Abhinav Grover *Editor*

Drug Design: Principles and Applications

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Editor

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1 Applications of Computer-Aided Drug Design

Joo Chuan Tong

1.1 Introduction

Computer-aided drug design (CADD) plays an instrumental role in the modern discovery of therapeutically important small molecules. It refers to computational methods that can help speed up the lead identification and optimization processes. In its broadest sense, CADD represents tools and resources for the storage, management, analysis, and modeling of compounds [1]. They are deployed in almost every step of the drug discovery pipeline, from the design of small molecule libraries, hits identification, to optimization of the affinity and selectivity of compounds. Digital repositories are useful resources for researchers studying important chemical interaction relationships [2]. Virtual combinatorial libraries can help minimize redundancy or maximize the number of discovered true leads by optimizing a library's diversity or similarity to a target [3]. They allow for both sequential and parallel selections of suitable compounds based on preferred molecular profiles. Many tools are now publicly available, with various methods and algorithms, to help identify protein binding sites and molecular functions [4, 5] as well as design compounds with interesting physicochemical properties for drug interventions [6, 7]. Some early successes of structure-based design include the carbonic anhydrase inhibitor Dorzolamide, and the HIV protease inhibitors Indinavir, Nelfinavir, Ritonavir, and Saquinavir [8]. Collectively, these tools and resources could help improve efficiency in new drug development and reduce costly late stage clinical trial failures. This chapter provides an overview of how various computational methods have been deployed to help expedite the drug design and discovery process.

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1.2 Virtual Combinatorial Libraries

Virtual combinatorial libraries offer the potential for improved design by optimizing a library's diversity or similarity to a target [9]. This approach can help identify molecules with desired makeup through systematical exploration of the compound property space. Molecular diversity, coverage, representativeness, physicochemical, and pharmacokinetic properties are concepts that are commonly applied to ensure a good sampling on product space using the minimum number of molecules. In recent years, much emphasis has been placed on designing libraries that allow the consensus selection of suitable molecules by optimizing multiple properties [10]. Such compounds are useful for investigating biological mechanisms and as leads for drug property optimization [11].

The design of a virtual library typically involves reaction encoding, selection of reagents and enumeration [11]. Two approaches are commonly used for enumerating molecular variants: Markush methods and reaction-based techniques. Markush methods enumerate libraries by varying the functional groups to be attached to a common scaffold [12]. While this approach can introduce diversity rapidly into the derived libraries, full (or implicit) enumeration of compounds is computationally expensive by nature. Reaction-based methods, on the other hand, offer a more flexible approach to library enumeration. This approach specifies which parts of the reacting molecules undergo chemical transformations and the type of transformations, allowing for the systematic generation of chemical products through the use of various reagents. However, the derived libraries tend to be smaller, thereby providing less diversity within the available chemical space.

1.3 Fold Recognition and Geometric Methods

Fold recognition is a method to model proteins that share the same fold as proteins of known structures, but do not have homologous proteins with known structure. Such method can help identify new binding sites and molecular function [13]. Commonly used methods include sequence comparison and protein threading [4].

Proteins are said to have a common fold if they share similar major secondary structures in the same arrangement and with the same topological connections. Sequence comparison methods typically begin by searching a protein sequence against a fold library of sequences with known three-dimensional structures, followed by assessing the alignment using substitution matrices, gap penalties, or propensity scales [5, 14, 15]. One good data source for such comparisons is the Structural Classification of Proteins (SCOP) database, which is a rich depository containing detailed structural and evolutionary relations between all proteins with known structures [16]. On the other hand, protein threading works by evaluating the goodness-of-fit of a target sequence on a source structure that is not evolutionarily conserved, followed by substituting the backbone coordinates of the template structure with the target sequence, and assessing the correctness of the model by means of a set of empirical potentials [17–19]. This approach is useful for identifying proteins that are structurally conserved but not evolutionarily related, and for modeling highly conserved molecular complexes.

Geometric algorithms predict active sites by locating cavities or "pockets" on the surface of a protein [20, 21]. Many computational methods have been developed that use geometric characteristics to detect protein pockets. There are several ways to identify pockets using protein geometry only. Computational tools such POCKET [22] and LIGSITE [23] map proteins onto a 3D grid and scan the grid points outside the protein for protein-solvent-protein and surface-solvent-surface events, respectively. SURFNET [24] identifies pockets by fitting spheres into the spaces between atoms. The clustered spheres with greatest volume define the largest pocket. CAST [25] detects pockets by merging neighboring empty tetrahedral that share a common triangle. In PASS [26], cavities in a protein structure are filled with a set of probe spheres, and potential pockets are identified as the probes with the most atom contacts. A benchmark on the performances of LIGSITE, $LIGSITE^{cs}$, $LIGSITE^{csc}$, SURFNET, CAST, and PASS showed that geometric methods can achieve a success rate of 71–77% when tested on a dataset of 48 proteins with unbound structures and 80–87% for 210 proteins with bound structures [27].

1.4 Molecular Docking

Molecular docking is commonly used to help understand drug–receptor interaction [6]. Predicting the binding mode of ligands to macromolecular receptors is nontrivial. The method must first identify the correct positioning of a ligand within the receptor binding site [28], and then evaluate how well the ligand can bind to the receptor [13]. A variety of molecular docking software is now available (Table 1.1). Incremental construction algorithms such as FlexX [29], FlexE [30], and DOCK [31] search for optimal binding poses by placing fragments in the receptor binding site and then extend the fragments to fill the space available. Monte Carlo methods such as ICM [32] randomly sample a conformational subspace, and then move to a new random position independent of the previous position, but according to the predefined continuous probability distribution. Ensemble docking methods such as those adopted by ICM [33] and FlexE [34] address the issue of receptor flexibility by using multiple conformations of the protein to dock the ligand [35]. Other methods, such as the use of genetic algorithms [36] for flexible docking of ligands to

Table 1.1 Some available molecular docking software

Name	URL
Autodock	http://autodock.scripps.edu/
DOCK	http://dock.compbio.ucsf.edu/
FlexX	https://www.biosolveit.de/FlexX/
FlexE	https://www.biosolveit.de/FlexX/
FITTED	http://fitted.ca/
FlipDock	http://flipdock.scripps.edu/
GOLD	http://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold/
Glide	https://www.schrodinger.com/glide
ICM	http://www.molsoft.com/

receptors, have also been described. In 2004, Kellenberger and colleagues [37] performed a comparative evaluation of eight docking tools (DOCK, FlexX, FRED, GLIDE, GOLD, SLIDE, SURFLEX, and QXP) for docking and virtual screening accuracy. Using the crystallographic structures of 100 small-molecular-weight ligands, the team found that molecular docking was capable of recovering 63% of cases at 1 Å r.m.s.d. threshold, with a maximum success rate of 90% at 2 Å r.m.s.d threshold.

Numerous methods have been developed for binding free energy estimations. These can be broadly classified into three groups: empirical scoring functions, knowledge-based potentials, and force field methods. Empirical-based potentials perform binding energy estimations by additive approximations of several energy terms such as van der Waals potential, electrostatic potential, hydrophobicity potential, among others [38]. The relationship between these terms and the binding affinity is obtained either by regression or machine-learning algorithms on a training dataset of receptor-ligand crystallographic structures with known binding affinity [39]. Tools that deploy empirical-based scoring functions include FlexX [29], SCORE [40], ICM [33], and VALIDATE [41]. Knowledge-based scoring functions, such as those implemented in Potentials of Mean Force (PMF) [42], DrugScore [43], and ASP [44], estimate binding free energies based on the frequencies of interatomic contacts. This approach is fast but unlike empirical scoring functions, it does not require binding affinity data for training [6]. Force field methods model free energies of binding by summing the strength of van der Waals and electrostatic interactions between all atoms of the two binding partners using established mathematical terms or high-level quantum mechanical calculations. This method had been implemented in AUTODOCK [45], GOLD [46], DOCK [31], and CHARMM [47].

1.5 ADME/Tox Assessment

Assessing small molecule compounds for their absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) properties is important for early stage drug discovery. It has been estimated that 40–60% of drug candidates fail due to unsatisfactory ADME/Tox properties [48]. Before a compound can exert a pharmacological effect in tissues, it has to cross the gastrointestinal barrier, the blood–brain barrier, and the microcirculatory barrier to reach the blood stream. From there, the compound is transported to its effector site for distribution into tissues and organs, degraded by specialized enzymes, and finally excreted from the body. Furthermore, some compounds may undergo metabolic activation and cause adverse reactions or toxicity in humans [49]. Accordingly, rapid screening of ADME/Tox properties plays a key role in the initial selection of a drug candidate, and for further optimization of potency and drug-like properties.

Many factors affect the membrane permeability of a compound, including compound size, aqueous solubility, ionizability (p*K*a), and lipophilicity (log *P*). The polar surface area (PSA), defined as the sum of surface contributions of polar atoms in a compound, has been shown to correlate inversely with lipid penetration ability

[50]. Compounds with PSA values of ≤60 Å^2 can be completely absorbed by our bodies, while compounds with PSA >140 Å^2 are known to be poorly (<10%) absorbed. Poor absorption and permeation are also more common for drugs with molecular weight of <500 g/mol, $C \log P < 5$, hydrogen bond donors <5, and hydrogen bond acceptors <10 [51]. These criteria constitute the Lipinski's "rule of five" to evaluate and prioritize compounds for properties related to "drugability" [51]. Extensions to this rule were proposed by other researchers, including a more stringent "rule of five" for compounds with molecular weight < 473 g/mol, $C \log P < 5$, hydrogen bond donors <4, and hydrogen bond acceptors <7 [52]. A "rule of three" for lead-likeness was also defined by Congreve and coworkers [53], for compounds with molecular weight < 300 g/mol, hydrogen bond donors ≤3, and $C \log P \leq 3$. While these are useful rules of thumb for evaluating drug-likeness, it should be noted that about 68.7% of compounds in the Available Chemical Directory (ACD) Screening Database (2.4 million compounds) and 55% of compounds in ACD (240 thousand compounds) do not violate the "rule of five" [54]. More complex computational and mathematical models have also been developed to assess ADME/Tox properties. These include methods based on genetic algorithms (GAs), ANNs, SVMs, and statistical models [54]. Collectively, these tools facilitate better understanding of the pharmacokinetics and pharmacodynamics of candidate compounds in the early stages of drug development.

