptor but do not elicit any biological response (no intrinsic activity/no signal). Sometimes they are also referred to as blockers; examples include calcium channel blockers, alpha blockers, and beta blockers. The majority of drug antagonists act by competing with endogenous ligands for orthosteric binding sites on receptors. Generally, antagonists prevent receptor activation and in turn blocks initiation of signals responsible for cellular functions. The drugs can antagonize/block or inhibit both cell-surface receptors well as intracellular receptors with a therapeutic significance.

9.4.1 Antagonists for Cell Surface Receptors

There are three general categories of cell-surface receptors: GPCR, enzyme-linked protein and ion-channel receptors. A number of drugs as antagonists targeting these cell-surface receptors have been developed for treating numerous diseases ranging from CNS to lifestyle to cancer.

CASE STUDY 5

α_1-Adrenoceptor Antagonists

The cell-surface receptor, alpha-1 (α_1) adrenergic receptor (or adrenoceptor) is a GPCR located on vascular smooth muscle. The α_1-adrenoceptors are located in vascular smooth muscle causing vasoconstriction. Endogenous ligand norepinephrine binds with the α_1-receptor, which results in vasoconstriction of smooth muscle of peripheral blood vessels and causes increase in blood pressure.

The α_1-adrenoceptor antagonists (Fig. 9.12) cause vasodilation by blocking the binding of norepinephrine to the smooth muscle receptors. Thus, α_1-blockers are used for treating hypertension as they block $G\alpha_q$/IP3/Ca^{2+} pathway, thereby leading to vasodilation.[2] Some of the clinically used blockers are prazosin (Fig. 9.11), terazosin, doxazosin, and trimazosin.

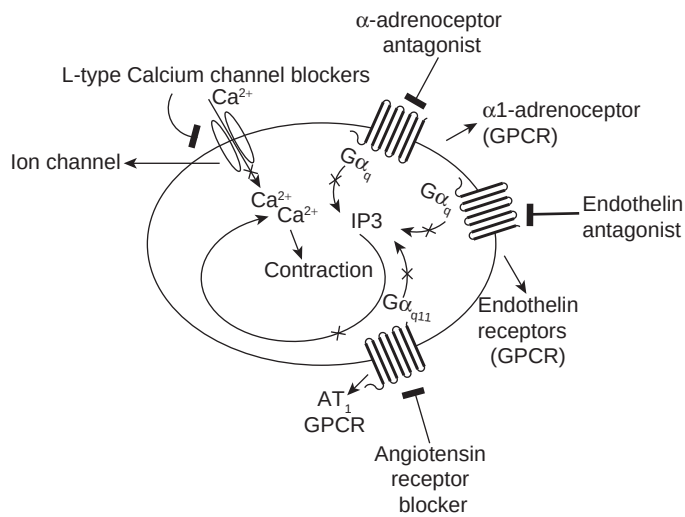


Figure 9.12 A diagrammatic representation of cell-surface receptor antagonists for hypertension (↓: activation of signaling; ⊥: inhibition of signaling).

CASE STUDY 6

Endothelin-1 (ET-1) Receptor Antagonist

Endothelin-1, a 21-amino acid peptide is a potent vasoconstrictor and has been implicated in the pathogenesis of hypertension, coronary vasospasm, and heart failure. It binds to smooth muscle endothelin receptors (two subtypes: ET_A and ET_B receptors) that belong to the family of G-coupled receptors and triggers vasoconstriction via $G\alpha q/IP3/Ca^{2+}$-mediated signal transduction pathway. An ET receptor antagonist (blocks ET_A and ET_B receptors) bosentan was designed to block the signal generated following the binding of endogenous ET-1 and is clinically used in the treatment of pulmonary hypertension (Fig. 9.12)

9.4.1.1 Angiotensin Receptor Blockers (ARBs)

Angiotensin II (RAS) produces the following action of ACE on angiotensin I, binds with AT_1 receptor, a GPCR, and leads to vasoconstriction via $G\alpha_{q11}/PLC/IP3/Ca^{2+}$ signal transduction pathway.[3] A series of clinically used antihypertensive sartans acts as AT1 blockers (antagonists) and prevents IP3 (second messenger)-mediated intracellular signal transduction pathway (Fig. 9.12).

9.4.2 Enzyme-Linked Receptor Blockers

The epidermal growth receptor (EGFR) belongs to the family of enzyme-linked receptors with an extracellular receptor component linked to an intracellular enzyme component. The EGFR is a transmembrane protein, and the receptor binds to family of endogenous epidermal growth factors as protein ligands. Human epidermal growth factor receptor 2 (HER2), a member of the EGFR family, has been implicated in breast and gastric/gastroesophageal cancer. The HER2 receptors are overexpressed on the surface of all cells in breast cancer. Herceptin (a hydrophilic biologics), a monoclonal antibody (also called *trastuzumab*) works as antagonist and binds to the orthosteric site of HER2 receptors on the surface of breast cancer cells. This, in turn, blocks HER2 receptors from receiving endogenous growth signals and slows down or stops the cellular functions of the breast cancer cells (Fig. 7.24 in Chapter 7).

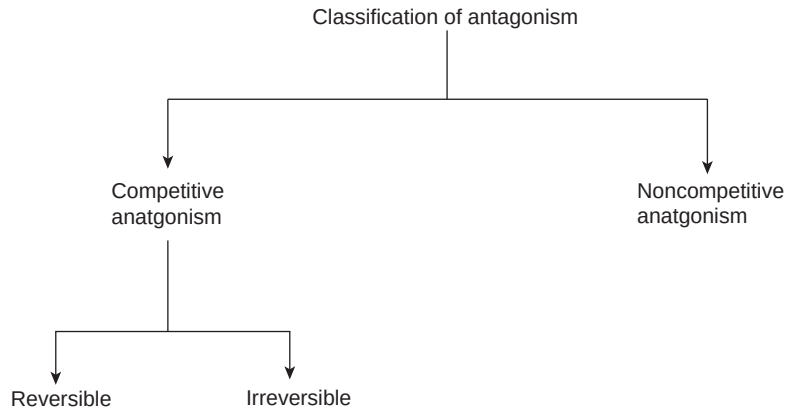
9.4.3 Ion Channel Blockers (Ionotropic Receptor Antagonists)

Calcium channel blockers are among most widely used drugs for regulating vascular tone. The impairment in the vascular smooth muscle tone due to the presence of high level of intracellular Ca^{2+} ion concentration has been implicated in hypertension, angina, and arrhythmias. L-type calcium channels (ligand-gated calcium channels also exist) are voltage-dependent ion channels located in vascular smooth muscle and cardiac muscle cells and display selective permeability for Ca^{2+} ions (Fig. 9.12). Thus, calcium channel blockers/antagonists block entry of Ca ion into cell and can stimulate vasodilation. The clinically used calcium channel blockers/antagonists comprise dihydropyridines (such as amlodipine, nitrendipine) and non-dihydropyridines (Verapamil; Fig. 9.11) used for treating cardiovascular diseases.

9.4.4 Antagonists for Intracellular Nuclear Receptor

In the majority of breast cancer (~70%) patients, there is an overexpression of estrogen receptor ERα in breast tissues (ERα-positive cancer). The natural endogenous ligand estrogen/estradiol for the estrogen receptor plays a crucial role in development and progression of breast cancer. Therefore, blocking estrogen receptor ERα by antagonists/drugs remains one of the best options for treating ERα-positive breast cancer. Tamoxifen has been used as estrogen receptor blocker/antagonist for the treatment of breast cancer.

9.4.5 Types of Antagonism



Flowchart 1 Classification of antagonism.

9.4.5.1 Competitive Antagonism

Competitive antagonism can be both reversible and irreversible.

Reversible Competitive Antagonism

Drug (agonist) is displaced from drug-receptor binding by another chemical by competitive antagonism (Fig. 9.13). Its main features are listed below:

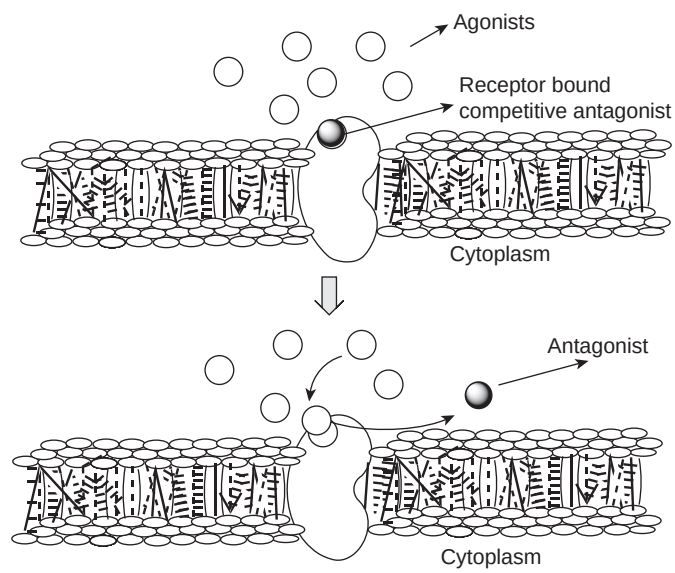


Figure 9.13 Reversible competitive antagonism; increase in the concentration of agonists displaces bound antagonist from the binding site.

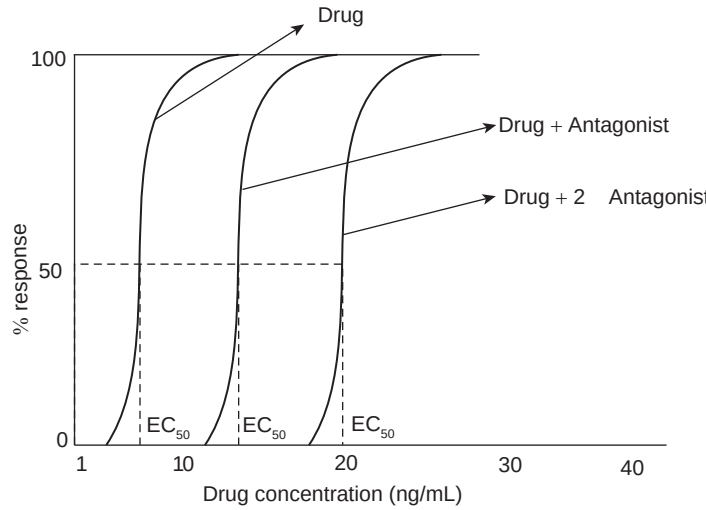


Figure 9.14 Competitive antagonism: Increase in concentration of antagonist increases EC_{50} and decreases potency of agonist (drug).

1. It is reversible and depends on the concentration of actual drug (agonist) and competitive antagonist concentration in the biophase. Increase in the concentration of agonists readily displaces bound competitive antagonist from the binding site. For some drugs, this is the route to end the action of the drug.
2. It follows the typical Law of mass action rule.
3. It competes with same receptor with agonist and reversibly binds to the receptor.
4. The maximum response remains same for any concentration of competitive antagonists (Fig. 9.14).
5. The presence of competitive antagonists, however, increases the EC_{50} and decreases potency of a drug with agonistic property. This is because the agonist and antagonist compete for the same receptors.

Example of Competitive Reversible Inhibition: Prazosin is a highly selective reversible competitive short-acting antagonist at α-1 adrenergic receptors. Blood pressure is decreased within 2 min which lasts for 10–15 min and the antagonist action is terminated following increase in the concentration of endogenous ligand/agonist norepinephrine.

Irreversible Competitive Antagonism

This is caused by compounds that either bind covalently with the receptor or bind so tightly that the compound-receptor complex cannot undergo dissociation (Fig. 9.15).

1. Generally, they are chemically reactive moieties.
2. Following the binding of antagonist to the receptor, the ligands chemically react with chemical moieties on receptor and forms irreversible covalent bonds. This makes the receptor unavailable and thus blocks the activation of receptor for a long period of time.
3. They have high affinity for the same receptor sites.
4. Due to covalent binding of the antagonist with the receptor, the blocking response cannot be regenerated even by increasing the concentration of agonists.
5. Shifts the agonist dose-response curve to the right and depresses maximal responsiveness with decreased potency (Fig. 9.15).

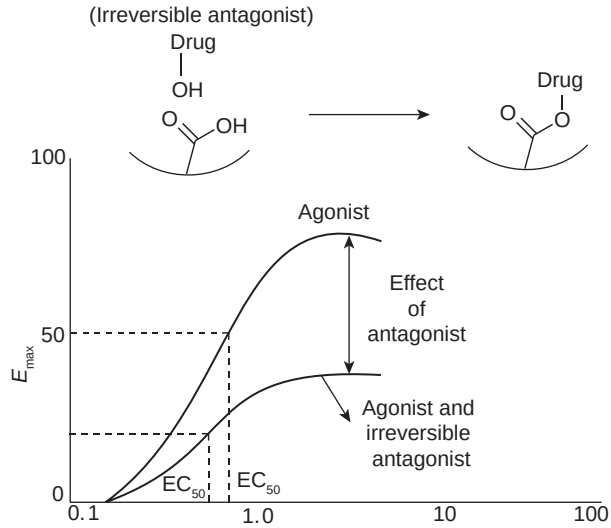


Figure 9.15 Irreversible antagonism: Both efficacy and potency of agonist is changed.

Example of Irreversible Competitive Antagonism: Phenoxybenzamine produces irreversible α-adrenoreceptor blockade (antagonism) via alkylation. The half-life of the drug $t_{1/2}$ is 14–48 h. thus has a long-lasting effect. The medication is used primarily for treating high blood pressure.

9.4.5.2 Noncompetitive Antagonism

Noncompetitive antagonists bind irreversibly (e.g., by covalent bonds) to a different site (allosteric site) and not to the active site and alter the conformation of the actual receptors for the agonist. This in turn prevents the binding of the agonist (Fig. 9.16).

1. Even high doses of agonist cannot overcome this antagonism. Thus, it is considered insurmountable. This is because the agonist and antagonist bind to two different sites.

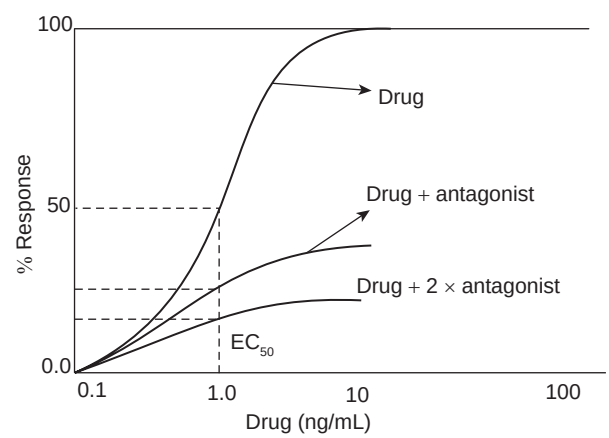


Figure 9.16 The presence of noncompetitive antagonism decreases the maximum response with no change in EC_{50}.

2. The maximum response is decreased; however, there is no change in EC_{50} because both drugs (the agonist and the antagonist) work on different receptors. The same dose of the agonist can still cause the effect, but it is a reduced effect due to availability of receptors.
3. The number of agonist-binding sites appears to be reduced.

Example of Noncompetitive Antagonism: N-methyl-D-aspartate (NMDA) receptors (NMDARs) are ion channels (Fig. 9.17) located in the membranes of post-synaptic neurons and are involved in the transmission of electrical impulse through chemical signals (neurotransmitters). NMDA has several binding sites that regulate the plasticity (ability to adapt to response) of neurons. The sites are as listed below:

1. Binding site for L-glutamate
2. Binding site for Gly
3. Binding site for extracellular Zn^{2+}
4. Binding site for extracellular Mg^{2+}
5. Binding site for polyamines
6. Binding site for drug antagonists: Phencyclidine, Ketamine, Dizocilpine.

In the resting stage of action potential (leading to transmission of electrical signal), the channel remains blocked (prevent ion permeation) following tight binding of Mg^{2+} ions with the NMDAR pore. When an electrical signal arrives from presynapse neuron, L-Glu is released from presynapse into the synaptic cleft. This together with Gly already present in cleft binds with Glu-binding site and Gly-binding site, respectively, on the NMDA receptor and combine their agonistic properties (coagonist) and generate a high-amplitude depolarization that dislodges Mg^{2+} ions from the pore and enable permeability of Ca^{2+} ions.

The internalization of Ca^{2+} ion triggers a host of activities inside neuron and allows movement of Na^{+} from cleft into post-synapse neuron to initiate depolarization leading to firing of neurons. This has important physiological consequences and plays a role in the improvement of memory, brain health and learning abilities. It is pertinent to add that both overexcitation and hypofunction of NMADR can have detrimental effect on CNS activities. Thus, NMDAR serves as an important therapeutic target for numerous CNS/neurodegenerative diseases such as Alzheimer's disease, mood swings, epilepsy, pain, stroke, etc.

Four kinds of antagonists for NMDAR have been studied that inhibit the action of receptor and induce analgesia, amnesia, and dissociative anesthesia. Competitive antagonists for Gly- and Glu-binding sites, allosteric antagonist for allosteric site, and uncompetitive antagonists bind to the site present in the pore, and thus block the pore of the channel. There are two non-competitive antagonists used clinically: memantine[4]

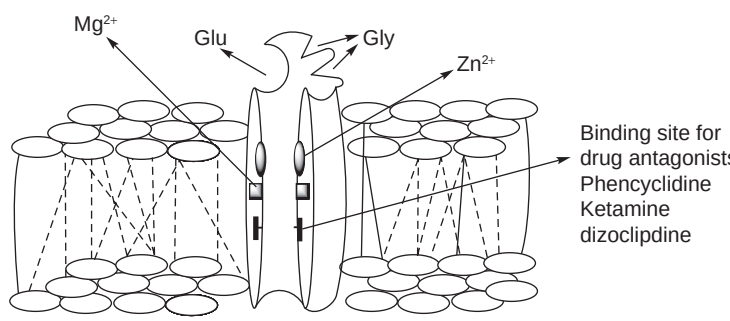


Figure 9.17 A diagrammatic representation of NMDA receptor.

and ketamine.[5] The former is used for treatment of Alzheimer's disease and the latter for inducing anesthesia and for relief from depression. Ketamine blocks the methyl-D-aspartate (NMDA) receptor as a non-competitive antagonist.

9.4.5.3 Salient Features of Antagonists

Reversible Competitive Antagonist	Noncompetitive Antagonist
Binds to orthosteric site	Binds to allosteric site
High concentration of agonist can displace the antagonist	High concentration of agonist cannot displace antagonist
Binds to orthosteric site with noncovalent interactions	Binds with allosteric site with covalent interactions
Presence of competitive antagonist raises the EC_{50} value of the drug (Fig. 9.18)	Presence of noncompetitive antagonist has no effect on EC_{50} value of drug (Fig. 9.18)

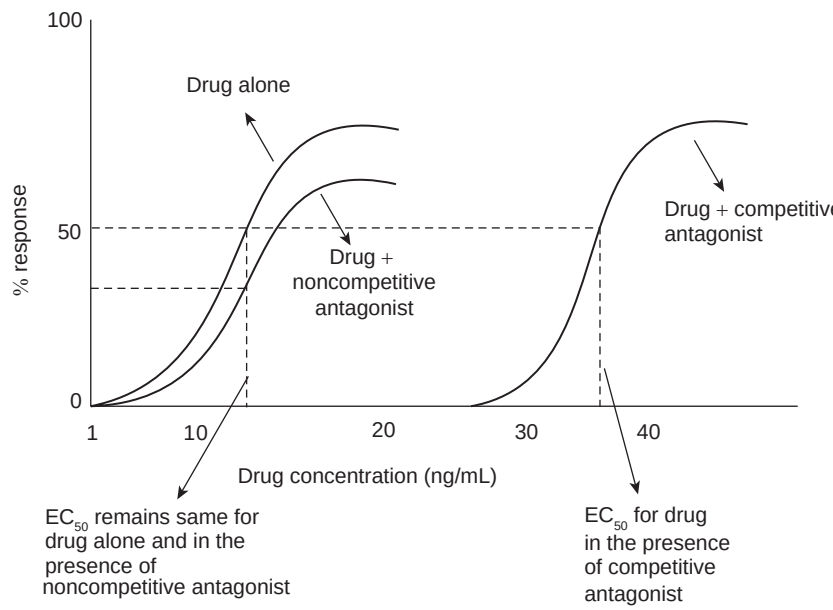


Figure 9.18 Effect of competitive and non-competitive antagonism on EC_{50}.

9.4.5.4 A Unique Receptor Antagonist for Serine Protease Enzyme Thrombin

In general, first messengers for receptors comprise endogenous substances neurotransmitters, hormones, steroids and xenobiotics including drugs. However, a unique phenomenon was observed when serine protease thrombin was found to activate GPCR-based receptor, called Protease activated receptor (PAR-1) and played an important role in platelet activation (Fig. 7.11 in Chapter 7). Thus, PAR-1 serves as an attractive therapeutic target to block to action of thrombin and this led to the development of Voraxapar as thrombin-receptor antagonist which is used clinically as antiplatelet drug.

Exercises

1. Explain different kinds of receptor antagonism.
2. Describe allosteric modulation of receptors.
3. Describe blockers as therapeutic drugs for cell surface receptors.
4. Describe antihypertensive drug antagonist for four cell surface receptors.
5. Write in short notes.
   - **(i)** Orthosteric binding
   - **(ii)** Allosteric site
   - **(iii)** Negative allosteric modulators
   - **(iv)** Antagonists and agonists
   - **(v)** Irreversible antagonism
   - **(vi)** Example of competitive inhibition
   - **(vii)** An example drug that acts as receptor antagonist
   - **(viii)** GABA receptors involved in firing of neurons
   - **(ix)** PAR1 antagonism
   - **(x)** Noncompetitive antagonism with example

References

1. Christopoulos, A., *Nat Rev Drug Discov*., **2002**, Mar; *1*(3):198.
2. O'Connell, T. D., Jensen, B. C., Baker, A. J., and Simpson, P. C., *Pharmacological reviews*, **2013**, *66*, 308.
3. Deng Fu Guo, Yu Lin Sun, Pavel Hamet and Tadashi Inagamil, *Cell Research*, **2001**, *11*, 165.
4. Rammes, G., Danysz W., and Parsons, C.G., *Curr. Neuropharmacol.*, **2008**, *6*, 55.
5. Orser B.A., Pennefather P.S., and MacDonald, J.F., *Anesthesiolog*, **1997**, *86*, 903.

CHAPTER

10

Drug–Target Interactions

KEY THINGS TO LEARN FROM THIS CHAPTER

- Structural features in compounds involved in binding with protein targets
  - Structure of protein targets
  - Role of water in drug-protein interaction
- Reversible drug-target interaction
  - Hydrogen bonds
  - Ionic interactions (salt bridge)
  - Cation-π interactions
  - Van der Waals forces
  - Dipole-dipole/Ion dipole/dipole-dipole induced
  - Hydrophobic interaction
  - π-π stacking interactions
- Irreversible drug target interactions (Covalent bonds)

10.1 Introduction

A drug once administered in our body has to pass through several physiological barriers in the body in order to exert its action by ultimately binding to the specific pharmacological targets (receptor or enzymes) associated with the disease. The ability of a drug to negotiate through these physiological barriers and bind selectively to the desired therapeutic target has been attributed to physicochemical properties of drugs such as lipophilicity, pK_a, molecular weight, polar surface area, solubility, etc.

In general, binding of a drug to a therapeutic target with high affinity for an optimal biological response is regulated by several non-covalent electrostatic and hydrophobic interactions. Thus, during drug design, polar and non-polar (hydrophobic) features need to be incorporated in the molecular architecture that will facilitate its tight binding with the receptor.

10.1.1 Structural Features in Compounds Involved in Binding

The polar and non-polar groups in a drug bind with the corresponding polar and non-polar groups in the binding site of the protein therapeutic target. The polar groups in the binding site comprise side chains of amino acids involving Arg, Lys, Asp, Glu, etc., while side chains of amino acids Phe, Trp, Val, Ile, Leu, etc., comprise non-polar groups (Fig. 10.1). The reactivity of functional groups in the drug/test compound

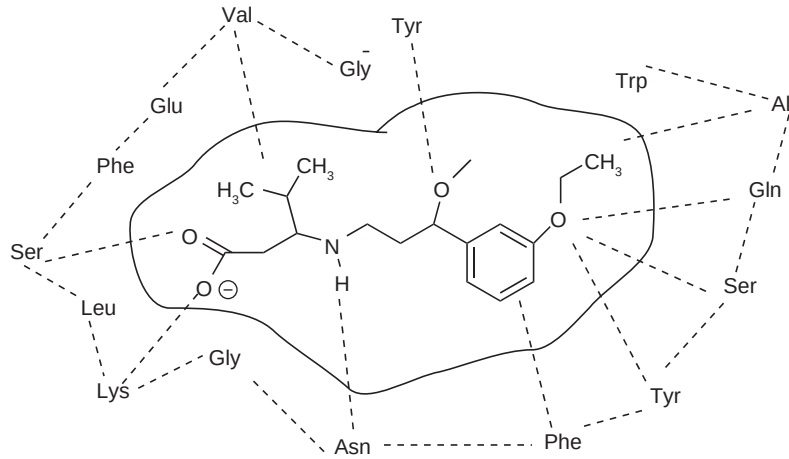


Figure 10.1 A diagrammatic 1D representation of a hypothetical molecule linked through weak interactions with amino acids at the binding site of a hypothetical target.

involved in the binding can be affected by modifying their electronic distribution either by introducing electron withdrawing or donating groups at suitable position(s) within molecules. Nonetheless, this can also affect other physicochemical parameters/activity of the molecule as well.

10.1.2 Structure of Protein Targets

The protein targets (receptors or enzymes) are distributed throughout the body in different tissues and play specific role in different physiological functions. Their biological functions depend on their interactions with specific endogenous ligands/substrates/drugs. The domain in a protein target that associates itself with a ligand/substrate is known as the binding site (active site). The latter are often present on the surface of the protein in the form of pocket or cavity. The binding site is made up of L-amino acids joined together via amide bonds (–CONH–) and are oriented in a three-dimensional format. The deeply embedded water molecules in the target proteins play an important role not only in the structure-function of proteins but also in the drug-target interaction. Depending on the three-dimensional orientation of the amino acids and the presence of water molecules (conserved in homologous proteins) in the binding cavity which is specific for every target, either the side chain of amino acids or the amide bonds may bind with the drug (Fig. 10.2). The drug-target interaction is assisted by desolvation (displacement of water molecules) of both protein target and drug, thereby influencing binding.

Other amino acids of the protein target that are buried in the interior of the protein although have no direct contact with the ligand/substrates; rather, they assist in the orientation of amino acids lining the binding cavity. This is evident from the fact that even a small change in one of the amino acids in the interior of a protein molecule (mutated proteins) can bring subtle change in the contours (shape) sufficient enough to destroy the binding site. For example, the genetic sickle cell anemia disease is caused by a single-point mutation in the β-globin chain of hemoglobin where hydrophilic amino acid Glu is replaced with a hydrophobic amino acid Val. An interesting feature of target proteins is that they are not rigid structures and have flexible regions often associated with protein folding/loops. The movement in the flexible regions (dynamic and not static) occurs in response to mechanical actions led by chemical events. Notably, binding of drugs with receptors induces conformational change in the receptors that may trigger or inhibit a signal transduction while no major conformational change occurs during the binding of drugs with enzymes which, in turn, activates or inhibits a reaction to form products.

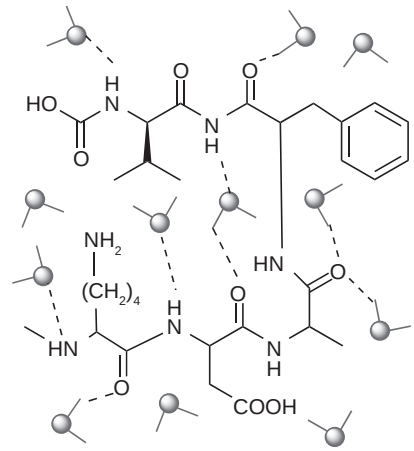


Figure 10.2 A diagrammatic 1D representation of a hypothetical binding site comprising water molecules (in red), amide bonds, and polar and non-polar side chains of amino acids.

10.1.3 Role of Water in Drug-Protein Interaction

The therapeutic protein targets are generally located either extracellularly in the interstitial fluid (including cell-surface receptors) or intracellularly within the cells in the cytoplasm. Since both interstitial fluid and cytoplasm have aqueous environment, water molecules play an important role in both the structure-function-stability of unbound proteins as well as in drug-target and protein-protein interactions. Ability of water molecule to form H-bond both as donor and acceptor allows it to interact with amino acids in the binding pocket via intermolecular H-bonds (interaction between the drug and the target) and also within the binding molecule via intramolecular H-bonds. Among homologous proteins water molecules at the ligand-binding sites have been found to be highly conserved, and most of them are present in deep grooves either at the target-drug interface or completely buried. The buried water molecules besides being rigid like other buried atoms of proteins form H-bonds with peptide (amide) backbone of loops (less buried in α-proteins), with other water molecules and with multiple deeply buried polar atoms of protein. Functionally, buried water molecules display stabilizing effect by filling internal cavities and may also play a role of lubricants during loop dynamics.

Thus, hydration pattern during drug-target binding significantly affects affinity and accompanies desolvation of both binding sites of target proteins and drugs. The expulsion of water molecules from the cavity during drug-target interaction affects binding affinity with favorable contributions from enthalpy and entropy.[1] The thermodynamic component enthalpy reflects changes in energy during H-bonding and Vander Waal interaction, whereas entropy reflects rearrangement of water molecules during the same two interactions. Thus, although water molecules play critical role in drug-target interaction, the exact mechanism involving the interplay between water, target protein, and drug has not yet been fully understood.[2]

10.2 Reversible Interaction

Generally, drugs bind targets reversibly with high degree of specificity. The forces that enable target protein to bind selectively and with high affinity to a ligand comprise a set of weak bonds and can be grouped

under reversible interactions. Besides, depending on the need, drug-receptor interactions may also involve irreversible binding due to covalent bond formation. The interactions have been discussed below:

1. Hydrogen bonds
2. Ionic bonds
3. Van der Waals forces
4. Dipole-dipole/ion-dipole/dipole-dipole induced
5. Hydrophobic interaction

10.2.1 Hydrogen Bonds

Hydrogen bonds are the strongest of all intermolecular forces among reversible interactions. It is an interaction, arising from the attraction between slightly positive hydrogen on one molecule (test compound/amino acid) and a slightly negative atom on another molecule (Fig. 10.3). The H-bond formation is generally depicted by dotted lines between the unshared electrons present on a nucleophile (electronegative atom) and the H atom.

Water, an important constituent of all living things, has unique solvation capabilities due to its H-bonding abilities. In fact, an adult human comprises up to 60% as water wherein the brain and heart are composed of 73% water, and the lungs constitutively have 83% water. Thus, in our entire body, water due to its unique H-bonding capabilities acts as a solvent for a variety of chemical reactions and also helps transport dissolved substances into and out of cells. The H-bonding abilities of water can be attributed to its oxygen atom that has two lone pairs of electrons and two hydrogen atoms, suggesting that up to four H-bonds can be formed by a single water molecule (Fig. 10.3). Besides H-bond forming abilities of water, disruption of H-bonding with water also occurs during the interactions between the biomolecules.

In living organisms, presence of multiple H-bonds contributes to the stability, structure, and function of numerous macromolecules involving proteins and DNA. They also play a critical role in the assembly of the three-dimensional structure of numerous intravital folded proteins including enzymes, receptors, and antibodies. All secondary structures α-helical, β-sheet, and turns (β, γ) in proteins[3] are stabilized by H-bond between –HN– and –CO– in the peptide backbone (Fig. 10.4). Similarly, one of the factors that stabilize the structure of DNA is H-bonds between base pairs (Fig. 10.4).

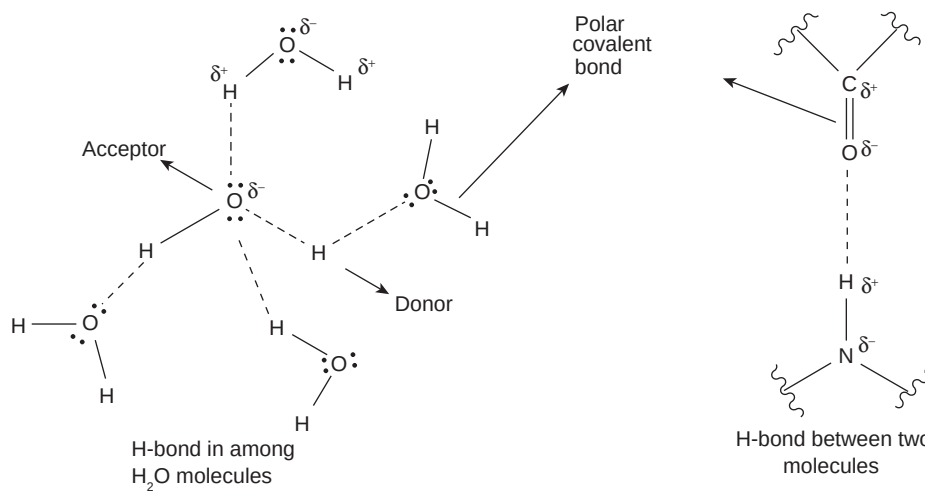


Figure 10.3 H-bonds among water molecules and between two molecules.

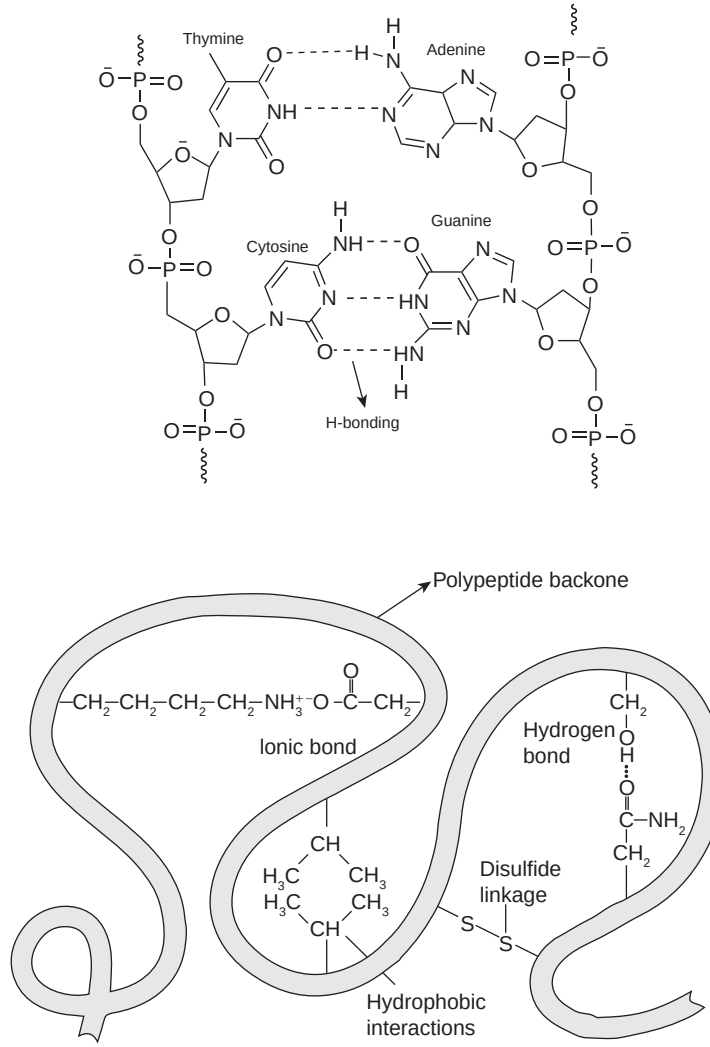


Figure 10.4 H-bonds in DNA and protein.

Although both the biomolecules are stabilized by H-bonds, water plays an important role in DNA-protein interaction. Later, has a fundamental role in the maintenance of genetic material and in the regulation of gene expression. Aqueous conditions not only facilitate interaction between DNA-protein via H-bonds but also result in a loss of H-bonds between respective biomolecule and water. Since the formation of H-bond occurs between two atoms, they are referred to as hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) (Table 10.1).

1. **HBD:** The HBD is the electronegative atom (e.g. N, O, F) of a functional group to which hydrogen(s) is covalently bonded and can readily donate the hydrogen(s) for H-bond formation. For example, hydrogen attached to a strongly electronegative atom such as N, O, or F acts as an HBD.

2. **HBA:** The HBA is the electronegative ion or functional group in vicinity that carries a lone electron pair in order to form an H-bond. In fact, partial positive charge on hydrogen attracts lone pair of electrons located on the neighboring atom to form an H-bond. Examples of electronegative atoms as H-bond acceptors are F, N, or O.

10.2.1.1 Intramolecular H-bonds

Although H-bonds involve HBD and HBA functionalities, they can be either part of the same molecule in proximity leading to intramolecular H-bond or part of two molecules resulting in an intermolecular H-bonding. Out of them, the formation of intramolecular hydrogen bonds has a pronounced effect on molecular structure and properties. A majority of intramolecular H-bonds involve formation of five- to six-membered ring, with reports in the literature for even upto the formation of seven- to eight-membered rings as well. However, there is a possibility that molecules with HBA and HBD in the same molecule may exist in equilibrium between a closed conformation with intramolecular H-bond and an open conformation in which the polar groups are exposed to the environment (Fig. 10.5).

Several groups have demonstrated the role of intramolecular H-bonds on the drug-receptor/enzyme interaction by hypothesizing that conformational restriction due to closed form allows favorable alignment of functional groups within the active site (hydrophobic pocket) of the target protein.

Table 10.1 Examples of HBD and HBA

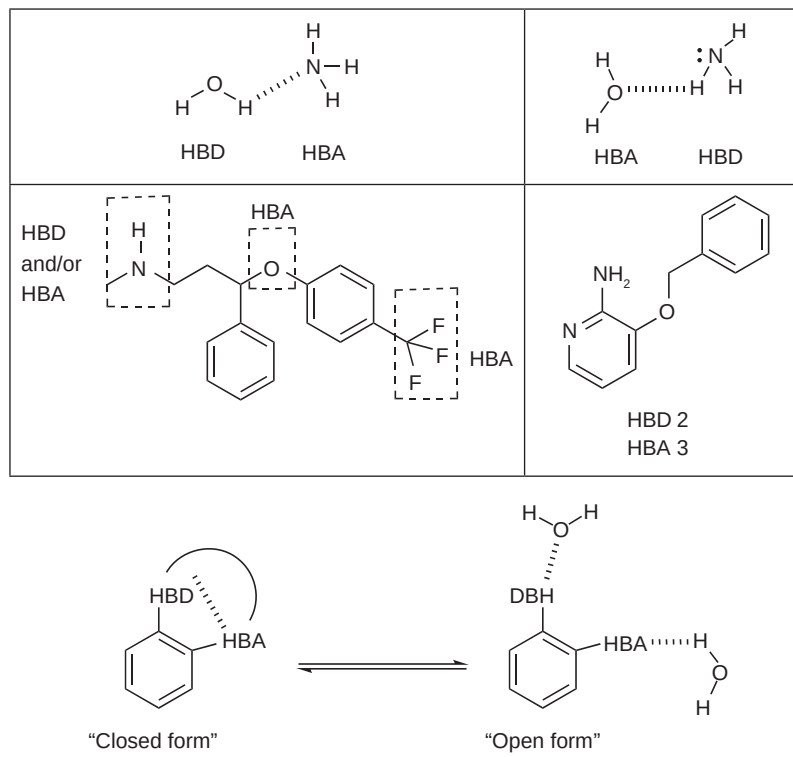


Figure 10.5 Thermodynamic equilibrium may exist between closed and open conformations.

CASE STUDY 1

Effect of Intramolecular H-Bond on Physicochemical Properties of Compounds

In a series of eight stereoisomeric compounds (Table 10.2) based on lactams (N-(5-(1-hydroxypropan-2-yl)-3-methyl-2-((methyl(4-phenoxybenzyl)amino)methyl)-6-oxo-3, 4, 5, 6-tetrahydro-2H-benzo[b][1,5]oxazocin-10-yl)isonicotinamide) (Fig. 10.6) exhibiting growth inhibiting activities against *T. cruzi*, physicochemical parameters: solubility, membrane permeability, lipophilicity, and pK_a were found to be associated with the presence of intramolecular H-bonds. There were two sets of compounds in the series: four of them were *cis* isomers (at C8–C9) with logD values between 3.4 and 3.9, and had higher solubility, whereas remaining four were *trans* isomers with poor solubility and higher logD values of 4.3–4.7. When these compounds were subjected to membrane permeability test in Caco-2 cell monolayer (a human epithelial cell line), increased epithelial permeability was observed for *trans* isomers with higher logD values while lower permeability was observed for *cis* isomers with low logD values. Since the difference in solubility, permeability, and lipophilicity could be due to difference in their pK_a values, determination of pK_a values revealed a difference of 1 unit between *cis* and *trans* isomers. The difference in pK_a values explains the difference in the structure-property relationship observed for *cis* and *trans* isomers. The difference in physicochemical parameters (Table 10.2) between *cis* and *trans* isomers with reference to pK_a and lipophilicity can be attributed to the presence of an intramolecular eight-membered H-bond between N28H and N15 (closed form).

In drug discovery, it is therefore important that among many approaches used for lead optimization, one could also introduce features that would facilitate the formation of intramolecular H-bond (closed form). This, in turn, will shield/hide the polar groups from environment (polar/non-polar solvent in the body) and consequently make the molecule more lipophilic and membrane permeable (enhance bioavailability), whereas the open form should be more water-soluble facilitating binding.

Table 10.2 Comparative physicochemical parameters of *cis* and *trans* isomers

Physicochemical Parameters	***cis* isomers (C8, C9)**	***trans* isomers (C8, C9)**
Solubility (at pH 7.4 in phosphate buffered saline containing 1% DMSO)	86–98 μM	1–2 μM
logD	3.4–3.9	4.3–4.7
Permeability	$<50 \times 10^{-6}$ cm/s	$>50 \times 10^{-6}$ cm/s
pK_{a1} (N34 pyridine)	2.97–2.98	2.76–2.77
pK_{a2} (N15 3′ amine)	7.07–7.16	6.08–6.14
Intramolecular H-bond	Absent	Present N28H–N15

(*Continued*)

Study 1 (*Continued*)

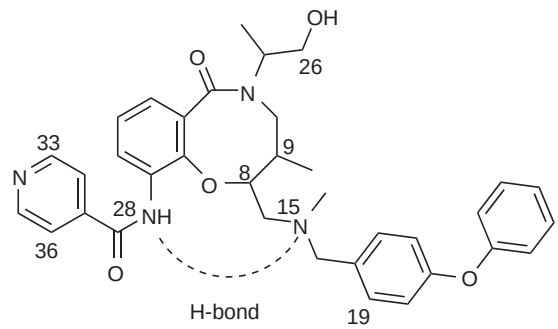


Figure 10.6 (N-(5-(1-hydroxypropan-2-yl)-3-methyl-2-((methyl(4-phenoxybenzyl)amino)methyl) -6-oxo-3, 4, 5, 6-tetrahydro-2H-benzo[b][1, 5]oxazocin-10-yl) isonicotinamide)

Effect of Intramolecular H-Bond on o-Hydroxybenzoic Acid Derivatives

Among the three constitutional isomers of disubstituted hydroxybenzoic acids, only the *ortho* isomer is stabilized through six-membered intramolecular hydrogen bonds. This also accounts for the greater acidity exhibited by the *ortho* derivative. As can be seen in Table 10.3, the pK_a of *ortho* is decreased to 2.98 in comparison to *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid. This gets further support from the fact that formation of an additional H-bond in 2,6 dihydroxybenzoic acid results in further increase in the acidity with pK_a value being reduced to 1.3. Similarly, lipophilicity due to the formation of intramolecular H-bond is also enhanced, thereby resulting in the significant reduction in the aqueous solubility (Table 10.3).

Russell et al.[4] demonstrated introduction of intramolecular H-bond in NK1 receptor antagonist by rational design that led to significant increase in lipophilicity, increased CNS penetration, and improvement in pharmacological activity. The formation of six-membered intramolecular H bond involved tertiary nitrogen and amide proton (Table 10.4).

Table 10.3 Effect of intramolecular H-bond on hydroxybenzoic acid derivatives

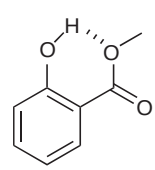
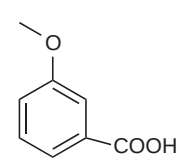
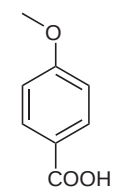
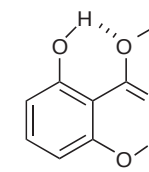
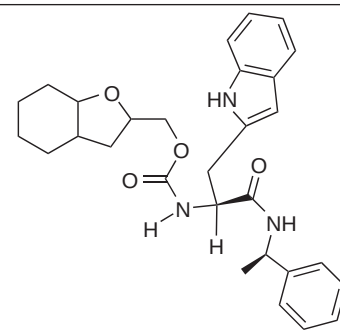
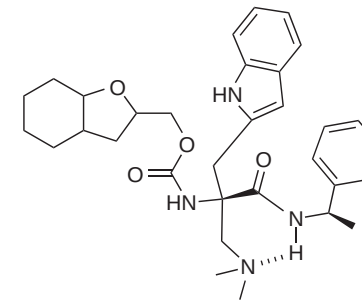
				
pK_a	2.98	4.08	4.58	1.3
Solubility	2.48 mg/mL	7.25 mg/mL	5.0 mg/mL	NA
logP	2.26	1.50	1.58	2.20

Table 10.4 A comparative profiles of lead molecule (left side) and its potent analogue (right side)

		
IC_{50}	0.73 nM	0.46 nM
Temperature dependence of amide proton*	2.78 ppb/K	8.43 ppb/K
Chemical shift of amide proton*	6.25 ppm	8.2 ppm
Intramolecular H-bond	Absent	Present between tertiary nitrogen and amide proton

**A very large ppb/K values indicates that the amide protons involved in H-bonding (Strengthening of H-bond with decrease in temperature).*

Effect of Intramolecular H-Bonds in Peptides on Bioavailability

There are several examples in the literature where presence of intramolecular H-bonds in cyclic (internal) and linear peptides has been attributed to higher lipophilicity, membrane permeability, and bioavailability. An immunosuppressant cyclic undecapeptide cyclosporine A (CsA), predominantly used in clinics as organ transplant drug is populated by conformations with an extensive intramolecular hydrogen bond network. This phenomenon has been attributed to good membrane penetration and lipophilicity (logP = 2.92) of cyclosporine as intramolecular H-bonds shield the polarity of the molecule. Notably, during the binding of CsA, the conformation in the physiological aqueous environment opens up exposing the polar groups that then bind to the target protein cyclophilin. Studies with cyclic hexapeptides as model compounds also support that ability to form intramolecular H-bond is critical for lipophilicity, and thereby passive membrane permeation.[5] In fact, the role of intramolecular H-bond is being looked as an additional parameter in drug likeness beyond '*Rule of Five*' (Lipinski rule) to account for oral bioavailability.[6]

10.2.1.2 Intermolecular H-Bonding

For the formation of H-bond between HBA and HBD, they should have directionality and placed within a distance varying from 1.5 to 2.5 Å. During the interaction of drug with target proteins, the drug enters into hydrophobic pocket (binding site) and among many weak interactions, H-bond predominantly assists in specific binding and recognition between a drug and its receptor/enzyme.

In one such example, the drug atorvastatin (Lipitor) that is used to treat high cholesterol, specifically binds to HMG-CoA reductase enzyme and blocks its action. The efficacy of the drug is evident from the fact that it not only binds to the enzyme very strongly but also exhibits extreme selectivity by avoiding interactions with other proteins that could lead to unwanted side effects. Binding is initiated by the shape of atorvastatin that is exactly complementary to the catalytic hydrophobic cavity of HMG-CoA reductase (an enzyme as target), followed by formation of at least nine specific hydrogen bonds with functional groups of the amino acid side chains at the active site of the enzyme (Fig. 10.7).

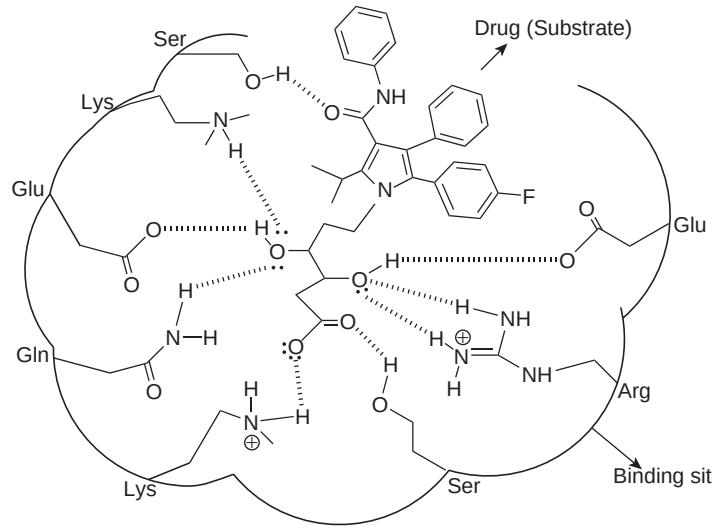


Figure 10.7 A diagrammatic representation of the binding of atorvastatin to the catalytic hydrophobic cavity of HMG-CoA reductase involving nine H-bonds.

10.2.2 Ionic Bond (Salt Bridge)

Ionic bonding is a type of chemical bond that involves complete transfer of valence electron(s) between atoms and results in the generation of two oppositely charged ions (cations and anions). The oppositely charged ions are held together by electrostatic interaction and is the primary interaction (also known as *salt bridge interaction*) occurring between ionic compounds (Fig. 10.8). Pivagabine, an antidepressant investigational drug binds with GABA receptor via ionic interaction. The salient features of ionic interaction have been summarized below:

1. The ionic interactions also referred to as *electrostatic interaction* arise as a result of attraction between two ions of opposite charges and are dependent on their distance.
2. They are ubiquitous and because they act across long distances, they play a prominent role in the actions of ionizable drugs.
3. The charge on each ion regulates the intensity of ionic interaction.
4. Solvents with high dielectric constant reduce the strength of ionic interactions.
5. Only polar test compounds exhibit ionic interactions.
6. The order of ionic strength is relatively strong (5 kcal/mole).

10.2.2.1 Cation–π Interactions

They are characterized as electrostatic interaction due to the involvement of clouds of π electron derived from aromatic ring and cations. Generally, aromatic rings present in drugs are source for π systems, whereas protonated basic side chains of Arg and Lys present in the binding site of protein are source for cations. Binding of GABA (neurotransmitter) and lisdexamfetamine (CNS stimulant; blocks the reuptake of norepinephrine and dopamine into the presynaptic neuron) with their corresponding receptors involving cation-π interaction between aromatic rings (from amino acids; Phe, Tyr or Trp) from the receptor and ammonium cation from ligands (Fig. 10.8).

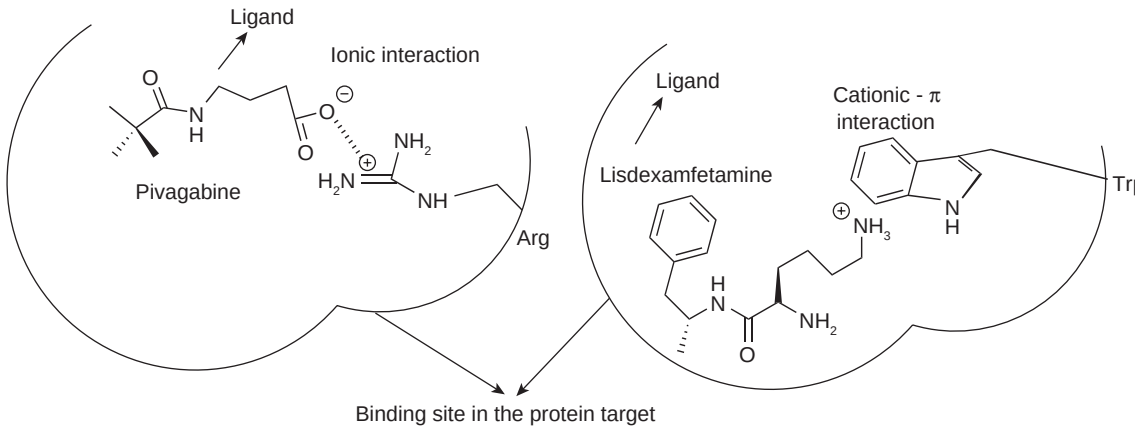


Figure 10.8 A diagrammatic representation of ionic and cation-π interactions.

10.2.3 Van der Waals Forces

Dispersion or London/Van der Waals attractions are weak forces of attraction between two or more electrically neutral molecules while passing very close to each other. It depends on the generation of instantaneous dipole arising due to slight fluctuations (asymmetric distribution) of the electron densities. The weak attraction arises due to the electrostatic attraction of the electrons of one molecule to the nuclei of the other but in order to affect this weak interaction, the molecules need to be in close proximity to each other. These bonds along with other weak bonds such as hydrogen bonds, hydrophobic and ionic interactions, not only contribute to the stability of macromolecules but also to the stability of drug-receptor/enzyme interactions (Fig. 10.9).

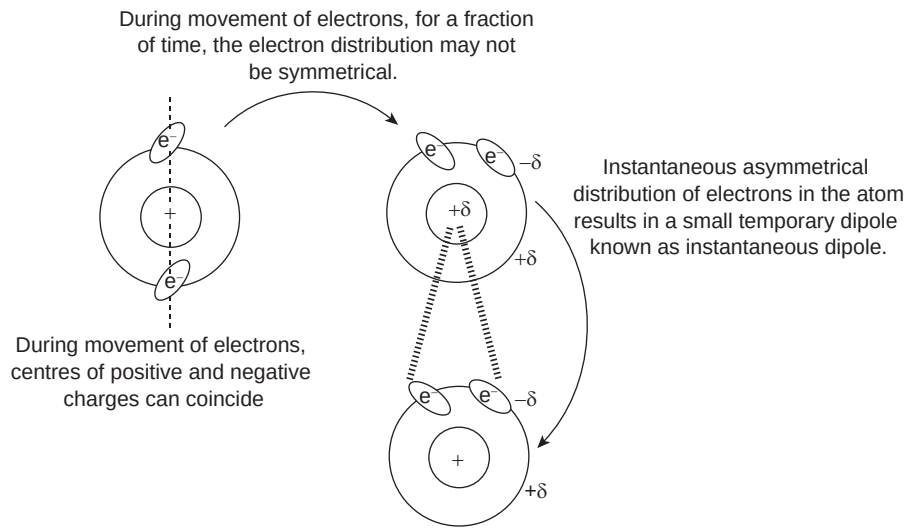


Figure 10.9 A diagrammatic representation of Van der Waals forces.

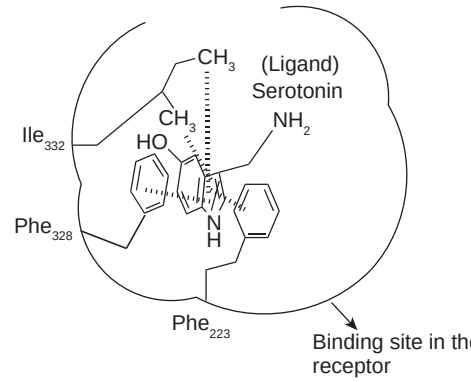


Figure 10.10 A diagrammatic representation of the binding of serotonin at GABA receptor via Van der Waals interaction.

The following factors affect the strength of Van der Waals forces:

1. Bigger molecules owing to much diffused charge clouds are able to produce instantaneous dipoles.
2. Increase in number of electrons too is able to produce instantaneous dipoles relatively better.
3. Increase in pressure.
4. Decrease in the temperature.

Serotonin, an endogenous agonist binds with its 5-HT_{2C} receptor via three weak interactions: the five-member ring in the indole of serotonin positions itself between two phenyl rings from two Phe residues 223 and 328 in the receptor favoring π-π stacking. Besides, five-member ring in the indole forms Van der Waals interaction with side chain of Ile residue in the receptor at 332 and phenyl rings of Phe residues at 223 and 328 (Fig. 10.10).

10.2.4 Dipole-Dipole/Ion-Dipole/Dipole-Dipole Induced Forces

10.2.4.1 Ion-Dipole Forces

An ion-dipole force is an attractive force arising from the electrostatic attraction between a neutral molecule that has a dipole and an ion. Thus, when a molecule with permanent dipole (polar molecules) is placed near an ionic compound, forces of attraction will come into existence, say between the negative end of the dipole to a positive cation, and vice versa. Such a force of attraction ultimately contributes to the binding affinity of drug to the receptor and can depend to it on three conditions:

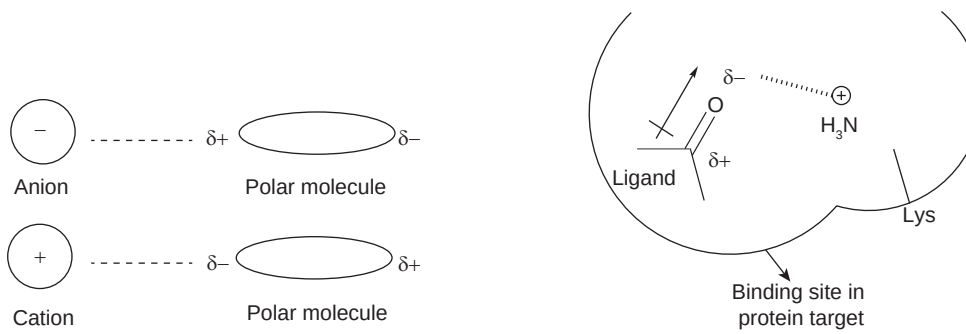


Figure 10.11 A diagrammatic representation of ion-dipole interactions.

1. The charge on the ionic component (higher the charge, stronger is the attraction/affinity.
2. The size or the magnitude of the dipole—greater the magnitude, greater will be affinity.
3. The distance between the two components means minimum distance between the two will lead to strong affinity/attraction (Fig. 10.11).

10.2.4.2 Dipole-Dipole Forces

This involves interaction between two molecules with permanent dipoles. A permanent dipole in a molecule leads to two opposite partially charged ends. This in turn leads to generation of dipole-dipole force between negative ends of one molecule with positive end of the other molecule, or vice versa (Fig. 10.12).

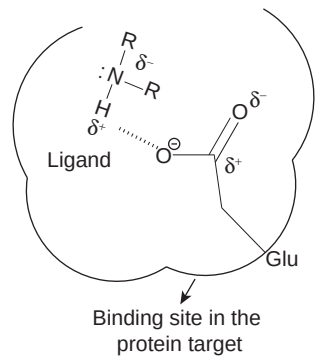


Figure 10.12 A diagrammatic representation of dipole-dipole forces.

10.2.4.3 Dipole-Induced Dipole Interactions

When two isolated molecules come together in close proximity, the one with a static dipole 'polarizes' the adjacent molecule. Thus, molecules with static dipole induce polarization in the adjacent molecule leading to dipole-induced dipole interaction (Fig. 10.13). The intensity of this interaction is regulated by two factors: magnitude of dipole present in one molecule and ability of the second molecule to get polarized following induction.

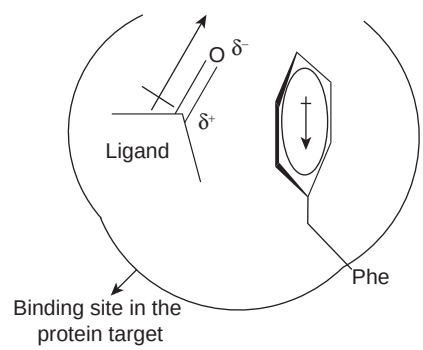


Figure 10.13 A diagrammatic representation of dipole-induced dipole interaction.

10.2.5 Hydrophobic Interaction

Hydrophobic interactions (forces of attraction) are highly prevalent and are major driving force during the binding of protein targets with drugs. It involves interactions between non-polar groups (hydrophobes) present in drug and target in an aqueous environment and is governed by thermodynamic parameters entropy and enthalpy. In an aqueous environment, hydrophobes are generally caged within water molecules leading to a decrease in the entropy. During drug-receptor interaction, when two such hydrophobes come into close range, spontaneous entropy-driven hydrophobic interaction is affected since non-polar residues tend to desolvate and aggregate in the water medium. In other words, both hydrophobes squeeze themselves out of the structured water molecules and in turn induce hydrophobic interaction between the hydrophobic molecules (Fig. 10.14).

The interaction is accompanied by an increase in enthalpy (ΔH) and entropy (ΔS) of the system as some of the H-bonds among water molecules in the cage are broken (ΔH is +ve) thereby increasing the disordered (ΔS is +ve) nature of water molecules. During drug-receptor interaction, hydrophobic interactions occur between two aliphatic moieties or between an aliphatic and an aromatic moiety. Aromatic moiety is highly prevalent among drugs and interacts with aliphatic residues present in the side chain of amino acids in the receptors such as Leu, Val, Ile, and Ala. Similarly, aromatic rings present in the side chain of amino acids Trp, Phe, and Tyr interact with aliphatic non-polar moieties present in the ligand such as methyl group. Other hydrophobic interactions involve the following:

1. Between non-polar residues aliphatic or aromatic moiety in the target and Cl and F in the ligand/substrate.
2. Between S atom present in the side chain of Met and a carbon of aromatic moiety from ligand/substrate.

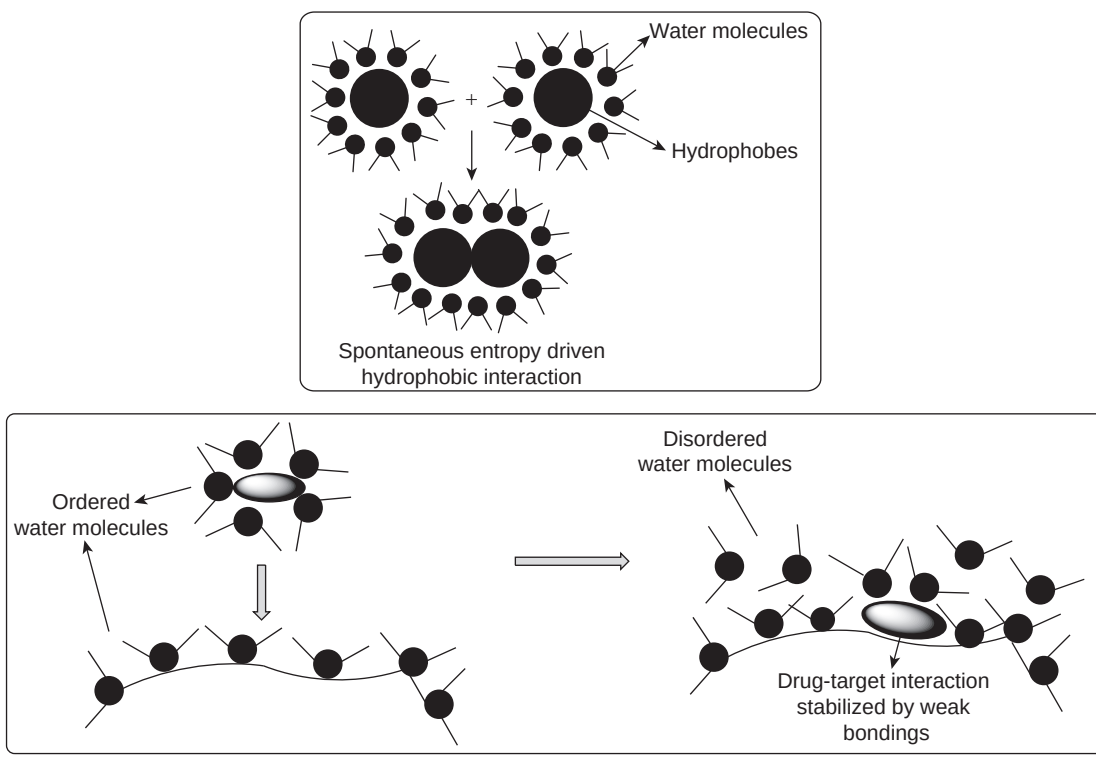


Figure 10.14 Spontaneous entropy-driven hydrophobic interaction.

10.2.5.1 π-π Stacking Interactions

A special case of hydrophobic interactions involves contacts between two aromatic moieties present in ligand/substrate and protein. In the protein, amino acids involved are Trp, Phe, Tyr, and His, with aryl rings in their side chains. The aryl rings in theses amino acids and ligands/substrates are generally oriented in a manner that they are available for binding in the binding site either as a part of receptor or ligand/drug (Fig. 10.15). For example, monoclonal antibodies (mAb used as drugs/biologics) when bind to their specific receptors, hydrophobic interactions between amino acids of mAb and receptor are highly prevalent and contribute towards their strong affinity for receptors. Four different modes of aryl-aryl interactions are T-shaped, edge to face, sandwich, and parallel displaced. However, the T-shaped edge-to-face and the parallel-displaced π-π stacking arrangement are more prevalent during drug-receptor interaction (Fig. 10.15). Lacosamide, an antiepileptic drug, binds with its receptor through π-π stacking between the aromatic ring from the receptor and the aromatic ring from the ligand.

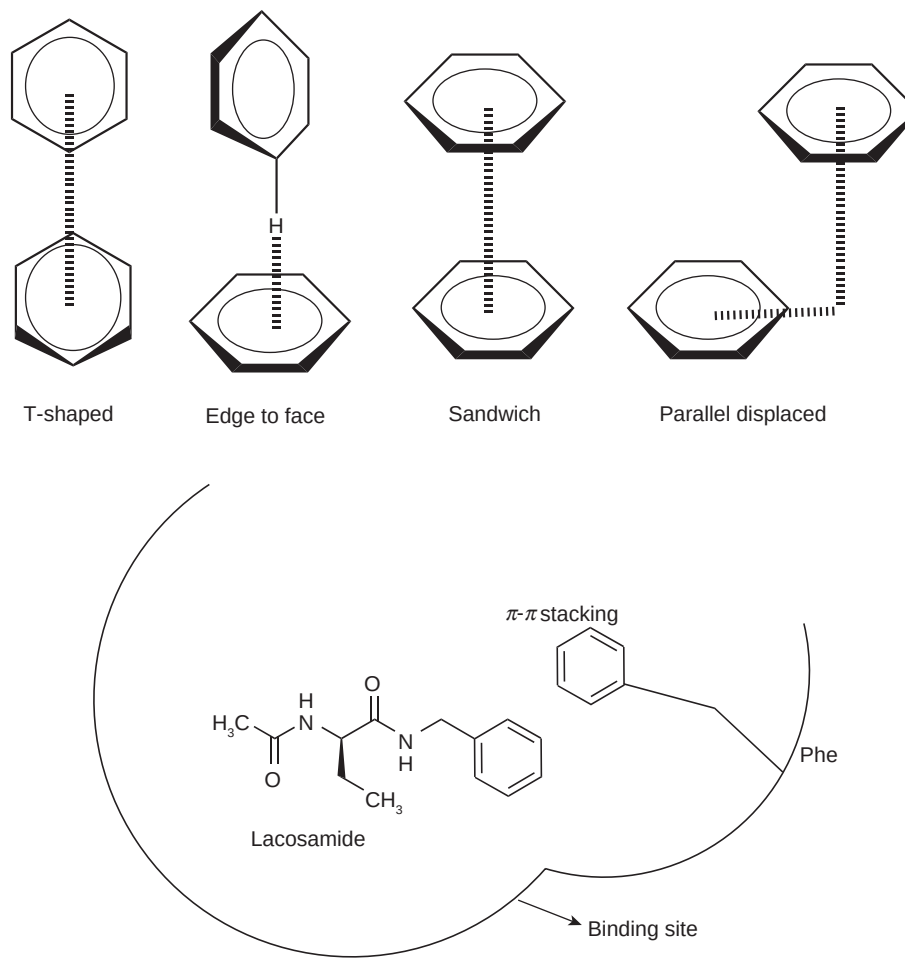


Figure 10.15 Hydrophobic interactions.

10.3 Irreversible Drug–Target Interaction (Covalent Bonds)

This leads to a strong, irreversible bond (–40 to –110 kcal/mol stability) and is generally more prevalent in drug-enzyme and drug-DNA interactions. The main objective behind the design of covalently bound irreversible inhibitor is to modify the amino acids present in the binding site through derivatization. Thus, the inhibitor must have reactive moieties in their structures that can covalently react with side chain of amino acids in the binding sites. The reactive functional groups such as ketones, aryl sulfonates, aldehydes, etc., can covalently react with the side chain of amino acids bearing nucleophilic functionality such as Cys, Thr, Ser, Tyr, etc., and inhibit the activity of enzyme. Penicillin for example undergoes irreversible binding with enzyme transpeptidase, thereby inhibiting bacterial cell-wall biosynthesis.

A classic example of irreversible covalent binding involves inhibition of acetylcholinesterase enzyme by organophosphorous compound diisopropyl fluorophosphate (DIFP). The enzyme has been implicated in the hydrolysis of acetylcholine bound to receptors on post-synapse neurons. The inhibition of acetylcholinesterase prolongs the duration of action of receptor-bound acetylcholine. The non-competitive and irreversible inhibition of acetylcholinesterase by DIFP occurs via formation of a covalent bond between the hydroxyl group of serine in the active site of the acetylcholinesterase and DIFP (Fig. 12.24 in Chapter 12). Formation during drug-receptor interactions is rare, and if it happens, it may involve formation of a covalent bond between a nucleophile derived from amino acids in the receptor such as –SH of Cys, –OH of Ser, –COOH from Gly, $–NH_2$ from Lys, and an electrophile component derived from reactive functional groups present in the ligand/drug such as alkyl group covalently bonded to halogen, epoxide ring, etc., present.

10.4 Summary of Drug–Target Interactions

The major non-covalent drug-receptor or drug-enzyme interactions have been depicted in Figure 10.16, and the energy associated with the formation of these weak and reversible bonds has been summarized in Table 10.5.

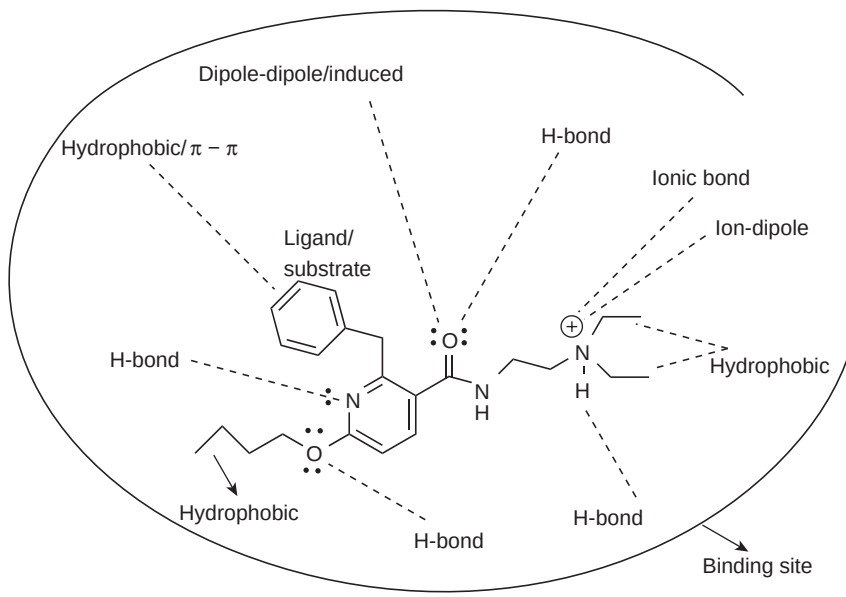
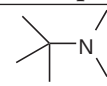
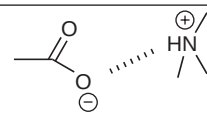
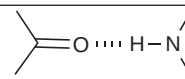
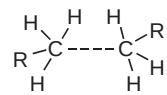
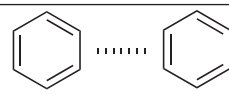
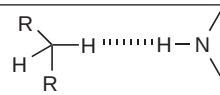


Figure 10.16 Summary of drug-target interactions in a hypothetical molecule.

Table 10.5 Major drug-target interactions

Example	Bond Type	Bond Strength (kcal/mol)
	Covalent	50 – 150
	Ionic	1 – 20
	H bonding	2 – 30
	Hydrophobic	10 – 14
	π-π aromatic stacking	0 – 10.0
	Van der Waals	0.1 – 1

10.4.1 Weak Interactions during Binding of Drug with GPCR

The weak interactions described above also occur during the binding of drugs with receptors. GPCR, for example, cell-surface transmembrane receptors comprise seven-membrane-bound helix TM-1 to TM-7. The binding site in these receptors comprises amino acids representing all the transmembrane strands. Depending on the physicochemical characteristics of test compounds, the binding may accordingly occur involving the amino acids from different TMs in the binding site (Fig. 10.17).

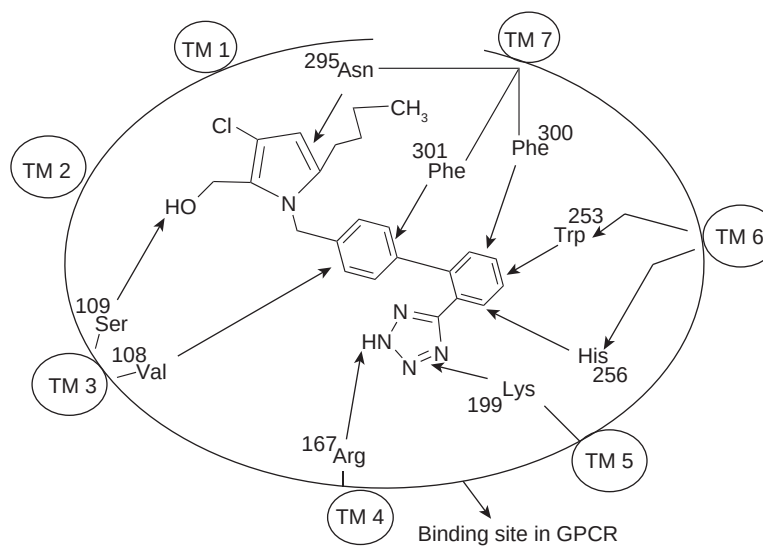


Figure 10.17 Weak binding interactions between sartan and GPCR.

Exercises

1. Write short notes on followings parameters during drug-receptor interaction:
   - **(i)** H-bonding
   - **(ii)** Ionic interactions: Ionic bonding
   - **(iii)** Van der Waals forces
   - **(iv)** Dipole-Dipole Forces
   - **(v)** Hydrophobic interactions
2. Draw a diagram depicting possible drug-receptor interaction using a hypothetical molecule.

References

1. Ernesto Freire, *Drug Discov. Today*, **2008**, *13*, 869.
2. Johannes Schiebel, Roberto Gaspari, Tobias Wulsdorf, Khang Ngo, Christian Sohn, Tobias E. Schrader, Andrea Cavalli, Andreas Ostermann, Andreas Heine, Gerhard Klebe , *Nature Communications*, **2018**, *9*, 3559.
3. https://en.wikiversity.org/wiki/Enzyme_structure_and_function.
4. Valerie A. Ashwood, Mark J. Field, David C. Horwell, Christine Julien-Larose, Russell A. Lewthwaite, Scott McCleary, Martyn C. Pritchard, Jenny Raphy and Lakhbir Singh, *J. Med. Chem.*, **2001**, *44*, 2276.
5. Taha Rezai, Jonathan E. Bock, Mai V. Zhou, Chakrapani Kalyanaraman, R. Scott Lokey, and Matthew P. Jacobson, *J. Am. Chem. Soc.*, **2006**, *128*, 14073.
6. Alexander Alex, David S. Millan, Manuel Perez, Florian Wakenhut and Gavin A. Whitlock *Med. Chem. Commun.*, **2011**, *2*, 669–674.

CHAPTER

11

Pharmacokinetics

KEY THINGS TO LEARN FROM THIS CHAPTER

- Pharmacokinetic parameters absorption (A), distribution (D), metabolism (M), and excretion (E).
- Affect of physiological barriers on drugs:
  - Gastrointestinal region as physiological barriers
  - Liver as a physiological barrier
  - Physiological barriers during systemic circulation
  - Aqueous/lipid environments as physiological barriers
  - pH of the aqueous environment as physiological barrier
- Affect of physicochemical properties of drugs/test compounds on ADME.
- Kinetics of ADME:
  - Absorption: Calculation of bioavailability, drug plasma concentration versus time curve (AUC), Absolute bioavailability, relative bioavailability, calculation of clearance (CL) using bioavailability/AUC data.
  - Distribution: Volume of Distribution, therapeutic implication of V_d values, one and two-compartment pharmacokinetic compartment models.
  - Metabolism: Therapeutic implication of biotransformation, role of CYPs in metabolism, redox partners cytochrome P450-reductase (CPR) and cytochrome b_5 (b_5), pharmacokinetic drug-drug interaction (DDI) owing to inhibition and induction of CYPs.
  - Excretion: Excretion through kidneys, pharmacokinetic parameters for excretion, rate of elimination, drug clearance, extraction ratio (ER) of drugs, blood flow, loading and maintenance doses and half-life.

11.1 Introduction

Once a drug is administered by a suitable route, it has to pass through multiple physiological barriers before it can reach its site of action to elicit the desired biological response. During this passage of drug within the body, the human physiology treats the drug as a foreign substance and, thus, makes all-out effort

to resist it from entering the system. It may be pertinent to add that these physiological barriers become more pronounced and complex when the drug is administered via oral route, a preferred route of choice. Following oral route of administration, physiological machinery makes a two-pronged simultaneous attack on the drug. On one hand, physiological barrier resists the absorption (A; bioavailability) of the drug into the blood stream and prevents its distribution (D) in tissues. On the other hand, it induces metabolism (M) of the drug so as to facilitate its rapid excretion (E) from the body. Pharmacokinetics is the study of change in concentration of drug with respect to time following absorption, distribution, metabolism, and excretion. Together, these four processes also make efforts to minimize the possibility of drug-induced toxicity (T) that can be caused by many factors. Thus, the five ADMET parameters together constitute the lifespan of drugs within the body from the administration to excretion. The rate at which these ADME phenomena occur inside the body comprises kinetics of drugs.

The absorption (A) process involves diffusion of drug through the epithelial cells in gastrointestinal region into the hepatic portal vein to reach liver. From the liver, the drug either goes into biliary secretion/enterohepatic circulation or enters systemic circulation. In systemic circulation, distribution (D) of drug is accompanied by diffusion from bloodstream into interstitial fluid through endothelial cells lining the walls of blood capillaries. In the interstitial fluid, drugs can access the tissues carrying the therapeutic target by either binding to the extracellular cell-surface receptors (target) or extracellular enzymes (target) or yet again diffuse through cell membrane to bind with either intracellular receptors (target) or intracellular signaling enzymes (target).

The phenomena of metabolism (M) and excretion (E) involve degradation of drugs by metabolic enzymes, i.e., cytochrome P450, into water-soluble metabolites that facilitate their excretion mostly through urine. However, depending on the physicochemical properties of the drugs, metabolite or unchanged drug may also be excreted through stool via gastrointestinal tract. Metabolism although occurs in all tissues, majority of the enzymatic degradation happens in liver and gut. Excretion (E) is largely carried out by kidney. Thus, gut, liver, and kidney work together not only for the metabolism and excretion of the drugs but also for the termination of their response. While drugs are going through ADME, toxicity (T) may be caused due to many reasons such as the binding of drugs to off targets (hERG protein), toxic metabolites, and increased exposure of body to the drugs. The high concentration of drugs in the gastrointestinal region (more than required) may saturate metabolic enzymes (CYP) and efflux transporter proteins, thereby not only delaying the process of metabolism and excretion but may also allow absorption of drug through gastrointestinal region in high concentration. This, in turn, may increase the concentration of drug in the bloodstream and may trigger nonspecific binding of drugs to off-target proteins resulting in toxicity. This complex movement of drug through the body against several barriers explains the fact that a high percentage (~80%) of failures of investigational drugs during clinical trials has been attributed to poor ADMET profile.

Thus, ADMET phenomenon happens parallely in the body whether in gastrointestinal region following oral administration or in the bloodstream following absorption. Two major factors (besides route of administration; discussed in Chapter 2) that regulate ADMET parameters are physiological barriers faced by the drug inside the body and physicochemical properties of the test compounds/drugs. Let us examine the physiological barriers that act on the drug and interfere with its ability to elicit its response.

11.1.1 Gastrointestinal Region as a Physiological Barrier

The majority of the barriers exists in the gastrointestinal region; some of them include first-pass metabolism, biliary excretion, repeated enterohepatic circulation, P-gp efflux transporter proteins (expelling the drug

back into lumen from inside the cell), phase I metabolism by cytochrome P450 located in the intestinal mucosa,[1] excretion through stool, gut microbiome (gut microbiota)[2] in drug metabolism,[3] toxicity,[4] etc.

11.1.2 Liver as a Physiological Barrier

After administration of a drug via oral route, it mostly undergoes first-pass metabolism in the gut followed by absorption of free native drug by the digestive system from where it enters liver through hepatic portal vein. In the liver, drug undergoes phase I and II metabolism and from there the free native drug (or active metabolite in some cases) may either enter systemic circulation or excreted into bile (in gall bladder) along with polar conjugate metabolites (Fig. 11.1).

Through biliary secretion, drug/conjugates from liver are secreted back into gut from where they may again undergo reabsorption (highly hydrophobic drug owing to their poor metabolism may be excreted from gut by stools). In addition, in small intestine conjugated metabolites may be hydrolyzed by enzymes to liberate the native drug which in turn may also be reabsorbed. The reabsorbed drugs from gastrointestinal region re-enter portal vein and are taken back into the liver via enterohepatic cycle which may be repeated several times. This repeated cycling of drug may be responsible for the enhanced duration of action of the drug and may also lead to toxicity. Thus, metabolism, biliary secretion, and enterohepatic cycle may act as major barrier, in liver, resulting in reduction in the bioavailability of drugs.

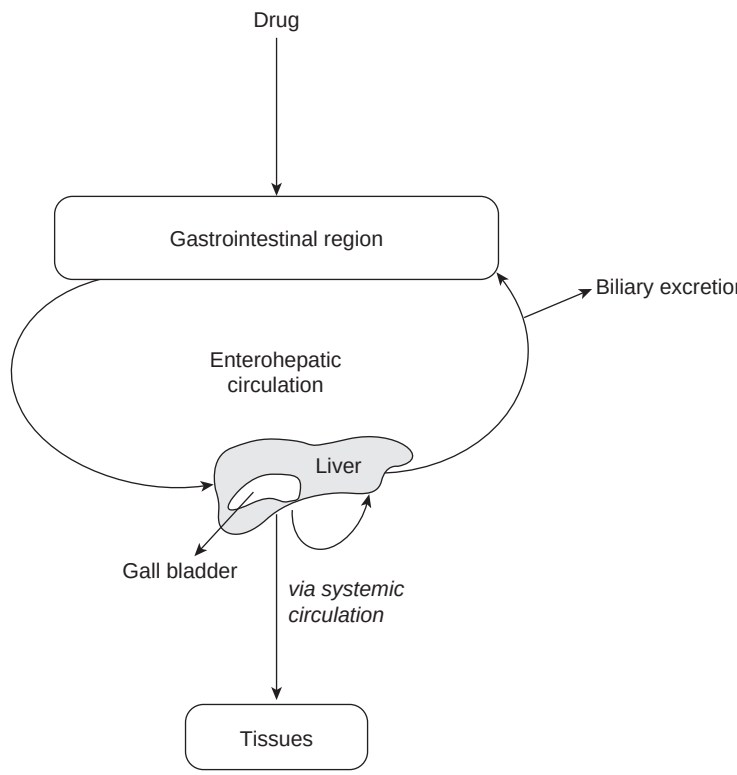


Figure 11.1 Gastrointestinal region and liver as physiological barriers resisting drug absorption/bioavailability.

11.1.3 Physiological Barriers during Blood Circulation

The drugs after passing through physiological barriers get absorbed through walls of gastrointestinal region, albeit in reduced concentration (less than the dose of administration), and then enter systemic circulation. Some of the physiological barriers faced by drugs in blood circulation involve plasma protein binding, binding with blood cells, diffusion in tissues with or without its target, re-entering liver for metabolism and back in circulation either as metabolite or in native form, metabolism in tissues and excretion via water-soluble metabolites through kidney, etc.

11.1.4 Aqueous/Lipid Environment as a Physiological Barrier

This is yet another barrier that regulates ADME parameters. Since the drugs move from gut lumen to blood stream to interstitial fluid to tissues, they face aqueous environment in lumen, blood, and ECF, whereas lipid environment during their diffusion through lipid bilayer cell membranes (gut, blood capillaries, and tissues). Lipophilicity (logP and logD) and pK_a are the two major physicochemical properties that deal with solubilization while passing through physiological aqueous and lipid environments. Unionized drugs diffuse readily through lipid membrane while ionized form of the drug helps in their movement through aqueous environment.

11.1.5 pH of Aqueous Environment as a Physiological Barrier

The pH of the aqueous physiological environment together with pK_a of the drug/test compounds plays an important role in the passive diffusion of drug through lipid bilayer membrane. The pH in the gastrointestinal region ranges from 1−3 in stomach, 5−6.5 in duodenum, 6.5−7.0 in jejunum, 7−7.5 in ileum, and 5.5−7.0 in colon. They comprise aqueous environments through which drug moves in the gastrointestinal region and is absorbed in the bloodstream. The physiological pH of bloodstream and interstitial fluid with aqueous environment through which drugs move is 7.4. Higher is the concentration of unionized drug at absorption site, higher the permeation (diffusion) of drugs through lipid bilayer membrane.

Let us now look briefly into the physicochemical properties of the drugs/test compounds that can affect ADME (details about physicochemical properties have been discussed separately in Chapter 6).

11.1.6 Effect of Physicochemical Properties of Drugs/Test Compounds on ADME

Lipophilicity/partition coefficients, solubility, PSA, ionization constant/pK_a, etc., are some of the important parameters that together regulate ionization of the drugs in the physiological environment (aqueous and lipid) and, in turn, affect ADME parameters.

Thus, pharmacokinetics refers to the time course followed by the drug from absorption to onset/duration of its biological response to excretion from the body. The physiological barriers coupled with physicochemical properties of drugs/test compounds not only shorten or enhance the life of the drug (duration of action) inside the body but are also accompanied by gradual fall in the concentration, and only a fraction of drug reaches site of action. In fact, concentration of drug in the bloodstream is associated with initial rise and fall in the concentration of drug in the body, and it follows a time course which is specific for each drug.

It may be noted that for lead optimization, the parameters of physiological barriers cannot be modified, but we can modify the physicochemical properties of the test compounds that will survive the physiological barriers. However, physicochemical properties vary from drug to drug, and it is impossible to draw general specifications pertaining to the physicochemical properties of test compounds required for drug development (Table 11.1).

Table 11.1 Drugs with variation in physicochemical parameters and their affect on ADME

Drug	MW	pK_a	logP	Half Life (hr)	Volume of Distribution V_d (L)	Bioavailability F (%)
Metformin	129	2.8 and 11.5	−1.8	4–8	~654	50–60
Atorvastatin	558	4.46	6.36	14	~380	12
Norfloxacin	319	5.77 and 8.68	−0.47	34	~210	30–40
Nifedipine	346	3.93	2.2	2	~25	40–77
Cyclopsorine	1,202	11.83	3.64	19	~350	10–89

In pharmacokinetics, the physiological barriers that affect the systemic survival of the drug are discussed separately under absorption, distribution, metabolism, and excretion toxicity (ADME) of drugs. In other words, the concentration of drug versus time course (kinetics) in the body follows ADME and can provide valuable information about the ability of drug to survive in physiological environment. It is pertinent to add that all the four ADME parameters occur simultaneously during the systemic circulation of the drug. The objective of optimizing PK properties (ADME) of test compounds is to regulate the lifespan of the drug while it passes through physiological barriers.

Thus, monitoring systemic concentration of drug/test compound versus time in the body forms the basis of PK studies. Generally, systemic monitoring of drug concentration is primarily carried out using blood plasma; other body fluids such as saliva and urine can also be sampled but are not preferred. Numerous laboratory methods have been designed for the analysis of drug concentration in blood plasma. It is to be noted that direct measurement of drug concentrations at the site of action (in tissue) is practically impossible as protein targets (receptors/proteins) are widely distributed in the body. The whole process involving movement of drugs (Details discussed in chapter 2) following drug administration over a period of time course has been summarized below:

1. Absorption of drugs is largely governed by route of administration. For example, absorption of drugs during oral route of administration is affected by first-pass metabolism, efflux of drugs by P-glycoprotein (transporter protein) in the intestine, gastrointestinal blood flow and pH, gastric emptying, etc.
2. Distribution of drugs depends on their lipid solubility properties for diffusion through cell membrane and on their ability to bind blood proteins.
3. Metabolism of drugs (biotransformation) involves chemical and enzymatic degradation within the body, predominantly by liver and gut.
4. Excretion of drugs is accomplished by release of drugs/metabolites into urine and stool.
5. Toxicity due to drugs/metabolites binding to off-target proteins.

11.2 Kinetics of ADME

The details about the four pharmacokinetic (PK) ADMET parameters, and their role in drug action have been discussed in Chapter 2. Along with this involvement of ADMET parameters in drug design/lead optimization studies have been discussed in Chapter 5. In this chapter, we shall discuss ADME kinetics (mathematical parameters) to quantify the PK parameters.

11.2.1 Absorption

It refers to the uptake of a drug from its site of administration into the bloodstream. For drugs administered via intravenous route, the phenomenon of absorption is not applicable as drugs directly enter the bloodstream. Many factors affect the absorption phase during administration by oral route:

1. **Physicochemical Characteristics of Drugs:** Molecular weight, ionization, solubility, lipophilicity, and formulation of drugs.
2. **Route of Administration** (Fig. 2.7; Chapter 2)**:** Gastric pH, contents of gastrointestinal region, first-pass metabolism, etc.

11.2.1.1 Bioavailability

Once a drug has been administered in the body, the human physiology treats the drug as a foreign substance and thus makes all-out effort to resist its entrance into the bloodstream via physiological barriers. These physiological barriers become more pronounced and complex during the administration of drug by oral route. Thus, bioavailability deals with the concentration of native drug (unchanged) that reaches systemic circulation following administration by oral route. Once the drug reaches the gastrointestinal region, both physiological barriers and physicochemical properties of molecules together regulate the bioavailability:

1. pK_a (ionization) of test compounds/drugs.
2. Lipophilicity/permeability (absorption) of drug through the gastrointestinal membrane lining the wall.
3. Solubility/dissolution of drug in the gastrointestinal milieu.
4. First-pass effect/metabolism/stability.
5. Efflux transporters.
6. Gastrointestinal pH.
7. Gastrointestinal blood flow.
8. Gastric emptying (Drug absorption is often facilitated when there is faster gastric emptying. Thus, factors that slow down emptying process may impair absorption of a drug. Hypothyroidism, presence of anticholinergics, $Al(OH)_3$, etc., can slow down the gastric emptying.
9. Faster gastric emptying is beneficial for the absorption of most drugs).
10. The drug besides gastrointestinal region is largely metabolized in the liver following oral administration and, therefore, affects bioavailability.
11. Biliary secretion and enterohepatic cycle.

In pharmacokinetics, bioavailability is fraction of drug dose reaching the bloodstream after overcoming the resistance put up by the gastrointestinal barrier. Significant difference between the dose of drug administered by oral route and the fraction of native drug reaching bloodstream has been observed. The concentration of this fraction of native drug entering bloodstream is represented by 'f', and the same when expressed in percentage is represented by 'F'. For example, when drugs are administered by IV route, the bioavailability is taken as 100%. However, it decreases by other routes of administration (Fig. 2.13; Chapter 2). This may be attributed to several physiological factors as well physicochemical properties of test compounds/drugs.

Bioavailability is measured by calculating the area-under-curve (AUC) of the drug concentration–time profile. It helps in calculating dosage of drugs for non-intravenous routes of administration since for IV route bioavailability is 100%. The fraction of the dose of a drug (F) that enters the general circulatory system can be represented by

$$F = \frac{\text{Amount of drug that enters systemic circulation}}{\text{Dose administered}} = \frac{\text{AUC}}{\text{Dose}} \quad \text{(Eq. 1)}$$

One of the objectives during drug design strategies is to enhance the bioavailability of test compounds as this will increase the fraction of drug reaching the drug target. However, bioavailability may not be the sole criterion as some of the clinically used drugs exhibit wide range of absorption by oral routes. For example, *F* for atorvastatin is only 14% while for metformin it is as high as 50–60%. Notably, both the drugs once prescribed have to be consumed every day for the rest of the life to manage hyperlipidaemia and diabetes, respectively.

11.2.1.2 Drug Plasma Concentration versus Time Curve

Following administration of a drug by oral route, its concentration in the blood serum peaks quickly and then slowly drops as the drug is degraded and/or metabolized by the liver and/or kidneys. For the estimation of drug concentration in blood plasma, the blood samples are collected after regular intervals from test animal subjects following the administration of drug. The blood samples are then processed and concentration of drug in these samples is determined using analytical methods, preferably LC–MS–MS.

Next, a typical plot of concentration of drug in plasma versus time is drawn, as depicted in Fig. 11.2. The total area under the curve called AUC reflects bioavailability (amount of drug in circulation) as it is equivalent to the total exposure of the body to the drug.

A number of important and crucial information pertaining to toxicology, biopharmaceutics (bioequivalency or bioavailability studies), pharmacodynamics, and pharmacokinetics can be derived between appearance and disappearance of drug in the blood plasma from a typical AUC curve (Fig. 11.3):

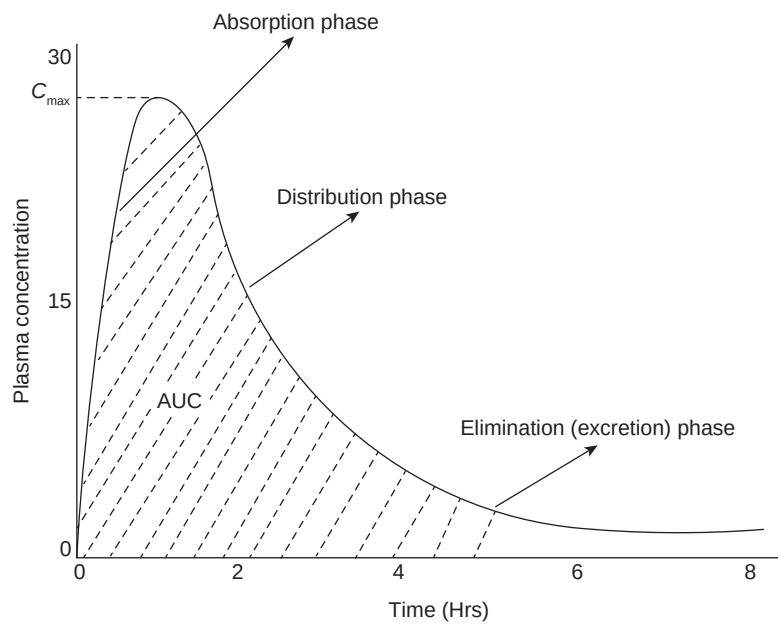


Figure 11.2 Typical drug plasma-concentration versus time curve. The shaded area is AUC.

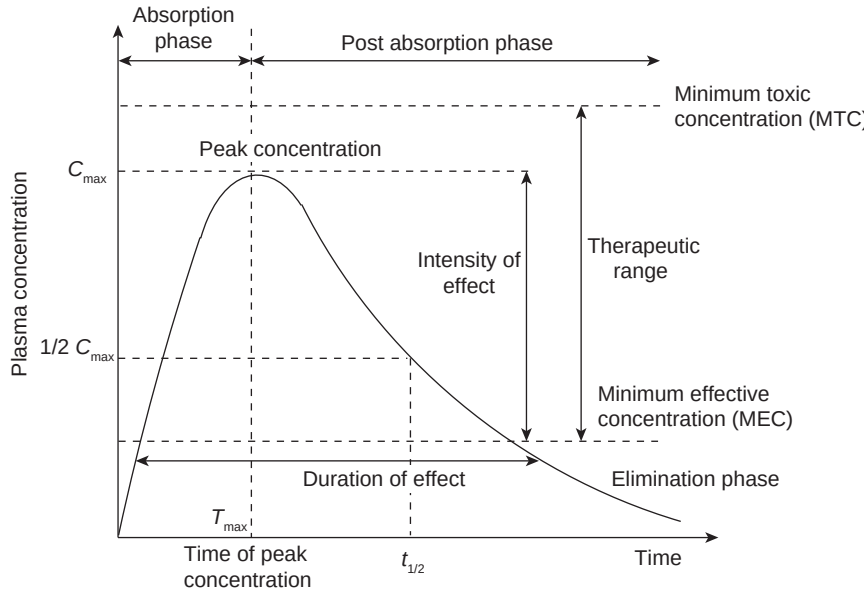


Figure 11.3 The plasma concentration-time curve after administration of drug.

1. Rise in concentration denotes absorption phase (ascending curve).
2. Drop in concentration denotes post-absorption phase (descending curve).
3. The post-absorption phase (decline in plasma drug concentration) can be attributed to either reversible distribution of drugs between plasma and tissues, and/or excretion from the body.
4. In the post-absorption phase, initially the decline in plasma concentration of drugs is rapid, which may be due to two factors, distribution and excretion, occurring simultaneously. However, once the equilibrium in distribution is achieved between plasma and tissues, the curve descends slowly (elimination phase) because of single factor in effect, i.e., excretion of drug from the body (metabolism/excretion). It is to be noted that the declining plasma drug concentration does not reflect route of elimination.
5. C_{max} corresponds to peak of the AUC. The maximum plasma concentration (highest concentration) achieved following drug administration. The peak also represents the point of time when absorption rate equals elimination rate of drug.
6. Peak concentration is often related to the intensity of pharmacological response and should ideally be above minimum effective concentration (MEC) but less than the maximum safe concentration (MSC).
7. T_{max} represents the time at which maximum concentration occurs.
8. MEC stands for minimum effective concentration that reflects the minimum concentration of drug needed at the target to elicit desired pharmacological response.
9. MTC stands for minimum toxic concentration that denotes the drug concentration sufficient enough to produce a toxic effect.
10. Therapeutic range is the range of concentration of drug between MEC and MTC.

11. Between appearance and disappearance of drug in plasma, the duration of action and intensity of effect can be readily determined.

12. The AUC is inversely proportional to the clearance of the drug.

13. The AUC indicates total concentration of drug in blood plasma, both free and bound forms.

14. Half-life of drug can be determined: The time taken by the drug to drop from C_{max} to half of its C_{max} is the half-life $t_{1/2}$.

15. Routes of administration affect bioavailability (AUC; Chapter 2; Fig. 2.11). The T_{max} increases in the order IV > IP > IM > *po* and C_{max} decreases in the order IV < IP < IM < *po*.

16. Depending on the route of administration, the concentration of drug in elimination phase drops down slowly or fast (by IV route the elimination is fast, whereas by p.o. route it is slow), thereby suggesting that the rate of excretion is proportional to the concentration of the drug.

17. The AUC of a concentration-time graph is also known as noncompartmental method of estimating the exposure to a drug.

18. AUC is a very important parameter in drug discovery, in defining how well the drug is absorbed, and has been mandatory by the FDA in the drug approval process.