Conclusion

A large variety of tools and resources are now available for computer-aided drug design. CADD is now widely accepted as a viable alternative and complement to high-throughput screening. The choice of suitable software is dependent on the availability of data and resources and varies across different targets of interest. Here, we have provided an overview of existing methods and tools for the discovery of new molecular entities. The review is by no means exhaustive, and more comprehensive surveys are available elsewhere [1, 4, 6, 10, 13, 28]. Over the past decade, much progress has been made in CADD. With the continuous developments in the fields of bioinformatics, high-throughput screening, chemical and structural biology, an increasing number of more sophisticated tools and methods can be expected in the future that can help realize the full potential of computer-aided discovery by design.

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2 Advanced Drug Discovery for Alzheimer's Disease: Challenges and Strategies

Rizwanul Haque and Aamir Nazir

2.1 An Introduction to Alzheimer's Disease

The progressive loss of organization and function of neurons leading to the death of neurons collectively constitutes the Neurodegenerative Diseases (NDs). Age-associated NDs have been a cause of significant health burden because of lack of treatment. NDs pose a great challenge for the elderly population, healthcare providers and caregivers. These diseases result from progressive loss of structure and/or function of neurons. Neuronal death within specific areas of brain predominantly cerebral cortex, hippocampus, and spinal cord results in deficiency of key neurotransmitters further affecting motor functions/movement (known as ataxia), and non-motor functions/mental functioning (known as dementias). Neurons in general don't reproduce or substitute themselves, when they are damaged they cannot be replaced in abundance under normal circumstances though recent studies on neurogenesis provide some hope on neuronal recovery too. NDs are incurable and debilitating conditions that result in progressive degeneration and/or death of nerve cells. A striking number of more than 600 disorders have been reported that affect the nervous system. The most common disorders include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, Spinocerebellar ataxia, Prion disease, and Amyotrophic Lateral Sclerosis (ALS). The cause of each one being believed to be dependent on a number of factors, some most important wherein causes range from particularly genetic or environmental factors [1]. The most common among all NDs is AD with an annual death toll of more than 500,000 people [2]. According to the World Health Organization (WHO) Global Burden of Disease Study in 2012, AD and other dementias are the top fourth cause of death in high income countries after heart disease, stroke, and lung cancer [3]. A 2014 report

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submitted by the US organization reported that more than 5.2 million Americans are currently living with the disease, which includes five million people above the age of 65 years (late-onset AD) and roughly 2 lakh individuals below 65 years of age (early-onset AD) [2], thus making AD the most expensive disease condition in the United States with an estimated $214 billion cost to the American Society. Worldwide, currently more than 25 million people are affected by dementia, most suffering from AD with five million new cases accruing up each year [4]. In Europe, the age-standardized prevalence in people more than 65 years of age is 6.4% for dementia and 4.4% for AD [5]. A new study predicts that the AD in the United States will get doubled by 2050 and the cost of caring will rise to $1.5 trillion per year.

AD has been named after a German physician Alois Alzheimer. On 3rd November 1906, while presenting his findings at the "37th meeting of the Society of Southwest German Psychiatrists" in Tübingen Germany, Alois Alzheimer for the first time described the symptoms of progressive cognitive impairment, focal symptoms, hallucinations, delusions, and psychosocial incompetence changes in a patient called Auguste D, a 51-year-old woman from Frankfurt hospital [6]. The disease was later named by a German psychiatrist Emil Kraepelin as "Alzheimer's Disease." AD is an age-related disorder which affects the population over 65 years of age (elderly population) and is not to be confused with the "normal ageing" phenomenon. Clinically, AD is characterized by progressive and irreversible decline in memory and cognitive functions. In later stages, motor and sensory functions are compromised which leads to drastic personality changes like aggression, apathy, agitation, paranoia, insensitivity to others, lack of initiative, delusional thinking, loss of interest in activities they previously enjoyed, inability to make decisions, and finally the person is socially withdrawn. The cognitive defects are reflected neuropathologically by demise of specific neuronal populations, synaptic loss, and brain atrophy in specific brain areas [7–9] and most importantly by the presence of senile plaques (amyloid plaques) and neurofibrillary tangles (Tau protein) which are formed by improperly processed proteins. These improperly processed proteins tend to form aggregates which are toxic to the neurons and ultimately result in their degeneration [10]. The diagnosis of AD can only be confirmed by autopsy after the death and in living patients it can be done on the basis of some cognitive tests [11]. Patients affected with AD tend to show cognitive decline which includes gradual memory loss, difficulty in performing daily tasks, declining physical coordination, lack of judgment making, personality changes, difficulty in learning, and loss of communication skills [12]. The disease eventually leaves its victims unable to care for themselves and in the final stages; victims are bedridden and normally die due to secondary infections like urinary tract infection, pneumonia, and/or bedsores. The molecular mechanism of the disease progression of AD has been a topic of debate for last several years, and there are two cardinal theories prevailing in the scientific community regarding mechanism of AD. Factors governing neuronal loss can be grouped into genetic, environmental, and endogenous ones. The main culprit is known to be the accumulation of abnormal extracellular protein plaques and neurofibrillary tangles of the microtubules formed by amyloid beta (Aβ) and tau protein, respectively. Aβ is a 40 or 42 amino acid peptide with approximate size of 4 kDa, derived from the precursor protein, namely amyloid

precursor protein (APP). In mid-1980s, APP was first reported to be the core component of amyloid deposits in AD and Down's syndrome patients [13–15]. APP is considered to play a protective role in glutamate excitotoxicity induced by neuronal stress or injury [16]. In diseased individuals APP undergoes abnormal cleavage by enzymes beta-secretase and gamma-secretase sequentially resulting in either a soluble 40 amino acid residue (Aβ40) or an insoluble 42 amino acid peptide (Aβ42) [17]. The resulting hydrophobic 42 amino acid fragments stick together to form clumps of senile plaque outside the neuronal surface causing the death of neurons. The other theory revolves around the tau protein, which plays a role in providing structural integrity to microtubules within neurons [18]. The hyperphosphorylated forms of tau have a tendency to bind among themselves rather than with the microtubules as a result hyperphosphorylated tau leads to formation of flame-shaped neurofibrillary tangles (a paired helical filament) thereby killing the neurons. Both the theories are equally recognized in the scientific world, as each of them is backed up by very important findings. It is possible that both theories are interconnected to each other via some junction points which are still to be uncovered. AD is subdivided into two forms: the rare early-onset AD, which affects individuals before the age of 65 usually in their 30s or 40s; and the second more common late-onset AD, where symptoms develop after 65 years of age. Early-onset AD tends to cluster within families affecting several generations and hence gets its name as "familial AD." These subforms of the disease show different patterns of genetic inheritance and genes associated with them. The familial forms of AD are reported to be linked with many genetic factors which are extremely rare mutations with the occurrence of 1 in 1000 cases. The genes implicated in causing early-onset AD include mutation in *PSEN1* (Presenilin 1) on chromosome 14, *PSEN2* (Presenilin 2) on chromosome 1, and APP on chromosome 21. Research findings suggest that *PSEN1* encodes for one of the four proteins in presenilin complex, *PSEN1/PSEN2* both function to regulate proteolytic cleavage of gamma-secretase [19], a predominant enzyme that cleaves the APP. Mutations in either *PSEN1/PSEN2* influence proteolytic property of gamma-secretase enzyme causing more Aβ42 peptide formation. APP is highly conserved protein whose function is yet to be discovered although it has been suspected to regulate synapse formation [20] and neural plasticity [21]. The gene for APP is located on chromosome 21 (triplicate in Down's syndrome) and mutation in APP gene results in abnormal APP protein that is preferentially cleaved by proteases to form more Aß42 [22]. It is likely that any person having mutant versions of *PSEN1, PSEN2*, or APP will develop AD at comparatively early age of 30s or 40s and will pass on these genes to their offsprings. The inheritance of late-onset forms of AD follows a more complex pattern because of its unpredictable behavior. At that place are some modest but rising gene variations that have been identified which affect—to different degrees—the prospects of developing late-onset Alzheimer's. The effects of these genes are subtle, with variations altering the likeliness of getting AD. The most important gene that is associated with and greatly influences the risk of developing late-onset AD is apolipoprotein E (APOE). APOE, primarily produced by the liver and macrophages, is a major cholesterol carrier that corroborates lipid transport through the bloodstream. Any deviation from the normal levels of cholesterol may cause disease like heart attack and stroke. The human APOE gene is found on chromosome 19 and occurs in

three common forms (polymorphic alleles): APOE ε2, APOE ε3, and APOE ε4. People carrying the ε4 allele are at expanding danger of getting AD, as compared with those carrying the more commonly found ε3 allele, conversely the ε2 allele is reported to diminish the hazard. Presence of APOE ε4 influences deposition of Aβ to form senile plaques and play as a risk factor for developing cerebral amyloid angiopathy. Aβ deposits as decrepit plaques are more plenteous in APOE ε4 transporters than in noncarriers [23]. Nowadays, scientists are targeting APOE ε4 which might offer an attractive alternative target for AD therapy in the near future [24].