Calculation of Bioavailability

Dose (D) administered is proportional to drug concentration in bloodstream, which can be derived from the AUC. Thus, the following equation was developed using D and AUC:

$$k_{el} = \text{dose}\frac{(\text{D})}{\text{AUC}} \quad \text{(Eq. 2)}$$

Significant difference between the dose of drug administered by oral route and the fraction of native drug reaching bloodstream has been observed due to resistance put up by physiological barriers. Thus, introducing F as bioavailability in equation (2) gives:

$$FD = k_{el}(\text{AUC}) \quad \text{(Eq. 3)}$$

$$k_{el} = \frac{\text{FD}}{(\text{AUC})} \quad \text{(Eq. 4)}$$

where, k_{el} = elimination rate constant

If the oral and IV doses are equal, F can be calculated using the following equation:

$$F = 100 \times \frac{(\text{AUC})_{oral}}{(\text{AUC})_{IV}} \quad \text{(Eq. 5)}$$

Bioavailability can also be measured in terms of 'absolute bioavailability' or 'relative bioavailability'

Absolute Bioavailability

It is always expressed with reference to the same drug administered by IV route as the drug administered by this route does not face physiological barriers. The range of absolute bioavailability remains between 0 and 100%. Thus, absolute bioavailability of a drug is determined by calculating the AUC through IV and non-IV routes and can be calculated using the equation (1) given below:

$$F_{abs} = 100 \times \frac{(\text{AUC})_{oral}}{(\text{AUC})_{IV}} \times \frac{(\text{D})_{IV}}{(\text{D})_{oral}} \quad \text{(Eq. 6)}$$

Relative Bioavailability

This compares the bioavailability between two different dosage forms by the same route. Again, the relative AUCs are used to make this comparison, and relative doses are used to normalize the calculation. For example, relative bioavailability of a drug at two different doses A and B can be determined by the following equation:

$$F_{rel} = \frac{(AUC)_{dosage\ A}}{(AUC)_{dosage\ B}} \times \frac{(Dose)_B}{(Dose)_A} \quad \text{(Eq. 7)}$$

Calculation of Clearance (C_L) Using Bioavailability/AUC Data

Clearance of drug from the body can be calculated using AUC as downfall of the peak in AUC (Fig. 11.1) reflects clearance of drug. Thus, from the plasma concentration–time curve following administration of a drug, it is evident that AUC should be inversely proportional to the clearance of the drug. Thus, if bioavailability and the dose of drug administered are known, the clearance of the drug from blood can be calculated by dividing the dose absorbed by the AUC.

$$AUC = \frac{F \times D}{C_L} \quad \text{(Eq. 8)}$$

Where,

AUC is the area under the concentration–time curve
F is bioavailability
D is the dose of drug
C_L is clearance

11.2.2 Distribution

It is a process that deals with equilibration (via movement) of the drug between the blood plasma and the rest of the organism. Once the drug reaches systemic circulation, distribution of drug begins following diffusion through blood capillaries out into interstitial fluid and then reaching the tissues carrying the therapeutic target. Once drugs reach tissues, they can either bind with the extracellular cell-surface receptors (target) or extracellular enzymes (target) or yet again diffuse through cell membrane to bind with either intracellular receptors (target) or intracellular signaling enzymes (target).

The process of reversible distribution accompanies the process of excretion; however, as soon as distribution equilibrium of drug is reached, the phenomenon of excretion slows down. Factors affecting drug distribution are as follows:

1. **Membrane Permeability of Tissues:** The drug needs to penetrate membranes (barriers) to reach the site of action.
2. **Plasma Protein Binding:** The extent of binding to plasma proteins influences drug distribution into tissues, which in turn, affects drug efficacy. Drugs bound to plasma proteins cannot cross membranes. Besides, half-life of the drug, an important parameter, is also influenced by protein binding. Sometimes, the protein-bound drug may act as reservoir/store for drug molecules and can be slowly released back into bloodstream in its native form. Continuous metabolism or excretion of unbound drug in bloodstream may cause the protein-bound drug to release free drug to maintain equilibrium.
3. **Lipophilicity of Drug (fat deposition of drug):** Lipophilic drugs accumulate in adipose tissue.

4. Rapid excretion of unbound drug.
5. Extensive metabolism of unbound drug.
6. The distribution of drug in various body tissues (apart from plasma) is known as volume of distribution and represented by V_d, with liters as unit.

Thus, a drug in blood depending on its pK_a may exist in either of the two bound and unbound forms or both with variation in their respective percentage (under Chapter 2; Fig. 2.16). The unbound drug is responsible for the activity. Therefore, it is important to determine the free fraction (f_u) of drugs in bloodstream at different stages of drug development, including discovery, preclinical, and clinical.

$$f_u = \frac{[D]}{[D]+[DP]}$$

Where,

[D] is the free drug concentration
[DP] is the concentration of drug protein complex
f_u is fraction of drug unbound

Thus, f_u can be also represented as ratio between concentration of unbound drug (C_u) and total concentration of drug (C_o) in plasma.

$$f_u = \frac{C_u}{C_o}$$

Similarly, percentage of drug bound to protein can be expressed as % protein binding:

$$\%PB = 100 \times \frac{[DP]-[D]}{[DP]} \quad \text{(Eq. 9)}$$

A drug's efficacy may be affected by the strength with which it binds to the plasma proteins. A weakly bound drug may increase the available concentration of unbound drug, which can then efficiently traverse cell membranes or diffuse in tissues bearing site of action. It has been hypothesized that a drug with poor binding for plasma proteins may increase the propensity of drug reaching/penetrating the tissue with site of action than those which bind with high affinity, but then metabolism/excretion for unbound drugs is also enhanced.

11.2.2.1 Volume of Distribution

The water present in human body is a major contributor to its body weight with more than 50% in females and more than 60% in males and more than 70% in infants. Water is part of fluids present in the body and pharmacologically has been categorized under five body fluids. Each of the five body fluids has distinct volume depending on the body weight and is called physiological compartments (Fig. 11.4). For a body weight of 72 kg, the entire volume of the body fluid is considered to be equivalent to 42 liters (total body water 60%). Accordingly, the volume of individual compartment is given below:

Extracellular fluids ~20% (1/3 of body water ~15 L)
The blood plasma ~5% (1/5 of extracellular fluids ~3 L)
Interstitial fluids ~16% (4/5 of extracellular fluids ~12 L)
Intracellular fluids ~35–40% (2/3 of body water ~25 L)
Transcellular fluids ~2% Present within organs, such as the gastrointestinal cerebrospinal, peritoneal, and ocular fluids, fetus fluid, etc.

Drugs in the blood move through the above pharmacological compartments starting from plasma resulting in equidistribution of drug throughout the system. During the distribution of drugs in blood plasma, depending on the lipophilicity of the molecule, it either binds with plasma proteins or diffuses (unbound drug) through the membrane to extravascular (comprising muscle, fat) bone, etc., compartments. The unbound drug moves from one compartment to other until equilibrium is reached (Fig. 11.5).

The distribution of the drug in various body tissues (rather than plasma) is expressed by the volume of distribution and represented by V_d, with liters as unit. The movement of drug follows first-order kinetics and a plot of concentration of drug versus time is in accordance with movement of a constant fraction/per unit time (Fig. 11.5).

In general, V_d value takes into account the total amount of drug present in plasma, irrespective of bound or unbound drugs. It is pertinent to add that not all drugs are evenly distributed between plasma and rest of the body. Thus, for drugs that are evenly distributed, the volume of distribution reflects distribution of drug in the entire body.

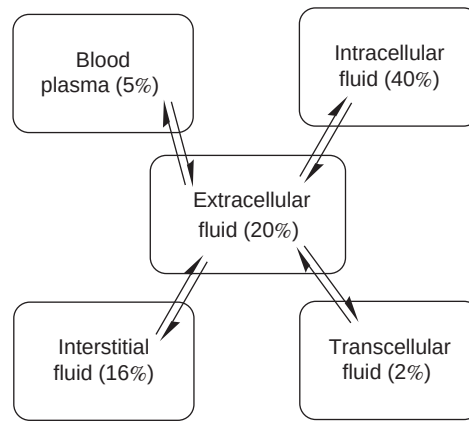


Figure 11.4 Physiological compartments within body, with respective volume of each container/compartment. Distribution of drugs among them occurs until equilibrium is reached.

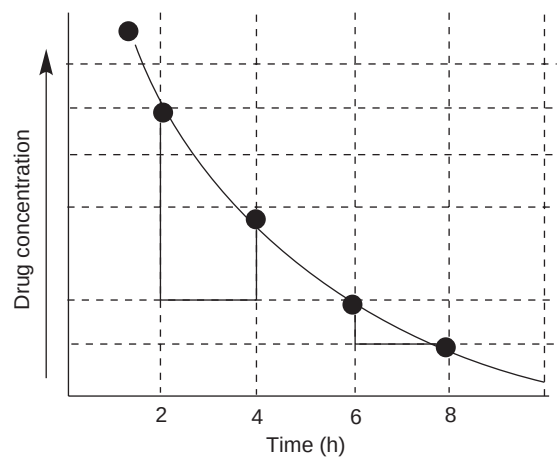


Figure 11.5 Movement of drug follows first-order kinetics.

11.2.2.2 Apparent V_d

The drug distribution in the body is represented by volume of distribution also known as apparent volume (theoretical) of distribution. In fact, it involves theoretical consideration in terms of volume visualizing concentration of drug in plasma to be same throughout the body. It is defined by the ratio of the amount of drug in the body to the concentration of drug measured in plasma. This PK parameter takes into account distribution pattern of drugs in plasma, tissues, and central and peripheral compartments of the body. Apparent volume of distribution can be calculated by dividing the amount of drug administered by concentration of drug in plasma. C_{max} in the AUC is largely affected by V_d.

Apparent volume of distribution can be calculated by dividing the amount of drug administered by concentration of drug in plasma.

$$\text{Volume of distribution} = \frac{\text{Amount of drug in body (Dose)}}{\text{Plasma concentration of drug}}$$

$$V_d\,(\text{Litres}) = \frac{\text{Dose (mg)}}{\text{Concentration (mg/mL)}} \qquad \text{(Eq. 10)}$$

1. The concentration of drug stands for the amount of drug in physiological containers/compartments. For example, if 50 mg of a drug is added to a beaker with a volume of 5 L, then concentration of the drug will be 50 mg/5 L.
2. If represented in mg/L, it should be multiplied with volume of human (for 72 kg weight) container ~42 L to get V_d or volume of the compartment (such as 5 L for blood plasma).
3. If represented in L/kg, then it should be multiplied with body weight to get V_d.
4. Alternatively, using Eq. 10, a dose of drug can be calculated if V_d and concentration of drug in plasma is known (in most instances, tissue drug concentrations parallel plasma drug concentrations):

$$\text{Dose} = V_d \times \frac{\text{Drug Plasma concentration}}{\text{target}} \qquad \text{(Eq. 11)}$$

V_d Calculation

Let us assume that a 50 mg of drug with optimal lipophilicity is administered intravenously, out of which 40 mg is diffused to extracellular region, 5 mg binds to plasma protein, and 5 mg is left as unbound drug in the blood volume of 5 liters. The V_d can be calculated using Equation 10.

Dose of drug = 50 mg
Concentration of the unbound drug = 5 mg in 5 L
Dose of drug/concentration, V_d = 50 mg/5 mg/5 L = 50 L

The V_d in this case is 50 L, which is more than the actual volume of blood plasma container, that is 5 L. Thus, 50 L is a theoretical volume of distribution, which suggests that concentration of free drug in plasma is equal to the concentration throughout the body, owing to the distribution equilibrium. Conceptually, in order to get concentration of free drug in blood, we need to place 50 mg of drug in a single container/compartment of 50 L.

A lower concentration of drug in blood plasma reflects higher volume of distribution.

1. Accumulation of drug in fat cells will decrease plasma drug concentration → high V_d
2. Accumulation of drug in bone tissue will decrease plasma drug concentration → high V_d
3. Accumulation of drug in tissue with site of action will decrease plasma drug concentration → high V_d
4. Enhanced binding of drug to plasma proteins will lead to higher concentration of drug in the plasma → low V_d.

Therapeutic Implication of V_d Values

1. An increase in V_d reflects enhanced distribution of drug in tissues. If the V_d for a drug exceeds the water volume of 42 liters in body, this reflects that distribution of drugs in tissue (e.g. chloroquine) is of very high order.
2. A V_d of drugs equal to or less than 4 L reflects lowest distribution with drug molecules being predominantly restricted to bloodstream since the volume of blood is 4.7 liters.
3. A V_d of drugs between 4 and 7 liters still reflects distribution being restricted to the blood plasma and blood cells.
4. A V_d value of water-soluble drugs is < 3 L/kg and their distribution remains limited to the extracellular compartments (interstitial fluid, plasma).
5. A V_d for lipid-soluble drugs is very high (> 400 L), probably because they have low ionization potential and low plasma protein binding. Besides, they can readily diffuse through cell membranes, hence are distributed in both the fluids (intracellular and extracellular).

Some drugs that bind too tightly to plasma proteins restrict themselves to the plasma compartment only and are unable to distribute well. Such drugs are generally characterized by a V_d equivalent to the size of the blood compartment > 0.1 L/kg. However, if the protein binding of drug is reversible, the unbound drug would diffuse from the plasma into tissues (it is not possible to obtain *in vivo* protein-binding data). This information is commonly obtained from *in vitro* studies.

11.2.2.3 PK Models

The distribution of a drug following its entry in the bloodstream involves binding to proteins (protein-bound drugs) and diffusion of unbound drug into the extracellular space followed by diffusion across tissues with/without its target. Initially, V_d keeps changing with time since mixing of drug in plasma followed by diffusion to tissues takes time. However, once a steady state is reached, there is no change in V_d with time. Although this distribution of drug from plasma (initial volume of distribution) to rest of the body has been discussed under five pharmacological compartments, pharmacokinetically it has been simplified and conceptualized into two compartments. The two-compartment concept comprises creating groups of tissues/fluids with more or less similar distribution pattern in each compartment. This is because distribution in renal tissue is expected to be quite different from adipose tissue. Besides, since the blood has unidirectional flow, it passes through highly perfused organs such as lung, heart, kidney, and liver along with muscle, fat, and cerebrospinal fluid, etc. Analyzing distribution separately among them may produce infinite compartments and complicate the outcome. Thus, a much simplified two PK compartments representing drug distribution from plasma into rest of the body was put into use and has been depicted in Fig. 11.6.

1. **Central Compartment:** This comprises of heart, liver, lungs, and kidneys as are highly perfused organs with very close distribution pattern of drug. These four organs along with plasma have been grouped under central compartment or can also be called highly blood-perfused compartment.
2. **Peripheral Compartment:** It comprises rest of the tissues/fluids (excluding perfused organs) and is considered to be less perfused. Muscle tissue, cerebrospinal fluid, fat tissue, etc., are part of peripheral compartment.

In pharmacokinetics, the drug distribution can occur either as one- or as two-compartment models:

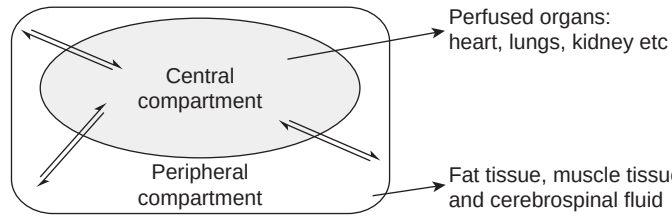


Figure 11.6 Diagrammatic representation of central and peripheral compartments.

One-Compartment Model

Drug enters the central compartment following administration of the drug and is immediately distributed in the entire body as a single giant vessel. The one-compartment model considers the entire body, including all of the organs and tissues as a single container. Drugs then leave the central compartment and are excreted (Fig. 11.7).

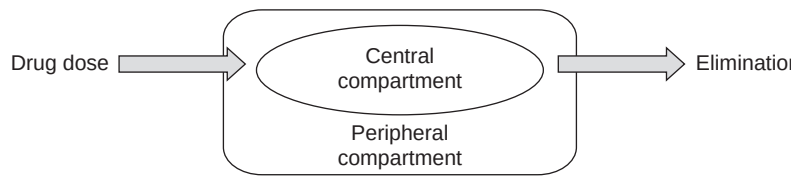


Figure 11.7 One-compartment model immediately following administration of drug.

Two-Compartment Model

The two-compartment model involves transfer of drug between central and peripheral compartments as two buckets. Drug enters the central compartment following administration of the drug (Fig. 11.8) and leaves the central compartment either to enter the peripheral compartment (referred to as compartment 2) or leave the body (excreted).

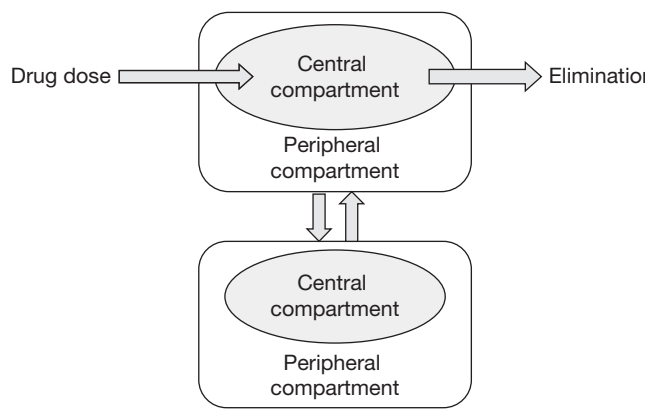


Figure 11.8 Two-compartment model after distribution equilibrium.

Significance of one-compartment model

The main objective of PK studies is to accurately measure the concentrations of drugs against time in bloodstream and tissues. In the one-compartment model since an instantaneous distribution of drug is taken into account, the PK parameters pertaining to drug outcome within the fixed distribution volume can be evaluated more easily and will be much more simplified. It is recommended that initially studies could be performed in single compartment model and if results are satisfactory, then it may not be necessary to use the complex two-compartment model.

11.2.3 Metabolism

Metabolism is a chemical process that occurs inside the body, with the sole objective to terminate the biological activity of drugs/xenobiotics within cells and facilitate their excretion from the body. The chemical process may involve oxidation, reduction, hydrolysis, hydration, conjugation, condensation, isomerization, etc. The metabolic enzymes cytochrome P450 (CYP) involved in the chemical processing (biotransformation) of drugs, although widely distributed in tissues, are predominantly more expressed in the liver. Thus, liver is considered as the primary site for drug metabolism. It is the most important organ in the body for the metabolism and detoxification of endo- and exogenous compounds.

The other major organ involved in metabolism is gastrointestinal tract (an important site for extrahepatic metabolism for drugs/xenobiotics), where following the non-parenteral administration of a drug, a significant portion of the dose gets metabolized in the intestinal epithelium followed by further metabolism in liver before reaching the systemic circulation. Thus, the oral administration of drugs leads to first-pass metabolism, which in turn reduces the bioavailability of drugs. Other organs involved in minor biotransformations are kidneys, lungs, adrenal glands, brains, placenta, brain, and skin.

Within cells, cytochrome P450 enzymes are located on the endoplasmic reticulum (involved in protein processing and transport) and in mitochondria (the energy-producing center of cells). The metabolic enzymes located in the mitochondria are involved both in the synthesis and metabolism of internal substances, while those located in the endoplasmic reticulum exclusively metabolize external substances (drugs), toxins formed within cells, and environmental pollutants.

Although biotransformation typically furnishes inactivated drugs (thus terminating the biological activity of drugs), some drug metabolites are therapeutically active substances or may sometimes be even more active than the parent compound.

11.2.3.1 Therapeutic Implication of Biotransformations (Metabolism)

Generally applicable to metabolism of all xenobiotics as well as endogenous compounds such as steroids, vitamins, and fatty acids

1. Generates water-soluble (polar) and inactive metabolites that are readily excreted from body through renal excretion.
2. Drugs that are not absorbed in the gut or are highly lipophilic are often excreted in stool directly from intestine via hepatic biliary excretion (along with enterohepatic recycle) process.
3. Metabolites with therapeutic activity or with toxic properties may be also produced.

11.2.3.2 Role of CYPs in Metabolism

Cytochrome (CYP) P450 is a family of heme (cofactor) containing monooxygenase enzymes. It carries out metabolic oxidative transformation of xenobiotics and endogenous substances via a complex electron transfer mechanism involving redox partner enzymes and molecular oxygen. Although CYPs are distributed

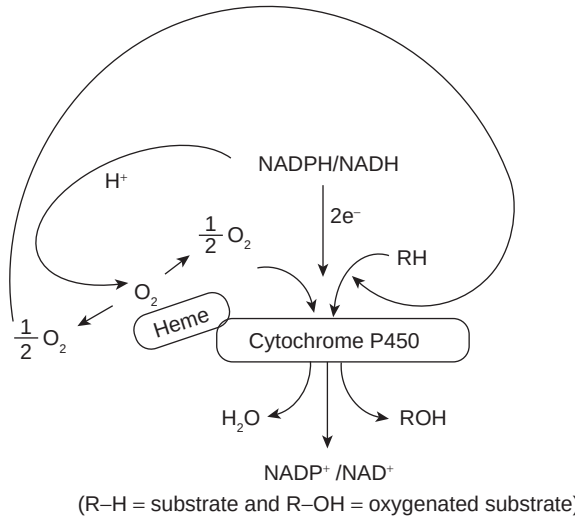


Figure 11.9 A typical CYP-mediated biotransformation.

throughout the cells in the body, they are found primarily as membrane-bound isozymes in endoplasmic reticulum (microsomes) of liver cells. While the catalytic site in the isozyme is present along the membrane, the binding site is present on either side of plasma membrane facing lumen or cytosol. Thus, substrates can access CYP450 both from cytosolic as well as exogenous side. In general, CYPs display poor selectivity for substrates and hence they are involved in both the biosynthesis and metabolic oxidation of numerous endogenous chemicals in cells. Besides, they are also involved in the metabolic oxidation and excretion (detoxification) of numerous xenobiotics. The objective of metabolic oxidation by CYP is to produce hydrophilic compounds for their elimination from body through urine. In general, the oxidation reaction catalyzed by CYP requires:

1. Two electrons provided by redox partner cytochrome P450-reductase and cytochrome b_5.
2. A molecular oxygen trapped by heme cofactor present in CYP450.
3. Protons obtained from cofactor NADH or NADPH (Fig. 11.9).

Redox Partners Cytochrome P450-Reductase and Cytochrome b_5 (b_5)

Cytochrome P450-reductase (CPR), also referred to as NADPH: ferrihemoprotein oxidoreductase, is an enzyme present on membrane (endoplasmic reticulum) and located next to CYP450 (Fig. 11.10). The enzyme has four binding domains, out of which three binds with NADPH: a cofactor, flavin mononucleotide (FMN or riboflavin–5′-phosphate): a prosthetic group, and FAD or flavin adenine dinucleotide: a coenzyme (cofactor). The second redox partner is cytochrome b_5 reductase, which is a low-molecular-weight flavoprotein and is involved in the transfer of electrons originating from CPR to CYP450 monooxygenase (Fig. 11.10). Thus, the flow of electrons involves NADPH to FAD to FMN in CPR, then to b_5 reductase, and finally to its place of action CYP450 monooxygenase (electron acceptor). Besides, flow of electrons originates from cofactor NADPH; the latter also releases proton which reaches the active site in CYP450 through amino acids in CYP. At the active site of CYP450, H^+ reductively splits molecular oxygen into two nascent oxygens: one of them forms water and another one is involved in the oxidation of substrate furnishing hydroxylated

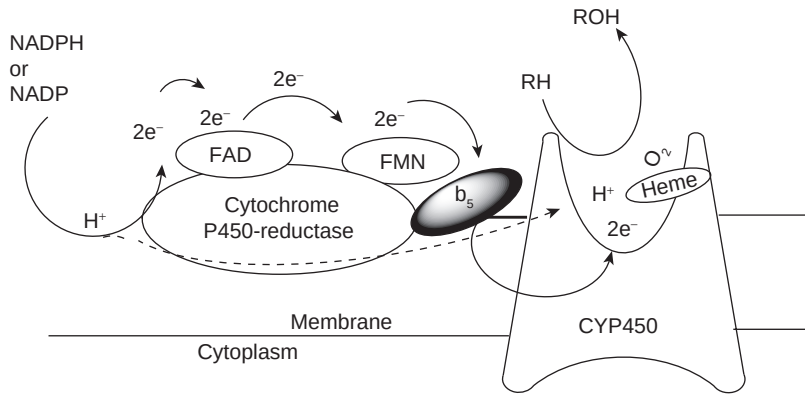


Figure 11.10 Redox partners cytochrome P450-reductase (CPR) and cytochrome b_5.

compounds. This phenomenon of forming two oxygenated compounds via catalytic action of CYP led to its naming as "mixed function oxidases". Some monooxygenases do not require assistance from NADPH; instead, electron is withdrawn from the substrate itself, e.g., flavin-dependent monooxygenases. Oxidation of drugs by CYPs in the presence of iron generally produces water-soluble metabolites to facilitate renal excretion and accounts for 70−80% of enzyme-mediated metabolism of drugs.

Interactions of Drugs with CYP450

There are two kinds of interactions between drugs and CYPs that play an important role in health care issues arising due to side effects of drugs and drug–drug interactions (DDIs). This particularly poses major problems in combination therapy that is becoming more prevalent for treating diseases.

1. **Interacting CYP as an Inhibitor:** Some specific drugs may inhibit the specific hepatic isoenzyme (CYP) and as a result may have an increase (accumulation) in the concentration of the drug in the body. This, in turn, may lead to drug toxicity due to prolonged exposure to the drug.
2. **Interacting CYP as an Inducer:** There are some drugs that can lead to an increase in the production of the metabolic enzyme (CYP) and as a result speed up the metabolism (oxidation) and clearance of the drug, and hence a higher dose of the drug may be required to achieve the desired therapeutic effect.

PK Drug–Drug Interaction Owing to Either Inhibition or Induction of CYPs

DDI comes into existence when the two drugs are administered together as part of polypharmacy and when both of them get metabolized by the same metabolic enzyme (metabolism of one or both the drugs may be delayed). This leads to major adverse drug interactions since alterations in the enzyme activity of CYP may affect the metabolism and clearance of the other drug. A rapidly metabolized drug will have little therapeutic effect as the effective concentration of drug in blood plasma/tissue is never achieved (reducing clinical efficacy), whereas slow metabolism may lead to toxicity/adverse drug reactions. This is because some drugs act as inducers while some act as inhibitors of the metabolic enzyme; however, in general, prevalence of enzyme inhibition is much more than induction. In combination therapy, if one drug inhibits a specific CYP that may be selectively metabolizing a second drug (substrate), then the concentration of the latter may increase leading to toxicity. A drug with enhanced concentration and prolonged stay in the body may bind to off-target proteins leading to toxicity (Fig. 11.11).

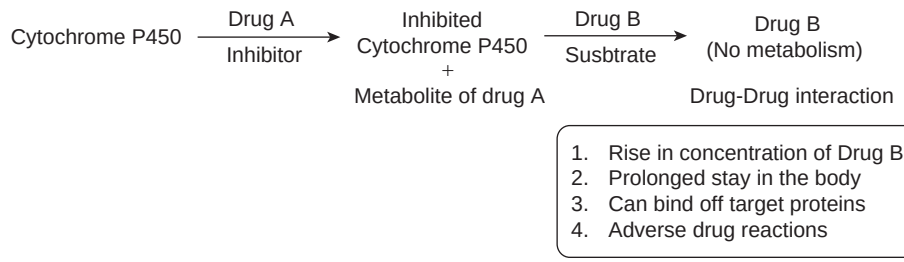


Figure 11.11 Drug–drug interactions either due to inhibition or induction of CYP.

Hence, these drugs may require dosage adjustments or selection of alternate drugs that do not interact with the CYP system. Grapefruit juice, for example, is a potent inhibitor of CYP3A4,[5] and therefore administration of drugs that are known to be metabolized by CYP3A4 along with the consumption of grape fruit juice may decrease first-pass metabolism and increase bioavailability of the drug. The dual phenomenon involving drug-CYP interactions also explains as to why drug metabolism rates vary among patients and why some patients metabolize drugs rapidly, whereas in some patients, metabolism is extremely slow. Erythromycin and midazolam both are substrates for 3A4 isozyme, and ultimately, metabolism of midazolam is inhibited, leading to enhanced level of drugs in the body.

In yet another DDI situation, one of the drugs while getting metabolized may induce (enhance) the production of the CYP, which in turn enhances metabolism of the other drug. Thus, enhanced production of metabolizing CYP may result in the reduced efficacy of the second drug. Rifampicin, for example, causes rejection of transplants in patients already under treatment with cyclosporine. This may be attributed to increased levels of CYP3A4 following administration of rifampicin.

Drop in the Efficacy of a Single Drug Due to CYP Induction

Following administration of drug in the body, sometimes therapeutic response is observed due to the active metabolite of drugs. In case metabolite is active, then induction of metabolic enzyme by drug while undergoing metabolism will enhance the concentration of enzyme and will facilitate complete biotransformation of the available drug molecules. This, in turn, will enhance the biological activity. However, if the native drug is responsible for activity, its ability to cause induction of the metabolizing enzyme will trigger fast metabolism of the drug. This will not only reduce the concentration of drug in bloodstream but also affect its efficacy, minimal exposure to drug, and duration of action (Fig. 11.12). Phenobarbital and carbamazepine, for example, act on CYP450 as inducers in elderly patients, thereby affecting efficacy of other drugs.

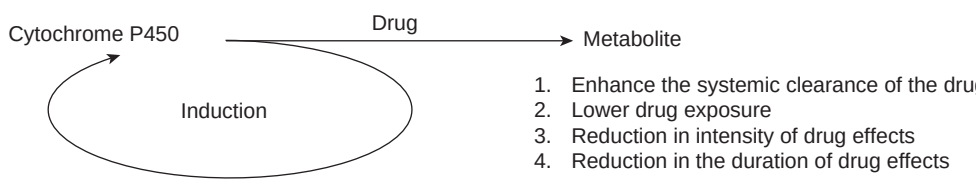


Figure 11.12 Drop in efficacy of a single drug due to CYP induction.

11.2.4 Excretion

Drugs once administered must be cleared from the body after it has exhibited the therapeutic efficacy. Two major organs that play an important role in excretion of drugs are kidney and liver. Other minor routes of excretion are sweat, saliva, milk, and tears. Pulmonary excretion (through breathing) involves elimination of anesthetic gases and vapors, as well as alcohol. Thus, drug excretion deals with irreversible removal of drug from the body by either of the major/minor routes of excretion (Fig. 11.13).

In general, there are two major pathways (besides sweat, saliva, tears, milk) for excretion of drugs/metabolites: hepatobiliary excretion where drugs after getting processed in liver are eliminated via stool or via systemic circulation may undergo renal excretion of water-soluble substances via urine. The drugs may be eliminated from the body either in its intact form (unchanged) or following metabolism/biotransformation. The drug metabolism/biotransformation is a degradable process by which the drugs are chemically converted into a metabolite inside the body. Biotransformation is both enzymatic as well as non-enzymatic process. Details of phase I and II metabolism of drugs have been discussed in Chapter 12.

The elimination route may largely depend on the lipophilicity of drugs/metabolites. The lipophilic drugs are often transformed into water-soluble (polar compounds) metabolites in the liver that can be excreted by the kidney. On the other hand, orally administered unabsorbed or highly lipophilic drugs or their metabolites undergo biliary excretion in the intestinal tract (for reabsorption of drugs) and if the drugs/metabolites still remain unabsorbed, they are excreted in the stool. Rifampicin is an example of drug which undergoes biliary excretion. Hydrophilic drugs (metformin), owing to their ability to dissolve in water, do not undergo metabolism and are therefore, often eliminated unchanged in the urine.

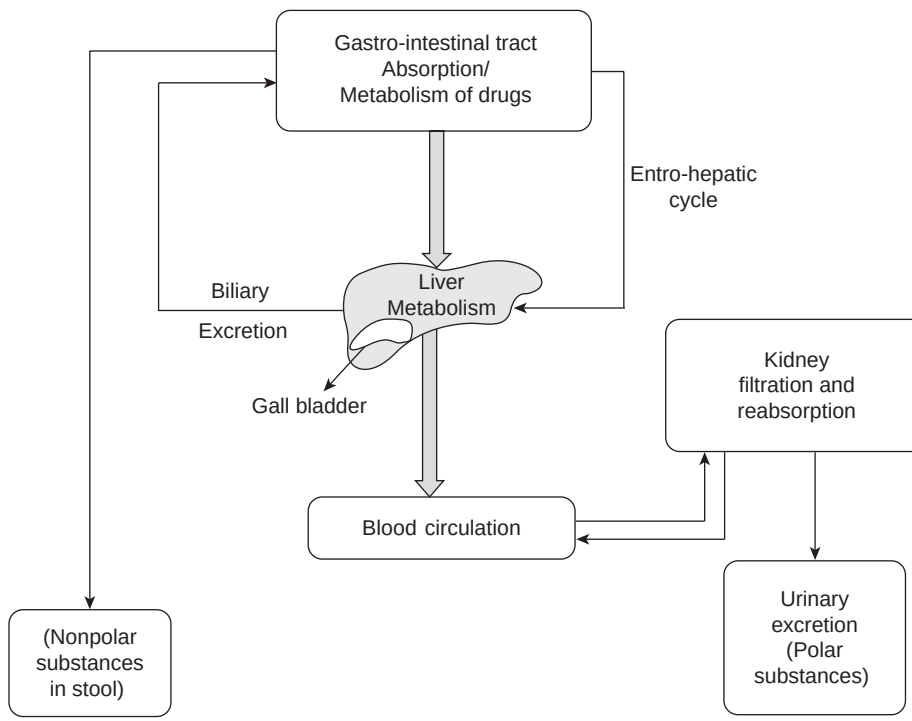


Figure 11.13 Diagrammatic representation of drug excretion.

11.2.4.1 Excretion Through Kidneys

For renal elimination of drugs/metabolites, they should be water soluble (hydrophilic/polar) and should have poor affinity (not to be bound too tightly) for plasma proteins. Besides, when it comes to excretion of intact drugs, only unbound drugs (not the ones bound to proteins) are excreted. For example, albumin-bound drugs in plasma are not excreted as albumin is too big a molecule to get filtered through kidney (and if it passes in the urine, it reflects damaged kidney).

There are three steps in renal excretion: filtration, reabsorption, and secretion of drug at the level of nephron (Fig. 11.14), a functional unit of the kidney. Drugs/metabolites enter through afferent arteriole and that with a particular size are filtered (both intact and metabolized) through glomerulus capillaries enveloped in Bowman's capsule and drugs that are not filtered leave through efferent arteriole and enter other capillaries called peritubular capillaries. Here drugs that escaped filtration get yet another opportunity to get secreted into the urine and also drugs (lipid soluble are preferred) from the urine may get an opportunity to be reabsorbed. In general, polar compounds (drug/metabolites) fail to get reabsorbed in bloodstream and are therefore readily excreted through urine. Thus, drugs that could not be filtered are reverted back into re-circulation along with other reabsorbed substances.

The ability of kidney to excrete hydrophilic drugs/metabolites is regulated by:

1. The acidity of urine (affects rate of excretion).
2. Rate of flow of the urine (decrease with age).
3. Flow of blood through kidneys (decrease with age).
4. Pathological conditions of kidneys.

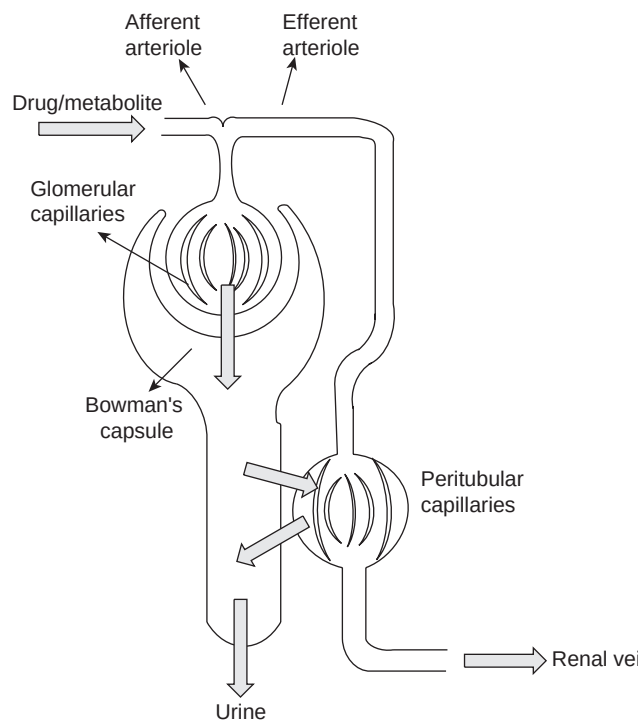


Figure 11.14 Renal excretion involving glomerular filtration and tubular reabsorption/secretion.

11.2.4.2 PK Parameters for Excretion

The PK parameters dealing with elimination analyze rate at which native drugs or its metabolites are excreted from the body.

1. Rate of elimination
2. Clearance
3. Flow of blood
4. Extraction ratio
5. Biologic half-life for first-order elimination of drugs

Rate of Elimination

It represents the amount of drug getting cleared from the plasma per unit time, thus reflecting fraction divided time. This is represented by k_{el} and can be derived by dividing clearance with volume of distribution.

$$\text{Rate of elimination}\,(k_{el}) = \frac{C_L}{V_d} \qquad \text{(Eq. 12)}$$

Drug Clearance

Once the drug reaches systemic circulation, its (unbound drug) clearance from the plasma commences immediately. Thus, removal of drug from the plasma is referred to as clearance represented by C_L. Clearance is quantified as volume of the blood plasma (fluid) from which drug is irreversibly cleared (filtered) and its unit is volume/mass/time depicted either as L/hr or L/hr/kg. It is defined as the volume of blood plasma that gets free (filtered) of drug molecules per unit time. Thus, it does not reflect the amount of drug that is getting removed; instead, it reflects the volume of plasma that is cleared of drug per unit time. The C_L is an important PK parameter used to determine maintenance doses of drugs required for treating diseases in multicellular organisms. Although blood plasma is generally sampled and analyzed for C_L, the value is taken as total clearances by all organs. C_L can be a function of distribution and elimination, and can be calculated using following equation:

Clearance (C_L)= Elimination rate constant (k_{el}) × Apparent volume of distribution (V_d) (Eq. 13)

If clearance of a drug is 10 L/hr and V_d is 100 L, the elimination rate constant can be calculated:

$$C_L = k_{el} \times V_d$$

$$k_{el} = \frac{10\ \text{Lh}^{-1}}{100\ \text{L}} = 0.1\text{h}^{-1} \qquad \text{(Eq. 14)}$$

The value of 0.1/hr can be expressed either as fraction of drug in the body removed per hour or as 10% of the 'Volume' that is cleared of drug per hour.

Since elimination occurs both in plasma and by individual organs, adding the individual clearances will furnish total systemic clearance (Fig. 11.15) and can be represented by:

$$C_{L\,\text{systemic}} = C_{L\,\text{hepatic}} + C_{L\,\text{renal}} + C_{L\,\text{p}} + C_{L\,\text{others}} + \ldots \qquad \text{(Eq. 15)}$$

Extraction Ratio of Drugs

It denotes fraction of test compounds/drug being cleared from the eliminating organ in a single passage, in other words rate of entrance vs. exit from the organ. It can be measured by determining the concentration of

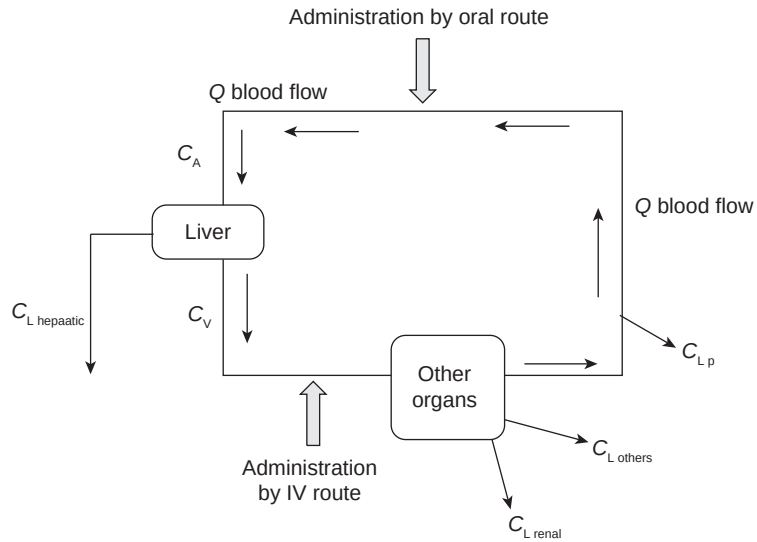


Figure 11.15 A diagrammatic representation of relationship between clearance of drug and concentration (C_{Lp}, clearance plasma; C_A, concentration of drug entering liver; C_v, concentration of drug exiting liver).

drugs/test compounds while entering (through artery) the organ and after exiting (through vein) the organ. The ER can be derived from the following equation and its value generally ranges between from 0 to 1.0 in different organs. The ER values depend on blood flow and functional abilities of organs. An ER value of 0.5 reflects 50% of the drug is cleared from the plasma while passing through the organ.

$$\text{ER} = \frac{C_A - C_V}{C_A} \qquad \text{(Eq. 16)}$$

Blood Flow

Flow of blood (Q) can affect elimination rate when an eliminating organ is able to clear drug in totality in a single passage, thus ER value is 1.0. However, when ER value is <1.0, changes in the flow of blood have no effect. The rate at which drugs enter the organ and exit the organ can be calculated by the following equation:

Rate of presentation of drug in organ $= Q \times C_A$

Rate of presentation of drug from the organ $= Q \times C_v$ (Eq. 17)

Where,

C_A = Concentration of drug entering the organ
C_v = Concentration of drug leaving the organ

Using the above rates of entrance and exit of drugs, clearance for individual organ can be calculated as follows:

$$C_{L\ organ} = Q \times C_A - Q \times C_V$$
$$= Q\left(C_A - C_V\right) \qquad \text{(Eq. 18)}$$

By taking extraction ratio into account clearance by single organ ($C_{L\ organ}$) can be also represented using following equation:

$$C_{L\ organ} = Q_{organ} \times ER$$

$$= Q_{organ} \times \frac{C_A - C_v}{C_A} \quad \text{(Eq. 19)}$$

Loading and Maintenance Doses

A loading dose deals with the administration of single or multiple set of doses with the view to attain optimal peak concentration of drugs required for the desired therapeutic effect. Notably, the optimal plasma concentration for a drug remains in competition with clearance. The volume of distribution V_d is a theoretical volume and its value can be used to determine the loading dose, whereas C_L is a constant and it can be used to determine the maintenance dose. Thus, loading dose can be derived by multiplying the desired peak concentration with the volume of distribution of the drug.

$$\text{Loading dose} = C_{P(Target)} \times \frac{V_d}{F} \quad \text{(Eq. 20)}$$

(*C_p can be taken as plasma drug concentration since the concentration at the target is presumed to be in equilibrium to plasma concentration mg/L and V_d in litres*)

A maintenance dose deals with maintaining a required steady state concentration of drug and is a small and fixed dose. If the dosing interval corresponds with the half-life of the drug, the loading dose will be twice the maintenance dose.

$$\text{Maintenance dose} = C_L \times \frac{C_p S S_{av}}{f} \quad \text{(Eq. 21)}$$

Where,

$C_p S S_{av}$ is the target average steady state drug concentration

F is bioavailability

C_L is clearance

Maintenance dose is expressed in mg/h, so in order to determine dosing regimen, the value needs to be multiplied by hours.

Alternatively, it can be also determined using dosing rate and interval:

$$\text{Maintenance dose} = \frac{\text{Dosing rate}}{F} \times \text{Dosing interval} \quad \text{(Eq. 22)}$$

Half-life ($t_{1/2}$)

It is one of the PK parameters that determines the duration (length) of action of a drug. This is the time during which the concentration or amount of drug in the body is reduced by half (takes to eliminate 50% of the drug). The half-life of a drug depends on how quickly the drug is cleared (eliminated) from the plasma. In general, concentration of drug drops in plasma due to continuous metabolism and the rate at which drugs are eliminated (follows either zero-order or first-order kinetics). The selection of either of the kinetics is largely governed by the availability of metabolic enzymes. Most drugs follow first-order kinetics (Fig. 11.16), which means higher the concentration of drug in the plasma faster will be the elimination of

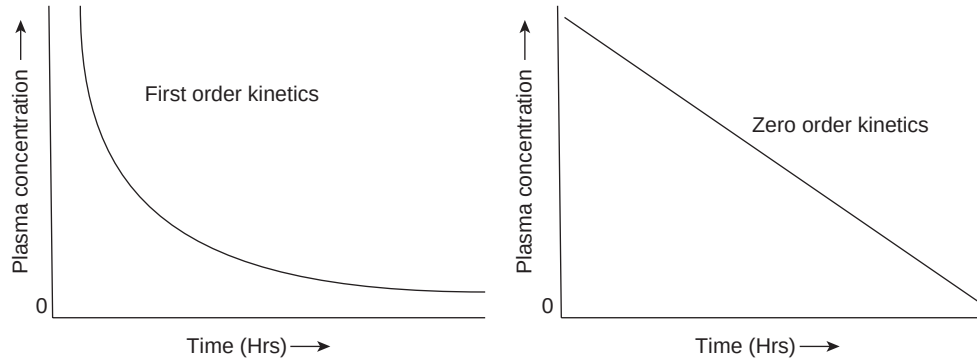


Figure 11.16 First order and zero-order kinetics excretion.

drug. The situation arises because concentration of metabolizing enzyme is more than the concentration of drugs and thus the enzyme is never saturated, and most drugs are metabolized. This although leads to same proportions (in %) of the drugs being eliminated per unit time, the amounts (quantity) will vary according to the concentration of drugs in the plasma. The rate at which the drug is eliminated slows down as the plasma concentration of drug drops. It is interesting to note that in the first-order kinetics with each succeeding half-life, the amount of drug eliminated is reduced by half and remains constant. For example, after the first half-life, the amount of drug remaining in the body is reduced to 50%, after second half-life the drug concentration is reduced by 25%, etc. A plot for the first-order kinetics between concentrations of drug against time, predominantly exhibits a linear curve (Fig. 11.17).

In situations, when drugs encounter limited quantity of CYP, most of the metabolic enzymes will be consumed (saturated) in metabolizing drugs and the presence of excess drugs will not affect the rate of excretion. Instead, same quantity of drug will be eliminated per unit time independent of drug concentration in the plasma. This constant rate of elimination is called zero-order kinetics. The plot for zero-order kinetics between concentrations of drug against time, predominantly exhibits a straight line (Fig. 11.17).

Generally, half-life of a drug is regulated by three factors: V_d, k_{el}, and C_L where the $t_{1/2}$ is inversely proportional to first-order elimination rate constant k_{el}, that means higher is the k_{el} shorter is the $t_{1/2}$. Thus $t_{1/2}$ can be derived using following equation:

$$t_{1/2} = \frac{\ln(2)}{k_{el}} = \frac{0.693}{k_{el}} = 0.693 \times \frac{V_d}{C_L} \quad \text{(Eq. 23)}$$

Overview of factors affecting duration of drug action are:

1. The absolute amount of drug given
2. The pharmaceutical preparation facilitating solubilization and passage in bloodstream
3. Gastric emptying time can vary from 0 to 3 hours for orally administered drugs
4. Removal of drug from plasma (C_L)
5. Volume of distribution (V_d)
6. The reversibility of drug action
7. The half-life of the drug
8. The time the drug is engaged on the receptor
9. Intracellular signaling

10. Effect on gene regulation
11. The slope of the concentration–response curve
12. The activity of metabolites
13. The effect of disease on drug elimination

11.3 Methods for Assessing Major Physicochemical Properties of Compounds that Affect ADME Parameters

There are a variety of physicochemical parameters that affect ADME properties of compounds: molecular weight, lipophilicity, ionization constant, solubility, polar surface area, H-bond donors and acceptors, permeability, etc. (Details discussed in Chapter 6). Methods for assessing major parameters have been discussed below.

11.3.1 Measuring Solubility

In physiological environment, drugs have to move through both aqueous and lipid medium before they can access the target for eliciting the desired response. Thus, solubility of drugs represents an important physiochemical parameter and plays an important role in the pharmacokinetics and pharmacodynamics of drugs. The basic requirement in the dissolution of a solid into solution (aqueous or nonaqueous) involves interactions among solute molecules and solvent molecules, and accommodation of solute molecules within the space of solvent molecules. Ultimately, a new stabilized interaction occurs between solvent and solute molecules during dissolution which is accompanied by slight change in entropy of the system. Two approaches generally used to establish solubility of drugs are: thermodynamic or kinetic solubility.

1. The thermodynamic solubility is based on traditional method wherein a solid test compound is exposed to the aqueous medium followed measurement of dissolved solute at equilibrium.
2. The kinetic solubility involves introduction of aliquots from a concentrated stock solution of the test compound in an organic solvent such as DMSO into aqueous medium.

Quantity of dissolved solutes that refers to solubility in both approaches can be determined by HPLC or LCMS-MS. Kinetic solubility can be readily performed in high-thoughput mode and is generally applied during early stages of discovery. On the contrary, thermodynamic solubility is generally carried out during formulation strategies for *in vivo* studies. One of the most important factors that affect solubility is the pH of the aqueous medium.

11.3.2 Measuring Lipophilicity

Lipophilicity plays an important role in all stages of drug discovery: starting with *in vitro* target-based assays wherein this has been attributed to facilitate tight binding, during generation of focused library with drug-likeness, during SAR and SPR studies leading to drug design. During absorption and distribution of drugs in physiological environment, during drug metabolism and pharmacokinetic modeling (DMPK), during formulation, etc. Lipophilicity of test compounds can be determined in high-throughput mode using either inexpensive computational tools or using HPLC, capillary electrophoresis, microtiter partitioning methods. The lipophilicity of test compounds represented by logP is generally determined by measuring the extent of distribution of neutral form of compound between an aqueous phase and a hydrophobic phase such as 1-octanol. However, for test compounds with ionizing abilities, the distribution of both unionized and

ionized compound between two phases is taken into account. This is generally expressed as logD and can vary with pH of the aqueous medium used. For example, $\log D_{7.4}$ at the physiological pH can be determined using octanol-buffer (pH = 7.4.) shake flask method followed by quantification using LCMS-MS.

11.3.3 Measuring pK_a

Along with lipophilicity, the acid dissociation constant pK_a is the second most important parameter that plays an important role in all stages of drug discovery. It reflects pH at which unionized and ionized moieties of acids are in equilibrium. A lower pK_a value reflects that Bronsted acids readily gives up its proton while higher pK_a values reflects that proton is held more tightly. Experimentally,[6] pK_a values for test compound is determined by titrating against base and using the famous equation proposed by Hasselbalch.[7]

$$\mathrm{pH} = pK_a + \log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]}$$

Where,
A^- is the concentration of dissociated acid
HA is the concentration of non-dissociated acid

11.3.4 Measuring Permeability

Permeability of test compounds regulate the ability of the active test compound to diffuse through lipid bilayer plasma membranes involving epithelial cells lining the gastro-intestinal tract, endothelial cells lining the blood capillaries and finally into the tissues if the therapeutic target is either nuclear receptor or signaling enzymes. Caco-2 colon carcinoma cell line and artificial membranes are used to estimate permeability.

11.4 Summary of Important PK Parameters

These studies are carried out as part of lead optimization/candidate selection process with the view to identify preclinical candidates (Table 11.2).

Table 11.2 Summary of some important PK parameters

Terminology	PK Parameter	Formula	Unit
Dose	Amount of drug administered	Dose regimen	mg
C_{max}	The peak plasma concentration of drug after administration	Can be directly measured by the concentration time curve	μg/L
T_{max}	Time taken to reach C_{max}	Can be directly measured by the concentration time curve	hour
Dosing interval (τ)	Time between dose administration		hour
Bioavailability (F)	Amount of drug absorbed/drug dose	$F_{abs} = 100 \times \frac{AUC_{po} D_{IV}}{AUC_{IV} D_{po}}$	%

(*Continued*)

Table 11.2 (*Continued*)

Terminology	PK Parameter	Formula	Unit
Concentration	Amount of drug in a given volume of plasma	$\frac{D}{V_d}$	mg/L
Apparent volume of distribution (V_d)	Amount of drug in body/plasma drug concentration	$\frac{\text{Dose}}{\text{Concentration in plasma}}$	L
Clearance (C_L)	Rate of drug elimination/plasma drug concentration, or elimination rate constant × apparent volume of distribution	$k_{el} \times V_d$	L/h
Elimination rate constant (k_{el})	Rate of drug elimination/amount of drug in body	$\frac{C_L}{V_d}$	h^{-1}
Half-life ($t_{1/2}$)	The time required for the concentration of the drug to reach half of its original value.	$t_{1/2} = \frac{\ln 2}{k_{el}}$	Minutes or hours

Exercises

1. Discuss significance of drug–receptor interaction.
2. Describe reversible interactions in the drug–receptor binding.
3. Discuss role of H-bonds in drug–receptor interaction.
4. Write a short note on:
   - **(i)** Ionic interaction
   - **(ii)** Van der Waals interaction
   - **(iii)** Hydrophobic interactions
   - **(iv)** Dipole–dipole/ion dipole/dipole–dipole induced interaction

Competitive Exam Questions

1. The loading dose of a drug is usually based on
   - **(a)** total body clearance of drug.
   - **(b)** percentage of drug bound to plasma proteins.
   - **(c)** fraction of drug excreted unchanged in urine.
   - **(d)** apparent volume of distribution and desired drug concentration in plasma.

   [***GATE Pharmacy 2001***]
2. The biological half -life of procaine in a patient was 35 min and its volume of distribution was estimated to be 60 L. The total clearance rate of procaine is
   - **(a)** 1.188 L/min **(b)** 0.115 L/min
   - **(c)** 11.5 L/min **(d)** 5.75 L/min

   [***GATE Pharmacy 2005***]
3. A drug (200 mg dose) administered in tablet form and as intravenous injection (50 mg dose) showed AUG of 100 and 200 microgram

h/mL, respectively. The absolute availability of the drug through oral administration is

(a) 125% **(b)** 250 %

(c) 12.5% **(d)** 1.25%

[***GPAT 2010***]

4. Inhibition/induction of which of the following cytochrome P450 enzyme system is most likely to be involved in important DDIs?

(a) CYP3A4 **(b)** CYP2D6

(c) CYP2C9 **(d)** CYP1A2

[***GPAT 2011***]

5. Regarding two compartment pharmacokinetics all are true except

(a) a drug is always removed from the peripheral compartment.

(b) a drug with a high volume of distribution is likely to be lipophilic.

(c) a drug can have a short duration of action while being eliminated very slowly.

(d) most anesthetic drugs are modelled well with a two-compartment model.

[***GPAT 2013***]

6. The range of absolute bioavailability is

(a) 0 to 1 **(b)** 0 to 100

(c) −1 to 1 **(d)** −1 to 100

[***GPAT 2015***]

7. What will be the AUC value of lidocaine if the administered dose is 0.2 g and the total clearance is 45 L/h ?

(a) 4.44 h mg/L **(b)** 0.0044 h mg/L

(c) 9.00 h mg/L **(d)** 9000 h mg/L

[***GPAT 2016***]

8. Grapefruit juice is P-glycoprotein and CYP40 enzyme inhibitor. If drug X is degraded by proteolytic enzymes, administration of grapefruit juice with X

(a) increase bioavailability of X.

(b) decrease bioavailability of X.

(c) does not affect bioavailability of X.

(d) cause unexpected action of X.

[***GPAT 2016***]

9. The co-administration of erythromycin with cyclosporine

(a) decrease bioavailability due to complex formation.

(b) increase bioavailability due to inhibition of microflora in the intestine.

(c) decrease bioavailability due to induction of hepatic metabolism.

(d) increase bioavailability due to inhibition of hepatic metabolism. [***GPAT 2019***]

References

1. Thelen K., and Dressman J. B., *J. Pharm. Pharmacol.*, **2009**, *61*, 541.
2. Hollie I. Swanson, *Drug Metab. Dispos.*, **2015**, *43*, 1499.
3. Ian D. Wilsonand Jeremy K. Nicholson, *Translational Research*, **2017**, *179*, 204.
4. Houkai Li, Jiaojiao He and Wei Jia, *Expert Opin. Drug Metab. Toxicol.*, **2016**,*12*, 31.
5. Bressler R., *Geriatrics*, **2006**, *61*, 12.
6. Jetse Reijenga, Arno van Hoof, Antonie van Loon, and Bram Teunissen, *Anal. Chem. Insights*, **2013***, 8*, 53.
7. Hasselbalch KA. Die Berechnung der Wasserstoffzahl des Blutes aus der freien und gebundenen Kohlensäure desselben, und die Sauerstoffbindung des Blutes als Funktion der Wasserstoffzahl. *Biochemische Zeitschrift*, **1916**, *78*, 112.

CHAPTER

12 Pharmacodynamics

KEY THINGS TO LEARN FROM THIS CHAPTER

- Biochemistry of enzymes: Enzymes as biocatalysts, binding and catalytic sites, cofactor: Apoenzyme and Holoenzyme, enzyme-substrate complex
- Enzyme-substrate interactions: Lock-and-key model and induced fit model
- Factors affecting enzyme activity: Effect of concentrations of substrates and enzymes, temperature and pH on rate of reaction, Michaelis-Constant and Michaelis Menten equation
- Effect of inhibitors on enzyme activity: Reversible, competitive, noncompetitive and uncompetitive inhibitors
- Allosteric enzymes: Allosteric activation (PAM) and inhibition (NAM), silent allosteric modulators (SAM), effect of allosteric modulation on reaction kinetics
- Nomenclature of enzymes: EC1, EC2, EC3, EC4, EC5, EC6 and EC7.
- Enzymes as therapeutic targets: Extracellular and intracellular enzymes.

12.1 Introduction

Pharmacodynamics (PD) is defined as the biological response (modulating a process in a system) observed following administration of a drug at different concentrations. The studies assist in establishing quantitative relationships between the concentration of drug available at the site of action and post binding pharmacologic (the efficacy) and/or toxicologic responses. Therapeutic response is primarily affected by affinity of drugs for protein targets, concentration of drug at the target site, and pharmacodynamic drug-drug interactions. These factors in turn are governed by the physicochemical properties of test compounds/drugs. However, other factors that can affect PD of a drug can be attributed to physiological effects and may involve pathogenesis of disease, aging, genetic makeup, gender, etc. The physiological disorders bring changes in target bindings, alter the availability of protein targets, or decrease the sensitivity of protein targets. Thus, there are two major factors through which PD of drugs/test compounds are regulated: physicochemical properties of compounds and physiological responses following binding with the protein targets.

The basic component of PD comprises a therapeutic target through which drug elicits its therapeutic response. In general, therapeutic targets comprise extracellular and intracellular receptors and enzymes involved in cellular/physiological functions. It is pertinent to add that while receptors only play a role in eliciting therapeutic response through cell signalling, enzymes on the other hand have a dual role to play. Depending on specific functions of enzymes, it can either act as therapeutic target or play a yet another important role involving metabolism of drugs. Fundamentally, both the actions involve interactions between enzymes and substrates resulting in product(s). Products generated following action of substrates on therapeutic enzymes regulate cellular/physiological functions while products generated following action of substrates with metabolizing enzymes are active or inactive metabolites for the purpose of excretion. In this chapter, PD principles with reference to enzymes will be discussed (PD with reference to receptors has been discussed in Chapter 8).

12.2 Biochemistry of Enzymes

Enzymes are protein molecules made up of amino acids and act as biocatalysts. They speed up chemical reactions in the body, but do not get consumed in the process. Almost all biochemical reactions in a living organism are catalysed by enzymes. Chemical reactions in the presence of an enzyme go much faster than they would in the absence of the enzyme.

Enzymes are known to catalyse thousands of intracellular and extracellular biochemical reactions via either catabolism or anabolism route. Besides, they are also involved in many signalling pathways involved in the regulation of overall physiological functions. Generally, most of the enzymes are derived from proteins; there are few derived from RNA molecules and are associated with enzymatic activity called ribozymes. Enzymes of digestive tract and those found in blood are present in their inactive form called zymogens or proezymes.

Catalytic behaviour of any enzyme depends upon its primary, secondary, tertiary, or quaternary structure. It is estimated that human body has the ability to generate 2 million different types of proteins, coded by only 20,000−25,000 of our genes. Notably, the estimate of the number of human genes is repeatedly revised.

There are four main types of proteins: globular proteins, fibrous proteins, membrane proteins, and disordered proteins.

1. Fibrous proteins provide structural support and protection to cells and tissues through formation of muscle fibre, tendons, bone matrices, and connective tissues.
2. Membrane proteins are those found in the lipid bilayer membrane of the cells. In animal cells, these proteins make up around 50% of the mass of the plasma membrane. They also act as transporters, anchors, receptors, and membranous enzymes.
3. Globular proteins are macromolecules with a ball-like (globular shape) tertiary structure induced by orderly folding of long polypeptide chains. They are folded into spherical shapes and are associated with the following properties.
   - **(a)** Display solubility in water.
   - **(b)** The polar groups in globular proteins are generally exposed on the surface and readily interact with water molecules in the environment through non-covalent interactions.
   - **(c)** Nonpolar groups on the contrary remain buried inside the globular protein away from aqueous environment.
   - **(d)** The round shape acquired by all globular proteins is regulated by orderly arrangements of peptide chains via α-helix section, β-sheet section, and β-turn (resulting in the folding of chains).

12.2.1 Enzymes as Biocatalysts

1. They enhance the rate of reactions by bringing down the energy required for activation.
2. They participate as catalyst to perform a chemical reaction but themselves remain unchanged.
3. It is due to their presence that cells can perform millions of reactions per second for carrying out their cellular functions efficiently.
4. They are reusable and can be used repeatedly (as long as they are not denatured).
5. Enzymes are specific to a particular endogenous substrate.
6. Often the activity of enzymes may be due to the presence of cofactors.

12.2.2 Active Site

In general, although enzymes have a large globular structure, the region on the enzyme with which a substrate binds are relatively very small. This small region (a special pocket or cleft) is called active site, as substrates following binding to this site elicit biological response. Thus, an active site is the part of an enzyme in the form of pocket that directly binds to a substrate and undergoes a chemical reaction to furnish products. In the active site, side chains of amino acids/amide bonds interact with the substrate via weak noncovalent interactions. The structure of the amino acid side chains within the active site enhances substrate binding and lowers the energy of the transition state (a temporary covalent bonded or noncovalent enzyme-substrate [*ES*] complex), which facilitates catalytic reactions (Fig. 12.1).

Thus, the active site aids both binding and catalytic hydrolysis of the substrate. Accordingly, the active site can be divided in two regions. Binding sites comprising amino acids that temporarily interact with the substrate and catalytic site comprising amino acids involved in the reaction (Fig. 12.2).

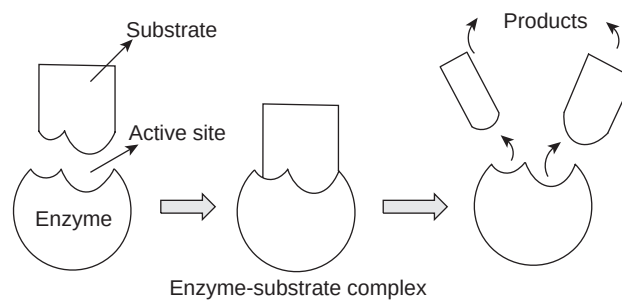


Figure 12.1 Substrate binds with enzyme and results in the formation of products.

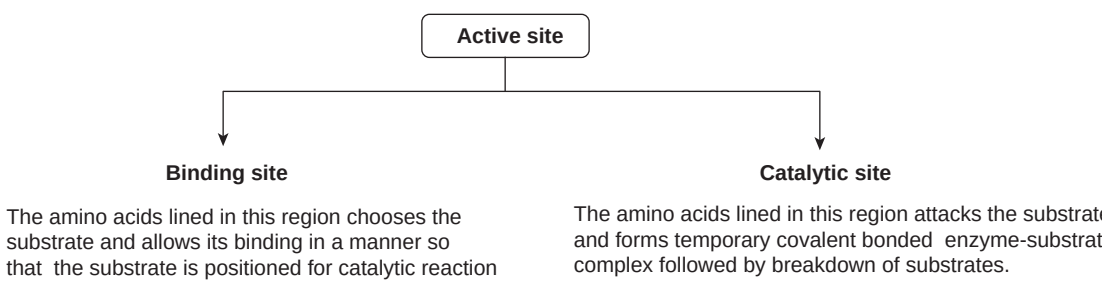


Figure 12.2 Active site comprises binding and catalytic sites.

12.2.2.1 Catalytic Site

The catalytic activity of an enzyme is generally restricted to the involvement of only few amino acids within the binding pocket and is one of the most conserved regions that survived evolution. Other amino acid residues present in the binding cleft called binding regions help in the orientation (positioning) of the substrate for catalytic action by the amino acids in the catalytic site. However, the residues present in remaining part of the enzyme do not survive evolution and the amino acid sequence keeps changing; this can be attributed to many factors.

During catalytic reactions, the amino acid residues in catalytic site interact chemically (through nucleophilic attack) with the substrate already oriented in position by binding region. This chemical interaction of amino acids in catalytic site with substrate triggers hydrolysis in the substrate via formation and breaking of bonds, leading to formation of enzyme-specific products. Following this, the enzyme is regenerated and is ready to perform again.

Thus, a detailed structure-function relationship of enzymes can help in the design of novel therapeutic drugs with the view to regulate the activities of enzymes in disease situations (Fig. 12.3).

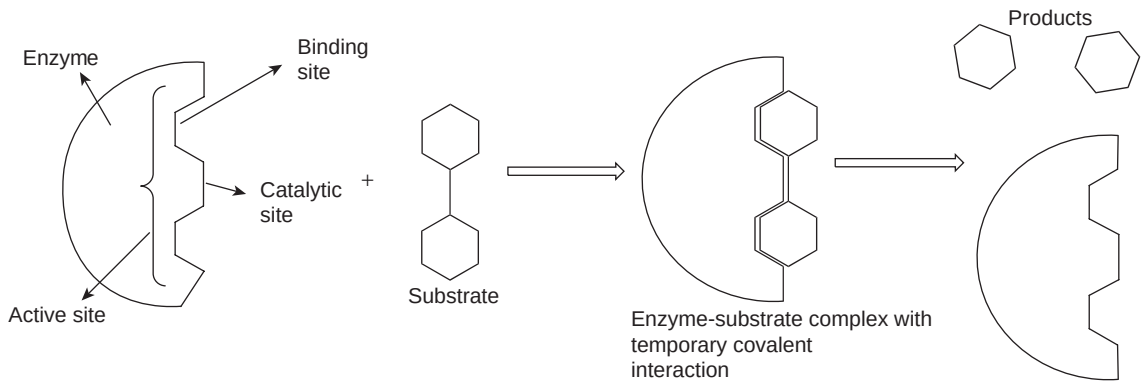


Figure 12.3 A diagrammatic representation of formation of products from substrates.

CASE STUDY 1

Catalytic Sites in Chymotrypsin

Chymotrypsin is a digestive enzyme belonging to a super family of enzymes called serine proteases.[1] It shows specificity for aromatic amino acids and thus cleaves the C-terminal residues: phenylalanine (F), tryptophan (W), and tyrosine (Y) from the peptide chains.

The active site of the chymotrypsin is lined with amino acids Gly at position 193, Ser at position 195, His at position 57, and Asp at position 102. The three amino acid residues present in this site—Ser, His, and Asp—are hydrogen bonded to each other in the binding pocket forming a catalytic triad and is called catalytic site. On the contrary, Gly at position 193 is involved in hydrophobic interaction with the substrate. Thus, the active site comprises a catalytic site and a hydrophobic binding site (Fig. 12.4).

In the catalytic site, all the three amino acids are oriented in a manner to trigger chemical reaction with substrates via nucleophilic attack on the amide bond. The hydroxyl functionality present in the side chain of Ser 195 acts as HBD and forms H-bond with tertiary nitrogen of the imidazole ring present in the side chain of His57. On the other hand, oxygen in the carboxyl moiety of the Asp102

(*Continued*)

Study 1 (*Continued*)

acts as HBA and forms H-bond with HN of the imidazole ting. Interestingly, out of the three amino acids in the catalytic site, ultimately it is only the Ser that gets involved in the chemical reaction with the amide bond in the substrate. The formation of H-bond of the hydroxyl group with tertiary nitrogen in the imidazole (Fig. 12.5) enhances the nucleophilicity of oxygen and facilitates attack via lone pair of electrons on to the carbon (with a partial positive charge) of the carbonyl group in the amide bond. It is this nucleophilic attack by Ser on the amide bond (−CO−NH−) of the substrate that triggers hydrolysis with the active participation of water and removal of C-terminal aromatic amino acids.

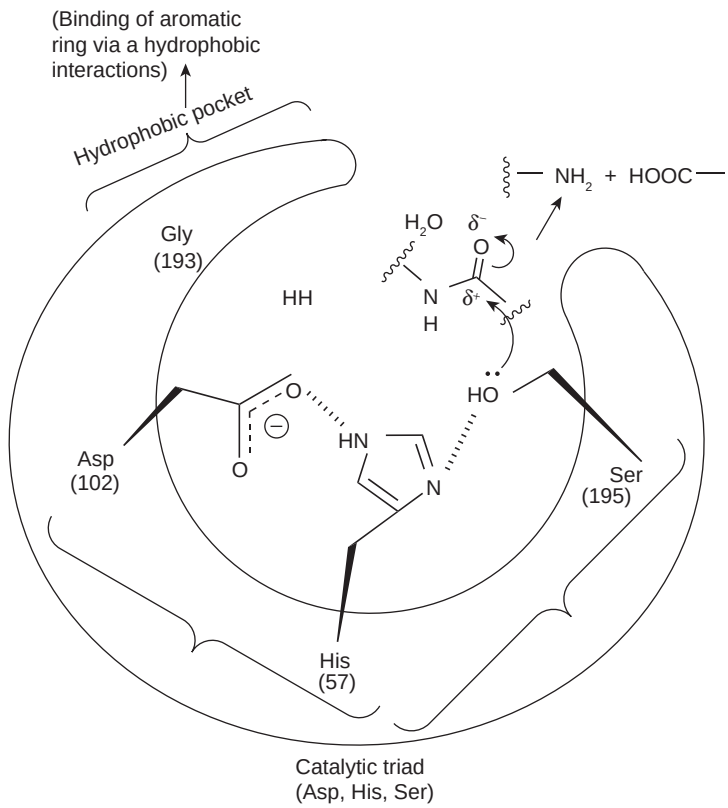


Figure 12.4 The active site of the chymotrypsin is lined with amino acids Gly at position 193, Ser at position 195, His at position 57, and Asp at position 102.

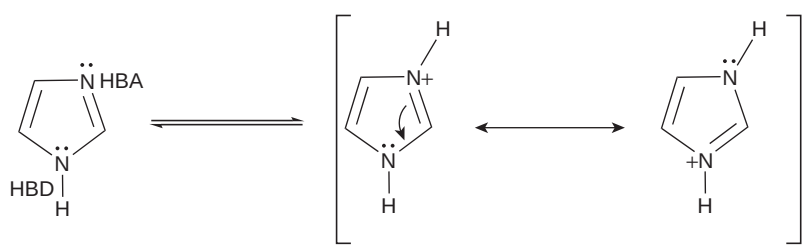


Figure 12.5 HBD and HBA in imidazole ring.

12.2.3 Cofactors

It is a non-protein substance (other than the substrate) or metallic ion whose presence is essential for the activity of the enzyme. It is bound non-covalently to the enzyme either tightly or loosely (in some cases permanently bound) and besides playing a role in the functioning of enzyme, it also assists in the stabilization of transition state formed during enzyme-catalysed reactions. When the non-protein component is weakly bound to an enzyme, it is called cofactor and in case it is tightly bound, it is called prosthetic group. Besides, when enzymes exhibit activity in the presence of metal ions, they are referred to as coenzymes. For example, soluble guanylyl cyclase, a unique intracellular cytosolic receptor (despite being an enzyme) following activation in the presence of heme, binds with NO, a second messenger to convert GTP into cGMP (another second messenger).

12.2.3.1 Apoenzyme and Holoenzyme

Some proteins demonstrate their catalytic activity only in the presence of a protein. Such enzymes without any component attached to it are called apoenzymes. However, when a protein part is attached with the protein, it is called holoenzyme (Fig. 12.6). Membranous adenylyl cyclase involved in the transfer of external signals from receptor to effector for downstream signalling is activated by cytosolic G-proteins.

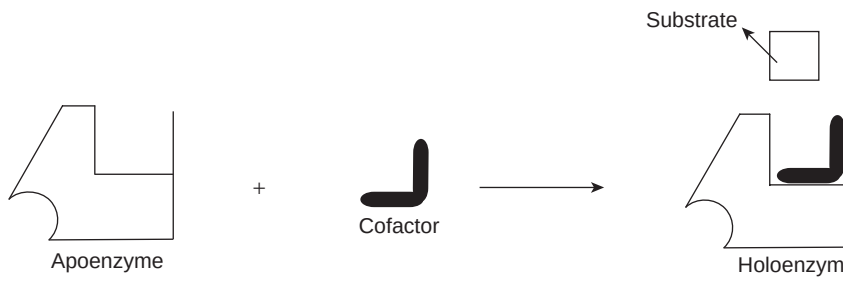


Figure 12.6 A diagrammatic representation of holoenzyme.

CASE STUDY 2

Nicotinamide Adenine Dinucleotide (NAD^+) as Cofactor

Nicotinamide adenine dinucleotide, NAD^+, is one of the most prevalent cofactors used by eukaryotes and plays a major role as an oxidative cofactor in many enzyme-catalysed reactions in cells. For example, alcohol dehydrogenase uses NAD^+ as cofactor to accelerate its catalytic reaction.

The human body produces the reduced form NADH from nicotinamide which is a major source for energy and oxidative cofactor for cells. The enzyme-catalysed cellular oxidations are accompanied by removal of hydrogen, which is picked up by NAD^+ to form NADH and thus gets reduced. NAD^+ as cofactor plays a role in cellular respiration such as redox reactions, glycolysis, and citric acid cycle in cells. Other common and important cofactors are heme made up of iron and four pyrrole rings, flavin adenine dinuleotide (FAD), derivatives of vitamins, etc.

$$NAD^+ + 2H \longrightarrow NADH + H^+ \qquad \text{(Reduction)}$$

$$NADH + H^+ \longrightarrow NAD^+ + 2e^- + 2H^+ \qquad \text{(Oxidation)}$$

12.2.4 Enzyme-Catalysed Reactions

The amino acids present in the catalytic site are primarily responsible for the hydrolysis of the amide bond in the substrate. The chemical reaction commences with the nucleophilic attack on the carbon of the polarized carbonyl group, which is then followed by formation and breaking of bonds via a transition state (TS) structure. The formation and stabilization of TS is largely catalysed by enzymes by reducing the activation energy (Fig. 12.7).

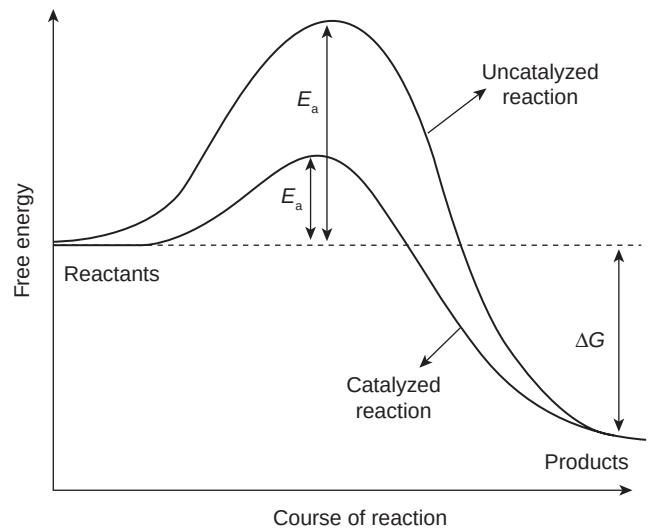


Figure 12.7 Enzyme catalyst reduces the activation energy required, thereby increasing the rate of reaction.

12.2.5 Enzyme–Substrate Complex

The formation of *ES* complex occurs following induced fit or lock and key model by the substrate at the binding site. The formation of *ES* is a reversible reaction; however, once the catalytic reaction is complete, formation of product via reaction occurs in forward direction only.

$$E + S \rightleftharpoons ES$$

$$ES \longrightarrow E + P$$

Once the product is released after a catalytic reaction, the enzyme is regenerated and is ready for next reaction. It is estimated that the enzyme-catalysed transformation of a substrate into product in the physiological conditions can be repeated a million times per minute. So, the overall reaction can be written as follows:

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

12.2.6 Enzyme-Substrate Interactions

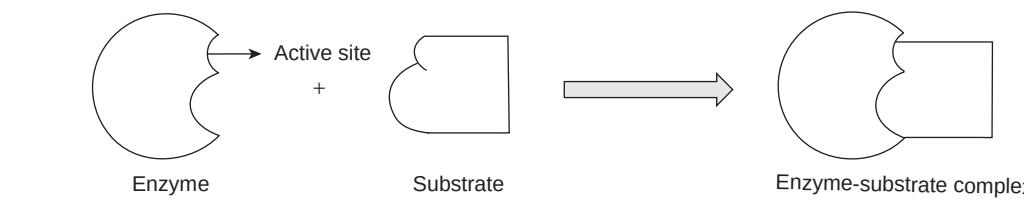
In general, the enzyme-catalysed reactions may recognize one or two substrates depending on the nature of chemical reaction. Besides, an enzyme-mediated catalytic reaction with substrates may produce one or more products. There are two kinds to interactions that can occur during enzyme-catalysed reactions: Lock and key model and Induced fit model.

12.2.6.1 Lock-and-Key Model

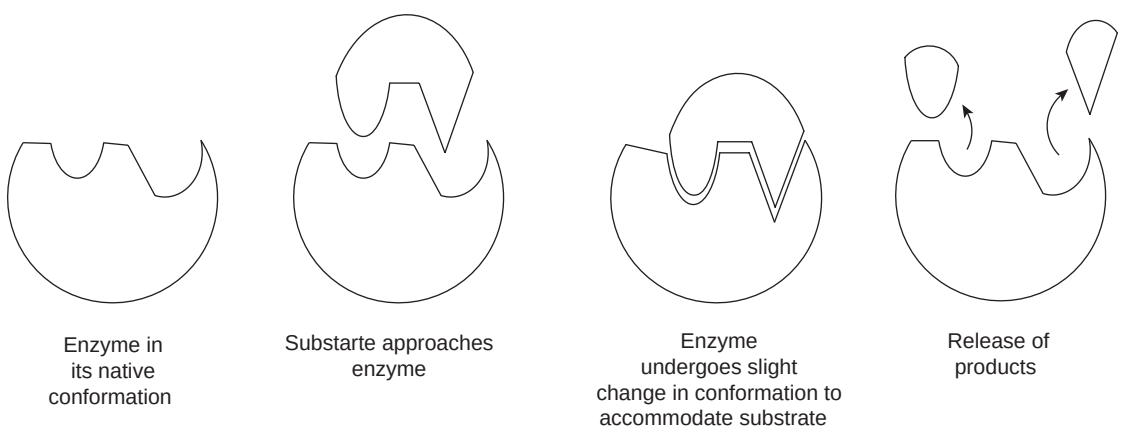
The primary requirement for this model is that shape of both binding site in the enzyme and substrate must be complementary to each other. In other words, during binding, the substrate can simply place itself in the grooves of the binding site and initiate catalytic reaction. Since the shapes for both of them are complementary, they are called lock-and-key model. During binding no change in the structure of enzyme occurs owing to the rigidity of the active site. This is an older model and is not prevalent among enzymes (Fig. 12.8 *a*).

12.2.6.2 Induced Fit Model

In this model enzymes are accommodative, flexible, and not rigid. In other words, enzyme undergoes minor conformational change to allow binding of substrate with a maximum fit. Particularly, amino acids in the binding region recognize the specific substrate and complement themselves with the shape of substrate. Thus, a range of substrates can bind if they can undergo specific enzyme-catalysed chemical reactions. A majority of physiological enzymes follow this induced fit model for binding as it allows a maximum fit and leads to an increase in the biological response (Fig. 12.8 *b*).



(a) Lock and key model



(b) Induced fit model

Figure 12.8 Enzyme-substrate interactions.

12.2.7 Factors Affecting Enzyme Activity

Several factors affect the rate of enzyme-catalysed reactions that have been discussed here.

1. Substrate concentration
2. Enzyme concentration
3. Temperature
4. pH
5. Product concentration (leading to feedback inhibition)
6. The presence of inhibitors
7. The presence of activators

12.2.7.1 Effect of Substrate Concentration on the Rate of Reaction

The rate at which an enzyme catalyses a biochemical reaction depends preferentially on substrate concentration and is expressed in terms of velocity of a reaction (Fig. 12.9). The initial velocity of V_0 commences rising with increase in concentration of substrate till a flat plateau is observed at high concentration of the substrate. The plateau reflects maximal rate of reaction and is also referred to as maximal velocity V_{max} when the entire available enzyme present in experiment has been saturated by substrates. Thus, the rate of reaction increases as substrate concentration increases (at constant enzyme concentration) and maximum activity occurs when the enzyme is fully saturated (when all enzymes are bound to substrates).

The enzyme-catalysed kinetics deals with studies pertaining to the rate of enzyme-catalysed chemical reactions under different experimental parameters. During *in vitro* screening pf compounds against enzymes, concentration of compounds required to either activate or inhibit the rate of enzyme-catalysed reaction is expressed as IC_{50} or EC_{50}.

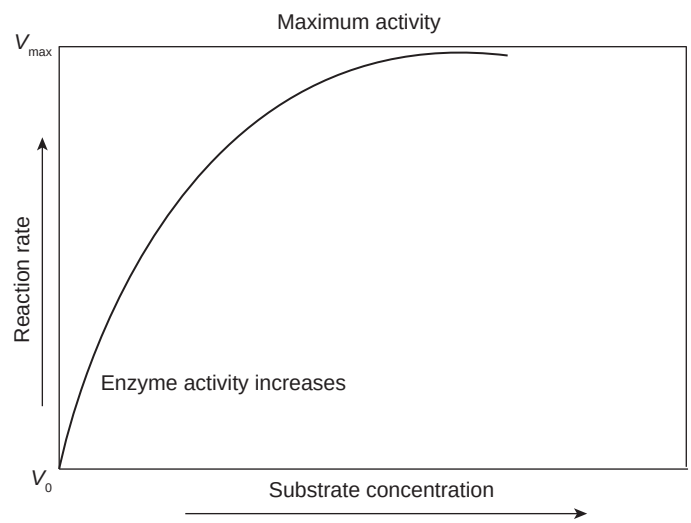


Figure 12.9 Maximum activity occurs when the enzyme is saturated (when all enzymes are bound to substrate).

Michaelis-Constant and Michaelis-Menten Equation

During kinetic studies of enzyme-catalysed reactions, it was observed that if concentration of the enzyme is kept constant and concentration of substrate is varied, the rate of reaction keeps on increasing till it reaches a maximum. After this, the increase in concentration of substrates has no effect on the velocity of reaction. This can be attributed to the complete saturation of enzymes with substrates or there is total conversion of enzymes into *ES* complex (Fig. 12.10).

In an enzyme-catalysed reaction when there is large excess of substrate and the enzyme concentration is held constant, a plot of concentration (S) against rate of reaction using velocity (V) as parameter gives a hyperbolic curve (Fig. 12.10).

K_m represents the Michaelis constant which reflects concentration of substrate that permits the enzyme to achieve half of the V_{max}. Thus, the **Michaelis Constant**, K_m can be used to derive useful information about enzyme-substrate interaction. K_m of the enzyme also reflects an inverse measure of affinity. A lower K_m indicates that less substrate is needed to reach a certain reaction speed. Hence, a low K_m reflects high substrate affinity. Thus, K_m can be represented by following equation:

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES \underset{K_{-2}}{\overset{K_{+2}}{\rightleftharpoons}} E + P$$

$$K_m = \frac{K_{-1} + K_2}{K_{+1}}$$

Michaelis developed a set of mathematical expressions to calculate enzyme activity in terms of reaction speed from experimental data generated in the laboratory. The mathematical equation that defines the quantitative relationship between the rate of an enzyme reaction and the substrate concentration is the Michaelis-Menten equation. It is the measurement of 'turn over' of number of substrates (into products) by the enzyme per second.

$$V_t = \frac{V_{max}[S]}{K_m + [S]}$$

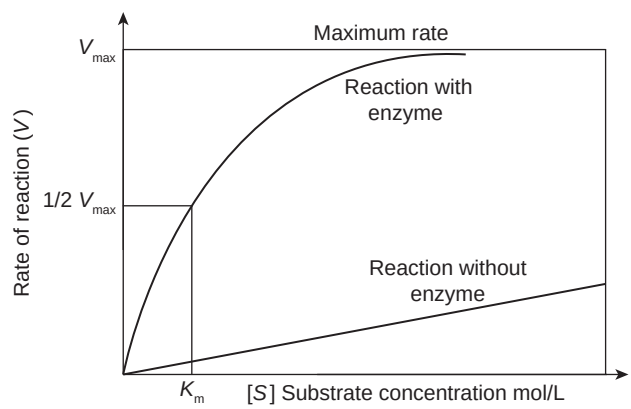


Figure 12.10 Calculation of Michaelis constant K_m with the help of substrate concentration.

Where,
V_t is the observed velocity at the given substrate concentration [S].
K_m is the Michaelis-Menten constant.
V_{max} is the maximum velocity at saturating [S] conc.

12.2.7.2 Effect of Enzyme Concentration

The effect of enzyme concentration on the rate of reaction is studied by keeping the amount of substrate in excess. In other words, rate of reaction is independent of concentration of substrate. Thus, concentration of product formation with time will be dependent on the concentration of enzymes. A graph of product concentration against time at different enzyme concentrations depicts a linear curve and is categorized under zero-order kinetics (Fig. 12.11). The rate of reaction is equal to constant K_2 as it is independent of substrate concentration. Increasing concentration of enzymes enhances the rate of reaction till the availability of substrates.

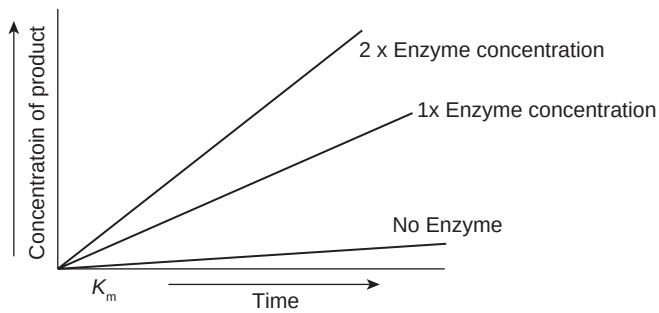


Figure 12.11 Effect of Enzyme concentration on rate of reaction.

12.2.7.3 Effect of Temperature on the Enzyme Activity

Most enzyme-catalysed reactions are dependent on temperature. An increase in temperature enhances the rate of enzyme-catalysed reaction, but then there is sudden fall in the rate of reaction to zero. This occurs due to denaturation of enzymes at certain temperature, thereby losing its activity. In general, most natural enzymes at temperatures above 40°C get denatured and lose their activity. Sometimes, prolonged storage of enzymes at moderate temperatures may result in deactivation of the enzyme, hence enzymes must always be stored at 5°C or below (Fig. 12.12).

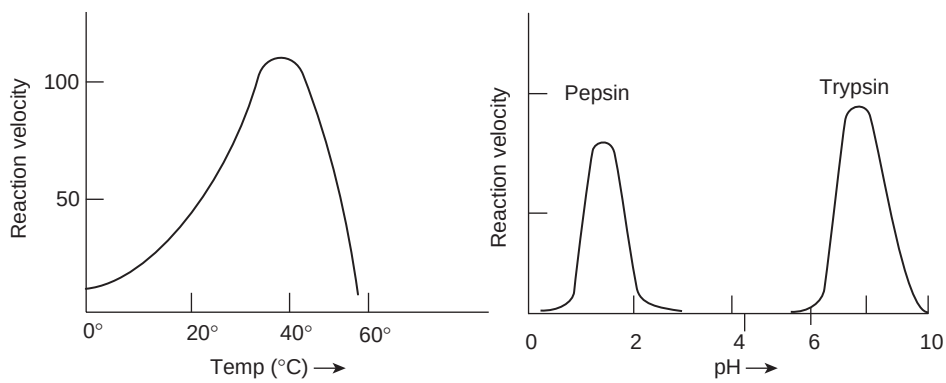


Figure 12.12 Effect of temperature and pH on enzyme activity.

12.2.7.4 Effect of pH on Enzyme Activity

In general, functioning of each enzyme is directly associated with pH of the environment/medium. A particular pH at which an enzyme exhibits maximum activity is known as optimal pH (Table 12.1).

If pH of the environment exceeds or decreases from the optimal pH requirement, the activity of the enzyme is completely lost (Fig. 12.13).

Table 12.1 pH for optimum activity by enzymes

Enzyme	pH Optimum
Lipase (pancreas)	8.5
Pepsin (stomach)	1.0–2.0
Trypsin	7.8–8.2
Maltase	6.1–6.8
Amylase (pancreas)	6.7–7.0

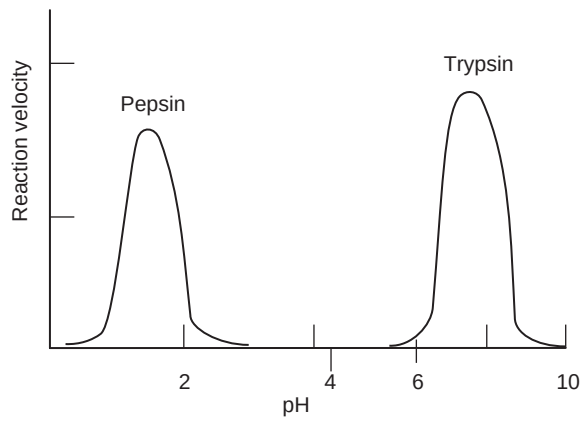


Figure 12.13 Pepsin, an endopeptidase with an optimum pH of activity at 1.6, hydrolyses 10–20% of the proteins in the meal.

12.2.7.5 Effect of Feedback Inhibition on Enzyme Activity (Product Concentration/ Termination of Drug Action)

Many enzymes catalyse sequential chain reactions and are part of a number of biochemical pathways involved in the maintenance of homeostasis. In these chain reactions, the product generated in the first step of enzyme-catalysed reaction becomes substrate for the second enzyme-catalysed reaction, and so on, till the final desired product is generated. The concentration of final product (*FP*) so synthesized in the enzyme-catalysed chain reactions is regulated naturally by feedback inhibition. This is achieved via allosteric binding of the *FP* to the enzyme that initiated the pathway. The allosteric-binding triggers

change in the conformation of the enzyme, which in turn disallows further binding of substrate, thereby closing down that pathway and blocking the further generation of *FPs*. The higher the concentration of the *FP*, the more likely that product will bind to the allosteric site of the enzyme, thereby shutting down that pathway/chain reaction.

Let's consider formation of a *FP* following binding of a substrate (*S*) to an enzyme (*E*1). The *E*1-catalyzed reaction results in the formation of intermediate product (*IP*-1) followed by a series of biotransformation involving enzyme 2 (*E*2)- and *E*3-catalyzed reactions to generate final product (*FP*) via intermediates *IP*-2, *IP*-3, etc. (Fig. 12.14).

As the concentration of the *FP* becomes more than the threshold value, the negative feedback imposes a halting effect on *E*1, by either slowing down production of intermediates *IP*-1 and *IP*-2, or completely shutting down the pathway. It is noteworthy that the feedback inhibition mechanism is affected only by the *FP* and not by any *IPs*.

For example, biosynthesis of amino acid residue His inside the cell involves ten biochemical steps (*E*1, *E*2, *E*3, … *E*10) with each step being catalysed by enzymes. When the concentration of His reaches the desired threshold, it binds allosterically with enzyme *E*1 and puts a break on the pathway via feedback inhibition. Notably, in our body numerous biochemical and signaling pathways are regulated by feedback inhibition via both hormones and enzymes.

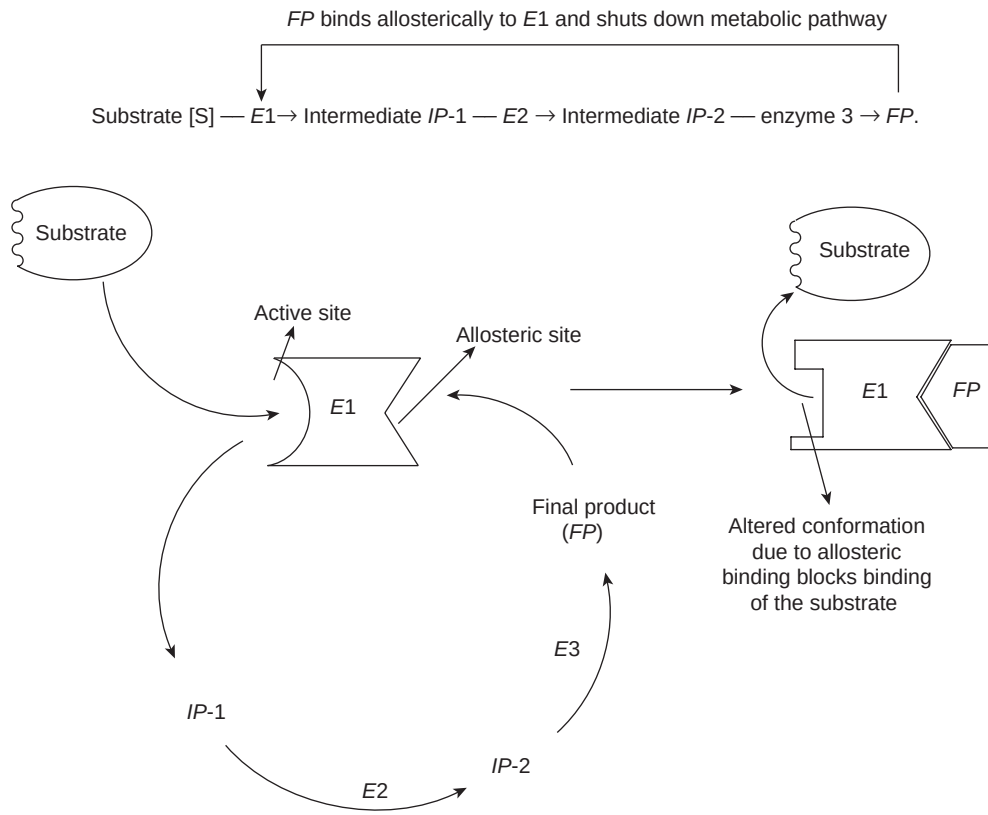


Figure 12.14 A diagrammatic representation of allosteric feedback inhibition.

CASE STUDY 3

Feedback Loop in Hypothalamus-Pituitary-Gonads Axis

The hypothalamus-pituitary-gonads (HPG) axis involving hypothalamus, pituitary, and gonads plays an important role in the reproductive system. Through this axis, hormones of peptide/protein origin and steroidal hormones regulate each other's release to carry out designated physiological functions. Starting from neuronal release of GnRH from hypothalamus in brain, the decapeptide hormone through bloodstream reaches pituitary glands. There it binds with receptors and stimulates secretion of two protein hormones LH and FSH, also referred to as gonadotropins. The latter, in turn, triggers the release of steroidal sex hormone from gonads: testosterone in males and oestrogen and progesterone in females. Since an increased release of hormones during either of the stages in the axis can cause significant impairment in physiological functions, a negative feedback inhibition occurs naturally within this axis. While gonadal hormones can block the secretion of gonadotropins from pituitary and GnRH from hypothalamus, gonadotropins can block the release of GnRH from pituitary (Fig. 12.15).

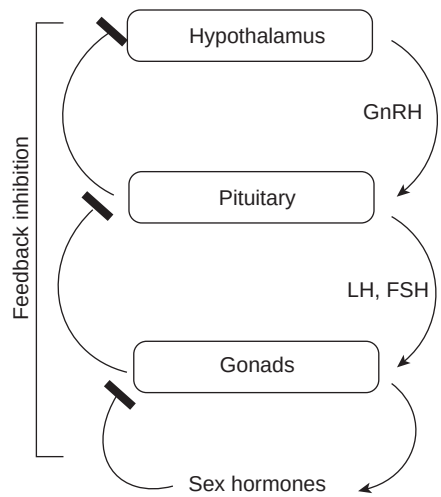


Figure 12.15 Feedback loop along HPG axis.

CASE STUDY 4

Feedback Loop with Insulin and Glucagon

Glucagon and insulin work in tandem to counterbalance each other's actions during the management of glucose levels in blood. About 4–6 hours after meals, the glucose levels in blood decreases, triggering pancreas to produce glucagon. The latter allows breakdown of stored glycogen in liver into glucose that is released into the blood in order to maintain normal glucose level. Similarly, immediately after meals, the glucose level in

(*Continued*)

Study 4 (*Continued*)

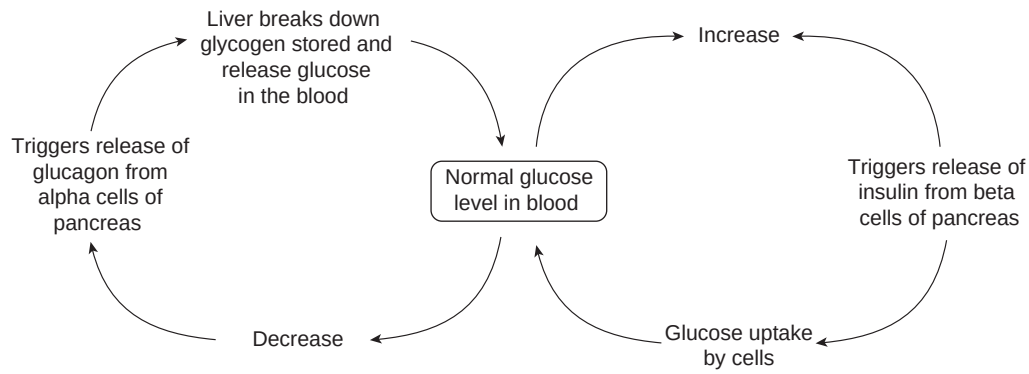


Figure 12.16 Feedback loop involving insulin and glucagon.

blood increases, which triggers the release of insulin from pancreas. The latter triggers glucose uptake by cells in order to bring down the levels of glucose to normal level in blood. This whole feedback loop with insulin and glucagon remains constantly in motion (Fig. 12.16) and keeps round the clock vigil.

12.2.7.6 Effect of Inhibitors on Enzyme Activity

Cells regulate their intracellular signalling enzyme activities to coordinate the cell-specific physiological functions through numerous biochemical pathways. This is achieved either by restricting the production of a particular enzyme or attenuating an enzyme-catalysed reaction in a feedback loop. However, any malfunction arising either due to uncontrolled increase or deficiency of signalling enzymes can lead to pathophysiological symptoms. Numerous diseases have been attributed to uncontrolled increase in the concentration of these signalling enzymes. One approach to address this issue involves use of enzyme inhibitors as drugs to alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop enzyme catalysis. Thus, enzymes remain second most sought-after therapeutic targets followed by receptors, for drug discovery programme. There are many drugs that are used clinically as inhibitors for these signalling enzymes. The process of enzyme inhibitions is of two types: Direct inhibition and Indirect inhibition.

Direct Inhibition

This involves binding of a substrate with active sites on the enzyme, thereby resulting in the inhibition of enzyme activity. The substrates are called direct inhibitors and may cause inhibition by two mechanisms. They can be accordingly classified as:

1. **Reversible Inhibitors:** As the name suggests, reversible inhibitors bind enzyme through weak non-covalent interactions: H-binding, hydrophobic interaction, ionic interactions, Vander Waals interaction dipole-dipole interaction, etc. The formation and dissociation of *ES* complex is generally rapid; however, slow release of substrate from *ES* complex may occur for some drugs. Rapid reversible inhibitions have been observed for fluoroquinolones antimicrobials and azole antifungals when bound to CYP3A4 isozyme (although not metabolized). On the contrary, slow reversible inhibition has been observed for lidocaine (local anaesthetic) and fluoxetine. A typical Michaelis-Menten scheme is followed by all reversible inhibitors. There are three kinds of reversible inhibitors:

(a) Competitive Inhibitors (raises K_m but V_{max} remains same): These inhibitors compete with endogenous substrate to bind with the same orthosteric site. In fact, these inhibitors mimic the shape and structural features present in the natural/endogenous substrate. Since both endogenous substrate and inhibitor compete for the same binding site, the activity of a competitive inhibitor depends on:

(i) Concentration of the inhibitor and substrate.