2.2 Diagnostics and Treatments Available

AD follows a complex etiology, additionally the cause of the disease is governed by multiple factors. It is believed that the clinical symptoms of AD are manifested many years after the initiation of pathological hallmark governing the disease. Researchers believe that since it is a complex ailment, it is impossible that a single treatment will avert or cure it. Broad exploration is creating and testing an assortment of conceivable medications for AD. While doctors can quite often figure out whether a person has dementia, it might be hard to decide the definite cause. There is no single test that can indicate or rule out, if a person has Alzheimer's. AD is analyzed through a complete medicinal appraisal; it is typically diagnosed with the help of healthcare providers that include neurologists, psychiatrists, and psychologists. They look at the patient's health history to figure out if a person's memory issue or other mental abilities have been declining over time. The first diagnostic test may include mental status testing via physical examination of memory, verbal skills, problem solving, thinking skills, and mood stability. The cognitive tests can be carried out by two commonly used tests; the mini-mental state exam (MMSE) [25] and the mini-cog test [26]. These tests depend upon a series of questions and simple remembering exercises intended to test the scope of ordinary mental abilities of the patient. Secondly, a physical and neurological exam of patient's urine, blood, and spinal fluid is carried out. Lastly, brain imaging techniques like computed tomography (CT) scan or magnetic resonance imaging (MRI) tests are carried out to rule out other causes of dementia-like symptoms that include strokes, tumors, or blood clots that might be the cause of dementia. Apart from this, there are some genetic tests available that check for some causative genes like Apo-E4, but this routine genetic testing is still not recommended for AD. This is because these genetic testings don't show whether a person will develop Alzheimer's or whether a person already has Alzheimer's. Furthermore, genetic tests may give us an idea of familial AD, which only accounts for 5% of all cases. The 100% confirmation of AD can only be done by performing autopsy that displays the amyloid plaques after the death of the patient [36]. In year 2012, the National Institute on Aging and the Alzheimer's Association published an article on the diagnostic guidelines for AD. In this article, they have defined the factors (molecular biomarker, epidemiological and neuropsychological evidence) which best predict the risk of progression from "normal" cognition to mild cognitive impairment and AD dementia, but this is only for research purposes and they do not have any clinical implications so far [27].

Table 2.1 List of drugs approved for Alzheimer's disease

Drug name	Brand name	Approved for	Function	Year of FDA approval
1. Donepezil	Aricept	All stages	Cholinesterase inhibitor	1996
2. Galantamine	Razadyne	Mild to moderate	Cholinesterase inhibitor	2001
3. Memantine	Namenda	Moderate to severe	NMDA (*N*-methyl-D-aspartate) receptor antagonist	2003
4. Rivastigmine	Exelon	All stages	Cholinesterase inhibitor	2000
5. Donepezil and memantine	Namzaric	Moderate to severe	Cholinesterase inhibitor + NMDA (*N*-methyl-D-aspartate) receptor antagonist	2014

2.3 Current Treatments

There is currently no absolute cure for AD, multiple FDA-approved drugs currently being prescribed (listed in Table 2.1) merely slow down the disease progression or bring down the symptoms. Pharmacological agents available in the market to treat AD provide only short-term symptomatic relief to the patients towards helping them in taking care of daily problems like Amnesia (memory loss), thinking, and judgment making. They either improve the cholinergic transmission within CNS or prevent the excitotoxic action that results from overstimulation of NMDA-glutamate receptors in selected areas of the brain. These drugs are characterized under acetylcholinesterase inhibitors and NMDA receptor antagonists.

2.4 Cholinesterase Inhibitors

Acetylcholine is the major neurotransmitter, a chemical messenger of the parasympathetic system that transmits signals across a synapse (the junction) from one neuron to another neuron, muscle cell, or gland cell. A defect in this cholinergic neurotransmission leads to the destruction of synapses and killing of neurons which is the characteristic feature of AD. Acetylcholinesterase enzymes are a class of serine hydrolase enzymes found mainly in neuromuscular junction and within cholinergic synapse that are involved in rapid hydrolysis of neurotransmitter Acetylcholine into choline and acetic acid, thus leading to its termination within the central and peripheral nervous system. Its active site contains two subunits—the anionic site that corresponds to catalytic machinery and its esteric site that holds choline binding pocket, on which many drugs target. Along these lines, inhibition of this protein Acetylcholinesterase is utilized as a key target towards managing the diminished acetylcholine in AD patients. Inhibition of Acetylcholinesterase leads to accumulation of the neurotransmitter acetylcholine and enhanced stimulation of postsynaptic cholinergic receptor. Currently, three drugs available in the market that include Donepezil, Rivastigmine, and Galantamine, work on the principle of inhibition of acetylcholinesterase. Another

acetylcholinesterase inhibitor, Tacrine, was the first drug that was used in the treatment of AD. It was potent in improving the memory and cognition but also resulted in cholinergic associated side effects like nausea, abdominal cramps, and hepatotoxicity because of which its utilization was suspended in the United States in 2013, due to concerns over safety. Donepezil acts as a reversible acetylcholinesterase inhibitor, provides symptomatic relief, and delays deposition of amyloid plaques within the brain [28]. It also imparts some cholinergic associated side effects like malaise, appetite loss, weight loss, sleep problems (insomnia), muscle cramps, weakness, nausea, vomiting, or diarrhea. Donepezil is prescribed for severe Alzheimer's dementia cases. Recently, it has also been used to improve speech in children with autism. Rivastigmine is a slow reversible carbamate inhibitor that blocks cholinesterase activity by binding to esteric part of the acetylcholinesterase. A transdermal rivastigmine patch with lesser side effects is available in the market that can be applied to the skin. Transdermal rivastigmine is also used to treat lewy bodies and dementia associated with PD. Galantamine is an alkaloid isolated from the plant *Galanthus woronowii.* Galantamine is a selective, competitive, rapidly reversible AChE inhibitor that acts at the anionic subunit of acetylcholinesterase. Due to the allosteric potentiating effect of nicotinic receptors, it has perturbing role on cholinergic, glutamate, GABA as well as monoamine neurotransmitter systems. Galantamine improves cognitive dysfunction and psychiatric disorder in patients of schizophrenia, depression, bipolar disorder, and alcohol abuse. Despite providing symptomatic relief none of the above medication imparts long-term benefits with superior efficacy to patients. AchE inhibitors have marked pharmacological application in various other neurodegenerative disorders but cholinergic side effects associated with them don't strongly support their prominent use. Thus, effective pharmacotherapy is still needed to combat AD that could target both cholinergic transmission hindrance and the protein aggregation associated with it.

2.5 NMDA (*N*-Methyl-D-Aspartate) Receptor Antagonists

NMDA (*N*-methyl-D-aspartate) receptors are glutamate receptors and ion channel proteins found inside the nerve cells. NMDA receptor is very important for regulating synaptic plasticity and memory function. It gets activated by docking of the glutamate present at cell surface which further allows influx of Calcium (Ca^{2+}) ion through the cell membrane. Over-activation of NMDA-type glutamate receptors results in excessive Ca^{2+} influx that leads to excitotoxicity leading to neuronal injury and death. Agents that block the NMDA receptor activity like partial antagonist Memantine are used for treatment of moderate-to-severe AD. Memantine, discovered as an antiviral drug, serves as an uncompetitive, open-channel blocker of excessive NMDA receptors without disturbing normal activity. The way to Memantine's restorative activity lies in its uncompetitive binding to the NMDA receptor. Its low affinity and rapid off-rate kinetics retain the normal physiological function of the receptor. Memantine is being

prescribed to patients of moderate and severe Alzheimer's dementia. Memantine is also in clinical trials, for additional neurological disorders that include dementia, depression, and severe neuropathic pain. In addition to this, researchers are currently working on a series of second-generation Memantine derivatives that may have better and safe therapeutic intervention properties as compared to Memantine.

2.6 Drugs in Clinical Trials

There are many new drugs that are under clinical trials for the treatment, prevention, immunization, or towards slowing the progress of AD. Past trials have tested an assortment of medications/drugs in individuals but no significant improvements have yet been demonstrated. The paradigm of drug discovery for AD has shifted from providing mere symptomatic relief towards targeting other parameters like inhibiting Aβ/tau aggregation, combinatorial drug therapy, inhibition of other enzymes and receptors involved in AD manifestation, modulation of pathways and vaccination/immunization therapy, and so on. Many drugs, as listed in Table 2.2, are in pipeline of phase III trials; these drugs focus on ways to treat the disease. These drugs may provide a critical opportunity for therapeutic intervention of AD in the near future.

Table 2.2 Drugs in phase III clinical trials

S.No.	Agents	Mechanism of action	Manufacturer
1.	Aducanumab	Antibody to protofibrillar Aβ	Biogen
2.	ALZT-OP1	Drug combination	AZTherapeutics
3.	AZD3293	BACE inhibitor	Astrazeneca
4.	Azeliragon	Inhibits receptor for advanced glycation end products	TransTech Pharma
5.	CAD-106	Vaccine against Aβ	Novartis
6.	CNP520	BACE inhibitor	Novartis
7.	Gantenerumab	Monoclonal antibody against Aβ	Hoffman-La Roche
8.	Insulin	Intravenous immunoglobulin	Grifols
9.	JNJ-54861911	BACE inhibitor	Janssen
10.	LU AE58054	5HT6 receptor antagonist	H. Lundbeck
11.	Masitinib	Inhibitor of c-KIT cell signaling	AB Science
12.	Nilvadipine	Calcium Channel Blocker	St. James Hospital
13.	Pioglitazone	PPAR-gamma activator	Takeda
14.	Sodium Oligo-mannurate	Inhibits Aβ aggregation	Shanghai Greenvalley Pharmaceuticals
15.	Solanezumab	Humanized antibody against Aβ	Eli Lilly
16.	TRx0237	Tau aggregation inhibitor	TauRX
17.	Verubecestat	BACE inhibitor	Merck

2.7 Challenges in Treating the Disease

With an astonishing improvement in science and medication, human life span has increased significantly; yet this has led to an increase in the age-related incessant ailment and AD is one of them. AD is a standout among the various forms of dementia in the elderly and seeking of an effective therapy against the disease is a serious concern. It is not a simple undertaking; however, a number of factors, as detailed below, make the goal of complete treatment hard to achieve:

1. *Complex etiology of the disease*: Scientists are trying to comprehend the actual cause for AD. There are several combinations of causes that contribute in the development of the disease but none of them are responsible for the disease progression as a sole factor.
   (a) *Genetic causes*: Pharmacogenetic profiling of AD patients has portrayed the inherited genetic differences in response to therapeutic potential of drugs. Variation in APOE gene greatly influences the development of late-onset AD. Presence or absence or a particular polymorphic form of APOE alleles greatly affects the therapeutic potential of the drugs. Similar drugs respond differently in APOE ε4 carrier and noncarrier. For example, bapineuzumab, a new immunotherapy against AD prevents Aβ deposition in the brains of APOE ε4 carriers with mild or moderate AD, but not noncarriers [29]. APOE ε3 noncarriers responded better to donepezil treatment than E3 carriers in Han Chinese patients with AD [30]. Thus, genetic make-up can increase the complication of therapeutic targets. People with different alleles respond to the same treatment differently. There are some specific genes that decide how effective a therapy would be, especially a gene which is involved in the drug metabolism and transportation, but none can be associated with the disease with certainty.
   (b) *Age*: Age is the biggest risk factor for AD. Age-related changes in the brain like atrophy, inflammation, mitochondrial dysfunctioning, and formation of free radicals may contribute to AD development. In AD, old brain is the easiest victim. Scientists are still trying to find answers of what factors and their amount is to be considered for the therapeutic development.
   (c) *Factors other than age*: Factors other than age may also play a role in the development of the disease, for example, gender, education, obesity, and other diseased conditions. These factors also decide the therapy that should be applied to a person, male or female, lean or fat, immunocompromised or hypersensitive. For example, if a person is immunocompromised, anti-inflammatory drugs are not prescribed.
2. *Multifactorial nature of the disease*: The conventional approach of the drug development has been "one molecule one target" but AD is a syndrome and has many contributing factors. There is no unitary theory to explain the molecular basis of the disease. There are varied opinions regarding different processes being the primary reason, some researchers believe in cholinergic hypothesis, some in tau hypothesis and others in amyloid hypothesis. Any of these or all of

them may contribute to the development of the disease, so we require a drug that could interact with several molecular targets of the cascade. All the presently marketed drugs although improve cognitive, functional, and behavioral impairments yet none of them inhibits disease progression, hence remain ineffective.

3. *Sensitivity of nervous system*: Our nervous system is highly sensitive. Researcher's trying to develop any CNS therapy have to be extra cautious. Neurons control almost every primal activity of our body be it movement, be it cognition, all senses and their related aspects are managed by neurons. Any interference in any of its function may result into serious damage; therefore, CNS drugs are more likely to fail than other therapeutic drugs. Most CNS drugs have side effects, 75% of all CNS drugs available to date have side effects. CNS drug takes longer to get into the market than other drugs. All these factors make it difficult to develop a therapy of Alzheimer's disease.
4. *Blood–brain barrier*: A huge number of 100 billion nerve cells communicate with each other in a very efficient way. In order to do so, it is very important to maintain its own microenvironment and integrity. Our body goes through chemical fluctuations all the time, for example, hormonal fluctuations, fluctuations in ions and others components, but to keep our brain unaffected from all these fluctuations our brain is provided with an additional protection that is blood–brain barrier. A network of blood vessels with tight junctions that is produced by several transmembrane proteins inhibit the entry of almost every molecule except the entry of essential nutrients like glucose, some amino acid, insulin, and other precursor molecules. Even if any molecule crosses the blood–brain barrier, it cannot stay inside for long, because of transporter proteins that extrude compounds from the brain, for example, P-glycoprotein which is a member of ABC transporter protein, it most effectively extrudes molecules from the brain. Even though we somehow develop any drug to cure AD, the problem is to deliver the drug into the brain.
5. *Cross talk with other diseases*: AD and its cross talk with other diseases make AD treatment more challenging. Some disease conditions increase the risk factors of AD, for example, diabetes significantly increases the risk of developing AD [31]. Insulin is the common factor that plays an important role in both of these conditions. Insulin regulates energy metabolism and homeostasis in various cells. It reaches brain by cerebral spinal fluid and transporters present at blood–brain barrier. It is thought to increase cognitive ability by activating insulin receptor in hippocampus region of the brain. The binding of insulin to its receptor activates extracellular signal-related kinase, mitogen-activated protein kinase, PI3 kinase/AKT pathway and inhibits GSK-3. Transforming growth factor-β signaling cascade also modulates Aβ aggregation and associated outcome as demonstrated in studies employing varius animal models [32, 33]. Dysregulation of these pathways may also lead to cardiovascular abnormalities, inflammation, and neuropathy. All of these have been associated with AD and further complicate the development of therapy against AD.

2.8 Progress Being Made

Transgenic mouse models for AD have been developed based on amyloid hypothesis and further improved with the understanding of specific genetic mutations. Like other disease models, AD mouse model also helps in better understanding of pathophysiology of the disease and has emerged as an invaluable tool in preclinical testing of potential therapeutics as well. Mouse serves as an efficient, evolutionarily close, and robust model for several diseases including AD. It has short life span as compared to other vertebrates. Invertebrate models of AD such as *Drosophila melanogaster* and *Caenorhabditis elegans* offer certain advantages over mouse model and have aiding in obtaining significant understanding of the disease; however, the downside of these models is that they are evolutionarily distant from the humans as compared to mice.

Triple transgenic mouse model of AD: The neuropathology of AD mainly revolves around the correlation of Aβ plaques and tangles. Therefore, in order to develop a better understanding of this correlation and its effect in synaptic function, triple transgenic mouse model (3×Tg-AD) has been developed having three transgenes $PS1_{MI46V}$, APP_{Swe}, and tau_{P301} [34]. Instead of crossing three independent lines, 3×Tg-AD is created by introducing two transgenes human APP_{Swe} and tau_{P301} (under the control of mouse Thy1.2 regulatory element) inside the single cell embryo isolated from $PS1_{MI46V}$ knock-in mice. The 3×Tg-AD mice deposit Aβ extracellularly prior to tangle formation mimicking amyloid cascade hypothesis [34]. These mice exhibit synaptic disjunction and deficit in long-term potentiation (LTR) in an age-related manner before plaque and tangle deposition which is found associated with intracellular Aβ immunoreactivity. Comparatively, double transgenic mice (2×Tg-AD) lack human APP as a result they neither deposit extracellular Aβ plaques nor exhibit intracellular Aβ immunoreactivity. Therefore, these 3×Tg-AD mice are very useful in studying the effect of Aβ and tau deposition in synaptic philology and to access anti-AD therapies in a more reliable and mechanistic way mediated by both hallmark lesions.

5XFAD mouse model: The 5XFAD model is a double transgenic mouse model of APP and PS1, which co-expresses five mutations (three mutations in APP and two mutations in PS1) which elicit overall Aβ production with $A\beta_{X-42}$ toxicity [35]. The model was developed by introducing APP Swedish (mutation at β-secretase cleavage site), London, and Florida mutations (mutation at γ-secretase cleavage site, APP 717 and APP 716, respectively) with human PS1 having M146V and L286V mutation under the control of mouse Thy-1 promoter. The Swedish mutation results into the higher level of total Aβ, whereas Florida, London, M146V, and L286V mutation enhances the production of $A\beta_{X-42.}$ This process is observed as an early onset of plaque deposition with astrocytosis and microgliosis parallely in 5XFAD mice. Interestingly, a gender-specific response is observed in this model system in response to stress, where female mice show significant deposition of plaques in the hippocampal area of brain as compared to male [36]. Apart from plaque pathology age-dependent synaptic degradation is observed in them. The 5XFAD model is also among the few models that show neuronal loss at the cortex and subiculum (by 9 months of age) mimicking AD pathophysiology relatively better.

A worm as AD model system: *Caenorhabditis elegans* (*C. elegans*), a nematode (round worm), is a very useful transgenic model to study neurodegenerative disease related to basic toxic mechanism [37]. The transparent nature of this worm facilitates to study cellular differentiation and other developmental processes as well. In the context of neurodegenerative diseases, using *C. elegans* confers many advantages like short life span; as a result, construction of transgenic model and assessment of any experiment can be done relatively faster. Researchers have developed transgenic AD model of *C. elegans* which expresses human Aβ fragment in the muscle cells [38]. A construct called PCL12 having DNA fragment which encodes for human $A\beta_{1\text{-}4}$ under the control of UNC-54 promoter was prepared . This construct was introduced inside the gonads of nematodes via microinjection to produce Aβ constitutively making animals undergoing paralysis. The assessment of transgenic strain was done by co-injecting pRF4 plasmid which encodes for a mutant collagen gene whose expression leads to the onset of roller mutation. The strain is temperature sensitive and maintained at 15 °C. Onset of paralysis and egg laying deficiency is introduced by maintaining the strain at 20 °C.

Drosophila as a model organism for AD: Comparative genomics has revealed that around 70% of disease-causing genes in humans have orthologs in the fly including the orthologs associated with AD genes with functional conservation. Drosophila has APP orthologs dAPPl, γ-secretase complex, and β-secretase-like enzyme which shows very low β-secretase activity; as a result, there is no endogenous production of Aβ in the fly. Overproduction of β-secretase-like enzyme has shown the cleavage of dAPPl which corresponds to the human Aβ which is able to aggregate and can induce behavioral and neurological impairments in an age-dependent manner.

Apart from endogenous Aβ production transgenic flies have also been generated to understand the AD in a better way. Greeve and coworkers have developed a transgenic fly which expresses human APP, human β-secretase (hBACE), and Drosophila presenilin (dpsn) with point mutations N14I, L235P, and E280A in order to mimic familial AD mutations [39]. These flies exhibit a correlatable association between neurodegeneration and age progression. Using Gal4/UAS inducible system another transgenic fly has been developed carrying Gal4-driven construct which encodes human APP and human BACE which is able to generate Aβ peptide in a tissue-specific manner. In this system, by using specific promoter yeast Gal4 protein is expressed in particular cells. As Gal4 is a transcription factor; it binds to the UAS and induces the expression of the gene of interest which is upstream to UAS in the construct. Such complex Aβ is ideal for the better understanding of APP processing. Such models are easy to handle as compared to models, where Aβ is fused with downstream of secretory peptide. Fly models overexpressing tau have also been developed, where human tau is overexpressed with increased activity of glycogen synthase kinase 3β (GSK3β) activity to form intracellular inclusions which resemble neurofibrillary tangles; this finding also confirms the previous notion that tau toxicity requires hyperphosphorylation and aggregation. Thus, Drosophila AD models provide a better insight of mechanism with two important AD hallmarks.