(ii) Difference in the relative affinities of the endogenous substrate and competitive inhibitor for the enzyme.

Thus, for a given enzyme concentration, if the concentration of inhibitor is higher than the concentration of endogenous substrate, then the binding of competitive inhibitor would be favoured. However, for the same concentrations of enzyme and the inhibitor, if the concentration of endogenous substrate rises, the latter will compete with the inhibitor at the binding site and will displace the inhibitor. This in turn reduces the effect of the competitive inhibitor. As can be seen in Figure 12.17, although the K_m value of the reaction increases (shifts on the right side) in the presence of an inhibitor, the value of V_{max} remains unchanged. In general, the inhibitor has affinity only for the enzyme and not for the *ES* complex. The kinetics for the phenomenon of competitive inhibition is represented by K_i as inhibition constant and can be derived following Michaelis-Menten equation. Although, during *in vitro* screening of compounds against enzymes, inhibitory activities of test compounds are expressed as IC_{50} values (Chapter 3), the values can vary in different experimental settings due to variation in the catalytic activity of the same enzyme from different batches. On the contrary, K_i values for a compound remains constant and independent of substrate concentration. It can be used to express potency of an inhibitor.

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES \underset{K_{-2}}{\overset{K_{+2}}{\rightleftharpoons}} E + P$$

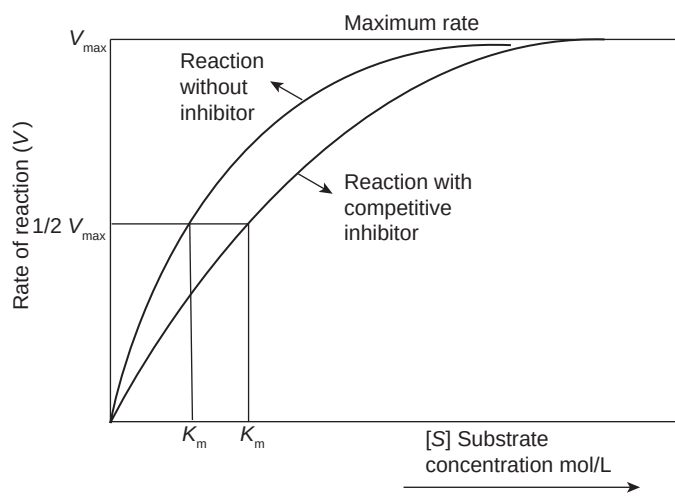
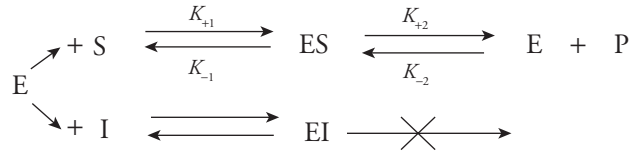


Figure 12.17 Competitive inhibitor increases K_m but does not affect V_{max}.

In the presence of a competitive inhibitor:



The dissociation constant for the *EI* complex represented by K_i is also referred to as inhibition constant and can be calculated from following equation:

$$K_i = \frac{[EI]}{[E][I]}$$

The Michaelis constant K_m for an enzyme-substrate interaction is represented by

$$K_m = \frac{[ES]}{[E][S]} = \frac{K_{-1} + K_2}{K_{+1}}$$

Since an increase in the concentration of substrate completely abolishes the effect of competitive inhibitor, the increase in apparent K_m by the competitive inhibitor can be expressed by the following equation:

$$K_{mapp} = K_m \left[1 + \frac{[I]}{K_i}\right]$$

Finally, the rate equation in the presence of inhibitor can be expressed as:

$$V = \frac{V_{max}[S]}{K_m\left[1 + \frac{[I]}{K_i}\right] + [S]}$$

For substrate-velocity curves generated in the presence of a competitive inhibitor at different concentrations, K_i, K_m, and V_{max} and are parameters common for all the curves with concentration of substrates and inhibitors as independent variables (Fig. 12.18). Finally, IC_{50} for a competitive inhibitor can be calculated by formula:

$$IC_{50} = K_i\left(1 + \frac{S}{K_m}\right)$$

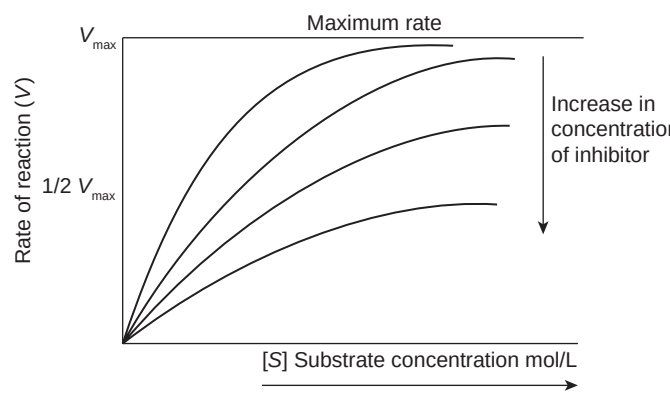


Figure 12.18 Velocity versus time for increasing concentration of substrates.

CASE STUDY 5

Acetylcholinesterase Inhibitors

The enzyme acetylcholinesterase (AChE) is responsible for the hydrolysis of acetylcholine (ACh) neurotransmitter bound to receptors in post-synapse. Inhibition of this enzyme can prolong duration of action of receptor-bound ACh. This may be a requirement for treating some diseases; however, if ACh remains bound to receptor for a long period of time, it can result in paralysis as observed with nerve gas. The inhibitors of this enzyme are referred to as anti-cholinesterase compounds and abbreviated as AChEI. Methacholine, a cholinergic drug reversibly inhibits the hydrolysis of ACh by competing for the enzyme AChE.

CASE STUDY 6

Direct Thrombin Inhibitors

Thrombin belongs to a family of serine proteases that plays a critical role in thrombus/clot formation and has been implicated in the pathogenesis of cardiovascular disease. Direct thrombin inhibitors bind directly to the thrombin and prevent fibrin formation as well as thrombin-mediated activation of Factors: V, VIII, XI, and XIII. Dabigatran etexilate is an oral prodrug that is metabolized by a serum esterase to dabigatran. It is a synthetic, competitive, and reversible direct thrombin inhibitor and exerts its effect by binding to the active site comprising catalytic triad Ser 195, His 57, and Asp 102 of the thrombin. Inhibition of thrombin disrupts the coagulation cascade and inhibits the formation of clot (Fig. 12.19).

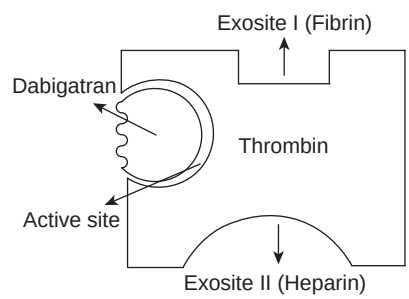


Figure 12.19 Dabigatran as competitive and reversible direct thrombin inhibitor.

(b) Noncompetitive Inhibitors (K_m remains same but lowers V_{max}): Non-competitive inhibitors generally bind to the allosteric and not to the orthosteric sites on enzymes. Binding of non-competitive inhibitors to allosteric sites results in the change in the conformation of the enzyme that prevents binding of the endogenous substrate to orthosteric site with changed shape. Even increasing the concentration of endogenous substrate has no effect on allosteric-bound inhibitor. Notably, non-competitive inhibitors have same affinity for both enzymes as well as ES complex and therefore have the same K_m, albeit with reduced V_{max} values (Fig. 12.20). A reversible inhibition can be competitive or non-competitive (Fig. 12.21). Notably, all non-competitive inhibition follows allosteric pathway but not all allosteric modulators are non-competitive inhibitors. IC_{50} for a non-competitive inhibitor can be calculated by formula $IC_{50} = K_i$.

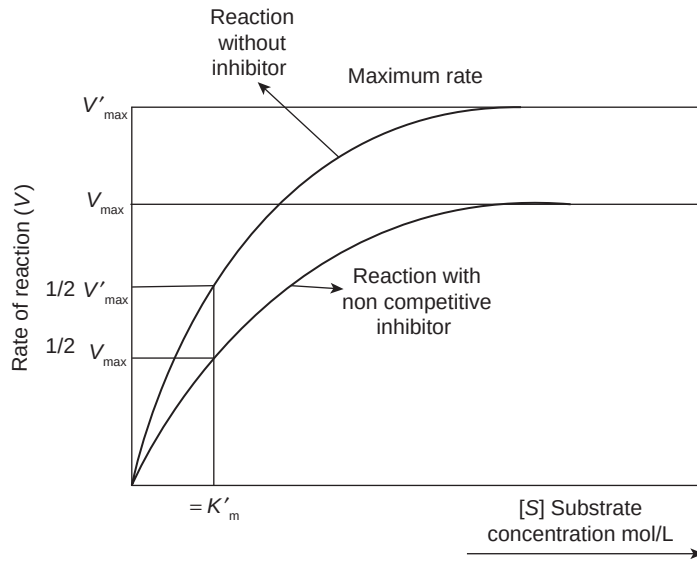


Figure 12.20 Non-competitive inhibitor decreases V_{max} but K_m remains the same.

(c) Uncompetitive Inhibitors. (raises K_m but lowers V_{max}): These inhibitors bind only to the ES complex and do not have the ability to bind to the free enzyme (unbound enzyme). Once bound, the uncompetitive inhibitors cannot be dissociated even by increasing the substrate concentration. The binding leads to reduction in both K_m and V_{max} values.

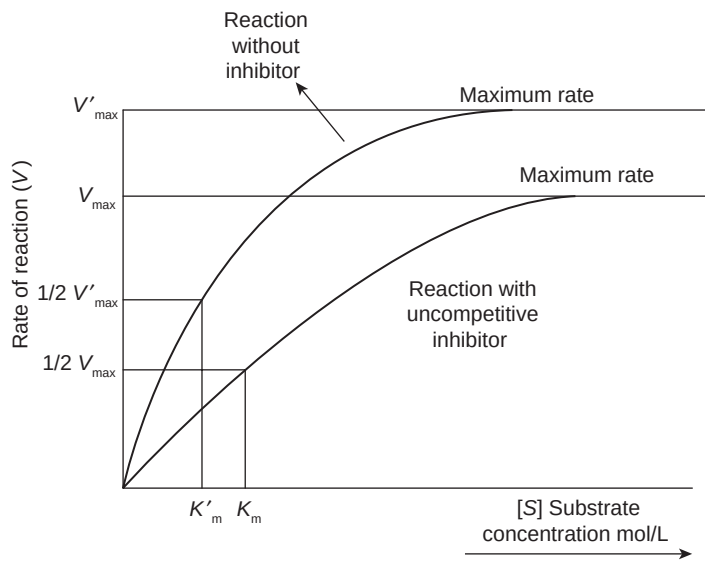


Figure 12.21 Uncompetitive inhibitor decreases V_{max} and increases K_m.

The reduction in K_m value can be attributed to enhanced affinity of inhibitor to *ES* complex, resulting in a stabilized complex that in turn makes dissociation of the inhibitors difficult (Fig. 12.21). Uncompetitive inhibitor generally binds to allosteric site causing changes in the conformation of the enzyme, thereby reducing the affinity of the endogenous substrate for the orthosteric site. IC_{50} for a non-competitive inhibitor can be calculated by formula $IC_{50} = K_i(1 + K_m/S)$. For example: isoniazid (an antitubercular drug) inhibits the metabolism (binds CYP allosterically) of phenytoin (anticonvulsant drug), carbamazepine (anticonvulsant or anti-epileptic drug), and ethosuximide (control seizure), and in turn, increases their therapeutic effect. Ideally, blood levels of these drugs should be monitored therapeutically for optimal response.

A comparative view of all the three enzyme inhibitors has been depicted in Figure 12.22.

2. **Irreversible Inhibitors:** Irreversible inhibitors generally bind covalently to the binding site and block the enzyme activity for good, even increasing the concentration of agonist cannot revive the enzyme activity. There are three kinds of irreversible inhibitions:
   - **(a)** Covalent binding of inhibitor with enzyme.
   - **(b)** Formation of tightly bound *ES* complex.
   - **(c)** Mechanistic-based irreversible inhibition.

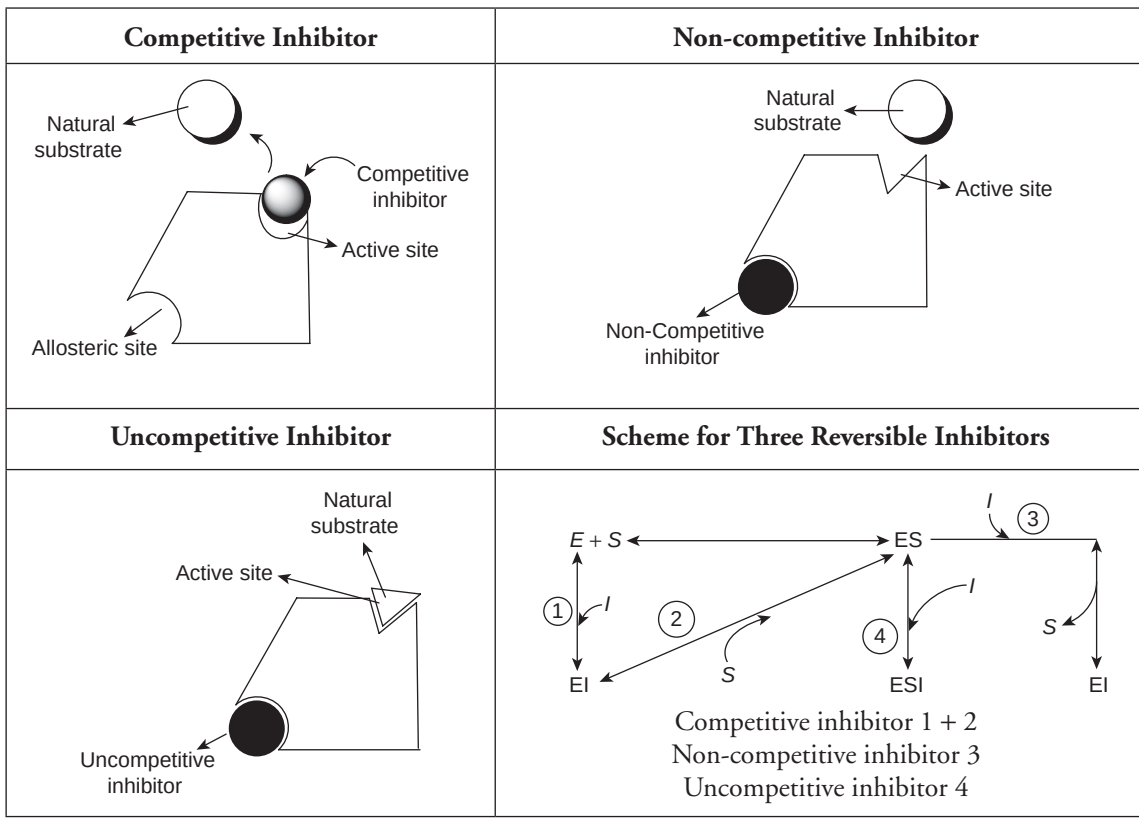


Figure 12.22 Reversible enzyme inhibitors (E = enzyme; S = substrate; I = inhibitor).

CASE STUDY 7

Cholinesterase Inhibitors

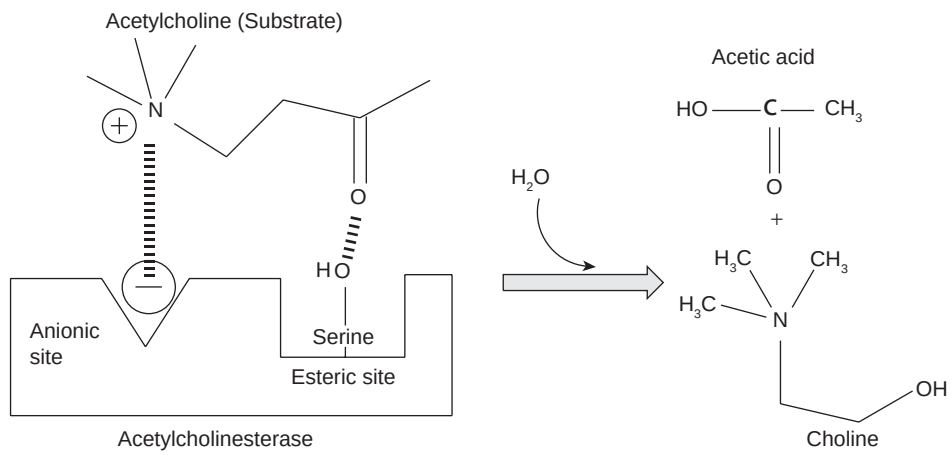
The main objective behind covalently bound irreversible inhibitor is to modify the amino acids present in the binding site through derivatization. Thus, the inhibitor must have reactive moieties in their structures that can covalently react with side chain of amino acids in the binding sites. The reactive functional groups such as ketones, aryl sulfonates, aldehydes, etc., can covalently react with the side chain of amino acids bearing nucleophilic functionality such a Cys, Thr, Ser, Tyr, etc., and in turn, inhibit the activity of enzyme. Penicillin for example undergoes irreversible binding with enzyme transpeptidase, thereby inhibiting bacterial cell wall biosynthesis.

The diisopropylfluorophosphate (DIFP) is an organic phosphorous compound and mimics lethal nerve gas (poisonous gas used as chemical weapon). It binds irreversibly with AChE enzyme and prevents hydrolysis of ACh. This causes ACh molecules to remain bound with its receptors in post-synapse neuron and enhances duration of action of ACh. The role of AChE located next to receptor is to hydrolyse ACh neurotransmitter in the synapse.

The mechanism of hydrolysis of ACh by the enzyme AChE: The hydrolysis of ACh involves non-covalent binding of acetate to serine residues within the esteratic subsite, forming a temporary acetylated form of AChE. A molecule of water then reacts with this intermediate, liberating the acetate group, which diffuses into the surrounding medium. What remains is an active form of the enzyme, ready to bind with a newly released molecule of Ach (Fig. 12.23).

Mechanism of inhibition of hydrolysis of ACh by AChE: The irreversible inhibition of AChE by DIFP occurs by the formation of covalent bond between the hydroxyl group of serine in the active site of the AChE and DIFP with release of HF (Fig. 12.24).

It may be interesting to mention that although DIFP binds irreversibly with the enzyme AChE, scientists have developed its antidote pralidoxime chloride (2-PAM Cl) as ACh regenerators. The antidote binds with anionic site and displaces DFIP from serine, thus regenerating AChE.



Figures 12.23 Hydrolysis of ACh by AChE enzyme.

(*Continued*)

Study 7 (*Continued*)

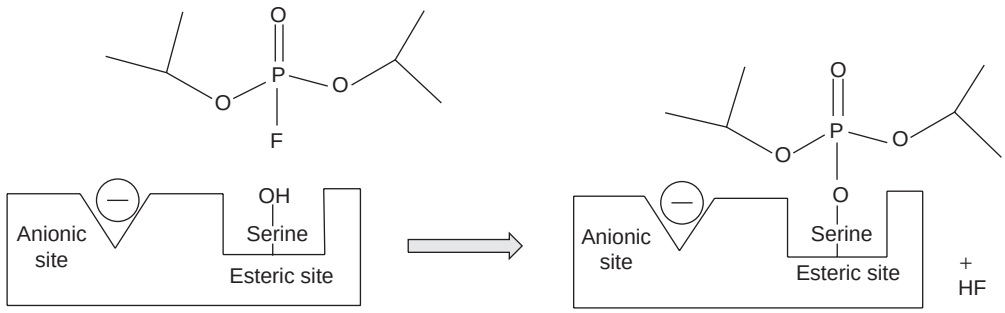


Figure 12.24 The irreversible inhibition of AChE by DIFP occurs by the formation of covalent bond.

Formation of tightly bound *ES* complex: Substrates can be designed by introducing changes in structural features and in the physicochemical properties of the molecule in a manner that it will bind with the target enzyme very tightly. The reaction kinetics of these inhibitors is similar to covalently bound irreversible inhibitors. The mechanism for tight-binding inhibitors proceeds with a rapid binding of the inhibitor to the enzyme. The resulting *ES* complex then rearranges via conformational change slowly into a tightly bound complex. Methotrexate and allopurinol bind to their enzyme as 'slow-binding inhibitors'.

Mechanism-based inhibitors: These inhibitions are highly prevalent among metabolic enzyme. The mechanism involves binding of a drug with a metabolic enzyme to form a reactive species (metabolite) which then covalently binds to the enzyme and in turns makes the enzyme inactive. These inhibitions have also been referred to as 'suicide inhibition'. Allopurinol, an antigout drug, acts as suicide inhibitor by blocking the action of an enzyme xanthine oxidase (non CYP family) via reactive metabolite oxypurinol (Fig. 12.25).

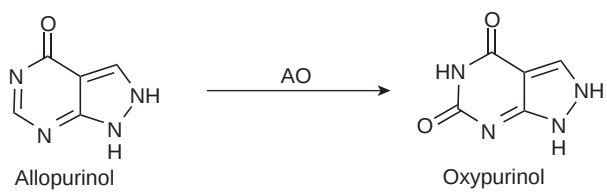


Figure 12.25 Oxypurinol as suicide inhibitors.

Indirect Inhibition

This occurs due to factors that indirectly affect enzyme concentration either by affecting the rate of enzyme synthesis/degradation or arising from the impaired physiological consequences.

"Repression", the term itself refers to the reduction in the concentration of enzymes. This can be attributed to decrease in the rate of synthesis as in the case of ethionine, which following its administration reduces the concentration of enzymes arginase and ornithine transcarbamylase. The deficiency of this enzyme can cause accumulation of ammonia in the blood to the toxic level.

12.2.7.7 Effect of Activators on Enzyme Activity

Enzyme activators are chemical substances that can enhance the velocity of an enzyme-catalysed reaction (Fig. 12.26). The activators can range from small organic molecules to ions to peptides and proteins. In general, these activators act as positive allosteric modulators and bind with the enzyme at allosteric site. Majority of enzymes are activated in the presence of ions; for example, phospholipase II enzymes are activated by Ca^{2+} and are involved in the hydrolysis of fatty acids. In fact, the enzyme has a binding site for Ca^{2+}, and the binding triggers conformational change in the enzyme and enhances the enzyme activity.

Adenylyl cyclase is an enzyme involved in the conversion of ATP to a second messenger cAMP which is responsible for intracellular downstream signalling through effectors. However, the enzyme is activated only after it binds with activator Gα proteins, a cytosolic component of G-protein-coupled receptor.

There are few activators that can activate the enzyme only after it is bound to another molecule to form a complex. The complex then binds to an enzyme and enhances the rate of reaction. An interesting example under this class is Ca-binding protein calmodulin. Initially, Ca^{2+} ion binds with calmodulin and induces change in its conformation, thereby resulting in the formation of a complex. The latter binds with enzymes calmodulin-dependent protein kinases and phosphatases to enhance their velocity of reaction.

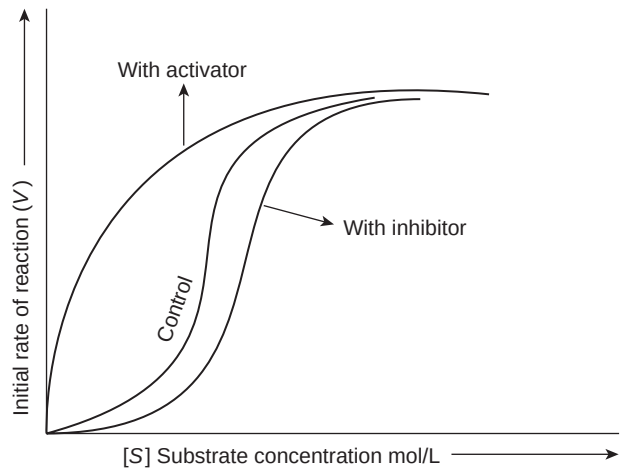


Figure 12.26 Activity of enzyme can be affected by both inhibitor and activator.

12.3 Allosteric Enzymes

These enzymes have an additional binding site called allosteric site other than the active site. A non-substrate molecule when binds to this allosteric site induces changes in the enzyme's conformation that in turn results in the activation/inactivation of the enzyme for its native substrate (Fig. 12.27).

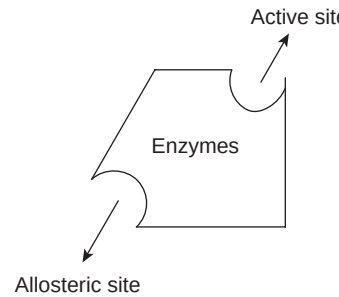


Figure 12.27 A diagrammatic representation of allosteric enzyme.

In general, allosteric binding of a substance to an enzyme is like a dimmer switch which is used to regulate the speed of the fan. The action of allosteric binding in the enzyme also regulates the activity of the enzyme. Allosteric binding occurs naturally in our body as part of our numerous physiological functions to turn on a pathway in case a substance is deficient and turns off when the same substance concentration is sufficient. In one such example, the FP formed following enzyme-catalysed reactions, when reaches a threshold concentration, the FP molecule binds allosterically with the enzyme to reduce its catalytic activity. Thus, allosteric enzyme regulation can result either in an inhibition or activation, and can be used in drug design strategies. In our physiological system, allosteric modulators play a major role in the regulation of activities of both enzymes and receptors.



Important Note

A new concept of drug discovery has emerged that involves the binding of the drug molecules to allosteric sites away from the orthosteric sites on targets. The amino acids lining the active site in the ortherosteric sites are conserved and has survived evolution, this causes selectivity issues due to isoform targets. Whereas amino acids lining the allosteric site are not conserved thereby provide selectivity.

12.3.1 Allosteric Activation (PAM)

Effectors that enhance the protein's activity are referred to as Positive allosteric activators (PAMs). The allosteric activation results in enhancement in the binding affinity of the substrate/ligand with the active site in protein (receptor or enzyme), thereby increasing the enzyme activity (Fig. 12.28).

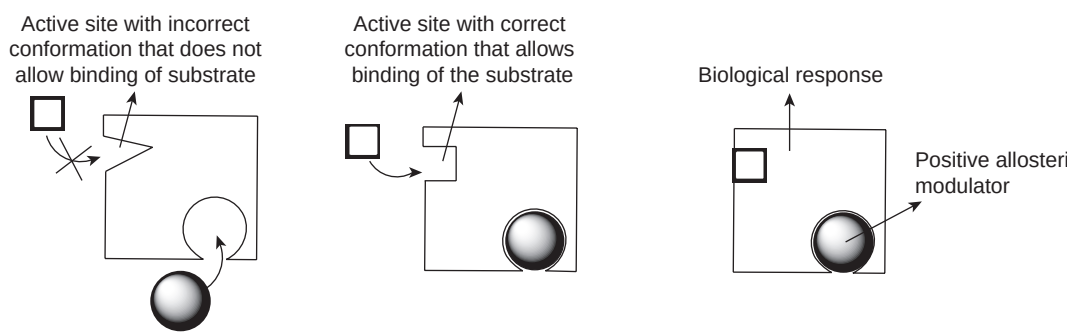


Figure 12.28 Effectors that enhance the protein's activity are referred to as PAMs.

CASE STUDY 8

PAM in the Activation of the Enzyme Adenylyl Cyclase

Adenylyl cyclase is an important effector involved in the intracellular signalling process. It is a crucial membrane-bound regulatory enzyme that essentially plays a role in every cell. It is activated allosterically by one of the Gα-proteins following activation of GPCR by a ligand. The allosteric binding of Gα with adenylyl cyclase triggers conversion of ATP into a second messenger cAMP (Fig. 12.29).

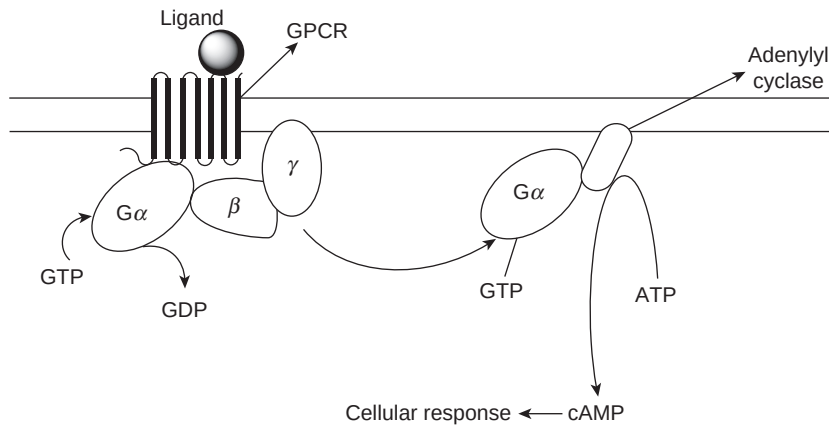


Figure 12.29 Adenylyl cyclase is activated only after its binds with activators Gα (PAM) proteins.

12.3.2 Allosteric Inhibition (NAM)

Effectors that decrease the protein's activity by decreasing the enzyme's affinity for the substrate at the active sites are called Negative allosteric modulation (NAM)/allosteric inhibitors (Fig. 12.30).

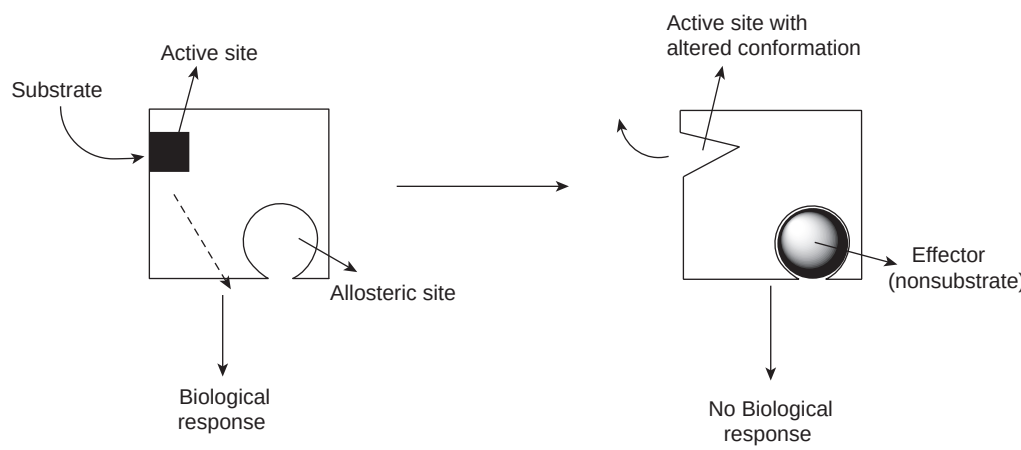


Figure 12.30 NAM/allosteric inhibitors.

Allosteric control involving both activation and inhibition also occurs in number of pathways to regulate homeostasis.[2] The hormone adrenaline (epinephrine) following its binding to GPCR activates intracellular membrane-bound enzyme adenylyl cyclase via allosteric activation. This in turn triggers the synthesis of cAMP as second messenger from ATP. The cAMP on one hand acts as an allosteric activator of the enzyme glycogen phosphorylase (via PKA; protein kinase) that speeds the metabolism of carbohydrate (glycogen to glucose) to produce energy (glycogenolysis). On the other hand, the same cAMP inactivates (via allosteric inhibition) the enzyme glycogen synthase (Fig. 12.31) leading to the inhibition of glycogenesis (no new synthesis of glycogen).

12.3.3 Silent Allosteric Modulators (SAM)

They are also referred to as neutral allosteric ligands as they bind to the allosteric site in the receptor but have no effect orthosteric agonist affinity or efficacy. They although are devoid of any therapeutic significance, are used as tool to demonstrate receptor mediate effects of PAM and NAM.

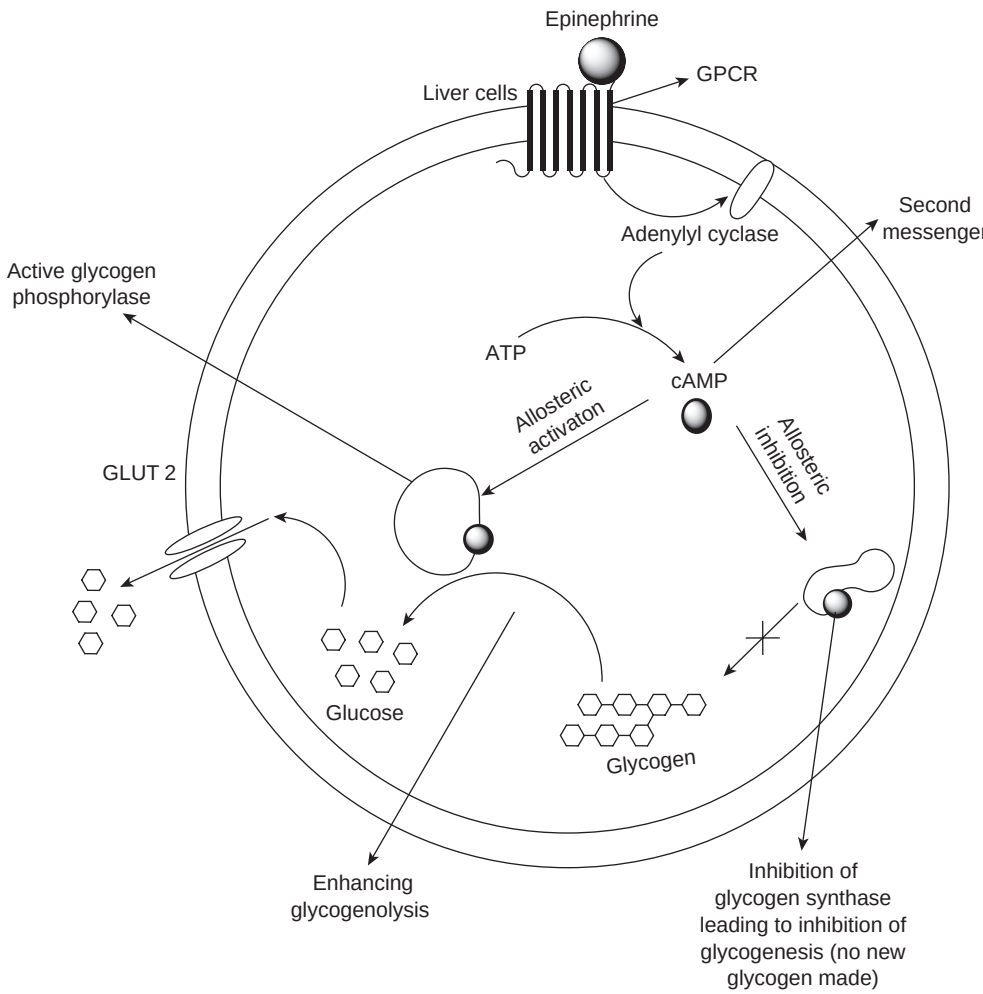


Figure 12.31 Allosteric control involving both activation and inhibition in the regulation of glucose homeostasis.

12.3.4 Effect of Allosteric Modulation on Reaction Kinetics

Allosteric enzymes in general do not follow Michaelis Menten scheme. In Figure 12.32, curve A represents an allosteric enzyme in the absence of allosteric modulator and has little sigmoid character. Addition of negative allosteric modulator results in curve B, which is relatively more sigmoid and is accompanied by increase in the K_m value. The observation suggests a decreased affinity for substrates; in other words, more substrate is needed to reach half of the maximum velocity. Addition of positive modulator results in curve C which is hyperbolic than sigmoidal and is accompanied with increase in K_m value, thereby suggesting that reduced concentration of substrate is needed to achieve half of the maximum velocity.

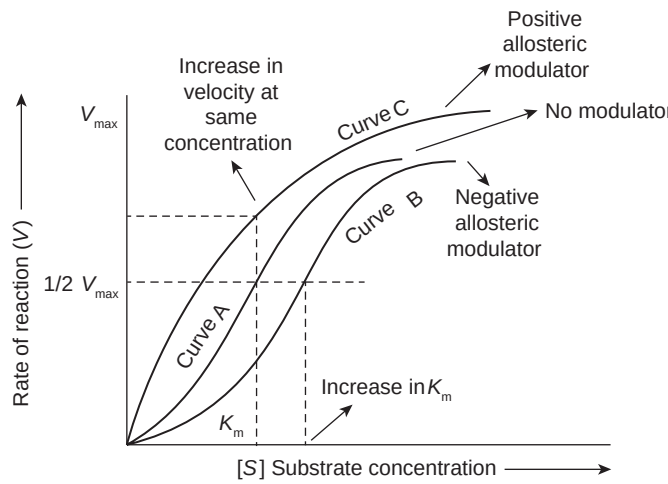


Figure 12.32 Effect of allosteric modulation on reaction kinetics.

12.4 Nomenclature of Enzymes

In 1984 a nomenclature committee, especially created by the International Union of Biochemistry (IUBMB) for enzyme nomenclature, laid down rules for naming each enzyme by allotting Enzyme commission (EC) numbers.

12.4.1 The EC Numbers

Each enzyme has been assigned a unique four-digit code separated by dots with EC as prefix (Fig. 12.33).

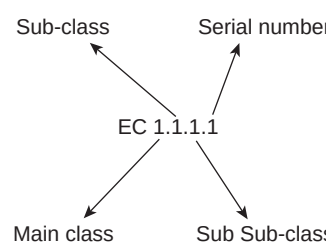


Figure 12.33 Four-digit code as EC numbers for enzymes.

For the classification of enzymes under the EC code, the enzymes were initially grouped under seven primary groups and were assigned codes EC 1 to EC 7 for each of the primary group. The seven primary groups of enzymes are as follows:

1. EC 1. Oxidoreductases
2. EC 2. Transferases
3. EC 3. Hydrolases
4. EC 4. Lyases
5. EC 5. Isomerases
6. EC 6. Ligases
7. EC 7. Translocases

12.4.1.1 EC 1. Oxidoreductases

These enzymes carry out redox (oxidation and reduction coupled reaction) reactions via transfer of either electrons or hydrogen/oxygen between two reactants. The phenomenon dealing with removal of electrons is called oxidation and phenomenon of gaining electron is called reduction. For example, in the reaction involving $4Al + 3O_2 \rightarrow 2Al_2O_3$, Al from zero valency is oxidized to Al^{3+} state in the product Al_2O_3 by losing electrons; on the contrary, oxygen from zero valency state is reduced to O^{-2} state in the product Al_2O_3. Similarly, when hydrogen gas is burned in the presence of oxygen gas $2H_2 + O_2 \rightarrow 2H_2O$, oxygen gets reduced as it accepts hydrogen and hydrogen gets oxidized as it gives away hydrogen.

Now let's examine a redox reaction catalysed by the enzyme dehydrogenase that reduces acetaldehyde into ethanol. The enzyme functions only in the presence of cofactor NAD^+ (produced from NADH), which is bound to the enzyme during its catalytic reaction. The redox reaction proceeds as follows:

$$NADH + H^+ \longrightarrow \underset{\text{Cofactor}}{NAD^+} + 2e^- + 2H^+ \qquad \text{(Oxidation)}$$

$$CH_3CHO + 2H^+ + 2e^- \longrightarrow C_2H_5OH \qquad \text{(Reduction)}$$

Other subcategories of enzymes carrying redox reactions under oxidoreductases are oxidases, oxygenases, hydroperoxidases, etc. Glucose oxidase plays a role in glucose metabolism in cells and converts glucose into gluconic acid in the presence of FAD as a cofactor (Fig. 12.34).

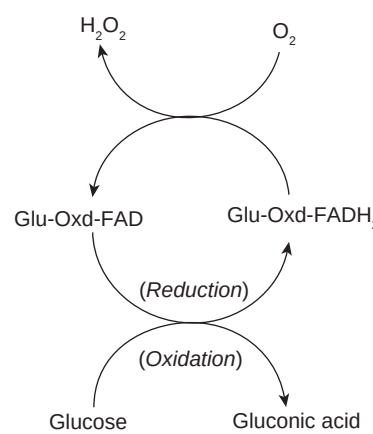


Figure 12.34 A typical reaction catalysed by oxidoreductase in glucose metabolism.

The subcategories under this group (EC1) are assigned using a four-digit code in the following manner:

EC 1	Reflects oxidoreductases groups of enzymes
EC 1.1	Acts on the CH–OH bond (donors)
EC 1.1.1	Reflects NAD^+ as acceptor
EC 1.1.1.2	Substrate is alcohol
EC 1.1.1.3	Substrate is L-homoserine

12.4.1.2 EC 2. Transferases

This involves enzyme-driven catalytic transfer of a chemical moiety between two participants. The best example involves transfer of amine/ketone from one participant to other (Fig. 12.35). Phase II metabolism of drugs in general is carried by transferases such as N-acetyl transferase, uridine diphosphoglucuronosyl transferase, etc.

Yet another interesting example is biosynthesis of catecholamine in which epinephrine (adrenaline) is formed from norepinephrine in the presence of methyltransferase (Fig. 12.36).

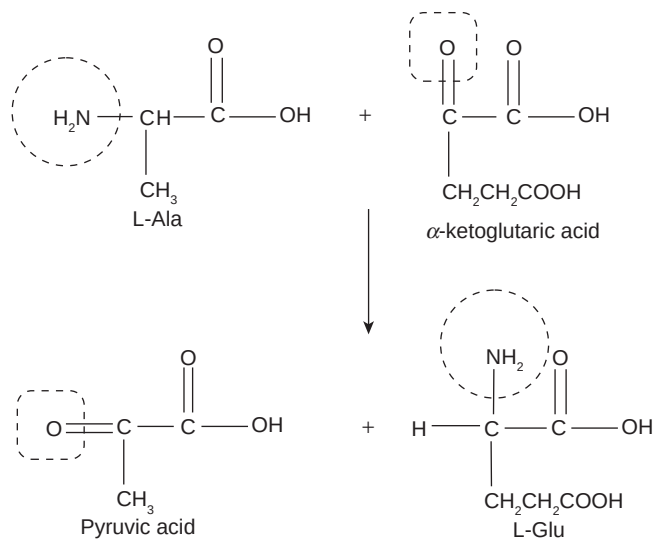


Figure 12.35 A typical reaction catalysed by transferase.

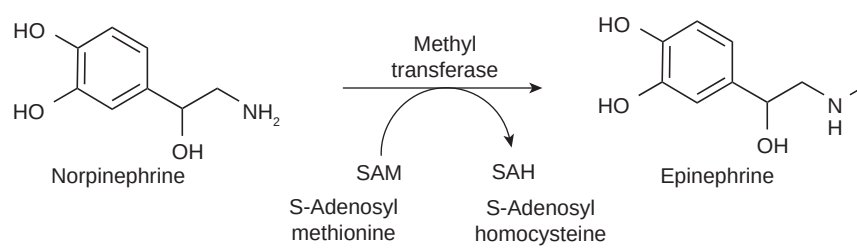


Figure 12.36 Biosynthesis of epinephrine.

12.4.1.3 EC 3. Hydrolases

This finds application in one of the most widely used enzyme catalysed biochemical process in living cells involving degradation (cleavage) of large molecules into small molecules by simple hydrolysis in the presence of water. Majority of them play a role in food digestion, biotransformation of drugs, several biochemical pathways, etc. Some of the most notable and widely used examples include proteases (proteolytic enzymes), lipase and esterases, etc. Esterase plays a role in the metabolic biotransformation of aspirin to salicylic acid while lipase transforms triacyl glycerol into diacylglycerol (Fig. 12.37).

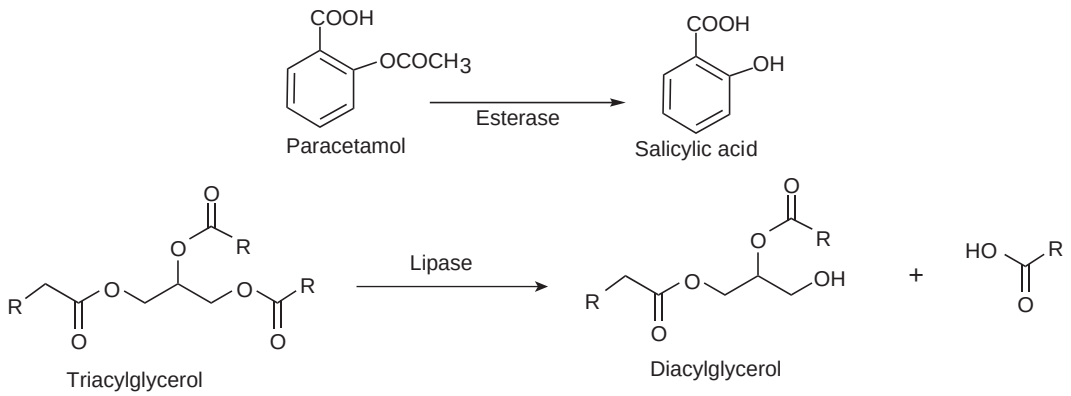


Figure 12.37 Biotransformation involving hydrolases.

The subcategories under this group (EC3) are assigned using a four-digit code in the following manner:

EC 3	Reflects hydrolases group of enzymes
EC 3.4	Enzyme is specific to substrates with CO–NH bonds
EC 3.4.11	Reflects they are aminopeptidases
EC 3.4.11.4	Cleavage of N-terminal amino acid from a tripeptide substrate

12.4.1.4 EC 4. Lyases

The enzyme finds application in the elimination of chemical moiety through the breaking of bonds barring hydrolytic cleavage. Pyruvate decarboxylase, a lyase, triggers cleavage of CO–COOH bond leading to the elimination (non-hydrolytic) of CO_2 and generation of a double bond. Yet another common biochemical reaction catalysed by lyase ATPase involves transformation of ATP to ADP (Fig. 12.38). Similarly, guanylyl cyclase (GC; effector molecule involved in signal transduction), is a lyase enzyme which catalyses conversion of GTP into second messenger cGMP. There are two kinds to GC, one linked with cell-surface receptor and second is a soluble form called sGC and is present in cytoplasm.

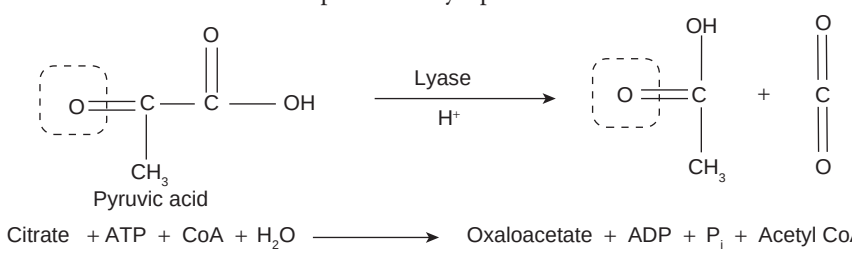


Figure 12.38 A typical reaction catalysed by lyase.

The subcategories under this group (EC4) are assigned using a four-digit code in the following manner:

EC 4	Reflects lyases group of enzymes
EC 4.2	Reflects breaking of C−O bonds
EC 4.2.1	Reflects elimination of water molecules
EC 4.2.1.20	Serine as substrate resulting in Trp formation
EC 4.2.1.28	Propane-diol as substrate furnishing propanal

12.4.1.5 EC 5. Isomerases

Generally, this class of enzyme catalyses unimolecular reactions involving conversion of structures. In fact, the conversion leads to simple isomerization via rearrangement of bonds with no change in molecular weight or atomic makeup. They play a major role in metabolism, and up to 4% of metabolic pathways[3] involve isomerase-catalysed isomerization (Fig. 12.39). A classic example for this class of enzyme is alanine racemase that transforms L-Ala into D-Ala. Another example includes mutarotation in glucose catalysed by the enzyme mutarotase.

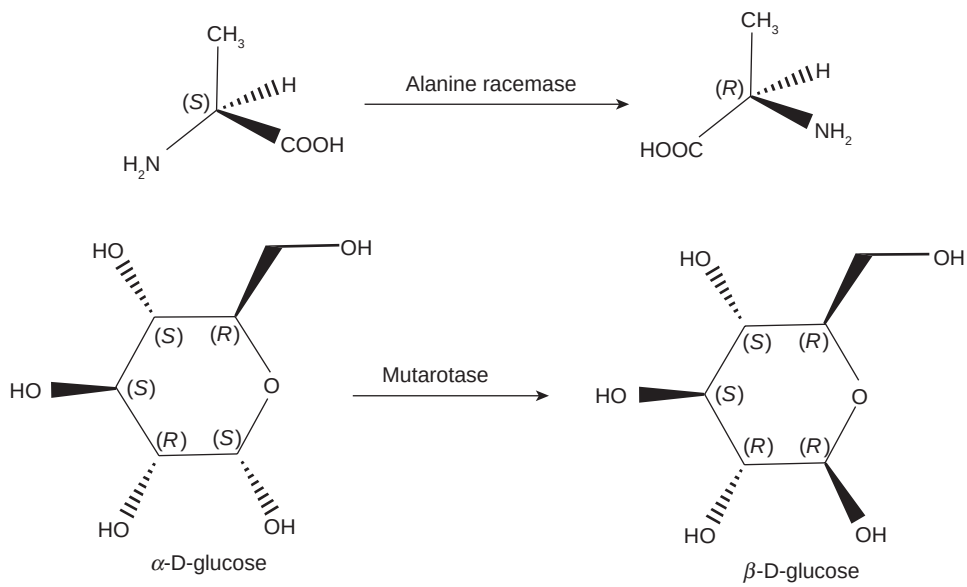


Figure 12.39 Biotransformation involving isomerases.

12.4.1.6 EC 6. Ligases

Its application involves energy (ATP)-driven catalytic reactions, linking two molecules via formation of new C-X bonds in which X may be O/S/N/C atom. Formation of C−O bonds occurs during condensation of amino acids with tRNA in the presence of tyrosine—tRNA ligase and also during linking of DNA fragments in the presence of DNA ligase (it acts as a glue to repair DNA or join their fragments). Besides, formation of C−N bonds occurs in the presence of synthetases resulting in an amide bond (Fig. 12.40).