Newer imaging techniques for diagnosis: Molecular imaging techniques have grown rapidly in the past few years. The advancements allow us to measure brain structural changes (like atrophy, volume, cortical thickness), pathology of the brain (fibrillary Aβ and tau), and functional changes (glucose metabolism and neurotransmitter activity). These new imaging techniques help us in diagnosing AD early and in evaluating the medical therapy in a better way [40]. Nowadays, the most commonly used molecular technique for diagnosis and treatment follow-up in AD is positron emission tomography (PET). This technique helps physicians in assessing abnormalities of the brain via a painless and safe method. PET scanning is a technique where three-dimensional images at both cellular and molecular level can be obtained. In a PET scan, very small amount of radioactive tracer is injected into the patient's body which has either affinity to bind to the desired target, usually a particular protein, to show the presence and extent of its deposition in the brain or the tracer can be metabolized to study the functional changes inside the brain.

1. *Aβ imaging in AD patients*: Pittsburgh Compound B (^{11}C-PIB) was among the first Aβ PET tracers. It has been found that as compared to the healthy individual in AD patients high ^{11}C-PIB is observed in cortical and subcortical areas of the brain [41]. Nowadays, ^{18}F-labeled tracers are thought to be more suitable because of their long half-life. The first ^{18}F-PET tracer that is used for visualizing Aβ plaque was ^{18}F-FDDNP. Though it was showing low affinity to Aβ as compared to ^{11}C-PIB but observations suggested that it also binds to neurofibrillary tangles. ^{18}F-flutemetamol, ^{18}F-florbetapir, and ^{18}F-florbetaben have been found as promising ^{18}F-PET tracer in AD patients [42].
2. *Imaging functional changes in AD brain*: In order to measure regional cerebral glucose metabolism rCMRglc, 2-[^{18}F]-fluoro-2-deoxy-d-glucose (^{18}F -FDG) is widely used [43]. Reduction in rCMRglc is observed in very specific areas of the brain (as the brain with specific dementia will consume less energy at specific area and therefore less sugar) which is more prevalent in early onset as compared to late-onset AD; whereas retention of ^{11}C-PIB is observed in larger areas of the brain with no variation in the regions specific in its retention in both early and late-onset AD. With the progression of the disease, a decline in the rCMRglc is observed.
3. *Imaging neuroreceptor and neurotransmitter*: Wreckage in cholinergic, dopaminergic, and serotonergic neurotransmitter system is usually observed in AD brains. Many PET tracers have been developed to study the different neurotransmitter levels and the receptors in the AD patient. Decline in cholinergic neurotransmission and nicotinic receptor has always been correlated to the reduction in attention and cognitive function.
4. *Tau imaging*: After successful implications of PET-Aβ imaging, substantial research is going on towards developing PET tracer that can detect tau inclusion bodies in order to understand the tau pathophysiology better. There are lots of hurdles in the development of such PET tracers highly specific for tau because of its structural variation and severe posttranslational modification. Despite these hindrances, several compounds have been developed which show tau binding ability [44]. Such compounds are under thorough investigation before they can enter into the clinical practice.

2.9 Strategies Ahead

Despite the fact that the pathophysiology of AD is not fully understood, it is majorly exhibited by the neurotoxic effect of amyloid plaques (Aβ) and deposition of neurofibrillary tangles (tau protein). Clinical advantages of the drugs available for the treatment of AD (to be specific cholinesterase inhibitors and NMDA receptor antagonist) are obvious, albeit they are merely restricted towards providing symptomatic and psychological treatment only. Over the past few decades, enormous amount of research worldwide has been directed towards the development of newer strategies in order to tackle or even prevent the pivotal pathological processes in AD. More than 200 pharmaceutical compounds are currently being tested in phase II and III clinical trials. The following are the cases of promising focuses for cutting-edge drug treatments under scrutiny in current research studies:

Anti-amyloid strategies: Misfolding of the Aβ protein leading to its aggregation is the characteristic hallmark of AD. During the past years, a great understanding has been developed about the molecular mechanisms through which they are formed. Drugs are being designed that are aimed at inhibition, prevention from overproduction or aggregation of Aβ or facilitating the clearance of Aβ from the brain. As shown in Table II numerous drugs that are in phase III clinical trials for their mechanistic ability to handle Aβ with the possibility of diminishing its load in the brain. The reported limitation is that these anti-amyloid agents should be administered in the early events of AD progression. Anti-Aβ vaccine, AN1792 has been proved effective for removal of brain Aβ via the use of anti-Aβ antibodies but its use has been shut down due to its side effects of causing acute meningoencephalitis. These vaccines work by eliciting immunological response against Aβ. The newer active and passive Aβ immunotherapies like bapineuzumab, humanized anti-Aβ monoclonal antibodies, aducanumab, CAD-106, and gantenerumab have been developed which are under clinical investigation with the aim of accelerating Aβ clearance from the brain of the AD patients [45]. For further reading, anti-amyloid agent has been reviewed in the work of Aprahamian et al. [46].

Beta-secretase (BACE) inhibitors: Apart from vaccines, therapeutic agents are being vigorously pursued that are aimed to block the action of β-site amyloid precursor protein cleaving enzyme (BACE) which is responsible for the production of the neurotoxic Aβ (reviewed in [47]). These therapeutic agents inhibit the ability of the BACE to make Aβ thus preventing Aβ clustering into plaques. The only difficult part for the researchers is to tackle the blood–brain barrier as BACE inhibitor drugs cannot pass through it and there also is toxicity associated with the molecules. Much of the BACE inhibitor drugs have been dropped out because of their toxicity. BACE inhibitor drugs, namely AZD3293 [48], CNP520 [49], JNJ-54861911 [50], and Verubecestat [51] are currently in phase III clinical trials.

Anti-tau strategies: Tau protein is the main component of neurofibrillary tangles, another hallmark associated with Alzheimer pathology. Tau proteins stabilize the microtubule and help in maintaining the structural integrity of the neurons. Analysts are developing drugs that can prevent formation, phosphorylation, and aggregation of tau protein to form neurofibrillary tangles. Strategies involved are the use of active and passive immunotherapy, tau protein kinase inhibitors,

microtubule-stabilizing agents, and tau aggregation inhibitors. Drug TRx0237, currently in phase III clinical trials, is a second-generation tau protein aggregation inhibitor [52]. TRx0237 is a more advanced formulation for Rember®, a formulation of methylthioninium chloride which is the purified form of methylene blue. TRx0237 is much more improved formulation than Rember® because it renders greater absorption, bioavailability, and tolerability. Another class of compounds is lithium salts that have been reported to prevent tau hyperphosphorylation and reduce its concentration; mode of their action is via inhibition of glycogen synthase kinase (GSK-3β), axonal degeneration, and release of transforming growth factor beta 1 (TGF-β1) [53–55]. Prolonged chronic lithium treatment may result in renal impairment and hypothyroidism. Safe treatment limits of lithium salts are still to be determined. Additionally, AADvac1, a vaccine against an abnormal form of tau protein is under research and may prove beneficial for the treatment of AD in the near future.

Anti-oxidative agents: In recent years, considerable amount of research data has provided evidence towards increased oxidative stress in the brains of AD patients. The theory of increased oxidative stress is supported by the fact that there is increased free radical generation, lipid peroxidation, protein and DNA oxidation, advanced glycation end products (AGE), and SOD-1 in neurofibrillary tangles present in AD patient brain. The increased ROS levels may be due to mitochondrial dysfunction, accumulation of transition metals, and accumulation of Aβ and tau proteins. These increased free radicals further lead to neuronal degeneration and death. Theoretically, therapeutic drugs aimed at getting rid of ROS are being designed to prevent propagation of tissue damage by ROS and improve neuronal survivals. Administration of very few antioxidants in the diet has shown some efficacy but its protective role against AD is still in question. The ROS formation may be a secondary event but its contribution to the initiation and progression of AD is not denied; hence, targeting it may be fruitful.

Anti-inflammatory agents: In the AD patient brain, Aβ and tau deposits are reported to elicit stimuli for inflammation, which result in degeneration and death of neurons. Inflammation results in activation of immune response in the brain primarily recruiting microglial cells. The presence of Aβ and tau microglial cells may become overactive and secrete toxic compounds thus damaging nearby neuronal cells. Recent clinical trials of readily available anti-inflammatory drugs like celecoxib and naproxen which were expected to improve cognitive function in elderly individuals were rendered insignificant along with slight adverse effects. In a new theory, inflammation may play a beneficial role towards the clearance of Aβ. CSP-1103 is another inflammatory drug in research which has recently undergone phase II clinical testing. CSP-1103 also acts via modulation of microglial cells in order to reduce inflammation in the brain [56]. Preliminary studies have been proven effective towards lowering Aβ levels and increased memory in phase II clinical trials. CSP-1103 is still in exploration and not accessible to people in general.

Targeting brain insulin signaling: There is an impairment of insulin signaling in AD patient brain. Decline in insulin level has been correlated with cognitive decline and the development of AD. A great interaction is reported to be present between

diabetes and Alzheimer's to an extent that some researchers are calling AD as type 3 diabetes [31]. An important role of insulin and insulin signaling in the treatment of AD has been proven. AD brain tends to show resistance to the normal effects of insulin known as insulin resistance, which may occur due to decreased expression of insulin receptors, or decreased levels of insulin or insulin-like growth factor in the brain. The insensitivity of insulin may also be due to impairment in the transport of insulin across the blood–brain barrier. Thus, targeting "brain" insulin signaling for the treatment of AD has now pulled in much consideration in the field of AD research and therapy [57]. Current drug in research that targets insulin resistance is the insulin molecule itself and drugs that improve insulin sensitivity, which include drugs like incretins, dipeptidyl peptidase IV inhibitors, thiazolidinediones, and metformin [58–61]. Intranasal insulin is given to counter the problem related to blood–brain barrier. Intranasal insulin therapy is currently under phase III clinical trials. Use of intranasal insulin is supporting therapeutic potential for patients with amnestic mild cognitive impairment and patients with AD [62].

Exploring of novel delivery routes: AD pathology is very specifically limited to the person's brain as the neurons degenerate in that area. Numerous drugs that have shown good efficacy in rodent models in the treatment of AD have failed in clinical trials. This may be attributed to the process of passing therapeutically active molecules across the blood–brain barrier. Passing of the drugs across the blood–brain barrier is a complex process that is mediated by special tight junctions present at blood–brain barrier. Several strategies have been employed to enhance the efficacy of the drugs being administered to AD patients. Some strategies include structural damage to the blood–brain barrier, nanobiotechnology transport/carriers-based delivery methods, and alternative route of delivery such as intranasal administration. Structural damage to the blood–brain barrier is done by forcibly opening it to allow diffusion of drugs or by direct introduction of drugs in the brain by surgical procedures. The drawbacks of structural damage to blood–brain barrier are that it results in damage to the barrier permanently. Another delivery method is the use of nanoscale particles transport/carriers [63]. The blood–brain barrier allows transport of molecules that are less than 1 nm. The advancement of nanobiotechnology has enabled us to synthesize very small nanoparticles/receptor-tagged nanoparticles that may just pass through the blood–brain barrier. In this approach, drugs are bound to a nanoparticle making the drug capable of passing through blood–brain barrier. The only limitation is the uncontrolled passage into the brain which may not be desirable. Nanobiotechnology researchers are exploring controlled ways of delivering nanoparticle-based drugs. The last approach is practical, simple, and noninvasive to tackle the blood–brain barrier by delivering drugs through nasal route [64]. The nasal cavity is rich in olfactory and trigeminal nerves that are involved in sensing smell. This olfactory epithelium in between the nasal mucosa and the brain serves as a link between brain and external environment that provides portal of entry of molecules directly to the brain. This route also allows drugs which do not cross the blood–brain barrier to enter the brain. A wide variety of therapeutic agents like insulin molecule are being rapidly delivered to the CNS using this intranasal approach.

Combinatorial therapies: AD is a multifactorial disease and is linked to the formation of aggregates of misfolded proteins (Aβ, tau) in neurons, degradation of neurotransmitters, increase in oxidative stress, mitochondrial dysfunction, excitotoxicity of neurons, genetic factors, insulin resistance, etc. As AD is governed by various factors, no single therapy has so far proven to be satisfactory for the effective treatment. One of the hypotheses for combating AD is the use of multiple agents/novel agents working through different mechanisms for targeting multiple factors associated with the disease [65]. The proposed hypothesis may offer the best hope for a future neuroprotective therapy. A combination of bioactive components, used against different pathomechanisms in neurodegenerative diseases may be fruitful. In addition to combinatorial therapy, researchers are also developing multitarget ligands, for example, benzofuran and chalcone hybrids bearing hybrid structures with a capacity to simultaneously interact with multiple targets against AD [66]. Phytomedicines are widely used across the globe as economical, effective, and safer alternatives to synthetic drugs. Phytomedicines exhibit the property of neuroprotection via free radical scavenging, abating misfolded protein aggregation, and enhancing dopamine (a neurotransmitter) level. Use of phytomedicines, multiple molecules or hybrid molecules may help in countering AD. It is still too early to comment whether combinatorial therapies will be effective for the prevention or treatment of AD.

Advent of early diagnostic tools via *employing newer technologies, looking for noninvasive/sensitive diagnostic tools*: The early diagnostic tools include high resolution neuro-imaging via MRI which is used for the quantification of the loss of brain volume. Analysis of Aβ and tau levels in cerebrospinal fluid by high resolution imaging, computed tomography (CT) may be used as an alternative for MRI when it is contraindicated [67]. Better tracer molecules like Amyvid (florbetapir F-18), Vizamyl (flutametamol F18), and Neuraceq (florbetaben F18) that bind to Aβ/plaques in the brain are being developed for use in living patients. Newer computerized cognitive testing is also being used for early detection of mild cognitive impairment via detection of changes in walking speed that reflect defects in motor neurons [68]. Current research is going on towards delivering an early diagnostic tool for AD via identification of several potential biomarkers that may appear before clinical symptoms [69]. Biomarkers are used to identify the state of disease, their progression, and predict possible treatment. BACE1 enzyme, still in its infancy, is considered as a biomarker in blood as one report suggests that it is enhanced in AD patients [70]. The plasma lipid, a lipid pathway in the brain particularly in cholesterol biosynthesis, is used as a noninvasive AD biomarker [71]. Small RNAs mainly miRNAs may serve as an upcoming class of biomarkers for AD as the regulation of amyloid production has been linked with some specific miRNAs [72, 73]. In spite of intense research efforts, currently there are no validated biomarkers for AD; however, significant progress has been made in the field. A very preliminary study known as saliva test has been presented at the 2015 Alzheimer's Association International Conference in Washington for the early detection of AD. In addition to this, identification of new markers, newer amyloid imaging, technology like smaller sensors, wireless technology, combining home computer, and use of proteomics assessment of protein signatures in the brain are underway for the early detection of AD.

Popularizing ways of increasing brain plasticity, encouraging healthy lifestyle: A standout among the most energizing science improvements of late years is the generation of knowledge regarding the plasticity of brain. The ability of the brain to form new neural connections is called neuroplasticity and the process to make new connections between neurons continues throughout the duration of our lives. A new study suggests that utilizing memory techniques can help the brain develop new neural pathways for learning and enhance memory, notwithstanding for individuals with early signs of AD. Nowadays, more and more neuroplasticity exercises like brain program, exercise, or game are becoming available on the Internet to enhance brain working. Brain's neural connections can be strengthened and regenerated by performing mental exercises on daily basis. Ongoing study shows that brains of people with MCI/Alzheimer's have plasticity and routine brain exercise increased their memory score by 33% [74]. Popularizing ways of increasing brain plasticity is a promising approach for delaying the effects of AD. Encouraging healthy lifestyle is another way of influencing AD improvement. Adherence to a healthy lifestyle throughout life may directly or indirectly help in preventing AD by reducing modifiable risk factors [75]. Promoting this knowledge can also help people and their families from the perils of AD.

Using information technology-based approaches towards recruiting larger number of patients for carrying out clinical studies: For carrying out clinical trials, recruitment of participants (human subjects) plays a vital role in the success of the research. Most of the studies fail due to less participation of human subjects. The failure of research frequently takes longer than foreseen that may be attributed to longer recruitment time which further increases the cost of projects than expected. The process usually involves identification of eligible candidate, obtaining informed consent, holding members until study fulfillment and following ethical norms. In one of the statistical surveys, it is reported that only 3–20% of the pool human subjects participate in the clinical trials [76]. The reduction in sample size diminishes the statistical analysis of the study and may give us a wrong impression of the drug under research. Among several ways, one way to tackle this situation is the use of information technology-based approaches towards recruiting larger number of patients for carrying out clinical studies. The approaches may include the use of automated clinical trial recruitment [77] that include digital platforms, social media, and mobile technologies. Internet is used to gather/share information about medical condition, experiences, and illness between caregivers and patients directly which minimizes the time and is less expensive. There are many online patient communities such as PatientsLikeMe®, TrialMatch®, and Ben's Friends which work on these principles to significantly boost success rates [78, 79]. These portals are used for phone calls, emails, or sending messages directly which minimize the burden on participants and make their participation more efficient and convenient for them. In fact, dynamic organizations have started to utilize e-enrollment systems effectively.

Improving outreach in hard-to-reach areas: One of the strategies to support people with AD and their families is by providing greater understanding of the disease and its symptoms so that it can be diagnosed and treated early.

Early detection may help the AD patients to slow down the disease by coordination of care and treatment. Recently, huge budget government projects have been sanctioned aimed at improving outreach in hard-to-reach areas. Outreach also helps the healthcare providers in recruiting human subjects for the clinical trials. It likewise increases support for individuals with AD and caregivers in the community with improved data collection and analysis to better comprehend the effect of AD on individuals with the disease, families and the well-being, and long-term care systems.

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Modern Approaches in Cancer Pharmacology

3

Sukriti Goyal and Abhinav Grover

3.1 Introduction

Cancer, also known as, carcinoma, malignancy, neoplasms and tumour is a collection of more than 100 different types of diseases. Cancer, a leading cause of death, is named according to the anatomical part of human body where it initiates such as lung cancer, pancreatic cancer and breast cancer. The burden of leading cause of death in economically developed countries is expected to rise worldwide owing to the present lifestyle, especially in less developed countries which constitutes 82% of the world population [1].

Several factors including internal (inherited mutations, hormones, etc.) and external (environmental, tobacco, diet, radiation and infections) are known to trigger this disorder [1]. The effect of diet consumed by residents of various countries has been revealed by significant difference in the types of cancer observed among them. For instance, residents of western countries possess 25 times higher incidence of prostate cancer and 10 times higher incidence of breast cancer than Asians [1]. Several similar factors and observations suggest that dietary routine, smoking, consumption of alcohol and infectious diseases have a profound impact on most cancers than hereditary factors (Fig. 3.1). Evidently, the hereditary aspect cannot be altered but lifestyle and environmental features can be modified. These changes might be a step towards preventability of cancer [2].