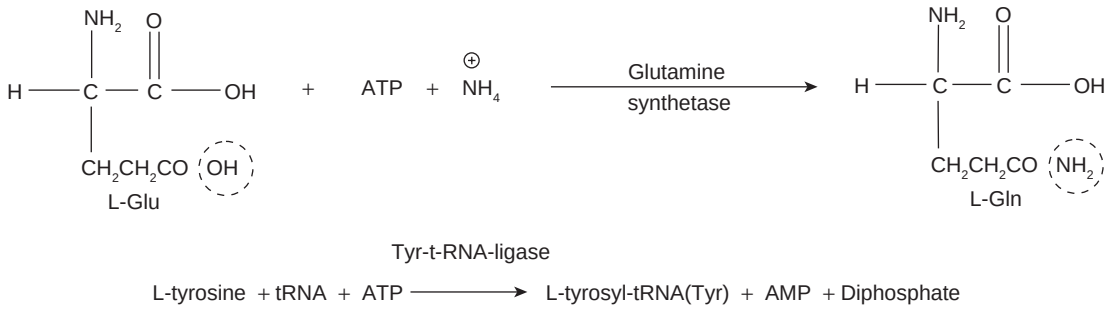


Figure 12.40 Ligase catalysed biotransformation.

The subcategories under this group (EC6) are assigned using a four-digit code in the following manner:

EC 6	Reflects ligases group of enzymes
EC 6.2	Reflects formation of C–S bonds
EC 6.2.1	Reflects acid-thio ligase
EC 6.2.1.1	Acetate as substrate
EC 4.2.1.3	Long-chain fatty acid as substrate

12.4.1.7 EC 7.0 Translocases

Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) introduced this enzyme in the year 2018 as EC7. It catalyses the movement of substances across the cell membranes.

12.5 Role of Enzymes

Enzymes play an important role in diagnosis, therapeutics, and metabolism.

12.5.1 Diagnostic Enzymes

Several enzymes in blood circulation are measured as a test to diagnose diseases. Lactate dehydrogenase for cell injury, creatinine (Cr) for kidney disease, and cardiac enzyme after heart attack, etc., are some examples used as diagnostics. Besides damages to the tissues in pancreas, liver, bones, and prostrate can be also estimated by detecting serum concentrations of specific enzymes. Recent findings have raised a possibility of detecting Alzheimer's disease using a blood test.[4]

12.5.2 Therapeutic Use of Enzymes

Therapeutically, enzymes have been used for treating a variety of diseases either by administering the enzyme alone or as apart of polytherapy.

1. They often bind and act on their targets with great affinity and specificity.
2. They convert multiple target molecules to the desired products.

These characteristics have resulted in the development of many enzyme drugs for a wide range of disorders. For example, lipase is used for the treatment of indigestion and acid reflux-created heartburns. Lipase is the

main enzyme that hydrolyses dietary fats in the digestive tract of humans. The enzyme has also been used therapeutically for the treatment of malignant tumours, dyspepsia, and gastrointestinal disturbances. The lipase enzyme isolated from *Candida* has been used as catalyst in the synthesis of lovastatin.

Thrombolytic enzymatic drugs streptokinase, urokinase, activase, etc. (plasminogen activators) have been used as curative therapy to dissolve thrombus/clot to maintain flow in obstructed blood vessels.

12.5.3 Enzymes as Therapeutic Targets

Like all proteins, enzymes are composed of amino acids as building blocks and act as biocatalysts in living organisms, regulating the rate of biochemical reactions in cells. Each enzyme possesses a unique characteristic sequence of amino acids that causes it to fold into a unique three-dimensional structure that plays a role in its structure and function. The characteristic amino acid sequence of enzymes in organisms is determined by a specific gene in the cell's nucleus. Enzymes are known to catalyse more than 5000 chemical reaction types in organisms. From medicinal chemistry point of view, two major groups of enzymes of interest are cell-signalling enzymes[5] and metabolic enzymes. Both are pharmacological protein targets against which drugs with either agonistic or antagonistic properties can be developed for treating a disease.

12.5.3.1 Extracellular Enzymes

These classes of enzymes are although produced inside the cell but following a signalling mechanism they are secreted by the cells and predominantly function outside the cells. They are generally involved either in the digestion of large macromolecules by breaking into smaller components that are absorbed by cells to maintain their cellular functions or act as signalling enzymes for a variety of physiological functions. Thus, they remain important therapeutic targets for drug discovery.[6] Among extracellular enzymes, proteases comprise a large family of enzymes which have been implicated and identified as drug target for a variety of diseases such as cardiovascular, lung diseases, cancer, etc. Captopril was the first structure-based drug launched in 1981 as inhibitor of a protease ACE.

ACE, a dipeptidyl carboxypeptidase (exopeptidase) cleaves His-Leu from the C-terminal of decapeptide angiotensin I and produces a potent vasoconstrictor (leading to increase in blood pressure) octapeptide angiotensin II that has been implicated in hypertension. Captopril inhibits the ACE enzyme, thereby blocking the formation of angiotensin II in renin angiotensin system (Fig. 12.41).

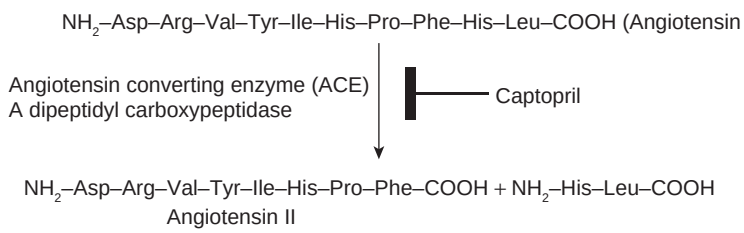


Figure 12.41 Clinically used drug captopril blocks ACE and prevents formation of angiotensin II.

Serine Proteases in Cardiovascular Disease

They are heterogeneous group of enzymes and play a crucial role in the formation and dissolution of blood clot via coagulation cascade and fibrinolytic system. The complex coagulation cascade employs several serine proteases thrombin and some prominent factors VI*a*, IX*a* and X*a*, and together they catalyse a variety of coagulation-related reactions. Ultimately, the clot formation involves thrombin-mediated conversion of

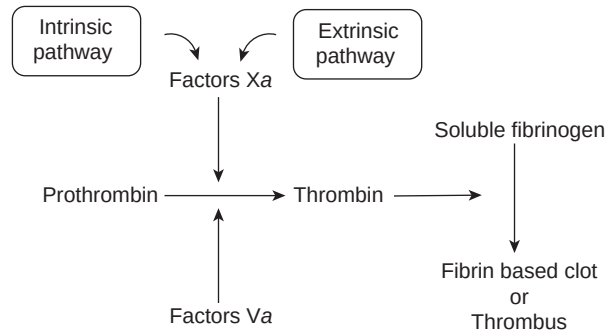


Figure 12.42 Role of serine proteases in coagulation cascade.

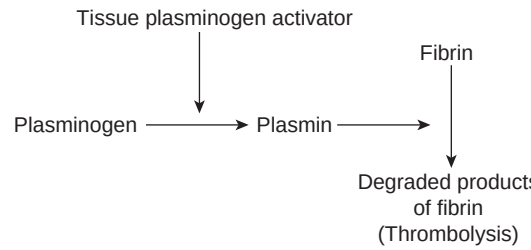


Figure 12.43 Role of serine proteases in fibrinolytic system.

soluble fibrinogen into solid fibrin-based clot/thrombus formation *(damages in vascular tissue is clot but its formation in healthy arteries/veins is called thrombus).* Thus, both thrombin and factors serve as important therapeutic target in cardiovascular diseases (Fig. 12.42).

In the coagulation cascade, there is yet another endogenous serine protease called protein C that by a natural mechanism counters clot formation by inhibiting formation of the thrombin. Clinically, protein C (Ceptotrin) has been used in patients suffering from protein C deficiency.

The fibrinolytic system also employs a serine protease called plasmin that degrades fibrin and thus dissolves fibrin blood clot (thrombolysis). In this system, formation of plasmin from plasminogen is catalysed by a tissue (*t*-)plasminogen activator, also a serine protease present in the endothelial cells lining the wall of blood vessels (Fig. 12.43). Interestingly, due to the excellent thrombolytic properties of *t*-plasminogen activator, its recombinant product is used for the treatment of ischemic stroke.

Role of Serine Protease in Diabetes

Dipeptidyl peptidase 4 (DPP4), a serine protease, has been implicated in the disease diabetes mellitus. It serves as an important therapeutic target for the development of inhibitors for disease management. The enzyme is expressed on the surface of cells throughout the body and inactivates GLP-1 responsible for the secretion of insulin. Sitagliptin is a potent inhibitor of DPP4 and is used clinically to manage diabetes.

Serine Proteases as Signalling Molecule

The extracellular proteolytic enzymes sometimes act as signal molecules and bind to receptors (GPCR) called protease activated receptors (PARs). One such receptor is present on platelets and binds with thrombin, a serine protease. However, unlike other endogenous ligand, thrombin binds with PAR1 (GPCR family) via a different mechanism (Chapter 7, Fig. 7.11). Voraxapar is a first PAR-1 antagonist used clinically as anti-platelet drug.[7]

Digestive Enzymes

The enzymes are involved in the digestion of food in gastrointestinal tract (GIT) and are released from cells in pancreas, liver, and from enterocytes in small intestine. There are three major enzymes carbohydrase, protease, and lipase involved in the breakdown of carbohydrates, proteins, and fats. Details have been discussed in Chapter 14.

1. Lipase – along with bile from the gallbladder, breaks down fats into glycerol and fatty acids
2. Protease – breaks down proteins into amino acids
3. Lactase – breaks down lactose, the complex sugar in milk products

Production of Enzymes

The first signal for the production of enzymes originates from DNA in the nucleus. The signal triggers transcription followed by post-transcription to produce mRNA that is exported out from the nucleus and gets attached to the ribosomes/polysomes present on endoplasmic reticulum (ER) or free ribosomes/polysomes. The ER-bound polysomes produce encapsulated proteins released in the form of transport vesicles. The latter unloads the proteins/enzymes in Golgi apparatus where the enzyme is processed packaged in secretory vesicles. The secretory vesicles are transported to the plasma membrane for secretion (exocytosis) of proteins/enzymes outside the cells. Secretory vesicles comprising hydrolases combine with endosomes and result in the formation of lysosomes (Fig. 12.44). The latter is responsible for the degradation of cytoplasmic wastes following which they may be recycled or excreted.

Free polysomes are exclusively involved in the production of proteins that are required by the cytosolic organelles—peroxisomes, mitochondria, nucleus, etc. Within cytoplasm these proteins do not require any transport vesicles and access cytosolic organelles through diffusion. Defunct cytosolic ribosomes are removed via autophagy pathways.



Important Note

Ribosomes

They are tiny organelles within cells involved in the production of proteins with diverse physiological functions. There are two kinds of ribosomes in the cytoplasm: one form remains bound to endoplasmic reticulum (ER) called rough ER and the other form is called free ribosomes that float freely in the cytoplasm.



Important Note

Polysomes (Polyribosomes)

They are cluster of ribosomes that are actively involved in the process of transforming mRNA into protein. In other words, they can be defined as active complexes held together by mRNA. In the cytoplasm they exist either as ER-bound or free-floating structures.

12.5.3.2 Intracellular Enzymes

Intracellular enzymes are also referred to as proteases. or proteolytic enzymes and are part of metabolic processes within cells that are essential for the survival of cells. Among many metabolic processes, the degradation of proteins (defective or surplus, as they are continuously produced/digested as part of

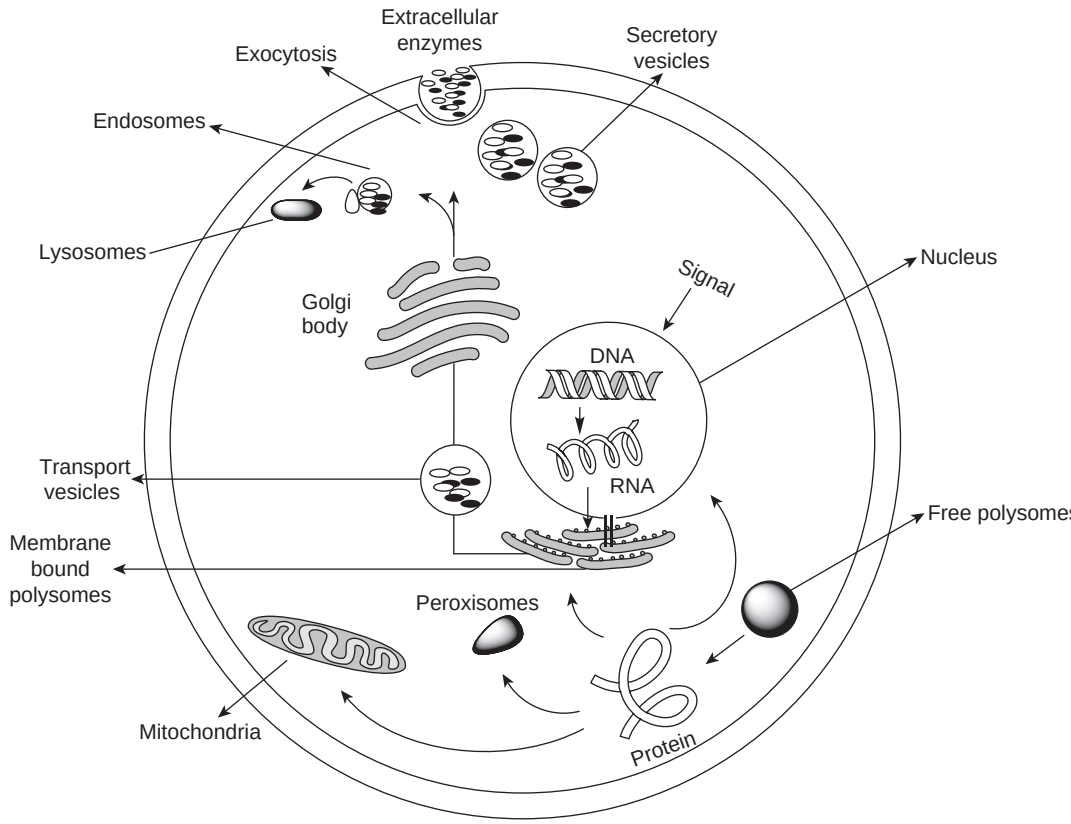


Figure 12.44 Production of extracellular and intracellular proteins/enzymes.

protein turn over) called proteolysis is a crucial phenomenon. Within cells, lysosomes[8] carry protease and play an important role in protein degradation and removal of cytoplasmic wastes from the cells. Lysosomes are tiny structures present within cytoplasm that carry proteases (aspartic, cysteine, or serine proteases, cathepsin) for the digestion of proteins. Lysosomes degrade both extracellular (from interstitial fluid) as well as intracellular proteins. The extracellular proteins are engulfed through endocytosis[9] in the form of small-bubble endosomes in the cytoplasm which then comes in contact with lysosomes and undergoes protein digestion (endolysosomal degradation). On the contrary, intracellular proteins are digested by several routes, and one of them involves autophagy (predominant during fasting or starving).

Researchers have established the role of endolysosomal degradation in the modulation and termination of extracellular signal transduction by dissociating and degrading (or recycled back to surface) first messengers (endogenous ligands) bound to their receptors.[10] Among many methods of degradation, one of them involves internalization of the receptor-bound ligands complexes.[11] Several reports have established endolysosomal degradation of endogenous ligands (first messenger) epidermal growth factor and insulin-like growth factor (IGF-1). Experiments have been carried out to prevent lysosomal degradation of IGF-1 using lysosomal protease inhibitors, particularly for cathepsin B; however, no drugs have yet reached clinics targeting lysosomal proteases.

Intramembrane Proteases (IMPs)

The first unexpected example of this class of proteolytic enzyme site-2 protease (S2P/IMP/ICLiPs) was reported in 1997.[12] This then led to the discovery of few more disease-linked intramembrane enzymes with the possibility of being a useful drug target and is still under investigation. These enzymes in general are impregnated in the lipid bilayer membrane. Other proteolytic enzymes of this class are rhomboid proteases aspartate IMPs and glutamate IMPs.[13] However, no drugs have yet reached clinics targeting these IMPs.

Intracellular Signalling Enzymes

These enzymes are involved in cellular/physiological functions and have remained therapeutic targets of great significance. They are activated following the binding of ligand to extracellular receptors which then undergoes change in conformation and transduces signal within the cells. The signal transduction goes through a process of propagation and amplification of the signal via second messengers and effectors, ultimately activating the intracellular signalling enzymes. Thus, intracellular signalling enzymes help in communication between extracellular cues and genes in the nucleus leading to cell-specific biological response. Activation of intracellular enzymes following signal transduction initiates numerous enzymatic modifications such as phosphorylation and dephosphorylation of proteins inside the cell by enzymes such as kinases and phosphatases.[14]

This phenomenon of intracellular cell signalling depending on the function of cell may activate or deactivate the enzymes and can be modulated therapeutically by drugs. Other intracellular signalling enzymes include adenylyl cyclases, caspases, phosphodiesterases, phospholipases, prenyltransferases, etc., and have been implicated as target for various diseases. An online data base on cell-signalling enzymes has been compiled by scientists from India[15] for research purpose.

Metabolic Enzymes

Metabolic enzymes are involved in cellular functions necessary for survival including proteolysis and digestion, cellular respiration, energy storage, transcription, etc. These reactions are catabolic or anabolic types resulting in breakdown or formation of substances within cells. In addition, digestive enzymes secreted in GIT are also involved in the breakdown of foods into small units for absorption or as waste materials.

Yet another class of metabolic enzymes is involved in the biotransformation of xenobiotics and endogenous substances for their elimination from the body. The biotransformation is carried out by microsomal and non-microsomal enzymes predominantly located in the liver but are virtually present in all tissues. Details about role of enzymes in metabolism have been discussed in Chapter 13.

Exercises

1. Discuss effect of pH and temperature on enzyme activity.

2. Describe Michaelis-Constant and Michaelis Menten equation.

3. Explain EC nomenclature of enzymes.

4. Discuss mechanism of enzyme inhibition.

5. Discuss effect of feedback inhibition on enzyme activity.

6. Discuss the role of enzymes as therapeutic target.

7. Discuss the role of serine proteases in cardiovascular disease.

8. Write a brief note on each of the following:

(i) Cofactor

(ii) Positive allosteric modulator

(iii) Binding site

(iv) Hydrolases
(v) Irreversible inhibitors
(vi) Suicide inhibitors
(vii) Therapeutic enzymes
(viii) Intracellular proteases
(ix) Enzymatic hydrolysis of ACh

9. Describe allosteric control involving both activation and inhibition in the regulation of glucose homeostasis.

Competitive Exam Questions

1. A term which describes a cofactor that is finally bound to an apoenzyme is
(a) holoenzyme. **(b)** coenzyme.
(c) prothetic group. **(d)** transferase.
[***GATE Pharmacy 2003***]

2. In the study of enzyme kinetics, V_{max} is said to be attained when
(a) there is an excess of free enzyme as compared to the substrate.
(b) virtually all the enzyme is present as the enzyme-substrate complex and concentration of the free enzyme is vanishingly small.
(c) the maximum velocity of the reaction in the presence of low substrate concentration.
(d) when the concentration of free enzyme equals that of the enzyme-substrate complex.
[***GATE Pharmacy 2005***]

3. Choose the correct match.

Group I *Type of inhibitor*	**Group II** *Description*
P. Competitive inhibitors	**1.** have affinity only for the [*E*–*S*] complex and not for the free [*E*]
Q. Non-competitive inhibitors	**2.** binding of the inhibitor and that of the natural substrate are mutually exclusive
R. Uncompetitive inhibitors	**3.** ultimately binds covalently to the enzyme
S. Suicide inhibitors	**4.** binds with the same affinity to [*E*] and [*E*–*S*]

(a) P–3; Q–2; R–1; S–4
(b) P–1; Q–3; R–2; S–4
(c) P–4; Q–1; R–3; S–2
(d) P–2; Q–4; R–1; S–3
[***GATE Pharmacy 2009***]

4. Which of the following statements about K_m, the Michaelis constant in Michaelis-Menten kinetics is correct?
(a) It is defined as the concentration of substrate required for the reaction to reach maximum velocity.
(b) It is defined as the dissociation constant of the enzyme-substrate complex.
(c) It is expressed in terms of the reaction velocity.
(d) It is a measure of the affinity the enzyme has for its substrate. [***GPAT 2017***]

5. RNA molecules having intrinsic catalytic activity are called
(a) Ribozymes **(b)** snRNA
(c) rRNA **(d)** mRNA
[***GPAT 2019***]

References

1. David M. Blow, *Acc. Chem. Res.*, **1976**, *9*, 145.
2. Michale Bouskila, Roger W. Hunter, Adel F. M. Ibrahim, Lucie Delattre, Mark Peggie, Janna A.van Diepen, Peter J. Voshol, Jørgen Jensen and Kei Sakamoto, *Cell Metabolism*, **2010**, *12*, 456.
3. Sergio Martinez Cuesta, Nicholas Furnham, Syed Asad Rahman, Ian Sillitoe and Janet M. Thornton, *Curr. Opin. Struct. Biol.*, **2014**, *26*, 121.
4. Schindler S. E., Bollinger J. G., Ovod V., Mawuenyega K. G., Li Y., Gordon B. A., Holtzman D. M., Morris J. C., Benzinger T. L. S., Xiong C., Fagan A. M. and Bateman R. J., *Neurology.*, **2019**, *93*, e1647.)
5. Oliver Schilling and Peter Findeisen, *Proteomics Clin. Appl.*, **2014**, *8*, 296.
6. Mare Cudic and Gregg B. Fields, *Curr. Protein Pept. Sci.*, **2009**, *10*, 297.
7. Dorothea M. Heuberger and Reto A. Schuepbach, *Thrombosis Journal*, **2019**, *17*, 1.
8. For review: https://www.ncbi.nlm.nih.gov/books/NBK6348/ Klaudia Brix. Lysosomal Proteases: Revival of the Sleeping Beauty (access date 21. 7. 2019).
9. Sabrina Müller, Julia Dennemärker and Thomas Reinheckel, *Proteins and Proteomics*, **2012**, *1824*, 34.
10. For review: Sabrina Müller Julia Dennemärker and Thomas Reinheckel, *Biochimica et Biophysica Acta*, **2012**, *1824*, 34.
11. Yanjie Xu, Suxuan Liu, Jixiang Xia, Sam Stein, Cueto Ramon, Hang Xi, Luqiao Wang, Xinyu Xiong, Lixiao Zhang, Dingwen He, William Yang, Xianxian Zhao, Xiaoshu Cheng, Xiaofeng Yang and Hong Wang, *Front Biosci (Landmark Ed)*, **2017**, *2*, 1439.
12. Rawson R. B., Zelenski N. G., Nijhawan D., Ye J., Sakai J., Hasan M. T., Chang T. Y., Brown M. S. and Goldstein J. L., *Mol. Cell*, **1997**, *1*, 47.
13. Steven H. L. Verhelst, *The FEBS Journal*, **2017**, *284*, 1489.
14. Ragul Gowthaman, Ramya Venugopal, and Dhiviya Dayanandam, *Bioinformation*, **2006**, *1*, 265.
15. Gowthaman R., Venugopal R. and Dayanandam D., *Bioinformation* **2006**, *28;1(7)*, 265.

CHAPTER

13

Role of Enzymes in Metabolism

KEY THINGS TO LEARN FROM THIS CHAPTER

- Role of microsomal enzymes in phase I metabolism and non-microsomal enzymes in phase II metabolism.
- Cytochrome P450 enzyme (CYP) mediated phase I metabolism reactions via functionalization reactions: Biotransformation via introduction of hydroxyl group, oxidation of compounds with carbon-heteroatom systems, unmasking' of existing polar functional groups and hydrolytic reactions.
- Non CYP mediated phase II metabolism via derivatization into water soluble products: Glucuronidation, sulfation, glutathione conjugation, acetylation.
- Induction and inhibition of CYP.
- Pharmacokinetic and pharmacodynamic drug-drug interactions.
- Drug-design approach for developing drugs with enhanced resistance to CYP metabolism.

13.1 Introduction

Following administration of a drug in the body, metabolism plays a crucial role in the elimination of drugs and, in turn, reduces the life span of drug in the body. The elimination may occur either as water-soluble metabolites or in its unchanged form depending on the physicochemical properties of drugs. For drugs administered by oral route, the metabolism begins in the form of first-pass metabolism from gastrointestinal region, and then in the liver, a major organ involved in metabolism. However, extrahepatic metabolism outside liver also occurs throughout the body in all tissues. Nitroglycerine (administered by palliative route) for example is predominantly metabolized outside liver cells.

The metabolic enzymes responsible for the metabolism of xenobiotics and endogenous substances can be broadly classified into microsomal and non-microsomal enzymes.

13.1.1 Microsomal Enzymes

The major component of microsomal enzymes is cytochrome P450 (CYP450) involved in phase I biotransformation of drugs into water-soluble metabolites. Xenobiotics and endogenous substances can induce or

inhibit microsomal enzymes. They are both soluble and membrane-bound enzymes predominantly present in the two cytosolic organelles of liver cells (hepatic tissues): endoplasmic reticulum (ER), and mitochondria (MT), a powerhouse for cells. The CYPs released by ER, in general, are involved in the metabolism of xenobiotics and endogenous molecules, whereas CYPs in MT are limited to synthesis and biotransformation of their in-house molecules. CYPs are also present in several extrahepatic tissues:

1. Gastrointestinal tract
2. Kidneys
3. Lungs/nasal membranes
4. Adrenal glands
5. Brains
6. Placenta
7. Brain
8. Skin

13.1.2 Non-Microsomal Enzymes

In general, they comprise non-specific enzymes primarily involved in phase II conjugation reaction (biotransformation). The phase II reactions generally occur in the presence of transferases except glucuronidation. Other enzymes are alcohol dehydrogenases, aldehyde dehydrogenase, xanthine oxidase, monoamineoxidase, and NADPH-quinoneoxidoreduxtase involved in hydrolytic, reductive, and oxidative reactions. They are located (other than ER) in the cytoplasm and mitochondria of liver cells, GIT, other tissues in the body, and blood plasma. Unlike microsomal enzymes, non-microsomal enzymes are never induced by substances.

13.2 CYP-Mediated Metabolism

The phenomenon of metabolism (M) involves degradation of drugs by metabolic enzymes called *microsomal enzymes*, Cytochrome P450. However, depending on the physicochemical properties of the drug, metabolites or unchanged drugs may also be excreted through urine or stool via gastrointestinal tract. CYPs responsible for the metabolism are though distributed in cells throughout the body, they are predominantly located in liver cells.Notably, this class has more than 50 enzymes (isoforms); only six of them are sufficient to metabolize up to 90 percent of drugs with the three most common enzymes being CYP2C8,[1] CYP3A4, and CYP2D6.[2] CYPs are a super family of hemoprotein enzymes as they have heme as a cofactor and function as monooxygenase. The most common CYP-mediated reaction involves the following:

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O$$

13.2.1 Types of Metabolic Reactions

The sole objective of CYP-mediated metabolism of xenobiotics and endogenous substances is to biotransform them into water-soluble metabolites so that they can be readily eliminated from the body. This is achieved either by introducing polar groups in the molecule or unmasking polar groups to enhance water solubility. Generally, there are two phases for enzyme-mediated metabolic reactions. The first phase involves CYP 450-mediated chemical reactions oxidation and hydrolysis leading to polar compounds and in the second phase (that does not involve CYP), the polar compounds obtained from phase I are derivatized (conjugation reaction) into

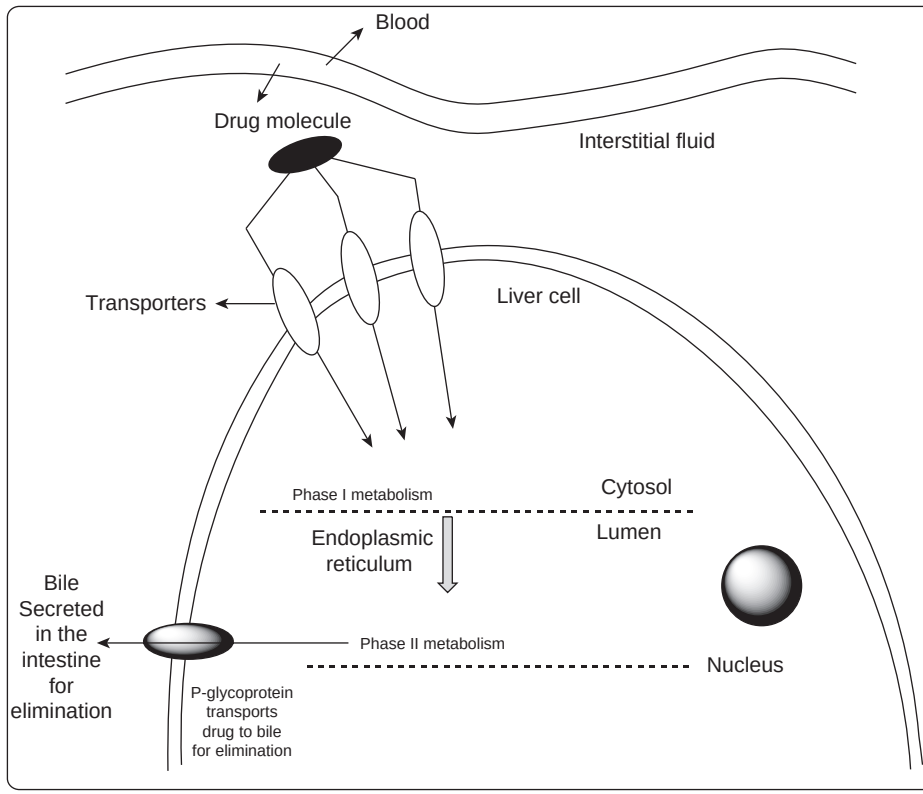


Figure 13.1 A diagrammatic representation of metabolism in a liver cell.

water-soluble conjugates (Fig. 13.1) in the presence of transferases. Generally, phase II metabolisms occur in the presence of reaction-specific transferase enzymes (non-CYP) such as glutathione S-transferase (GST) that derivatizes phase I metabolite with tripeptide glutathione, sulphotransferase (ST) involved in the derivatization via sulfonation reaction, and uridine diphosphoglucuronosyl transferase (UGT) which catalyzes glucuronidation of phase I metabolites. Cytochrome P450-mediated phases I metabolism occurs on the surface of the endoplasmic reticulum cytosolic side whereas membranous transferase catalyzed phase II occurs in the luminal side of endoplasmic reticulum just opposite[3] to the location of CYP 450 (Fig. 13.1).

13.2.1.1 Phase I Metabolism: Detoxification or Functionalization Reactions

A degradative reaction involving functionalization with polar groups is carried out with the view to prepare the compound for phase II metabolism. The objective of introducing polar functional groups in phase I is to enhance the polarity of the resulting metabolite. The products of phase I may or may not have enough polarity and act as precursors for phase II conjugation reactions furnishing water-soluble metabolites for renal excretion. The resulting polar metabolites in phase I are generally devoid of pharmacological activity (termination of therapeutic activity) but in some cases may be equally or more active than the parent drug. Some of the functionalization reactions performed by the cytochrome P450 system involve oxidation, reduction, hydrolysis, hydration, etc. These diverse functionalization reactions are carried out by several CYP 450 located in microsomes within cells.

Types of Phase I Biotransformation Reactions

This involves introduction of polar functional groups into xenobiotics. Some of the functional groups that are introduced during phase I metabolism are –OH, –NH_2, –SH, and –COOH. Examples of CYP-mediated phase I biotransformation reactions have been summarized in Tables 13.1 and 13.2.

Table 13.1 Some CYP-mediated functionalization reactions

Functional Groups Present in the Drug/Xenobiotics	Phase I Transformation	Category of Reaction
Ketones, aldehydes	Alcohols	Reduction
Alcohols	Carboxylic acids	Oxidation
Esters, amide	COOH, NH_2, OH	Hydrolysis
Azo, nitro	NH_2	Reduction

Table 13.2 Some common phase I biotransformation reactions in clinically used drugs

Reactions	Drug
Hydroxylation of arenes	Amphetamine and phenobarbital
Hydroxylation of aliphatic moieties	Cyclosporine and ibuprofen
N-Dealkylation	Diazepam
O-Dealkylation	Codeine

Biotransformation via introduction of hydroxyl group

The introduction of hydroxyl group in a molecule is a CYP 450-mediated process of oxidation. It can be represented by a general formula:

$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + R-OH$$

Where R represents a substrate such as a steroid, fatty acid, oracompound with an alkene, alkane, aromatic ring, or heterocyclic ring as substituent that serves as a site for oxygenation.

Nicotinamide adenine dinucleotide phosphate (NADPH) functions as electron carrier and its main job in the cell is to shuttle electrons during enzyme-catalyzed reactions. Besides shuttling electrons, hydrogen is added and removed from the nicotinamide parts. When a H is present, NADPH is in a reduced form, because whenever a molecule receives a hydrogen or electron it issaid, in chemistry, to have been reduced. Similarly, if they lose a hydrogen or electron, the molecule $NADP^+$ is oxidized thus, NADPH acts as a reducing agent.

1. **Oxidation of Unhindered Methyl Group:** Methyl groups present in a molecule with no hindrance can be readily oxidized in the presence of CYP to alcohols (Fig. 13.2).



Figure 13.2 Oxidation of unhindered methyl group.

2. **Hydroxylation of Arenes:** Aromatic hydroxylation via oxidation of arenes occurs at the *para* position as it is the most-favored position. The reaction proceeds with formation of epoxide intermediate, which then rearranges to form arenols (Fig. 13.3).
   - An aromatic-based drug phenobarbital undergoes aromatic hydroxylation during phase I metabolism in the presence of CYP 450 (Fig. 13.4).
   - A subset of CYP 450-mediated reactions that occurs during phase I reactions for the purpose of biotransformation has been summarized (Fig. 13.5).

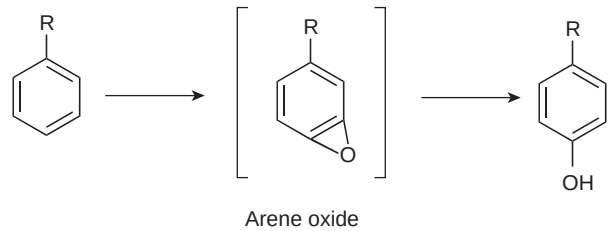


Figure 13.3 CYP-mediated phase I aromatic hydroxylation.

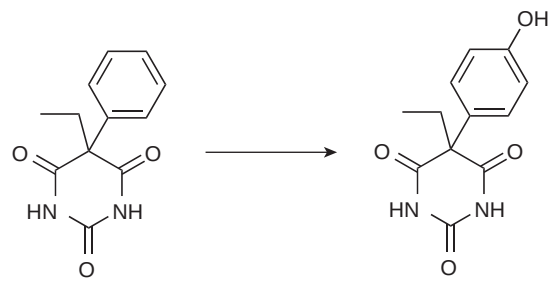


Figure 13.4 Hydroxylation of phenobarbital.

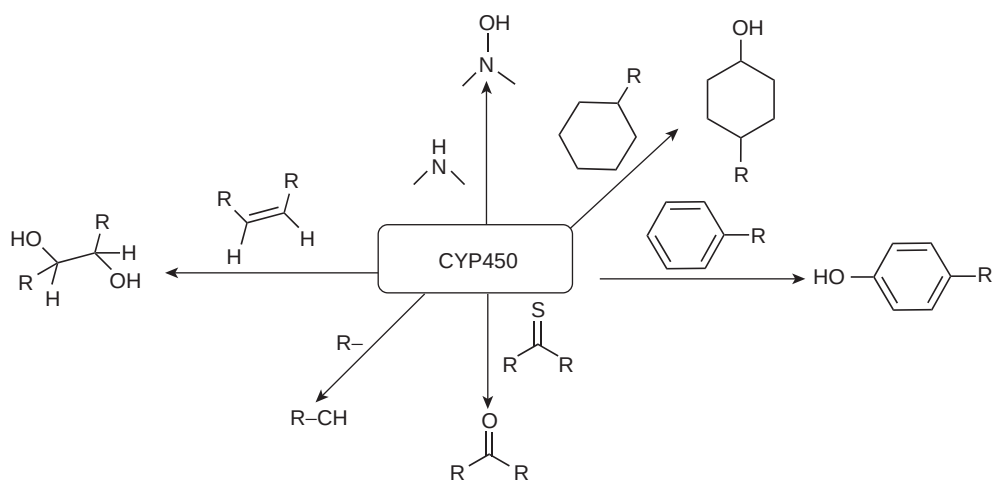


Figure 13.5 CYP-mediated phase I biotransformation.

3. **Minimizing Phase I Hydroxylation in Aromatic Compounds:** Since aromatic compounds have a tendency to undergo metabolism via hydroxylation, depending on the need, this metabolism can be blocked by introducing F at the *para* position of the aromatic ring as an isostere of H. This can assist in improving the concentration of drug in plasma and could be an important tool during drug design strategies (Fig. 13.6).

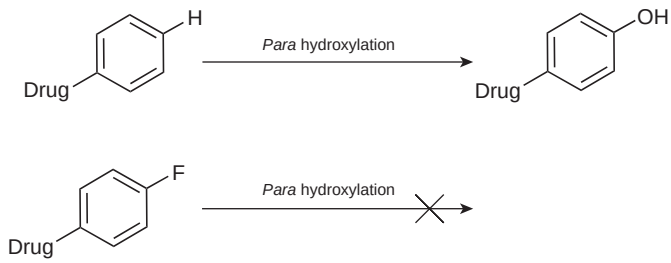


Figure 13.6 Phase I biotransformation/metabolism can be reduced by replacing hydrogen at *para* position with F as a bioisostere.

Biotransformation via oxidation of compounds with carbon-heteroatom systems

Some of the phase I CYP 450-mediated reactions in carbon-heteroatom systems may involve N-oxidation, N-hydroxylation, S-oxidation, N-dealkylation, etc.

1. **N-Hydroxylation**: Drugs/xenobiotics containing non-basic aryl amines, non-basic nitrogen atom (such as amides), and basic amines are metabolized in phase I by N-hydroxylation (Fig. 13.7) reactions.
2. **N-Oxidation:** This reaction in phase I metabolism is prevalent in compounds with tertiary amines (Fig. 13.8).
3. **S-Oxidation:** Compounds with C–S bonds are prone to CYP 450-mediated S-oxidation resulting in either sulfoxides or sulfones (Fig. 13.9).

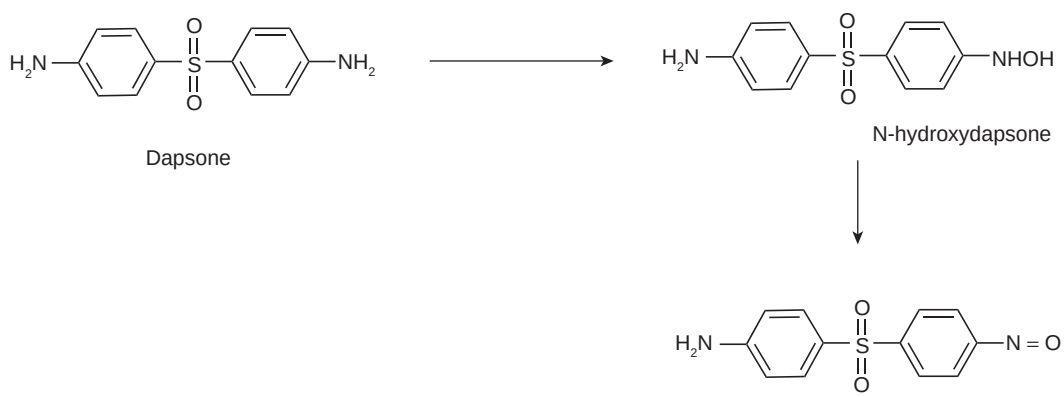
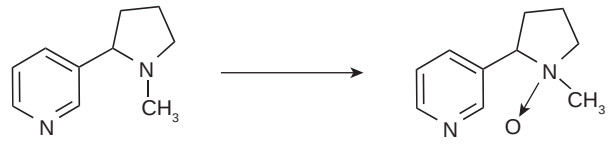
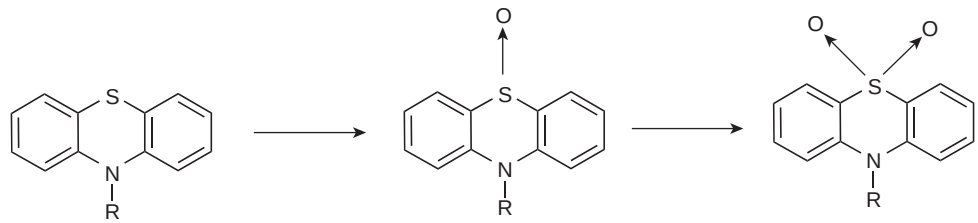


Figure 13.7 CYP-mediated phase I N-hydroxylation.



Nicotine Nicotine-1'-N-oxide

Figure 13.8 CYP-mediated phase I N-oxidation.

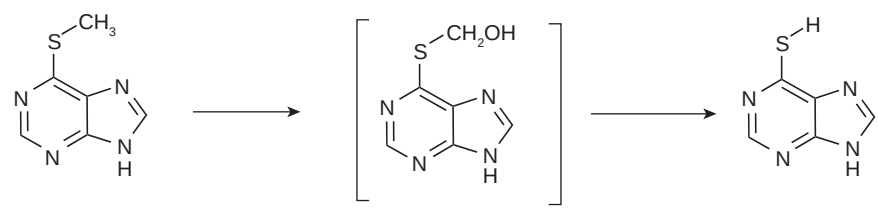


Phenothiazine Phenothiazine sulfoxide Phenothiazine sulfone

Figure 13.9 CYP-mediated phase I S-Oxidation.

Biotransformation via 'unmasking' of existing polar functional groups

1. **S-Dealkylation:** This involves CYP 450-mediated removal of substituent to expose polar groups. Oxidative cleavage of S-alkyl group furnished a polar thiol group (Fig. 13.10).
2. **N-Dealkylation (1°/2° Amines):** CYP 450-mediated dealkylation in primary or secondary amines led to polar compounds (Fig. 13.11).
3. **N-Dealkylation (3° Amines):** CYP 450-mediated dealkylation in tertiary amines furnished polar compounds (Fig. 13.12).
4. **O-Dealkylation:** CYP 450-mediated O-dealkylation of drugs possessing C–O–C furnishes hydroxyl compounds.CYP 450-mediated dealkylation of phenacetin produced paracetamol (Fig. 13.13).
5. **Oxidation of Benzylic Carbons:** CYP 450-mediated oxidation of benzylic carbon produced –COOH as polar group in the molecule with two intermediacies: alcohol and aldehyde (Fig. 13.14).



6-(methylthio)-purine Hydroxylated intermediate 6-Mercaptopurine

Figure 13.10 CYP-mediated S-dealkylation.

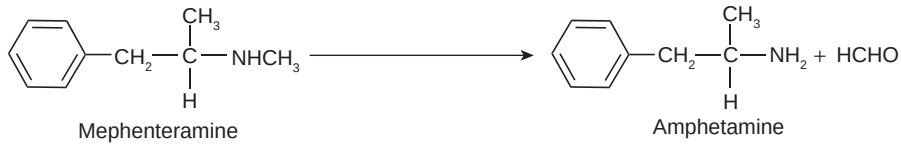


Figure 13.11 CYP-mediated N-dealkylation.

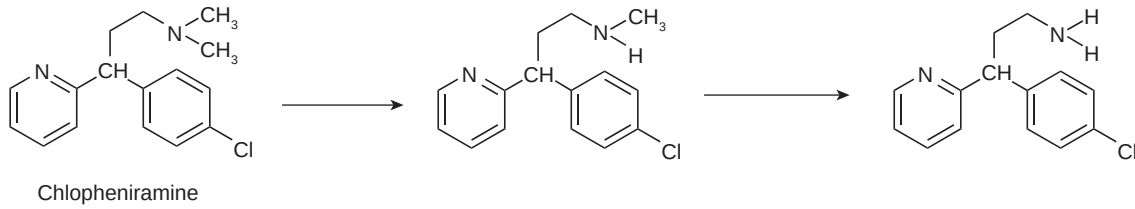


Figure 13.12 CYP-mediated dealkylation of amines.

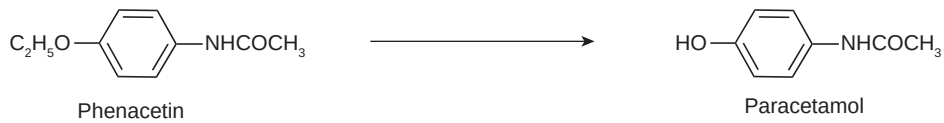


Figure 13.13 Dealkylation of phenacetin produced paracetamol.



Figure 13.14 CYP-mediated oxidation of benzylic carbons.

Hydrolytic biotransformation

Hydrolytic enzymes present in plasma, tissues, and organs metabolize drugs with amides or esters functionalities. For example, amidases hydrolyze amide bonds, whereas esterases hydrolyze esters. Biotransformation of aspirin to salicylic acid is a classic example of ester hydrolysis (see Fig. 13.19, later in the chapter).

13.2.1.2 Phase II Metabolism: Conjugation Reactions (Non-CYP Drug Biotransformation)

Phase II metabolism involves reaction (covalent bond formation) between functional group on the parent compound or phase I metabolite with either of the endogenous reactants (present in the body) such as sulfate, glucuronic acid, acetate, or an amino acid/di-tri-peptide. Phase II reactions are not regulated by metabolic cytochrome P enzymes, but instead catalyzed by non-microsomal enzymes, primarily transferases. The phase II chemical conjugation reactions include sugar conjugation, sulfation, methylation (methylation is an important biochemical event, and all the common purines and their nucleosides occur

naturally in mono- or poly-methylated forms), acetylation (metabolism of isoniazid occurs by acetylation), amino acid conjugation, glutathione conjugation, etc. Among these transferase-catalyzed phase II reactions, glucuronidation alone accounts for >40–60% phase II derivatization reactions. The resulting end products are biologically inactive, highly water soluble (polar compounds) and rapidly excreted in urine. The various phase II conjugation reactions that occur in the presence of enzymes (not CYP) have been summarized in Table 13.3 and Figure 13.15.

Table 13.3 Phase II conjugation reactions occur in the presence of enzymes (non CYP)

Phase-II Conjugation Reaction	Catalytic Enzyme Involved (Non CYP)
Glucuronidation	UDP-glucuronosyltransferase
Sulfation	Sulfotransferase
Glutathione Conjugation	Using glutathione-S-transferase
Acetylation	Acetyltransferase
Amino acid conjugation	Aminotransferase
O and S-methylation	Methyltransferase

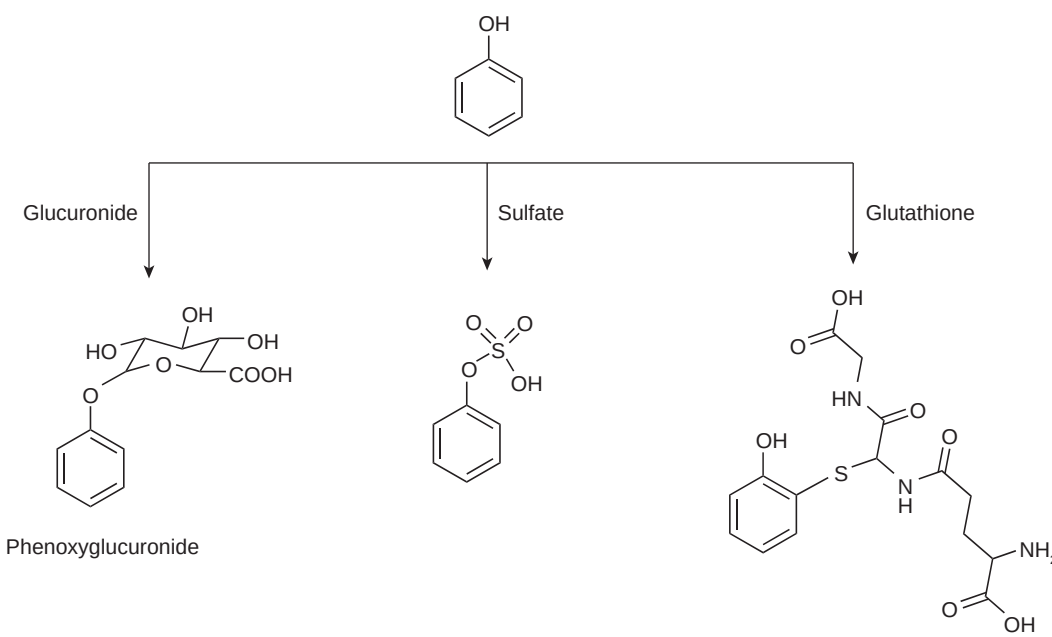


Figure 13.15 Some typical phase II conjugation reactions.

13.3 Examples of Phase I and II Drug Metabolism

Figures 13.16 to 13.20 describe summary of phase I and II reactions along with metabolism for some drugs.

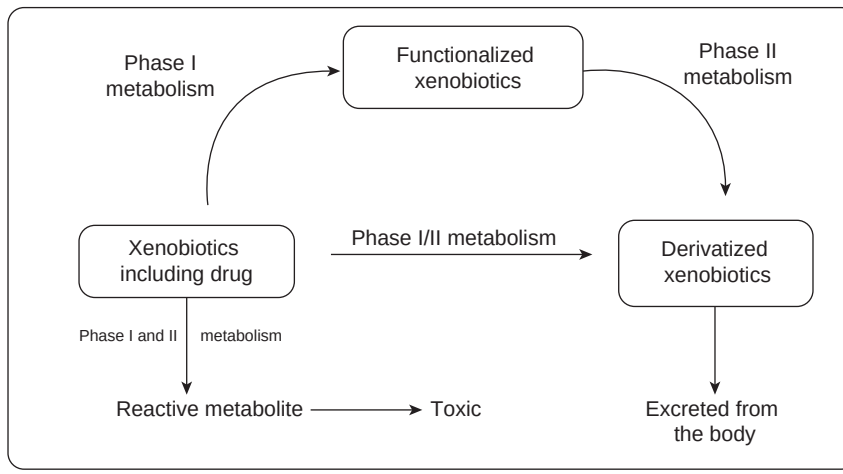


Figure 13.16 A summary of phase I and phase II metabolism of xenobiotics.

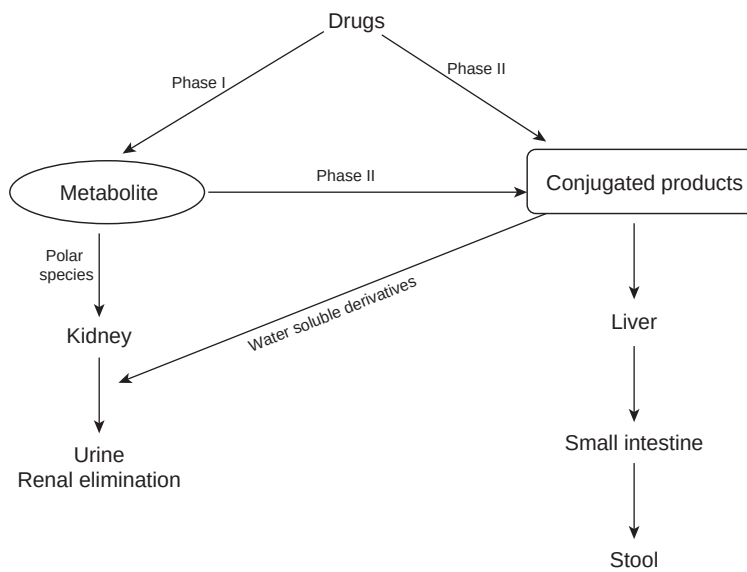


Figure 13.17 A diagrammatic representation of metabolism and excretion.

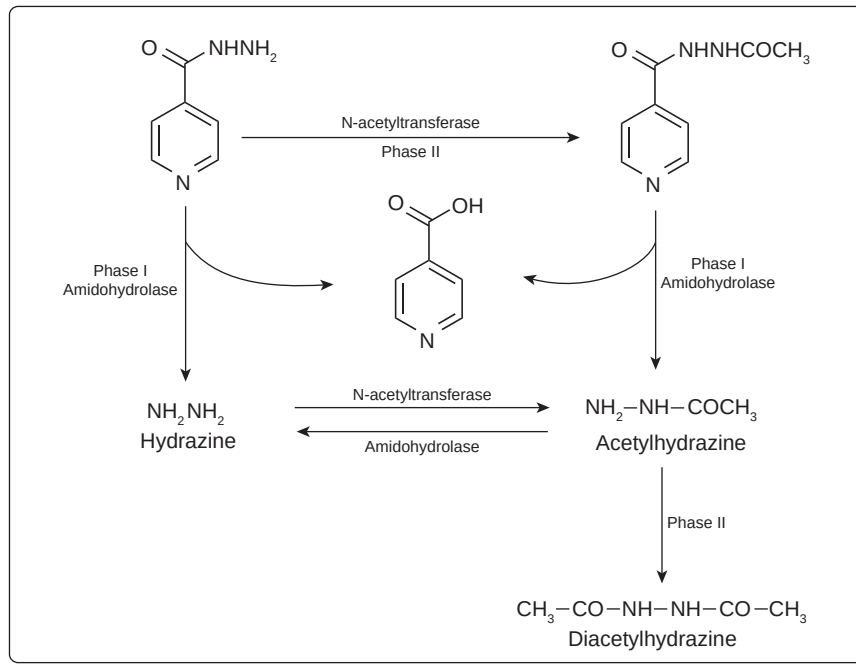


Figure 13.18 Metabolism of isoniazid.

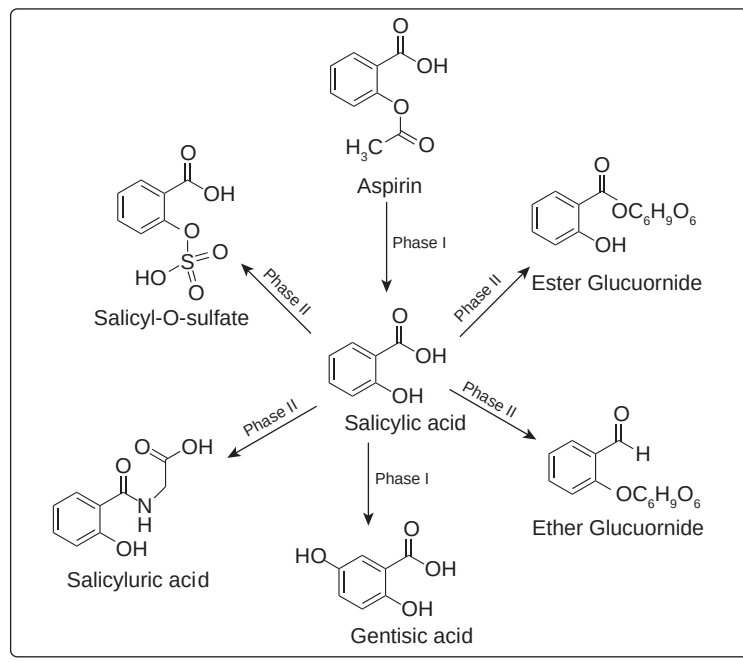


Figure 13.19 Metabolism (Biotransformation) of aspirin.

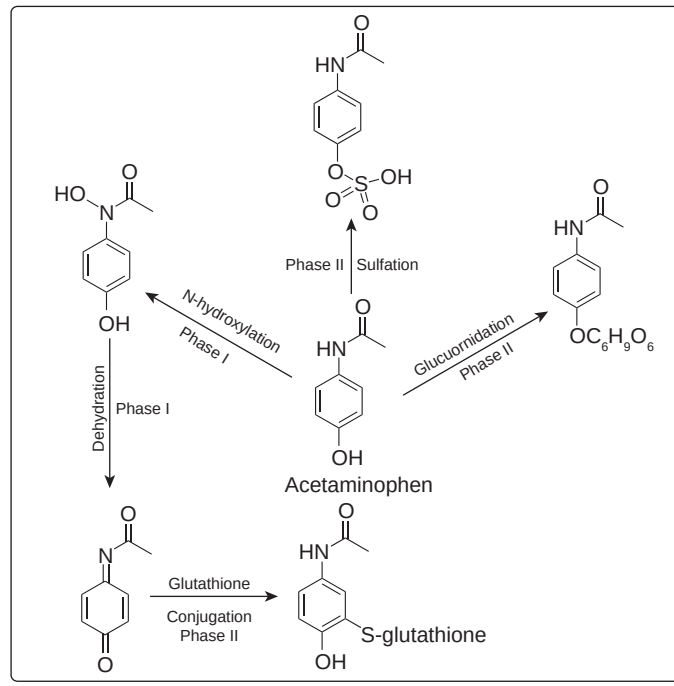


Figure 13.20 Metabolism (biotransformation) of acetaminophen.

13.4 Cytochrome P450 Enzyme Induction and Inhibition

There are two kinds of interactions between substances and CYPs that can lead to either induction or inhibition of CYP (Fig. 13.21).

13.4.1 Enzyme Induction

There are some substances that can lead to an increase in the production of the metabolic enzyme (CYP) and as a result speed up the metabolism (oxidation) and clearance of the drug, and hence a higher dose of the drug may be required to achieve the desired therapeutic effect.

13.4.2 Enzyme Inhibition

Some substances may inhibit the specific hepatic isozyme (CYP) and as a result may lead to an increase (accumulation) in the concentration of the substances in the body. This in turn may lead to toxicity due to prolonged exposure of the body to the substance.

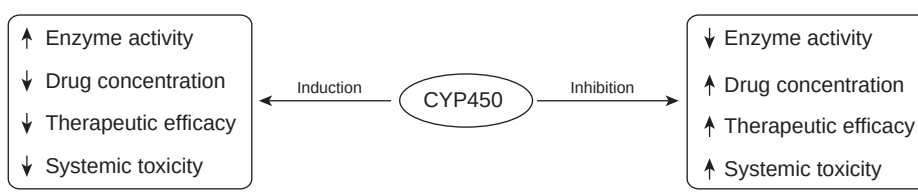


Figure 13.21 Induction and inhibition by CYP may lead to drug-drug interaction.

CASE STUDY 1

Bilirubin Clearance and CYP Enzyme Induction

Bilirubin (BR) is an end product of heme degradation and plays a role of oxidant protecting cells from oxidative free radicals. However, when the intracellular concentration of BR rises above threshold value, it becomes toxic for the body and is therefore needs to be cleared from the body. The elimination involves hydroxylation of BR by phase I metabolism followed by derivatization of the hydroxyl group with glucuronic acid by phase II conjugation. Any impairment in this physiological process such as reduction in the clearance of BR by phase I and II metabolism leads to neonatal jaundice. The endogenous metabolite BR may induce several subtypes of CYP enzymes and in turn remove BR from blood serum by enhancing biliary excretion of hydroxylated BR (Fig. 13.22).

In a newly born babies, infant jaundice is highly prevalent since the liver in babies is not matured enough to eliminate bilirubin from the bloodstream. This can be treated by phototherapy which through photo-oxidation adds oxygen to the bilirubin and results in a water-soluble oxidized product.

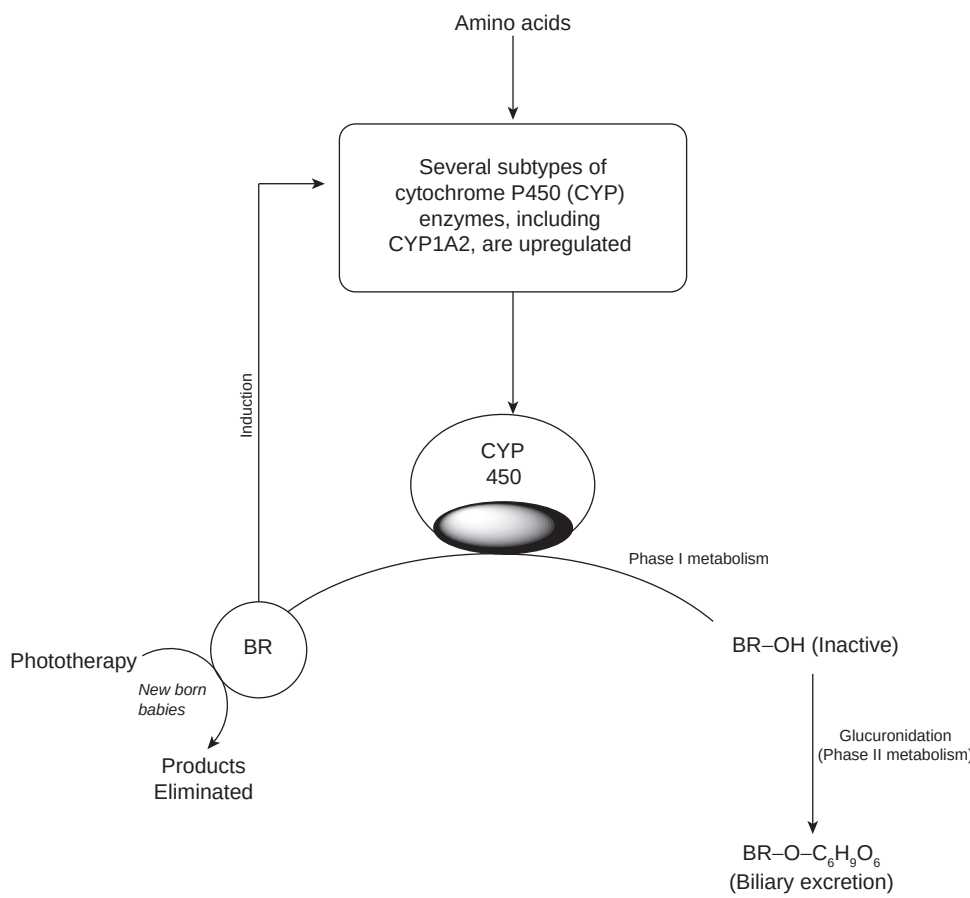


Figure 13.22 Clearance of bilirubin (endogenous substance) via cytochrome induction.

13.5 CYP Enzyme Induction by Exogenous Substances (Pharmacokinetic Drug-Drug Interactions)

Xenobiotics including drugs and environmental pollutants can lead to induction of the metabolic enzyme CYP, which can have serious consequences. Xenobiotic that triggers its own metabolism via CYP induction is called *autoinduction* (e.g. carbamazepine) and in case the CYP induction is caused by other compounds, it is referred to as *foreign induction*. Consumption of medicines with some foods may also affect efficacy of a drug. Solid and liquid food such as olive oil, kava, wine, beer, black tea, etc., may modulate CYP activity and in turn can affect drug metabolism.

1. **Scenario 1:** Drugs causing CYP induction may result in enhanced concentration of CYP, which in turn will facilitate rapid elimination of the drug from the body. This, in turn, would decrease the efficacy of the drug and reduce its duration of action.
   (a) Example 1: Phenobarbitone (phenobarbital; a CNS depressant/anticonvulsant), not only induces the metabolism of itself, but also metabolizes other drug such as phenytoin (anticonvulsant), warfarin (oral anticoagulant), etc.
   (b) Example 2: Rifampicin, an antitubercular drug, when given to patients already on cyclosporine (to prevent rejection of their transplanted organs) causes acute rejection of the transplant. This has been attributed to the CYP induction by rifampicin, which in turn induces rapid metabolism of cyclosporine.
2. **Scenario 2:** Sometimes, an induction of CYP 450 may enhance the concentration of reactive metabolites, which in turn could lead to enhanced toxicity by the metabolites. For example, CYP induction by rifampicin increases the biotransformation of isoniazid, an antitubercular drug, into isonicotinic acid and hydrazine, and both are toxic to liver.

13.5.1 Physiological Consequences of CYP Enzyme Induction

1. Drugs may not be able to elicit their therapeutic response due to their rapid metabolism and excretion.
2. In situations where metabolite of drug is responsible for biological activity, the activity is expected to be enhanced.
3. Enhanced metabolism of endogenous substances may cause severe pathophysiological conditions.

13.6 Pharmacodynamic Drug-Drug Interactions

It deals withthe drug-drug interaction (DDI)[4] in which one drug can either potentiate (via synergistic/additive route) or antagonize the therapeutic effect of the other drug.[5] In contrast, pharmacokinetic drug-drug interaction deals with effect on ADMET parameters of the drugs (life span of drug in the body) caused by CYP induction or inhibition by either of the drugs during metabolism.

The pharmacodynamic DDI particularly is of great importance when elderly patients in sixty plus age groups on an average consume 3–4 drugs and 2–3 supplements on daily basis. As a consequence, there can be an increased risk of interactions among drugs and supplements resulting in either serious side effects or may be even reducing the therapeutic efficacy of drugs. Although drug-drug interactions can prevail at any age, but the risk becomes more pronounced with age.

Several fluoroquinolines due to their association with QT prolongation resulting in cardiotoxicity were withdrawn as their use in elderly patients with cardiac problems can cause cardiac arrhythmias either when consumed alone or with other antiarrhythmic/hERG inhibitory drugs. Besides, inagiven scenario, a combination of erythromycin and fluoroquinolines can be fatal.

Similarly, a combination of ACE inhibitor and amiloride, potassium sparing diuretics, in diabetic patients led to life-threatening hyperkalaemia where potassium level went beyond normal level.

Patients suffering from coronary heart disease and consuming aspirin as prophylactic are advised not to take NSAID drugs ibuprofen or naproxen as these will trigger cardiac issues.

1. Co-administration of NSAIDs can have increased risk of gastric bleeding due to increased inhibition of COX-1.
2. Co-administration of acetaminophen with reduced dose of NSAID for patients who are dependent on NSAID can have synergistic effect in the management of pain.
3. Co-administration of citalopram, a serotonin uptake inhibitor with NSAIDs can cause impairment in the functioning of platelet and enhance the risk of bleeding.

Combination therapy has been a story of immense success in the highly active antiretroviral therapy (HAART) for treating AIDS. There are six categories of drugs that attack different stages in HIV cycle. The regimen comprises at least three drugs administered as cocktail: two of them are nucleoside reverse-transcriptase inhibitors (NRTIs) as a 'backbone' along with one non-nucleoside reverse-transcriptase inhibitor (NNRTI) such as inhibitors of integrase/protease. This kind of success involves extensive pharmacodynamic (PD) interaction (DDI) studies with drug combinations in order to establish optimal dosing for each drug required in the 2/3-drug regimen for maximal response.

Thus, PD interaction studies deal with the design of sequential and combination mechanism-based protocols including variations in doses to examine the additive/synergistic outcome on efficacy. In fact, a combination of drug metabolism and pharmacokinetics (DMPK) with PD DDI studies should be the integral part of drug discovery programme.

13.7 Drug-Design Approach for Making Drugs More Resistant to CYP Metabolism

The phenomenon of metabolism (M) is one of the physiological barriers faced by the drug before it can elicit its biological response. The CYP-mediated metabolism of drug may be rapid or slow, which may be due to numerous physiological factors. Among non-physiological factors, physicochemical properties coupled with structural features of test compounds/drugs can play a significant role in regulating its metabolism. Numerous methods can be applied to improve sustainability of drugs against CYP metabolism. Yet the question always remains, as to what extent one can make the drug either resistant or susceptible to CYP-mediated metabolism. Too much resistant may lead to toxicity and too much susceptible to metabolism may decrease its efficacy. The strategies used for decreasing CYP-mediated drug metabolism have been discussed in Chapter 5 under Section 5.11. Some of the widely used methods for minimizing CYP-mediated metabolism have been summarized below:

1. Metabolic drug stability by introducing steric shields.
2. Modulating electronic effects by introducing bioisosteres.
3. Introducing stereoelectronic modifications in test compounds.
4. Introducing metabolic blockers.

5. Removal of susceptible metabolic group(s).
6. Providing electronic shielding to NH_2 groups.
7. Introducing ring variation.

13.8 Examples of Other Non-Microsomal Metabolisms

Besides phase II conjugation reactions, non-microsomal enzymes also catalyze reactions involving reduction, oxidation, and hydrolysis.

1. **Alcohol Dehydrogenase (ADH) in Non-microsomal Phase I Reaction:** They are group of metalloenzymes located in the cytoplasm of gut and hepatic cells. They catalyze dehydrogenation (oxidation) of alcohols to aldehydes/ketones and are accompanied by reduction of cofactor NAD^+ to NADH (Fig. 13.23). Cimetidine, used for treating gastroesophageal reflux disease, is a non-competitive inhibitor of gastric ADH *in vitro*.
2. **Aldehyde Dehydrogenase (ALDH) in Non-microsomal Phase I Reaction:** They are group of $NAD^+/NADP^+$ (as cofactor) dependent enzymes predominantly located in liver and in many other tissues in the body. Two major isoforms in liver cells are ALDH1 and 2 located in cytoplasm and mitochondria. They play an important role in the removal of electrophilic toxic aldehydes by biotransforming (metabolism) them into non-toxic acids (Fig. 13.24).
3. **Xanthine Oxidase:** The reactive oxygen-producing enzyme is located in blood plasma and lungs. The level of this enzyme is enhanced during damage of liver or infection caused by influenza A virus. The enzyme catalyzes oxidative conversion of hypoxanthine to xanthine to uric acid. High level of uric acid causes gout, arthritis disease.

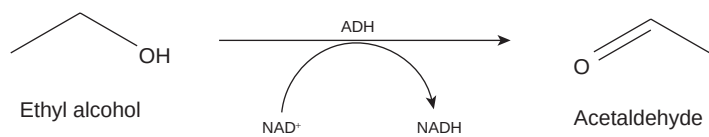


Figure 13.23 ADH-catalyzed reduction of alcohol.

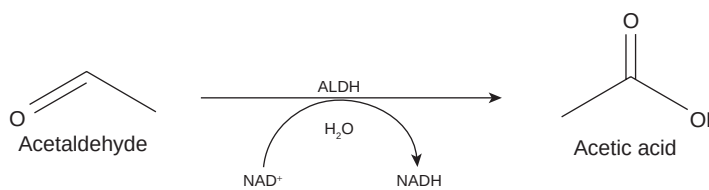


Figure 13.24 ALDH-catalyzed oxidation of alcohol.

Exercises

1. Describe role of enzymes in drug metabolism.
2. Discuss role of phase I and II metabolism in drug metabolism and excretion.
3. Discuss pharmacodynamic drug-drug interaction.
4. Discuss pharmacokinetic drug-drug interaction.

5. Describe bilirubin clearance.

6. Write the products formed following metabolism of aspirin or isoniazid.

7. Name advanced *in vitro* assays for pharmacokinetic properties.

8. Discuss consequences of cytochrome P450 enzyme induction and inhibition.

9. Write short notes on:

(i) Role of NADH

(ii) CYP mediated hydroxylation of arenes

(iii) Glucuronidation

(iv) Minimizing *para* hydroxylation during phase I metabolism in arenes

(v) Nonmicrosomal metabolism

(vi) Microsomal metabolism

(vii) Metabolism of alcohol

(viii) Role of Xanthine oxidase in metabolism

Competitive Exam Questions

1. Many xenobiotics are oxidized by cytochrome P450 in order to

(a) increase their biological activity

(b) increase their disposition in lipophilic compartments of the body

(c) increase their aqueous solubility

(d) all of the above. **[*GPAT 2010*]**

2. Which of the following is a characteristic of cytochrome P450?

(a) Located in the lipophilic environment of mitochondria

(b) Catalyzes aromatic and aliphatic hydroxylations

(c) Catalyzes O–, S–, N-methylation reactions

(d) Catalyzes conjugation reactions **[*GPAT 2017*]**

3. Numerous isomers of human liver P450 enzymes have been identified. It is noteworthy that _____ alone is responsible for the metabolism of over 50% of prescription drugs metabolized by liver.

(a) CYP2B6 **(b)** CYP3A4

(c) CYP1A2 **(d)** CYP1A11

[*GPAT 2019*]

References

1. Janne T. Backman, Anne M. Filppula, Mikko Niemi and Pertti J. Neuvonen, *Pharmacological Reviews*, **2016**, *68*, 168.

2. Tom Lynch, Amy Price, *Am. Fam. Physician.*, **2007**, *76*, 391.

3. Shingo Oda, Tatsuki Fukamib, Tsuyoshi Yokoia and Miki Nakajima, *Drug Metabolism and Pharmacokinetics*, **2015**, *30,* 30.

4. Jin Niu, Robert M. Straubinger and Donald E. Mager, Doi: 10.1002/cpt.1434.

5. Cascorbi I: *Dtsch. Arztebl. Int.*, **2012**, *109*, 546.

CHAPTER

14 Digestive Enzymes

KEY THINGS TO LEARN FROM THIS CHAPTER

- Digestive enzymes: Various parts of digestive system release enzymes and assist in the breakdown of food components: carbohydrates, protein and fats. They are mostly produced in stomach, pancreas and intestine.
- Digestion of proteins (Protein degradation): Exopeptidases and endopeptidases.
  - Zymogens (Proenzymes): Gut secretion pepsinogen and pancreatic zymogens.
  - Protein homeostasis.
- Digestion of lipids: Emulsification and digestion, bile salts, pancreatic lipase, micelles and absorption of cholestrol.
- Digestion of carbohydrates.

14.1 Introduction

Our body derives essential nutrients from food for the maintenance and proper functioning of the physiological processes. The nutrients are derived from carbohydrates, proteins, and fats, as well as vitamins and inorganic components for nutritional balance. Unlike inorganic components which are absorbed as such in blood circulation, carbohydrates, proteins, and fats cannot be absorbed directly as they are either macromolecules, e.g., protein and polysaccharides, or hydrophobic molecules like fats (triglycerides). Therefore, digestive enzymes are present in our gut, which break down the dietary substances into small oligomeric or monomeric components through enzymatic hydrolysis. The latter are membrane-permeable, soluble, low-molecular-weight fragments (organic molecules) (Table 14.1).

Table 14.1 Breakdown of macromolecular dietary components into membrane permeable fragments

Large Dietary Components	Site of Breakdown	Soluble Components
Carbohydrates	Mouth and small intestine	Simple sugars
Proteins	Stomach and small intestine	Amino acids
Lipids (fats)	Small intestine	Fatty acids + glycerol

14.1.1 Release of Digestive Enzymes

Secretion of digestive enzymes occurs either in response to food or even in anticipation and smell of food. It begins from mouth and continues through stomach and intestines. There are three major enzyme classes: carbohydrase, protease, and lipase, specifically involved in the digestion of proteins, carbohydrates, and fats, respectively (Table 14.2). The major sites of secretions of the digestive enzymes are mouth, stomach, pancreas, and brush-border membrane in small intestine. They break down large, insoluble molecules into smaller, soluble fragments so that the latter can be absorbed into the blood circulation.

Table 14.2 Digestive enzymes used in the degradation of three major food components

Digestive Enzymes	Site of Secretion
Carbohydrase (e.g. amylase)	Salivary glands, pancreas, and small intestine
Protease (e.g. pepsin)	Stomach, pancreas, and small intestine
Lipase (e.g. gastric lipase)	Pancreas and small intestine

14.1.1.1 Digestive Enzymes in Stomach

Chief cells in gut release pepsinogen, chymosin, and gastric lipase enzymes. Pepsinogen is activated in response to acidic conditions and catalyses digestion of proteins, whereas gastric lipase catalyses degradation of triglycerides into fatty acids (FAs) and mono or diglycerides.

14.1.1.2 Digestive Enzymes Secreted from Pancreas

The secretory products present in the pancreatic juice are bicarbonate and digestive enzymes. While the bicarbonate is secreted from the epithelial cell linings in the pancreatic duct, digestive enzymes are released form exocrine acinar cells.

14.1.1.3 Brush-Border Enzymes

The intestinal digestive enzymes are called brush-border enzymes (Fig. 14.1). Unlike digestive enzymes in stomach, brush-border enzymes,[1] instead of getting released, remain anchored to the plasma membranes. However, their catalytic site remains exposed to chyme for binding with carbohydrate/protein substrates. Examples of brush-border enzymes for digestion of carbohydrates are dextrinase, glucoamylase, maltase, and sucrose. Lactase is absent in most adult humans and for them lactose (like most polysaccharides) is not digested in the small intestine (Table 14.3).

Table 14.3 Brush border digestive enzymes attached to inner lining in small intestine

Substrate	Digestive Enzymes	Action
Carbohydrates	Lactase, sucrase, glucoamylase	Oligosaccharides to monosaccharides
Proteins	Aminopeptidase, carboxypeptidase, dipeptidase enterokinase	Small polypeptides into amino acids

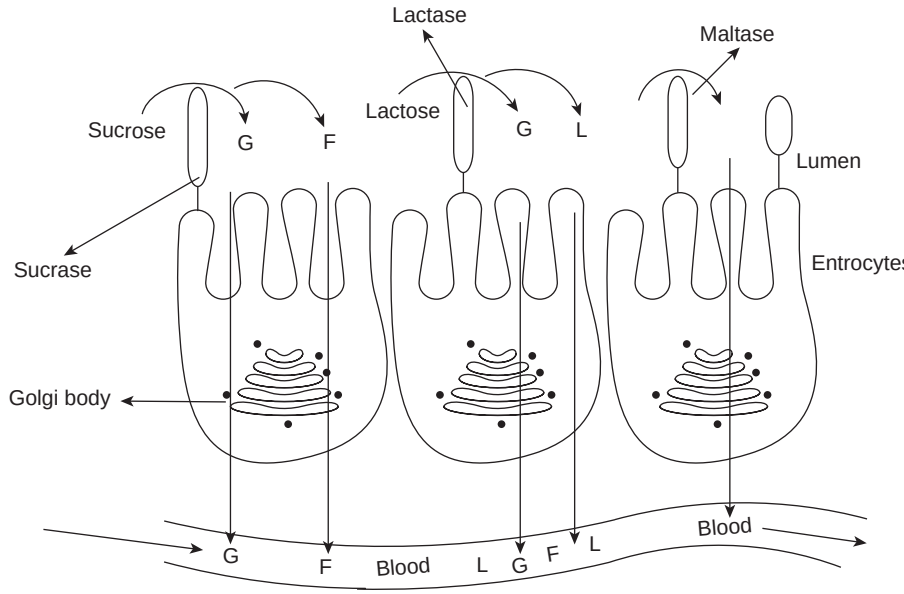


Figure 14.1 Brush-border digestive enzymes in small intestine (G – Glucose; F – Fructose; L – Galactose; P – Protein; AA – Amino acids).

14.2 Digestion of Proteins

In contrast to digestion of carbohydrates that begins in mouth, initiation of protein digestion occurs in the stomach; but major hydrolysis of proteins to amino acids, di- and tripeptides occurs only in intestine from where the degraded constituents are readily absorbed through intestinal wall into blood.

14.2.1 Proteases

Protein degradation is carried out mainly by proteolytic enzymes also called proteases[2] or proteinases or peptidases. These enzymes catalyse the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis. Digestive enzymes catalyse the hydrolysis of –CO–NH– (peptide) bond (Fig. 14.2).

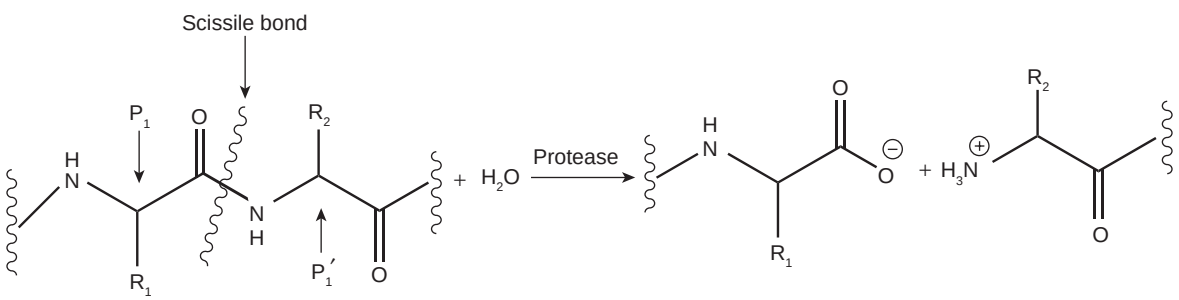


Figure 14.2 Hydrolysis of proteins into peptide fragments by proteases.

A scissile bond is a covalent chemical bond that can be broken by an enzyme, and the residues on either side of bond are called P_1 and P_1'. The residue with the carbonyl group in the scissile bond is P_1 and residue with NH group is P_1'.

14.2.1.1 Exopeptidases

These enzymes have the ability to hydrolyse single amino acid at a time either from N- or C-terminal regions of proteins. In fact, one amino acid is cleaved off at a time either from N- or C-terminal side. Exopeptidases are divided into carboxypeptidases and aminopeptidases.

Aminopeptidases

They selectively catalyse the hydrolysis of N-terminal amino acid form of proteins or peptide chains (Fig. 14.3). They are widely distributed both among plant and animal kingdoms, either present in cytosolic organelles or as brush-border enzymes. They are also secreted in the pancreatic juice. Many, but not all, of these peptidases are zinc metalloenzymes (Zn as cofactor).

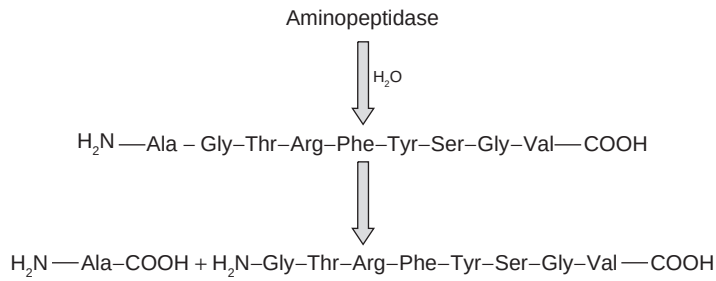


Figure 14.3 Removal of N-terminal amino acid in a model peptide.

Carboxypeptidases

These enzymes are produced in the pancreas and secreted into the small intestine. It is also present in the small intestine as brush-border digestive enzyme (Fig. 14.1).

It removes a C-terminal amino acid at a time from proteins and peptides. It has a stronger preference for hydrophobic amino acids with either aromatic ring or branched hydrocarbon chains as their side chain (Fig. 14.4). The enzyme contains zinc (II) ions as metal ion cofactor, which is at the heart of the active site. The major exopeptidases present in the pancreatic juice are carboxypeptidase A and B.

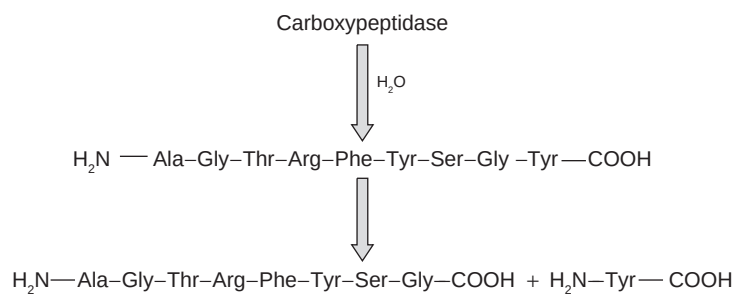


Figure 14.4 Cleavage of C-terminal amino acid residue by carboxypeptidase.

14.2.1.2 Endopeptidases

These are enzymes that catalyse the cleavage of peptide bonds within a polypeptide or protein. They are usually very specific for certain amino acids (Fig. 14.5).

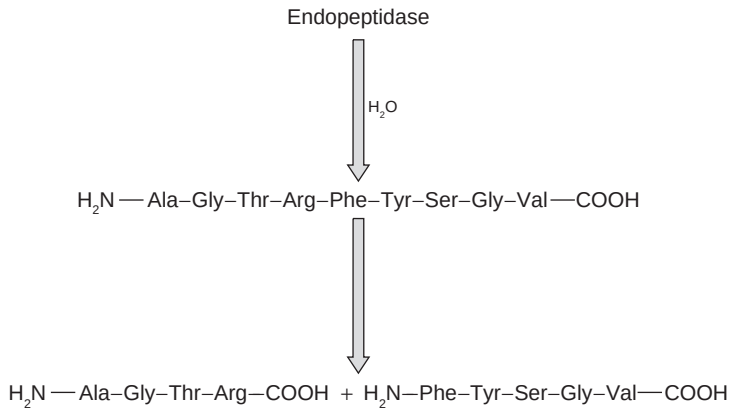


Figure 14.5 Cleavage of peptide bonds within a model polypeptide.

Endopeptidases in Stomach

The first stage of protein digestion that occurs in the stomach involves an endopeptidase called pepsin (only protein-digesting enzyme in the stomach). It is a potent enzyme in the gastric juice that digests almost all of the structural proteins such as those in the meat, eggs, seeds, or dairy products into soluble smaller peptides. The enzyme is secreted from the mucosal lining of the stomach as its inactive precursor pepsinogen. Under acidic conditions, pepsinogen is converted into pepsin. It particularly cleaves Tyr/Phe/Trp-amino acid amide bonds. It functions only under acidic conditions, as proteins present in the food are denatured under acidic pH, thereby exposing the amide bonds for proteolytic hydrolysis by pepsin. The catalytic hydrolysis of diet proteins initiated in stomach continues till it reaches the small intestine from where smaller peptides are absorbed in the blood circulation. Besides pepsin, other two endopeptidases are chymotrypsin and trypsin.

Endopeptidases in Pancreas/Small Intestine

Pancreatic juice secreted by the pancreas in small intestine contains a variety of enzymes, including trypsin, chymotrypsin, elastase, carboxypeptidase, pancreatic lipase, nucleases, and amylase. Out of this trypsin, chymotrypsin, and elastase are endopeptidase and are together classified under chymotrypsin family. Although hundreds of endopeptidases are known, they all catalyse the hydrolysis of amide bond in proteins internally but differ in specificity and catalytic action.

Classification of Endopeptidases

The three major criteria used for the classification of the peptidases are:

1. Prototype of the reaction catalysed.
2. Presence of nucleophilic amino acids in the catalytic site.
3. The evolutionary relationship, as revealed by structure.

Proteases can be classified into six broad groups (Table 14.4):

Table 14.4 Proteases can be classified into six broad groups

Catalytic Classes	Nucleophile	Acyl-enzyme Intermediate	Catalytic Site
Serine proteases	His activated hydroxyl of Ser	Yes	Ser, His, Asp; as catalytic triad
Cysteine proteases	His activated thiol of Cys	Yes	Cys, His, Asp as catalytic triad or Cys, His as catalytic dyad
Threonine proteases	Amine activated secondary hydroxyl group of Thr	Yes	A conserved N-terminal Thr bonded at the active site is a single active site residue.
Aspartic proteases	Aspartate activated water molecule	No	Asp, Asp as catalytic dyad
Glutamic proteases	Glutamate activated bound water molecule	No	Glutamic acid and glutamine as catalytic dyad
Metallo proteases	Metal activated water molecule	No	Zn^{2+} coordinated to 2 × His

Serine proteases

These enzymes have Ser amino acid in the catalytic triad that serves as a nucleophile and hydrolyse the amide bond in proteins.[3] Over one third of all known proteolytic enzymes are serine proteases that belong to the family of endopeptidases. They are involved in the degradation of proteins present in the extracellular matrix (ECM) such as collagen, elastin, etc. They are also known to exert their effect through intracellular transduction of signal produced by the binding of messengers to G-protein coupled receptors (GPCR) located on surface of the membrane. The two prototypes of serine proteases are discussed below.

1. **Trypsin-like Serine Proteases:** These enzymes have Ser amino acid in the catalytic triad that serves as a nucleophile and hydrolyses the amide bond in proteins.
   - **(i)** Trypsin: Cleaves Arg/Lys-amino acid bonds
   - **(ii)** Chymotrypsin: Cleaves Tyr/Phe/Trp-amino acid bonds
   - **(iii)** Elastase: Cleaves Ala/Gly/Ser-amino acid bonds
2. **Subtilisin-like Serine Proteases:** These have been isolated from *Bacillus subtilis* and do not bear any structural analogy with chymotrypsin except for the presence of same catalytic triad.

Catalytic Triad in Serine Proteases: Serine proteases have independently evolved and carry a similar catalytic site characterized by the presence of amino acids Ser, His, Asp triad (Fig. 14.6):

1. Ser195, His57, Asp102 in chymotrypsin, trypsin, and elastase
2. Ser221, His64, Asp32 in the bacterial subtilisin

Besides their role as digestive enzymes, several activated clotting factors also belong to the family of serine proteases, and they too carry the same catalytic triad Ser195, His57, Asp102. Examples are factors X, XI, and XII, thrombin and plasmin.

In the catalytic triad, the hydroxyl group from Ser195 is H-bonded to the tautomeric nitrogen in the imidazole ring of His57, which is further H-bonded via the secondary NH in the imidazole ring to the carboxyl group of Asp102. The hydrolysis of amide substrates begins with the nucleophilic attack by Ser oxygen at 195.

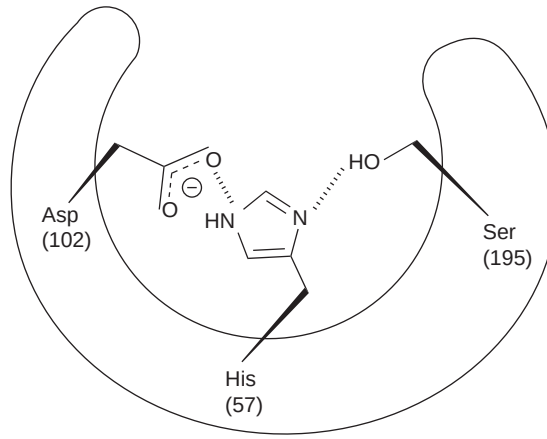


Figure 14.6 Catalytic triad in (endopeptidases) serine proteases.

CASE STUDY 1

Chymotrypsin

Chymotrypsin is a serine peptidase produced by the canard cells of the pancreas. It cleaves the amide bond on the C-terminal side of Phe (F)/Trp (W)/Tyr (Y) in any substrate (both natural and synthetic). It shows specificity only for aromatic amino acids because of its hydrophobic pocket. At the active site of chymotrypsin, the substrate (proteins undergoing digestion) is hydrolysed by the catalytic triad Ser195, His57, and Asp102. The nucleophilic Ser195 located in the catalytic site of the enzyme attacks the unreactive carbonyl group of the amide bond following F/W/Y, which temporarily becomes covalently bonded to the substrate, thereby forming a tetrahedral enzyme–substrate complex (Fig. 14.7).

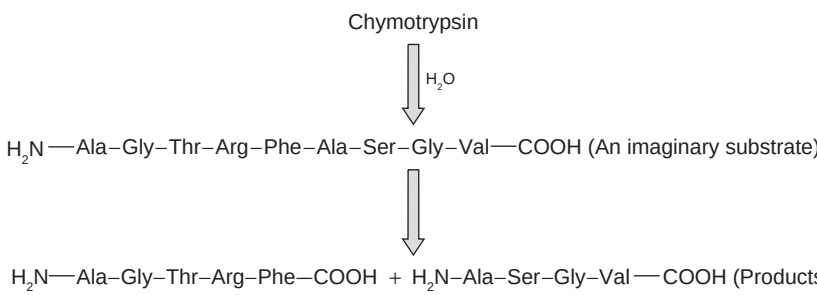
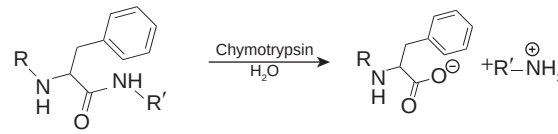
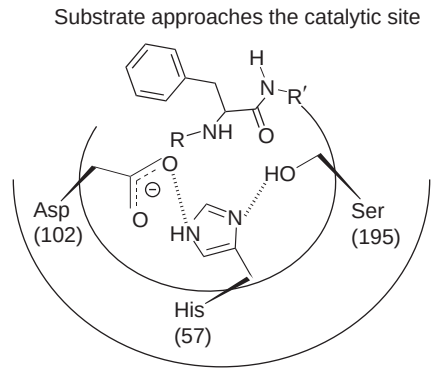


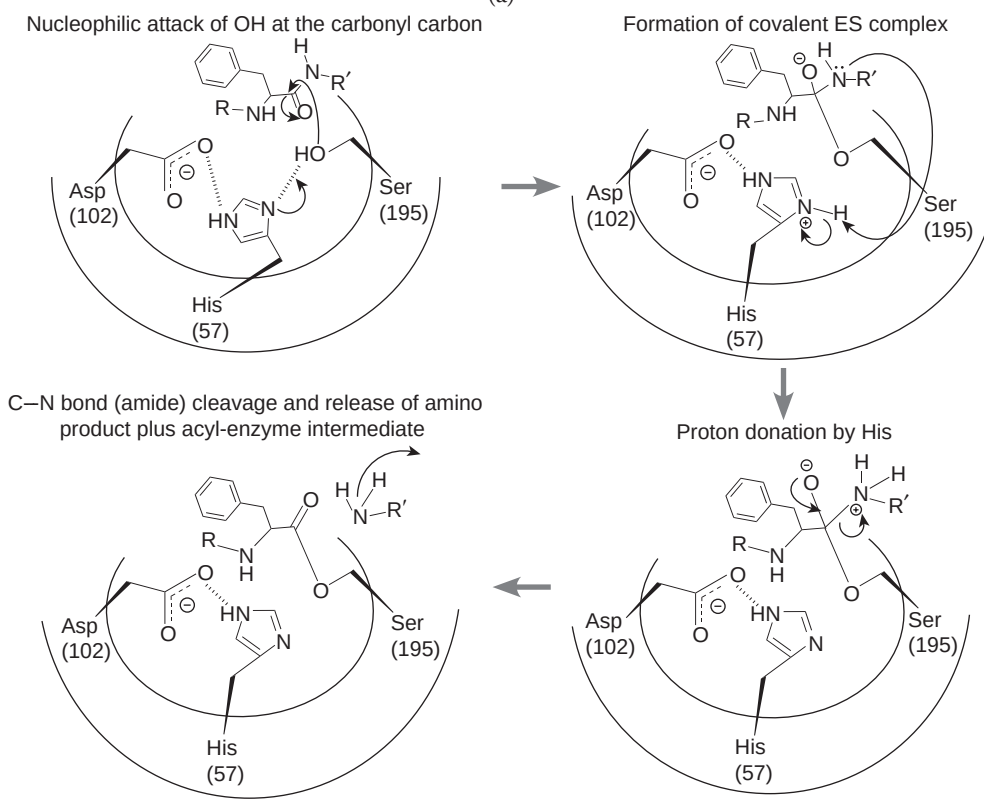
Figure 14.7 Hydrolysis of a model peptide by chymotrypsin.

A mechanism for the hydrolysis of a substrate represented by R–NH–CH(CH_2Ph)–CO–NH–R has been depicted in Fig. 14.8. At the catalytic site, the hydrolysis proceeds with nucleophilic attack of serine oxygen onto the carbonyl carbon of the peptide bond.

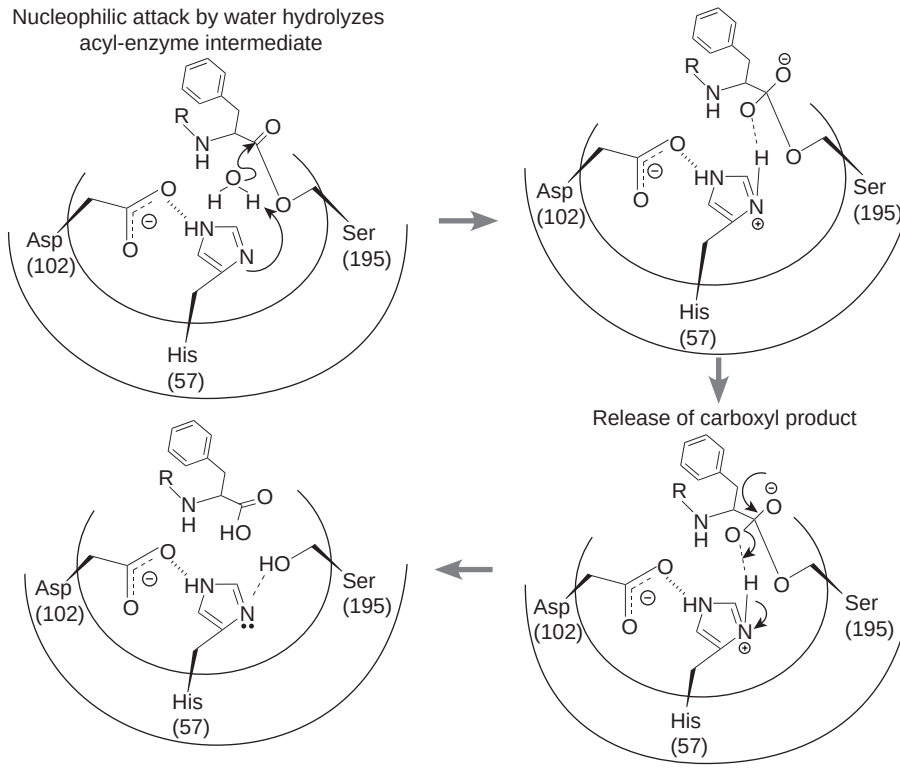




(a)



(b)



(c)

Figure 14.8 Mechanism for the hydrolysis of a model substrate by serine proteases.

Cysteine proteases

These enzymes have Cys amino acid in the catalytic sites that serve as a nucleophile and hydrolyse the amide bond in proteins.[4] They can be found in both plants (papain from papaya) as well as in mammals (calpains). They carry His and Cys in their catalytic site and hydrolyse amide substrate by initiating deprotonation of the thiol group in the side chain of Cys by the basic moiety imidazole present in His of the enzyme. The resulting nucleophile following deprotonation of thiol attacks the carbon of the carbonyl group in the amide bond (substrate) to induce hydrolysis (Fig. 14.9).

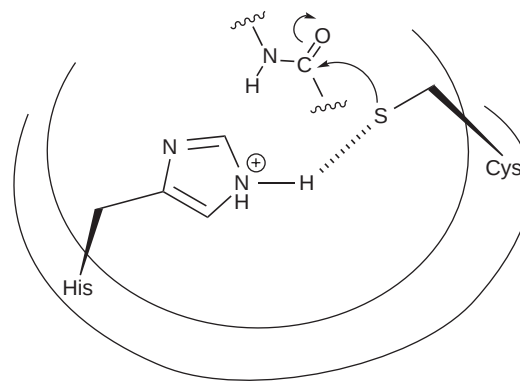


Figure 14.9 Catalytic site in cysteine protease.

Aspartic protease

These are a group of proteolytic enzymes which carry two Asp residues in their catalytic site (Fig. 14.10).[5] They are found in stomach, lysosomes, and kidney and are inhibited by a hexapeptide isolated from the cultures of actinomyces called pepstatin. The enzyme functions under acidic conditions and uses active site water molecules to hydrolyse the amide bond in peptide substrates. The hydrolysis is a one step catalytic process and does not involve the formation of acyl-enzyme intermediate.

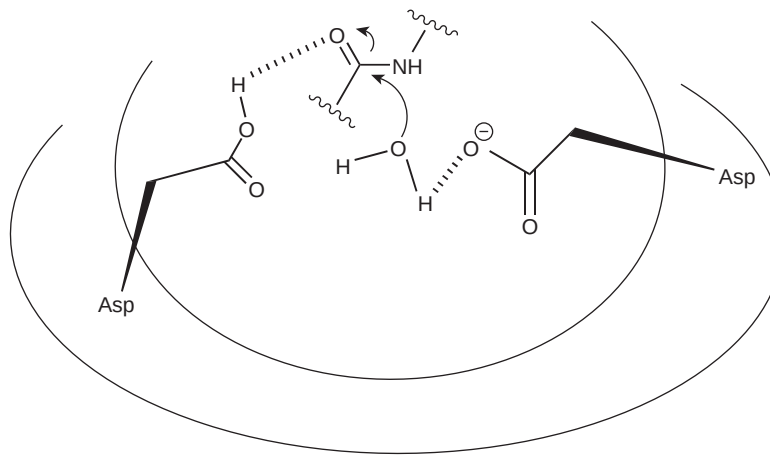


Figure 14.10 Catalytic site in aspartyl proteases.