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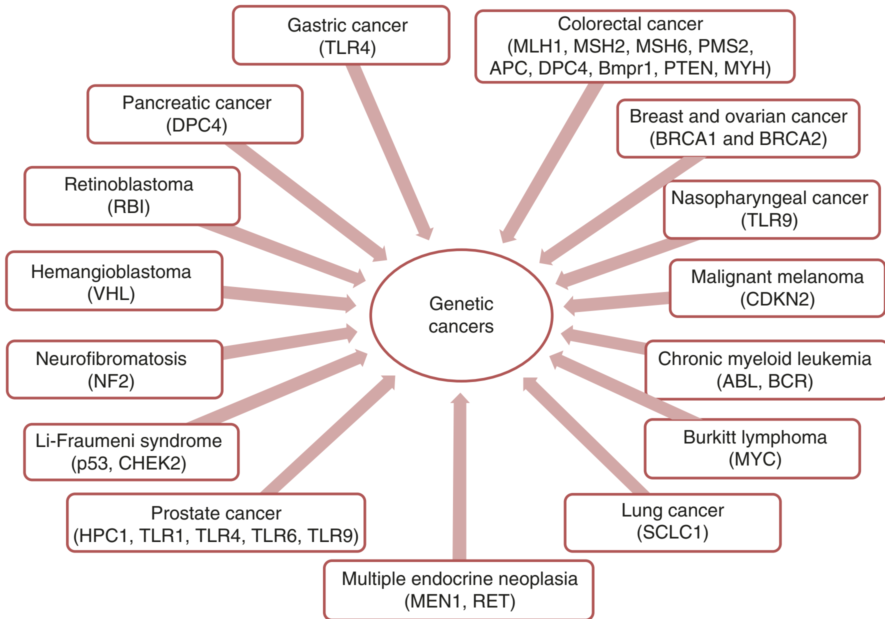

Fig. 3.1 Genes associated with risk of different cancers

The first test widely performed for cancer screening was Pap test, developed by George Papanicolaou [3]. He identified the potential of this test for identification of cervical cancer. Later in 1960s, mammography was exploited for detection of breast cancer [4]. Subsequently, early identification of various cancers such as breast, rectum, endometrium, thyroid, oral cavity, skin, lymph nodes and ovaries cancers were detected and performed in the clinic. This chapter will give an overview of various treatment methods employed to combat cancer and the modern approaches being considered to counter the various problems associated with cancer.

3.2 Methods for Treatment of Cancer

3.2.1 Surgery as a Treatment Method

Surgeons in the ancient era knew about the resurgence of cancer after surgical removal of cancer. This belief, that numerous cancers are incurable, is still prevalent among people delaying consultation of doctor at an early stage. In 1846, after invention of anaesthesia, operations involving removal of complete tumour along with lymph nodes were led by surgeons Bilroth, Handley and Halsted. However, report of metastasis by surgeon Paget and lack of information of mechanism of cancer led to identification of limitation of cancer operations [5].

3.2.2 Chemotherapy

Fifty years post invention of anaesthesia, Roentgen discovered X-ray which formed a new method of treatment of cancer [6]. These radiations were used in combination with surgery for treatment of cancer during last decades of twentieth century [7]. This method, owing to the nature of treatment known as chemotherapy, has yielded successful treatment of various cancers. Recent research on this treatment method aims to reduce the numerous side effects caused by chemotherapy.

3.2.3 Hormonal Therapy

Influence of hormones on growth and development of cancer has given rise to a new class of drugs, namely, aromatase inhibitors and LHRH analogues. These drugs are being used for treatment of prostate and breast cancers [8].

3.2.4 Immunotherapy

Application of bio-agent which mimics the natural signalling process and is used by body to curb tumour growth is known as immunotherapy [9]. Interferons, cytokines, interleukins, antigens and endogenous angioinhibitors are such natural biological agents that are created in laboratory. Monoclonal antibodies rituximab [10] and trastuzumab [11] target lymphoma and breast cancer cells were produced by scientists in 1990s for their therapeutic application.

By the end of twentieth century, majority of cancer drugs worked by destroying cancer cells. Unfortunately, in addition to the cancerous cells, these drugs also destroyed a number of normal cells. Owing to this reason, a new approach known as Targeted Cancer Therapy (TCT) [12] was developed, which will be explained in a separate section.

3.3 Targeted Cancer Therapy

Blocking the growth and development of cancer by inhibiting specific molecules involved in its growth, development and progression using drugs and other substances are TCT [12]. This therapy varies from conventional chemotherapy in numerous ways. While chemotherapy acts by killing rapidly dividing normal and cancerous cells, TCT targets specific molecular proteins involved or associated with progression of cancer (Fig. 3.2). TCT is cytostatic in nature which means it inhibits tumour cell growth, whereas chemotherapy exhibits cytotoxic (toxic to cells) nature. Owing to the several advantages offered by TCT over chemotherapy, former is currently the focus of research in the field of anticancer drug designing.

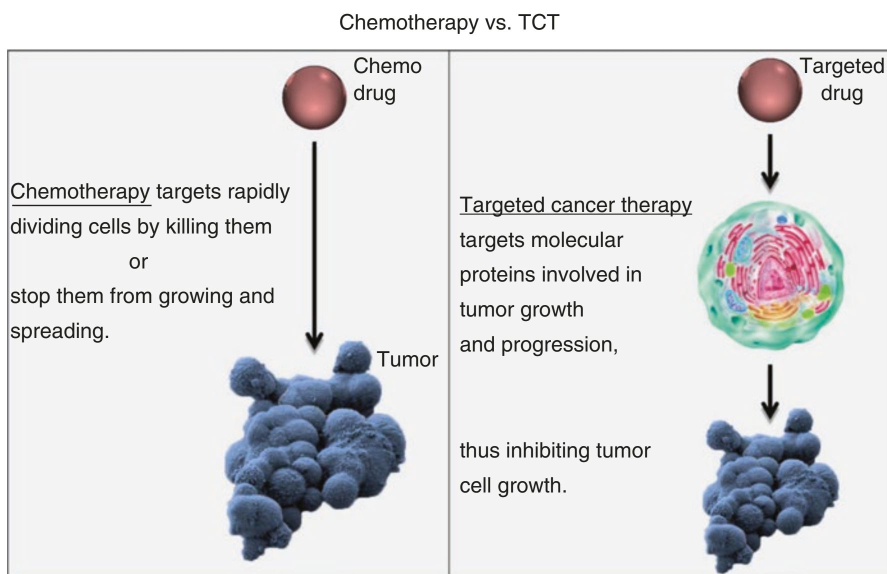

Fig. 3.2 Difference in approach of chemotherapy and targeted cancer therapy

The foremost necessity for development of TCT is identification of good molecular targets that are known to play a key role in growth, progression and survival of cancerous cells. Depending on the target, inhibitors used in TCT can be of various types. Although many approaches are being adopted by researches for this purpose, a few of these shall be discussed in length.

3.3.1 Growth Signal Inhibitors

Chemicals or substances which regulate the growth and division of normal cells are known as growth factors (GF). They attach to receptors on a cell which in turn sends a signal to inside of cell initiating a series of reactions that makes the cell grow or divide. Depending on the function they perform, GFs can be categorised into many classes. Some GFs indicate the specialisation of the cell, some make the cells grow and divide while some GFs stop the growth of cell. Examples of GFs include epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor (PDGF) and fibroblast growth factor (FGF) [13–15].

Around 1960s, role of GFs was identified in foetal growth and tissue repair which made the scientists comprehend contribution of abnormal levels of GFs to the growth of cancerous cells. Later during 1980s, changes in GF signalling were identified by scientists, which led to anomalous behaviour of cancerous cells. GF inhibitors function by either lowering the levels of GFs in the body or blocking the growth factor receptor (GFR) present on the cell or blocking the signals

inside the cell once GF interacts with the respective receptors. Cancer growth inhibitors are of many types depending on the type of chemical or substance they block [15].

The first category of growth inhibitors is tyrosine kinase inhibitors (TKIs) [16, 17]. This class of inhibitors works by blocking chemical messengers or enzymes, called tyrosine kinases, known to be involved in sending growth signals in cells. The function of these enzymes makes them a suitable target since inhibiting their function stops cell growth and division. Examples of TKIs currently in use or in clinical trials include Afatinib (Giotrif), Dasatinib (Sprycel), Erlotinib (Tarceva), Gefitinib (Iressa), Imatinib (Glivec) and Lapatinib (Tyverb) [18]. These inhibitors are oral inhibitors usually taken in the form of tablets or capsules.

Another class of growth inhibitors is proteasome inhibitors (PIs) [19]. Proteasomes are small structures present in the cell that help in breaking down of unwanted proteins. The broken down proteins are then utilised by the cell to prepare new proteins. Since it plays a crucial role in cell proliferation, therapeutic regimes that can alter the proteasomal activity can potentially repair the homeostasis in cancer patients. PIs cause a build of unwanted proteins forcing the cell to undergo apoptosis. Brotezomib is one such PI used for treatment of myeloma and is administered through an injection into the vein [20].

The third category of growth inhibitors includes GFR inhibitors. This class of drug as the name suggests interferes with the binding of GF with the corresponding GFR, disrupting cell growth and division. Cetuximab (Erbitux) is an example of GFR inhibitor.

3.3.2 Angioinhibitors

A key event in invasive tumour augmentation and tissue maintenance is establishment of sufficient blood supply by the formation of new blood vessels, a process known as angiogenesis [21]. In normal conditions, this process plays a crucial role in healing wounds and repairing damaged tissues and organs; however, in cancerous condition it becomes unregulated supporting tumour growth by providing it with blood supply and nutrients in order for it to sustain.

Inhibiting this unregulated process is known as angioinhibition that utilises drugs and chemicals to impede tumours from getting blood supply [22]. Judah Folkman from Harvard Medical School was the one to suggest this notion and in 2004, bevacizumab was the first angioinhibitor to be approved for clinical trials. There are various types of anti-angiogenesis drugs including the ones that inhibit blood vessel GF, block inter-cell (within cells) signalling and inhibit intra-cell (between cells) signalling. Bevacizumab (Avastin) [23] is an example of monoclonal antibody that inhibits VEGF, Sunitinib (Sutent) [24] is type of TKI that obstructs growth signals within the cells of blood vessels and Lenalidomide (Revlimid) [25] blocks the formation of blood vessels by acting on chemicals used for signalling between cells.