Threonine proteases

Threonine proteases have the conserved amino acid threonine at the N-terminal in the active site. The proteases are predominantly located in the cytosolic organelle proteasomes that is largely responsible for the degradation of proteins (Discussed chapter 1 under Section 1.11.4). The secondary alcoholic group of the N-terminal threonine acts as a nucleophile and attacks the carbonyl group of the amide bond in the target proteins. The presence of N-terminal amine in the threonine acts as a general base and facilitates polarization of water molecules that deprotonates the secondary alcohol and increases its nucleophilicity. The catalytic action following nucleophilic attack on the substrate results in a covalent acyl-enzyme intermediate and release of the first product (amine component) of the protein hydrolysis. Next the acyl–enzyme intermediate undergoes hydrolysis to regenerate the enzyme and second part of the protein hydrolysis product.

Glutamic proteases

The group of proteolytic enzymes carry amino acid residue glutamic acid in their actuve site. The catalytic hydrolysis of protein substrate involves protonation of carbonyl oxygen in the amide bond by glutamic acid. This is followed by nucelophilic attack by the hydroxyl group supplied by one or two water molecules. Next protonation of nitrogen in the amide results in the cleavage of the amide bond. The neighbouring amino acid glutamine then allows the glutamic acid to return its native state. The hydrolysis is a one step catalytic process and does not involve the formation of acyl-enzyme intermediate.

Metalloproteases

These proteolytic enzymes are characterized by the presence of a metal ion in their catalytic site.[6] These metal ions are generally complexed to a water molecule required for the hydrolysis of the amide bonds in protein substrates. Generally, proteases carry out their function through Zn^{2+} ion but in few cases Co^{2+} and Mn^{2+} ions have been found to catalyze the action of enzymes. The metal ions in these proteases are present in a tetrahedral arrangements bound to three charged amino acids and one water molecule. Various metalloproteases depending on their specificity for the hydrolysis of proteins have been grouped under metalloendopeptidases and metalloexopeptidases. Thermolysisn is an example of metalloendopeptidase and it hydrolyzes internal amide bonds involving amino acid-Leu/Ile-Phe bonds. During catalytic hydrolysis, Zn^{2+} assumes a pentacoordinate geometry from tetrahedral format involving three amino acid residues: His142, His146, and Glu166, the oxygen of the nucleophilic water, and the carbonyl oxygen of the amide bond in protein substrate. Similarly, carboxypeptidase is an example of metalloendopeptidase containing Zn^{2+} ion as cofactor in their catalytic site. The ion is bound to His69, Glu72, and His196 and is additionally bound by a water molecule involved in catalytic hydrolysis. Zn^{2+} is stabilized within the active site through interactions with His69, Glu72, and His196 and is additionally bound by a catalytic water molecule that has been shown to interact with Glu270. The hydrolysis is a one step catalytic process and does not involve the formation of acyl-enzyme intermediate.

14.3 Zymogens (Proenzymes)

Digestive enzymes are usually synthesized as larger inactive precursors – zymogens. Otherwise, they would digest tissues that synthesize them. (Acute pancreatitis: Premature activation of digestive enzymes produced by pancreas results in the auto-digestion of pancreas.)

Zymogens are activated by proteolysis in the lumen of the gastrointestinal tract (GIT). Examples of zymogens are as follows:

1. Pepsinogen
2. Trypsinogen
3. Chymotrypsinogen
4. Proelastase
5. Procarboxypeptidases
6. Prophospholipases

14.3.1 Pepsinogen

The chief cells in the gut secrete the inactive zymogen pepsinogen following arrival of food components. Along with this, HCl is also secreted by parietal cells in the lumen. Generally, pepsin, a peptidase is activated in stomach only under acidic environment (pH 2–3) and can be inactivated above pH 5.0. Under acidic conditions, pepsinogen is partially activated, which then activates other pepsinogen into active pepsin by cleaving the N-terminal 44 amino acid precursor chain (Fig. 14.11). The digestion of protein is initiated by pepsin at very low pH (high acidity), which indeed is not a common phenomenon for enzymes.

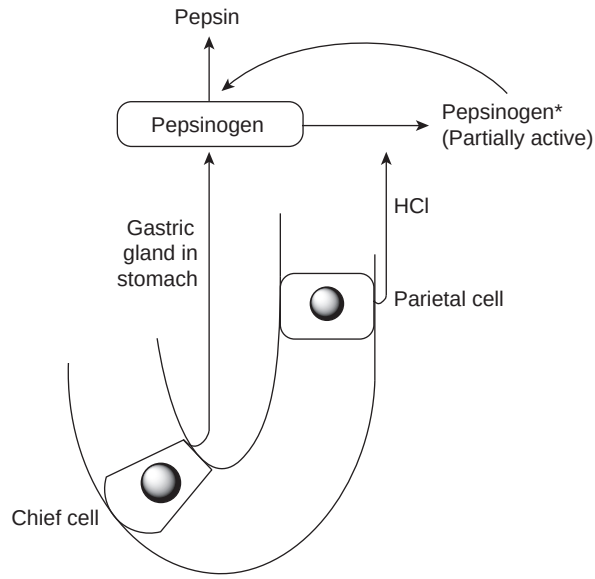


Figure 14.11 Activation of pepsinogen in the stomach.

14.3.2 Pancreatic Zymogens

Pancreas is the major organ that synthesizes the digestive enzymes in its inactive form or precursors (zymogens). They are secreted in the duodenum in response to stimulation by a hormone cholecystokinin (CCK) secreted by duodenum. The arrival of food components in duodenum triggers the cells in the duodenum to release CCK. The latter also triggers gall bladder to squeeze and release its stuff in the duodenum.

Inactive trypsinogen released from pancreas is generally activated in the small intestine by the brush-border enterokinase into the active enzyme trypsin. The latter then activates other pancreatic inactive enzymes and converts them into active enzymes such as chymotrypsin, elastase, etc. (Fig. 14.12). Any impairment in the pancreatic duct prevents flow of these zymogens and can cause pancreatitis.
A window view to overall protein digestion and absorption has been depicted in Fig. 14.13.

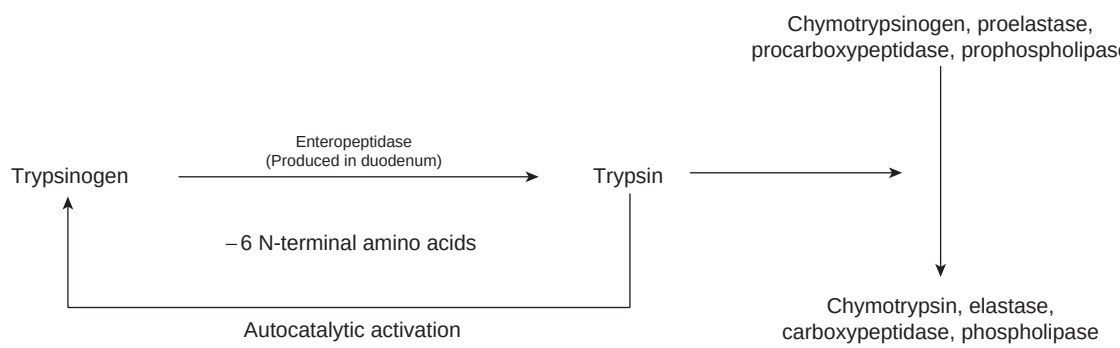


Figure 14.12 Activation of pancreatic zymogens in the lumen of the small intestine.

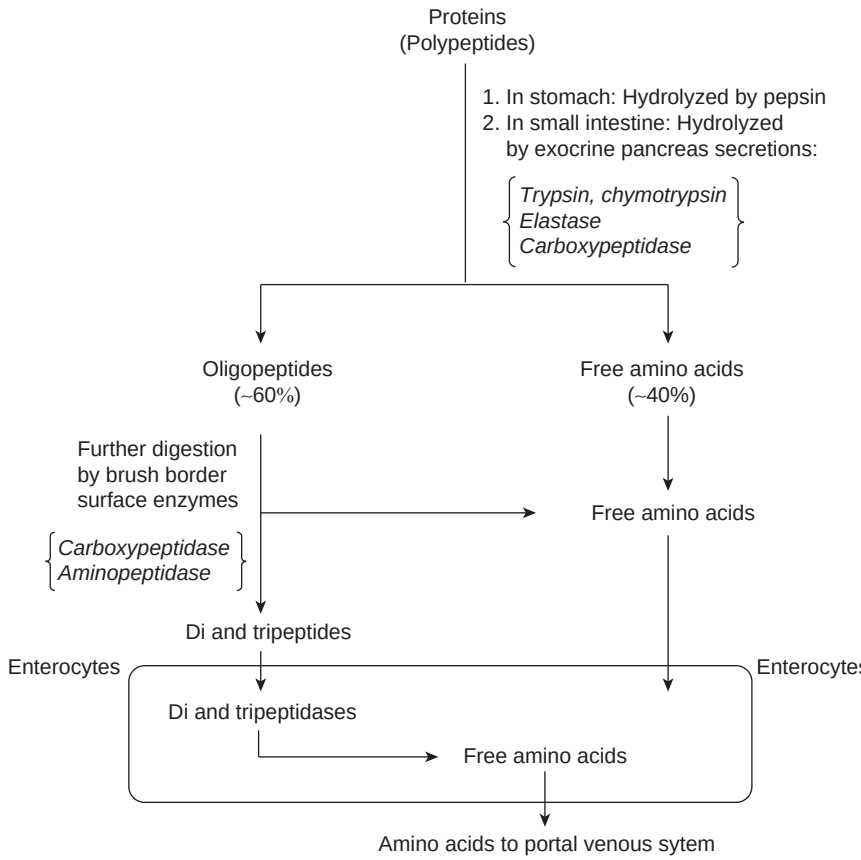


Figure 14.13 A window view to protein digestion and absorption.

14.4 Protein Homeostasis

Proteins are major constituents of the body. They not only play a vital role in the maintenance of body's structure but are also involved in a variety of cellular/physiological functions.[7] Some of the important functions of proteins have been summarized below (Fig. 14.14).

Given the magnitude of the role of proteins in the body in different capacities, proteins are continuously faced with wear and tear resulting in nonfunctional/damaged proteins, both within and outside cells. Under a recycling process, these defunct proteins are digested into their building blocks (amino acids) that are then reused by cells for protein synthesis (Fig. 14.15).

Protein homeostasis (proteostasis) or turnover regulates maintenance of equilibrium between protein biosynthesis and their degradation in the body. The phenomenon is regulated by exogenous and endogenous signals, and impairment in protein turnover has been directly linked to aging. This has been primarily associated with the inability of cells to remove defective proteins, thereby interrupting with the supply of functional proteins. Neurodegenerative diseases such as Alzheimer[8] and Parkinson's,[9] cataract,[10] and malfunction in cardiovascular system[11] are some examples of age-related diseases[12] arising from loss of protein

homeostasis. The degradation of obsolete proteins within cells occurs either via lysosomal or ubiquitin–proteasome pathways; in contrast, biosynthesis of proteins occurs only in ribosomes and are regulated by variety of genes.

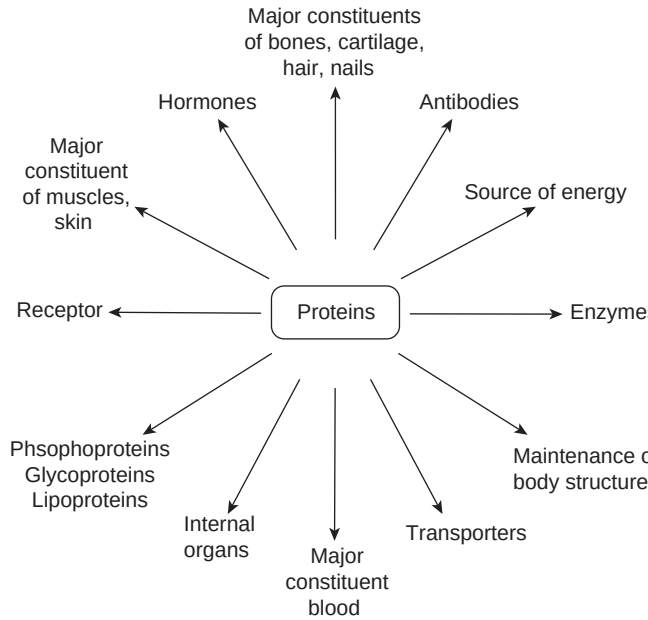


Figure 14.14 Role of proteins in human physiology.

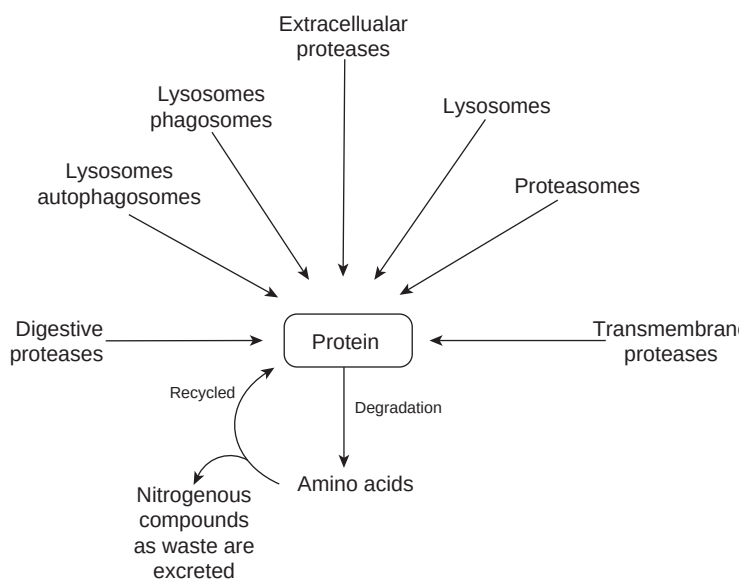


Figure 14.15 Protein homeostasis.

14.5 Digestion of Lipids

The major fat in diet comprises neutral fat or triglyceride. Lipids are hydrophobic and are thus sparingly or not at all soluble in water. This may either lead to poor accessibility of the substrate to the enzyme or initiate aggregation of products of hydrolysis to larger complexes that are hard to absorb. This is overcome by emulsification of fats that generally occurs in the presence of bile salts and pancreatic lipase.

14.5.1 Emulsification and Digestion

The dietary triglycerides, due to its hydrophobicity and insolubility in aqueous environment, undergo absorption by two processes:

1. The aggregates of dietary triglyceride are dispersed into tiny emulsified suspension through motility movement of gut.
2. The triglyceride molecules need to be hydrolysed into their constituent components monoglycerides (MAs) and FAs for further absorption in blood circulation.

Two factors that play an important role in the above two processes are bile acids and pancreatic lipase.

14.5.1.1 Bile Salts

These assist in the digestion of fats in duodenum under alkaline conditions. The major constituents of bile are phospholipids, cholesterol, bile acids, and salts.

14.5.1.2 Pancreatic Lipase

The digestive enzyme pancreatic lipase digests the fat globules by attacking its surface and breaks them into tiny emulsion droplets (Fig. 14.16).

The latter then gets coated with bile salts and phospholipids in order to prevent re-association of droplets. The water-soluble lipase present in these emulsion droplets proceeds with enzyme-catalysed hydrolysis of TAGs into MAs and FAs. Another amphipathic protein colipase also helps in the digestion of TAGs by anchoring to the surface of the droplets (Fig. 14.17).

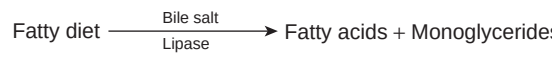


Figure 14.16 Digestion of fatty diet.

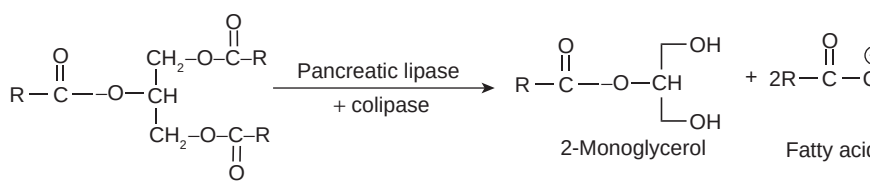


Figure 14.17 Digestion of fatty diet.

14.5.1.3 Micelles

MAs and FAs resulting from the digestion of fatty diet combine with bile salts and phospholipids and are transformed into micelles. The latter are absorbed through the surface of enterocytes, following which MAs and FAs are again reconstituted into triacylglycerol (TAG) that are then packaged into droplet of fats called chylomicrons with the help of proteins, phospholipid, and cholesterol. Finally, chylomicrons are released from the basolateral side of the enterocyte into lymph. The reconstituted TAGs in chylomicrons are again hydrolysed into MAs and FAs in the presence of lipoprotein lipase located in the endothelial cells (Chapter 3; Fig. 3.8) and is available for diffusion into tissues. Thus, one strategy to prevent absorption of fat from diet is to target the enzyme lipase.

14.5.1.4 Absorption of Cholesterol

It has great clinical consequences as increased concentration of cholesterol in blood circulation following intestinal absorption can increase chances of heart attack or stroke. Generally, in body, there are two sources for cholesterol, one from the dietary source and second is liver, which releases cholesterol though bile in the intestine. Fortunately, due to nature's own defence mechanism, out of the total cholesterol that reaches intestine from two different sources, 50% is excreted through stool and remaining 50% is absorbed through intestinal walls into portal vein that takes it to the liver. In fact, release of cholesterol through bile is part of biliary secretion for hydrophobic substances for excretion via stool (Fig. 14.18).

The drug ezetimibe binds and blocks the transporter protein in the intestinal cells that is responsible for the absorption of cholesterol through the lining of the intestine wall. When the drug is co-administered with statins (that interfere with cholesterol biosynthesis), a significant reduction in the levels of LDL cholesterol occurs, thereby reducing the risk of stroke and heart attack (Fig. 14.19).

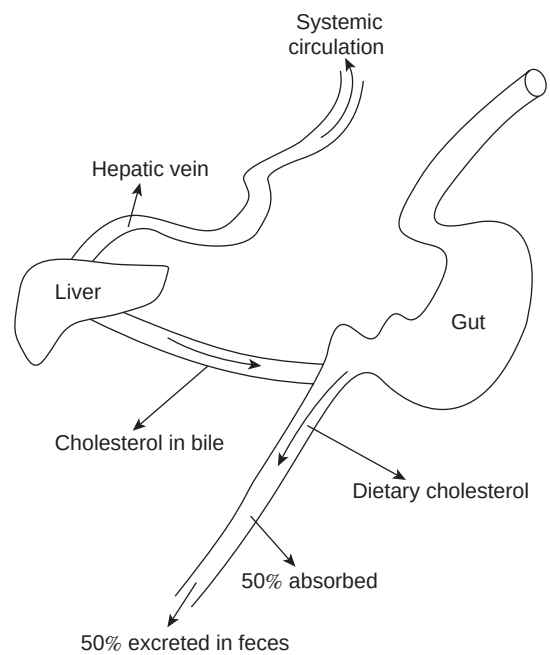
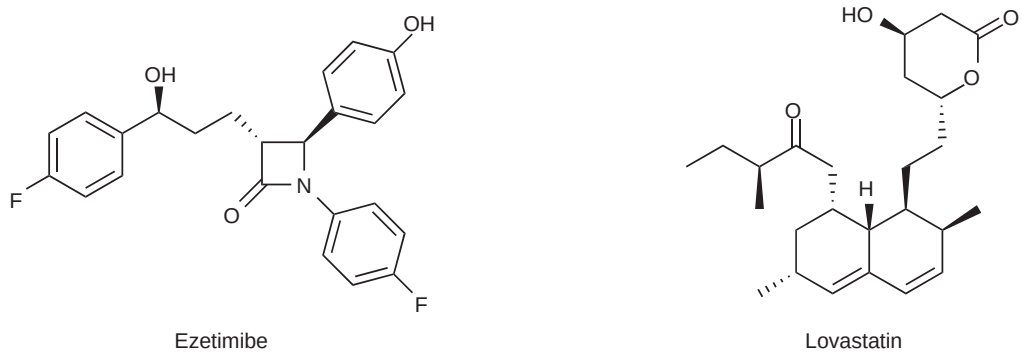


Figure 14.18 Total cholesterol reaching GIT from bile and dietary sources (50% of it is excreted in stool).



Ezetimibe Lovastatin

Figure 14.19 Drugs used clinically for lowering the levels of LDL cholesterol.

A window view to lipid digestion and absorption has been depicted in Fig. 14.20.

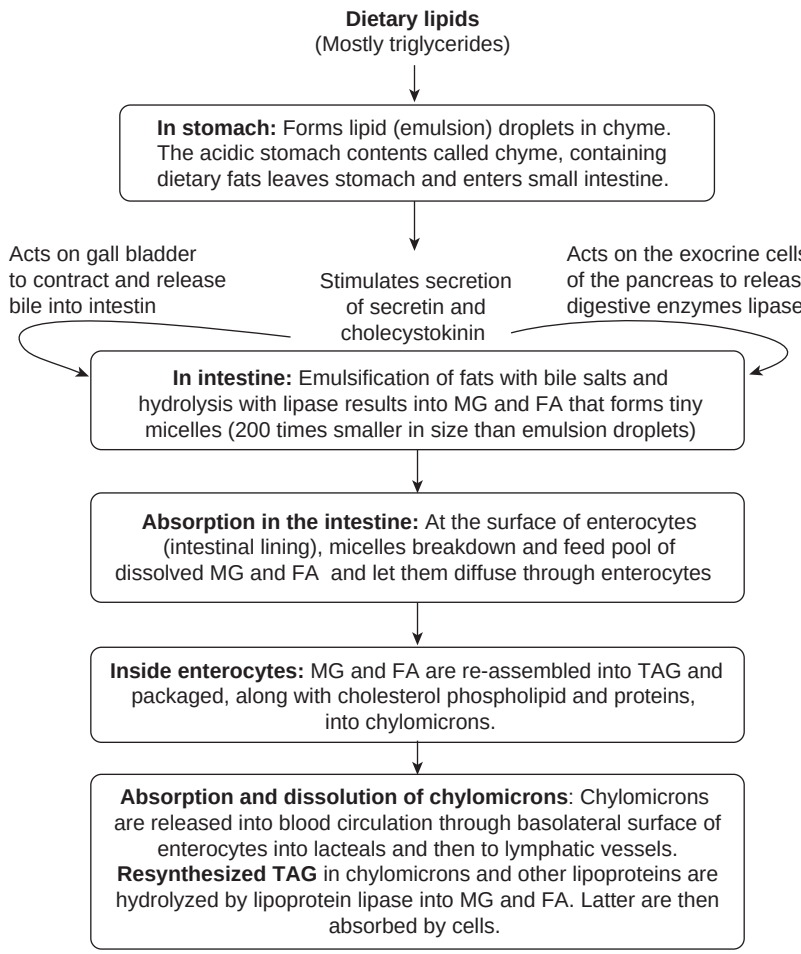


Figure 14.20 A window view to lipid digestion and absorption.

CASE STUDY 2

Effect of Statin on Cholesterol Biosynthesis

The intracellular cholesterol biosynthesis occurs via mevalonate pathway, which is a complex pathway regulating the concentration of cholesterol including triglycerides, LDL, HDL, etc., in bloodstream (Fig. 14.21).

Since high levels of cholesterol lead to numerous life-threatening diseases, the enzymes involved in the biosynthesis pathway remain an attractive therapeutic target. The two important steps in this pathway involve generation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl-CoA followed by HMG-CoA reductase (HMGR) catalysed reduction of HMG-CoA to mevalonate in the presence of NADPH. Clinically used drug statins mimic and compete with the natural substrate molecule, HMG-CoA, thus competitively inhibit HMG-CoA reductase (Fig. 14.21), thereby inhibiting cholesterol synthesis. This reduced level of cholesterol production is compensated by liver cells, by expressing more cell-surface LDL receptors, thereby facilitating LDL (bad cholesterol) uptake from serum (decreasing LDL levels in blood serum). Following internalization (uptake) of receptor-bound LDL particles into liver cells, the cholesterol is converted into bile salts that are either eliminated or recycled.

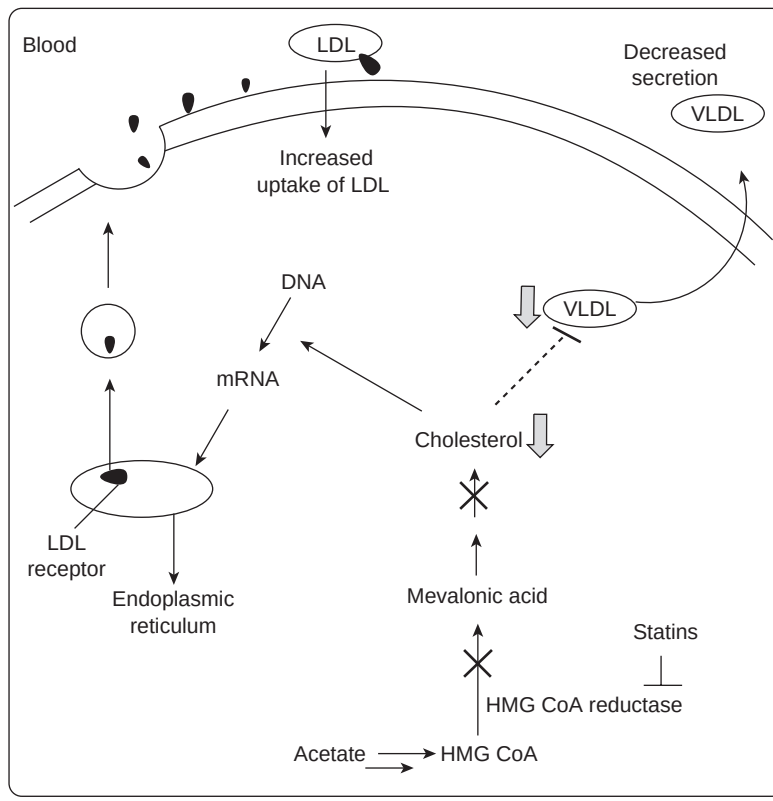


Figure 14.21 Effect of clinically used drug statin on cholesterol biosynthesis. (↓ Decrease ⊥ Blocks/inhibits)

14.6 Digestion of Carbohydrates

The digestion of carbohydrates commences in the mouth and continues in stomach and small intestine. The digestive enzyme salivary amylase commences with the degradation of starch and maltose present in food. Next, the chewed food moves into stomach through oesophagus, which is devoid of any digestive enzymes except for the secretion of mucus for lubrication. In the stomach, no significant digestion of carbohydrates occurs as the acidic pH destroys the activity of enzymes. Next the chyme moves from stomach to duodenum, where it is exposed to digestive enzymes and secretions from liver and pancreas. The incomplete degradation of carbohydrate that started in mouth continues with pancreatic amylase converting starch into maltose and glycogen, while maltase produced from intestinal lining catalyses hydrolysis of maltose into glucose.

Other disaccharides, such as sucrose and lactose, are broken down by sucrase and lactase, respectively. Digestive enzymes sucrases and lactases are also present in the brush border of the small intestinal wall. Sucrase breaks down sucrose (or 'table sugar') into glucose and fructose, and lactase breaks down lactose (or 'milk sugar') into glucose and galactose. Thus, enzyme-catalysed digestion of carbohydrates results in three monosaccharides: glucose, fructose, and galactose, which are transported through the intestinal wall into the portal vein and then taken to the liver. The transportation of glucose and galactose occurs through active transport, whereas fructose is transported through facilitated diffusion.

A window view to digestion of carbohydrates and absorption of membrane permeable components has been depicted in Fig. 14.22. Similarly, a summary of carbohydrate digestion by digestive enzymes in GIT has been summarized in Table 14.5.

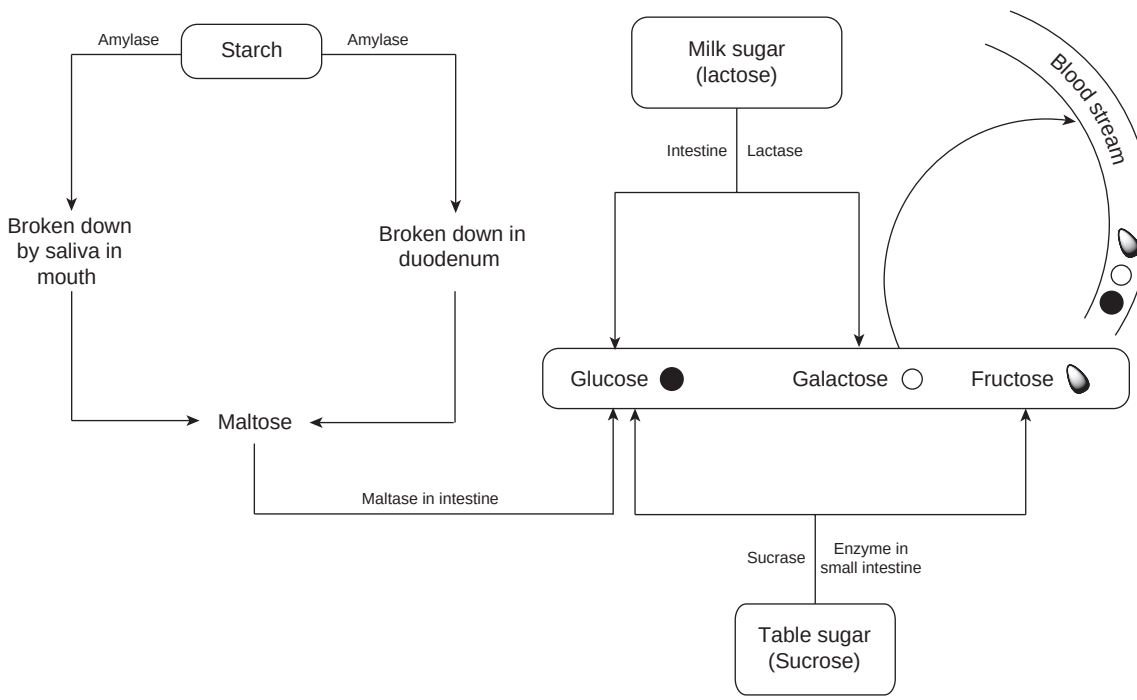


Figure 14.22 A window view to digestion of carbohydrates and absorption of membrane permeable components.

Table 14.5 A summary of carbohydrate digestion by digestive enzymes in GIT

Enzyme	Produced by	Site of Action	Substrate Acting on	End Products
Salivary amylase	Salivary glands	Mouth	Polysaccharides (Starch)	Disaccharides (maltose) Oligosaccharides
Oligosaccharides	Brush border membrane in the intestine	Small intestine	Disaccharides	
Pancreatic amylase	Pancreas	Small intestine	Polysaccharides (Starch)	Disaccharides (maltose) monosaccharides

Exercises

1. Discuss digestion of carbohydrate.

2. Discuss the role of proteases in drug discovery.

3. Describe digestion of proteins in diet.

4. Discuss classification of endopeptidases.

5. Describe the role of enzymes in the digestion of fatty diet.

6. Write a brief note:

(i) Brush border enzymes

(ii) Carboxypeptidase

(iii) Catalytic triad

(iv) Pancreatic zymogens

(v) Chylomicrons

(vi) Pepsinogen

Competitive Exam Questions

1. Simvastatin belongs to

(a) HMG-CoA reductase inhibitor type of antilipidemic agents.

(b) HMG-CoA reductase inhibitor type of anticoagulant agents.

(c) fibrate type of anticoagulant agents.

(d) fibrate type of antilipidemic agents.

[***GATE Pharmacy 2004***]

2. Which one of the following amino acid residues is specifically recognized by chymotrypsin during peptide bond cleavage?

(a) Phe **(b)** Leu

(c) Val **(d)** Asp [***GPAT 2013***]

3. The enzyme HMG-CoA reductase is involved in the pathogenesis of

(a) Atherosclerosis.

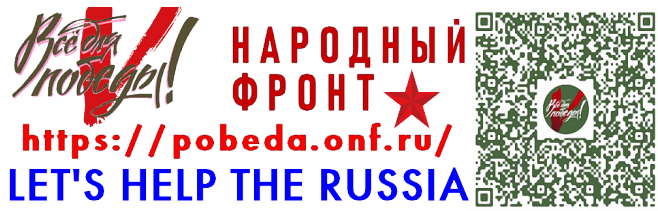
(b) Alzheimer's disease.

(c) Renal failure.

(d) Parkinson's disease. [***GPAT 2018***]

References

1. Hooton D., Lentle R., Monro J., Wickham M., Simpson R., *Rev. Physiol. Biochem. Pharmacol.,* **2015**, *168*, 59.
2. Judith S. Bond*, The Journal of Biological Chemistry*, **2019***, 294,* 1643.
3. Neitzel, J. J., *Nature Education*, **2010**, *3*, 21.
4. Verma S., Dixit R., Pandey K. C., *Front Pharmacol.*, **2016**, *7*, 107.
5. Edited by Rollinson D. and Hay S.I., *Advances in Parasitology*, **2012***, 79*, 1.
6. Chang C., and Werb Z., *Trends Cell Biol.*, **2001**, *11*, S37.
7. Calamini B., and Morimoto R.I., *Curr. Top Med. Chem.*, **2012**, *12*, 2623.
8. Cheng J., North B. J., Zhang T., Dai X., Tao K., Guo J., and Wei W., *Aging Cell*, **2018**, *17*, e12801.
9. Lehtonen Š, Sonninen T. M., Wojciechowski S., Goldsteins G., and Koistinaho J., *Front Neurosci.*, **2019**, *13*, 457.
10. Ke Liu, Lei Lyu, David Chin, Junyuan Gao, Xiurong Sun, Fu Shang, Andrea Caceres, Min-Lee Chang, Sheldon Rowan, Junmin Peng, Richard Mathias, Hideko Kasahara, Shuhong Jiang, Allen Taylor, *Proceedings of the National Academy of Sciences*, **2015**, *112*, 1071.
11. Wang Z. V., and Hill J. A., *Cell Metab.*, 2015; 21(2): 215–226. doi:10.1016/j.cmet.2015.01.016.
12. Kikis E. A., Gidalevitz T., and Morimoto R. I., *Adv. Exp. Med. Biol.*, **2010**, *694*, 138.


Всё для победы!
НАРОДНЫЙ ФРОНТ
https://pobeda.onf.ru/
LET'S HELP THE RUSSIA

Index

A

absorption (A) process, 338
acetylcholine receptor (AChR), 6
acid secretion in stomach, regulation of, 33–35
adenylyl cyclase (AC), 6
ADMET phenomenon, 97, 100, 110–111, 115, 122, 151–153, 171, 181–182, 184, 192, 201–202, 211, 218, 221, 224, 228, 230–231, 234, 338
  absorption (A), 54–55, 342–346
  distribution (D), 55–61, 346–352
  elimination (E), 61–62, 356–362
  factors affecting, 64–91
  kinetics of, 341–362
  metabolism (M), 61, 352–355
  pharmacokinetic (PK) parameters, 342–364
  physicochemical parameters affecting, 362–363
  toxicity (T), 62–63
agonists, 292
  full, 293–297
  inverse, 298
  partial, 297–298
AIDS, 42
alkylating agents, 51
allosteric ligand, 304
allosteric site, 304–308
alpha-1 adrenergic receptor, 311
anabolic processes (anabolism), 30–31
angina pectoris, 9
angiotensin receptor blockers (ARBs), 312
antacids, 51
antagonism
  competitive, 313–314
  irreversible competitive, 314–315
  noncompetitive, 315–317
antidiabetic drugs
  that target enzymes, 17
  that target receptors, 18
antimetabolites, 207–210
  drug 6-mercaptopurine (6-MP), 209–210
aspartame, 125–126
aspartic protease, 430, 434
aspirin (acetylsalicylic acid (ASA)), 147
atrial natriuretic peptide (ANP) receptor A, 7
autophagy, 44
Azathioprine, 199

B

binding of drugs, structural features, 319–320

C

calcium homeostasis, 28
cAMP/PKA cascade, 10
cAMP-responsive element-binding protein (CREB), 15
carbohydrates digestion, 443–444
catabolic pathway (catabolism), 30–31
cell damage (injury), 38
cell-signaling pathways, 1–2
cellular metabolism, signaling in
  metabolic pathway, 30–31
  metabolites, 31–32
  metabolomics, 32–37
cGMP-response element-binding protein (GREBP), 15
cGMP signaling pathway, 6–7, 260–263
  in angina/male reproductive dysfunction, 8
chemical libraries, 94–95, 100–103, 121–123, 139–141, 219
chemical messenger, 2–9
  first/second messenger, representation of, 13
chlorpromazine, 125–126
cholesterol biosynthesis, 442
chromosome abnormalities, 40
chymotrypsin, 370–371, 431
Cimetidine H2 receptor antagonist, 35

CNS drugs, 125–126
cyclic AMP, 6–7, 260–263
  in fat cells, 10–11
cyclosporine (CsA), 87–88
cysteine proteases, 430, 433
cytochrome P450 (CYPs), 87
  induction and inhibition, 418–420
  mediated metabolism, 408–415, 421–422

D

1,2-diacylglycerol (DAG), 14
Diclofenac, 196
digestive enzymes, 401, 425
  initiation of protein digestion, 427–435
  release of, 426–427
dipeptidyl peptidase (DPP-4) enzymes, 3
dipole-dipole/ion-dipole/dipole-dipole induced forces, 330–331
diseases
  classification of, 40–43
  main causes of, 38–40
  signaling pathways and pathogenesis of, 43–44
disordered proteins, 368
diuretics, 51
DPP-4 inhibitors, 17
drug design strategy
  hit to lead, 152
  lead optimization strategies, 153–212
  preclinical candidates, 152
  structural features and biological activity (SAR), 155–164
drug discovery and development, 94–97
  clinical trials, 99
  endogenous sources (humans or animals), 126–134
  FDA monitoring, 99
  FDA review, 99
  initial steps, 99–115
  microbial origin, 135–136
  preclinical research, 97–98
  screening for drug targets, 102–118. *see also in vitro* screening of compounds; *in vivo* screening of compounds
  timeline of, 212–213
drug–receptor complex formation and dissociation, 288–289
  binding affinity, 290
  calculation of receptor occupancy, 291
  dose–response relationship, 289
  equilibrium dissociation constant (K_D), 290
  K_D and EC_{50}, 291–292
  mathematical equation, 290–291
drug–receptor interaction, mechanism of
  induced fit model, 288
  lock and key hypothesis, 288
drug–receptor occupation theories
  activation aggregation theory, 287
  Ariens and Stephenson's hypothesis, 286
  Clark's hypothesis, 286
  macromolecular perturbation theory, 287
  mobile receptor hypothesis, 287
  rate theory, 286
  receptor cooperativity model, 287
  simple occupancy theory, 286
  two-state receptor theory, 287
drug research, 45–46
drug (s)
  actions, 53–63
  bioavailability, 68–70, 72–74, 88
  bioequivalence, 74
  definition, 49–50
  diffusion of, 78–81
  distribution, physiological factors affecting, 74–86
  elimination, physiological factors affecting, 89–91
  hydrophilic, 69
  hydrophobic, 69
  metabolism, physiological factors affecting, 87–88
  naturally occurring compounds, 52
  pharmacological activity of, 87
  physicochemical parameters, 63, 362–363
  physiological parameters, 64–65
  plasma concentration–time curve, 73–74
  routes of administration, 65–72
  structurally nonspecific, 50

structurally specific, 50–51
synthetic, 53
transportation of, 75–79
drug-target interaction, 335
irreversible, 334
reversible, 321–333
role of water, 321

E
effectors, 13–15
endocytosis, 85–86
endogenous ligands, 250–252
endogenous toxicity, 38–39
endopeptidases, 429
Endothelin-1, 312
enzymes, 368–389
active sites of, 369
allosteric, 389–393
apoenzymes, 372
as biocatalysts, 369
catalysed reactions, 373–374
catalytic activity of, 370–371
coenzymes, 372
cofactors of, 372
diagnostic, 398
digestive, 401
extracellular, 399–401
factors affecting activity, 375–389
holoenzyme, 372
intracellular, 401–403
metabolic, 403
microsomal, 407–408
nomenclature of, 393–398
non-microsomal, 408, 422
production of, 401
-substrate complex, 369, 373
therapeutic use of, 398–403
epidermal growth receptor (EGFR), 312
exocytosis, 86
exogenous signal molecules, 4
exogenous toxicity, 38
extracellular receptor, 16
Ezetimibe, 62

F
feedback (negative feedback) inhibition, 28–29
fibrous proteins, 368

G
genetic disorder, 39
genotype, 17
globular proteins, 368
GLP-1 agonists, 18
glucagon-like peptide (GLP-1), 3–4
glucagon-mediated signal transduction pathway, 28–29
glucagon signaling, 10–11
glucose homeostasis, 28–29, 36
glucose uptake, in cells, 9–10
α-glucosidase inhibitors, 17
GLUT4, 9
glutamic proteases, 430, 434
G-protein-coupled receptors (GPCRs), 13–14, 255–260, 263–266, 272–273, 308–309, 335
gramicidin, 136
guanine trisphosphate (GTP), 6
guanylyl cyclase, 14

H
Hexobarbitone, 195
high-throughput screening (HTS), 121–122
hit molecules
characteristics of, 122
computer-aided drug design (CADD) approach for identifying, 145–146
definition of, 121
differencesw between fragments, leads and, 147–148
fragment-based drug discovery for identifying, 143–144
phenotypic screening for identifying, 147
repositioning of drugs for identifying, 147
sources of, 123–124, 136–138
strategies for identifying, 123, 139–147
homeostasis, role of signaling pathways, 27
hydrogen bonds, 322–328
hydrophobic interactions, 332–333

hypothalamic–pituitary–thyroid axis, feedback control in, 29

I
inositol 1,4,5-trisphosphate (IP_3), 14
insulin, 18
insulin-receptor substrate (IRS) proteins, 9
insulin secretion, regulation of, 36–37
intercellular signaling, 18–25
intracellular receptor, 16
intracellular signaling pathway, 2
intramembrane proteases (IMPs), 403
in vitro screening of compounds
  absolute inhibition constant K_i, 108–109
  assays employed, 110–115
  EC_{50} values, 109–110
  ED_{50} values, 109–110
  IC_{50} values, 108
  LD_{50} values, 109
  pIC_{50} values, 109
in vivo screening of compounds
  functional assays, 115–116
  model organisms for, 116–118
ion channel blockers, 312
ionic bond (salt bridge), 328–329
isosorbide dinitrate, 9

K
kinase inhibitors, 12

L
lactone prodrugs, 200–201
lead optimization strategies, 153–212
  absorption via modulating polarity, 184–186
  allosteric modulators, 204–205
  antimetabolites, 207–210
  bioisosterism, 174–180
  design strategies for targeting drugs, 190–192
  functional group modification, 164–165
  'Me too' classes of drugs, 205–207
  pharmacophore identification, 154–155
  prodrugs, 192–201
  selection of preclinical candidates, 211–212
  stability of drugs, 186–190
  stereoisomerism, 181–184
  structural features and biological activity (SAR), 155–164
  structure-based, 165–173
  structure-property relationship (SPR), 201–204
lipid digestion, 439–441
Losartan, 199–200
lysosomes, 44

M
medicinal chemistry protocol, 100
meglitinides–nateglinide, 18
membrane proteins, 368
metabolism
  CYP-mediated, 408–415, 421–422
  non-microsomal, 422
  phase I and II reactions, 416–418
metabolites, 31–32
metabolomics, 32
metalloproteases, 430, 435
Metformin, 17, 138
minoxidil, 125–126
monoclonal antibody therapy, 134–135
monogenetic or Mendelian inheritance, 39
multifactorial genetic inheritance, 39–40

N
negative allosteric modulation (NAM), 306–307
neuropeptides, 3
neurotransmitters, 3, 105–106
nicotinamide adenine dinucleotide (NAD^+), 372
nitric oxide (NO), 9
  NO synthetase (NOS), 9
nitroglycerin, 9
norepinephrine (NE) inhibition of insulin, 37
nutritional deficiency, 39

O
orthosteric binding site, 303–304

P
paclitaxel, 136
peroxisomes (POs), 44
P-glycoproteins, 82

phagocytosis, 85–86
pharmacodynamic drug-drug interactions, 420–421
pharmacokinetics of drugs, 342–364. *see also* ADMET phenomenon
pharmacophore, 154
phenotype, 17
phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$ or PIP_2), 14
phosphodiesterase (PDE), 6
phospholipase C (PLC), 14
phospholipase signaling interactive pathway, 14
pH partition theory, 77
physicochemical properties of drugs, 217–220
  of antihypertensive drugs artans (angiotensin-receptor blockers), 242
  aromatic rings, 236–237
  of CNS drugs, 242–243
  distribution of compound, 230–231
  electron density, 238–239
  hydrogen bond acceptor (HBA)/hydrogen bond donor (HBD), 234–235
  Lipinski's rule, 220–221
  lipophilic efficiency (LiPE), 231–232
  lipophilicity, 228–230, 362–363
  molecular size, 238
  permeability, 230, 363
  pK_a (acid-base dissociation constant), 221–226, 233–234, 363
  polar surface activity, 239–240
  of preclinical candidate drug, 243–2444
  rotatable bond, 236
  solubility, 226–228, 362
  steric factor, 237–238
  surface activity, 235–236
  undesirable functional groups (Alert), 237
pinocytosis, 85–86
polymyxin, 136
positive allosteric modulators (PAMs), 306
Prednisolone, 197
prodrugs, 192–201
protease-activated receptors (PARs), 4, 318
protein homeostasis (proteostasis), 437–439
protein kinases, 6, 9, 11–12, 14
protein targets, 320–321
PTP 1B inhibitors, 17

R

receptor antagonists, 311–318
receptor guanylate cyclase (rGC), 274
receptors
  cell-surface, 253–266
  difference between enzymes and, 249–250
  as drug target, 248–253
  enzyme-linked, 267–281, 312
  gamma-amino butyric acid (GABA) receptor ($GABA_A$), 305
  G-protein-coupled receptors (GPCRs), 255–260, 263–266, 272–273, 308–309
  intracellular, 281–282, 312
  ionotropic, 274–281
  muscarinic ACh receptors, 276–277
  nicotinic, 277
  protease-activated receptor (PAR), 257–258
  spare, 299–300
  TK activity, 269–270
routes of administration for drugs, 65–72
  barriers, 66–67, 338–340
  inhalation, 70–71
  intramuscular (IM), 70
  intravenous (IV), 70
  oral, 65–66, 68–69
  subcutaneous (SC), 70
  topical, 71–72

S

serine proteases, 430–433
serotonin (5-hydroxytryptamine, 5-HT), 83
Ser (Serine), 12
sGC/NO/cGMP signaling pathway, 7
signaling molecules, 3–4
signaling pathways
  classification of drugs based on, 45–46
  defect in, 15–16
  and pathogenesis of diseases, 43–44
signal transduction, 2, 11, 252–253, 265–266, 271–272

drug targets in, 17
enzyme, 11–12
modulators, 15–17
Sildenafil citrate, 8
silent allosteric modulators, 307–308
single gene inheritance, 39
smooth muscles, 260–261
solute carrier (SLC), 82–84
statins, 136–137
sulfonylureas, 18

T
thiazolidinones, 18
threonine proteases, 434
thrombin-receptor antagonist, 318
Thr (Threonine), 12
thyroid homeostasis, 29
transporter proteins, 84–85
Tyr (Tyrosine), 12

V
Van der Waals attractions, 329–330
Verapamil, 278
Viagra (sildenafil citrate), 125–126

W
warfarin, 125–126

X
xenobiotics, 4, 15, 18, 60–61, 68, 75, 82, 87, 318

Z
zymogens (proenzymes), 435–437

About the Book

In recent years, there has been significant decrease in the number of new drugs reaching clinics. This is despite the fact that there have been numerous technological innovations in drug discovery including computational tools and "omic" approaches such as genomics, proteomics, metabolomics, bioinformatics, etc. The failure of a drug discovery program has been largely attributed to poor pharmacokinetic (PK) parameters and toxicity (such as cardiotoxicity) exhibited by drug candidates during clinical trials. This involves poor absorption and distribution, metabolic susceptibility, excretion, and toxicity arising due to adverse drug reactions or drug-drug interactions. This led to the integration of PK studies during initial stages of medicinal chemistry. Thus, for a medicinal chemistry program, simply designing active compounds against a therapeutic target based on structure-activity relationship (SAR) studies alone is not sufficient; structure-property relationship (SPR) of test compounds needs to be integrated with SAR. SPR is dependent on physico-chemical properties of test compounds and plays an important role in overcoming physiological barriers thereby facilitating access of drug candidates to the target.

The present book *An Introduction to Medicinal Chemistry* is written with the view to describe basic components of medicinal chemistry involving identification of hit/lead molecules and their transformation into preclinical candidates.

Salient Features

- Components of inter- and intra-signaling pathways involved in the pathogenesis of diseases: first and second messengers, receptors, effectors, signal transduction enzymes and cellular/physiological response.
- Drug actions involving absorption (A), distribution (D), metabolism (M), excretion (E), toxicity (T).
- Validation of therapeutic target, quality of chemical library, *in silico*, *in vitro*, and *in vivo* screening, identification of hit/lead, lead optimization: SAR and SPR studies, drug design strategies.
- Role of physico-chemical properties of test compounds in the regulation of kinetic, metabolic and toxicological parameters.
- Pharmacokinetic and pharmacodynamic parameters highlighting physiological barriers, ADME kinetics, access to target, drug-target interaction, affinity for target and therapeutic response in the form of efficacy.

follow us on
facebook.com/wileyindia twitter.com/wileyindiapl linkedin.com/in/wileyindia

READER LEVEL
Undergraduate/Graduate
SHELVING CATEGORY
Engineering

WILEY

Wiley India Pvt. Ltd.
Customer Care +91 120 6291100
csupport@wiley.com
www.wileyindia.com
www.wiley.com

ISBN: 978-81-265-9988-2