3.3.3 Drugs that Induce Apoptosis

A complicated programme of cell death with various interconnected biochemical and genetic pathways known to play a crucial role in growth and homeostasis of normal tissues is called apoptosis. This process ensures removal of unwanted cells in order to regulate the balance between cell survival and death. Evidently, inadequate apoptosis manifests cancer, marking impaired apoptotic signalling an imperative therapeutic target for anticancer drug designing. Tumour necrosis factor-α related apoptosis inducing ligand (TRAIL) belongs to the super-family of tumour necrosis factor (TNF) of cytokines is one such promising candidate that can induce cellular apoptotic mechanism in cancerous cells.

3.4 Development of Drug Resistance

In 1961, a clinical trial involving high dose four-drug combination was pioneered by Frei and Freireich for treatment of paediatric leukaemia [26]. This treatment plan attained great results with 11 out of 16 patients (enrolled for this trial) showing complete remission. However, within a short period of time most of these patients relapsed exhibiting a more aggressive form of cancer insensitive to the treatment. This account of cancer treatment and its aggressive nature towards the same explains the major obstacle, i.e. drug resistance, faced in the field of oncology drug designing.

Effectiveness of chemotherapeutics is restricted by drug resistance towards chemotherapy, one of the main treatment modes of cancer. Drug resistance can be sorted into two classes, namely, intrinsic and acquired. Presence of pre-existing resistance causing factors in cancerous cells prior to receiving chemotherapy comprises intrinsic resistance. However, development of drug resistance during or after treatment owing to factors such as mutations, over-expression of drug target or alternative signalling pathways compensating for the lost target encompasses acquired drug resistance [27].

Modern technological applications in the field of genomics, proteomics and functional analysis has led to a significant rise in our potential to recognise new genes and networks regulating the sensitivity of tumour cells to its corresponding treatment. Additionally, combination of high-throughput technology with bioinformatics and systems biology has supported the evaluation of clinical samples and aided in recognition of molecular signatures and genotypes that delineate responses to chemotherapeutics. Identification of novel drug targets for therapeutic benefits in order to overcome drug resistance is also possible using the above-mentioned approaches.

A wide variety of molecular mechanisms associated with drug resistance include increased drug efflux rates, mutation of drug target and modification in metabolism of drug.

The high adaptability of tumours, initiation of signalling pathways for survival and the inactivation of downstream death signalling pathways are also factors that

contribute to drug resistance. In addition to the above mentioned, many more factors and mechanisms facilitate drug resistance. However, since information and knowledge regarding mechanism of drug resistance has already been studied extensively in case of cytotoxic chemotherapy and since mechanism of drug resistance in chemotherapy mainly overlaps with that of TCT, earlier research can be used to predict and explain mechanism of resistance for new TCT targets.

3.4.1 A Case Study Comprehending the Structural Changes Upon Mutation and Designing a New Inhibitor for Resistant Models of EGFR

In this study [28], the structural changes in EGFR due to a drug-resistant mutation T854A were analysed and studied. Molecular dynamics simulations (MDS) and essential dynamics were employed for this purpose. Parameters such as radius of gyration (Rg), solvent accessible surface area (SASA), root mean square deviation (RMSD), root mean square fluctuation (RMSF) and ED analysis were assessed and analysed for this purpose.

The T854 residue is located in the depth of ATP binding cavity [29]. The side chain of threonine at position 854 is known to be lying in the contact vicinity of erlotinib or gefitinib (TKI) (Fig. 3.3). The wild-type (WT) and mutant (T854) structures of EGFR kinase domain was simulated for a period of 40 ns. Post analysis of the structural alterations leading to drug resistance, a QSAR study was performed to design a novel inhibitor against WT and T854A forms of EGFR.

A 3D-QSAR model that gives a statistical correlation between the structures and inhibitory activity of ligands by calculating 3D molecular descriptors (hence 3D) such as steric, electrostatic and hydrophobic points located on the 3D spatial grid was developed. For this study, a set of 38 thiazolyl-pyrazoline derivatives with inhibitory activity against EGFR were selected. The set was divided into two sets, namely, training (27 compounds) and test (11 compounds). Stepwise forward multiple regression protocol was applied for generation of the model. The model generated comprises six descriptors, namely, E_337, S_335, E_832, E_424, S_151 and

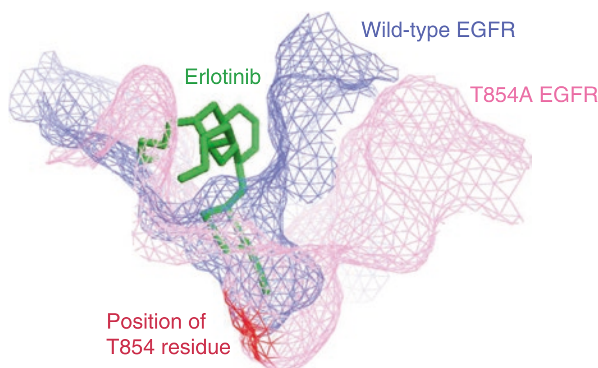

Fig. 3.3 Position of erlotinib in ATP binding cavity and effect of T854A mutation

E_721 where 'E' stands for electrostatic field energy of interactions while 'S' for steric field energy of interactions. The numbers associated with these descriptors correspond to their respective spatial grid points. Four of these grid points (E_337, E_832, S_335 and E_424) exhibited a positive contribution (8.09%, 17.77%, 5.29% and 13.37%, respectively) towards inhibitory activity against EGFR, while the remaining two descriptors (S_151 and E_463) displayed negative contribution of 24.05% and 31.44%, respectively. The generated model can be used for prediction of inhibitory activity of novel compounds based on their structure. Steric descriptors stand for the class of bulk descriptors which illustrates the size and shape of the chemical entities and fragments. Consequently, a positive contribution of a steric descriptor at the specific grid point indicates the importance of a bulky group at that position. Electrostatic descriptors, the second category of descriptors, signify the importance of electronegative and electropositive groups at a particular position of the lead compound. Thus, descriptors with positive contribution give the importance of occurrence of electropositive moieties while those with negative contribution indicate the significance of presence of electronegative groups.

The results of MDS analysis of T854A structure implied loss of stability leading to increase in flexibility. This flexibility in turn leads to structural changes rendering the T854A mutant drug resistant against erlotinib. Radius of gyration (Rg) is defined as 'the mass-weight root mean square distance of collection of atoms from their common centre of mass was helpful in giving further insight into the structural changes due to mutation and the overall dimension of the protein'. Significant deviation in Rg calculated for mutant (T854A) structure throughout simulations suggested structural alteration in the mutant structure. SASA is the surface area of a biomolecule accessible to a solvent. This parameter was observed to be stable throughout the simulations indicating that not a large change was observed in the protein structure with respect to cavity, which is supportive of a point mutation. RMSD is the change observed in the backbone of protein structure with respect to the initial structure. Evidently, a larger RMSD indicates significant changes while smaller indicate stable structure for entire period of simulations. For this study, WT showed backbone RMSD of ~0.13–~0.27 nm till 14 ns of simulations, after which WT structure exhibited minimum deviation till the end while mutant structure exhibited deviation till the end of simulation resulting backbone RMSD of ~0.13–~0.47 nm. In order to determine the effect of mutation on the individual behaviour of residues, the RMSF values for WT and T854A mutant was analysed. The results obtained suggested higher degree of flexibility in T854A mutant than WT.

In this study, two novel compounds possessing high predicted inhibitory activity for EGFR TK domain against both WT and T854A structure were reported. The reported compounds were observed to possess better predicted inhibitory activity than BIBW2992 (a second-generation T854A EGFR inhibitor) indicating their potency for further experimental studies. This study presents a compendious view of the relationship between the chemical structure and inhibitory activity of experimentally reported dataset molecules. Results of this study will also prove to be useful in designing potent anti-tumorals based on EGFR TK inhibition to further develop drugs against cancer.

3.5 Modern Cancer Treatments

The field of oncology has witnessed a remarkable advancement in early cancer detection, management and prevention of this disease. The modern treatment methods developed recently have been briefly described below.

3.5.1 Nanotechnology

Cancer nanomedicines are advancing at a steady rate while the research and growth in this area has witnessed an exponential growth since 2000s [30]. The path of drug designing and development of oncology drugs is a lengthy and tedious process while also carrying considerable risk; however, nanotherapeutics might be a successful contributor to the field of oncology drug development. A significant growth at which pharmaceutical companies are developing partnerships in order to exploit proprietary nanotechnologies has been observed [31]. Nanotechnology can contribute to develop differentiated products and upgrade clinical outcomes by augmenting the efficiency of novel lead candidates.

Nanotherapeutics furnishes the advantage to encapsulate drugs with poor solubility [8, 9], shield therapeutics and alter the blood circulation and tissue distribution [11, 12]. Such characteristics for encapsulating cytotoxics possessing widespread physiological characteristics and toxicities are favourable in oncology. Another advantage offered by nanotechnology is that they can expedite the combination protocols usually followed in cancer treatment. Encapsulation of the pharmaceutical ingredient by one nanoparticle might potentially provide coordinated effects in order to elevate the efficiency of treatment therapies, by reducing the chances of resistance. Each drug performs its own different pharmacology when multiple drugs are delivered separately. Owing to the difference in pharmacokinetic and pharmacodynamic properties of drugs, the fact that target cells and tissues will receive optimum levels of the pharmaceutical entity at the same time is uncertain. On the contrary, simultaneous exposure of optimum amount of multiple drugs can be ascertained and controlled when combined in a single nanoparticle which may lead to a synergistic action. The timely co-delivery to cancer cells of multiple agents inhibiting distinct, essential pathways could provide improved anticancer effects.

3.5.2 Personalised Cancer Therapy

Over the past decade, a slow and steady change has been observed in the approach of cancer therapy from a 'one size fits all' to a more individualised approach, which treats each patient based on the particular genetic defect in the tumour. The foremost requisite for this personalised therapy is identification and development of biological indicators (also known as Biomarkers) that assist the doctors in deciding the patients to be treated and the therapy most probable to be beneficial for the patient [32].

Hence, even though integrative genome characterisation can produce all genomic and statistical support for novel cancer therapeutic targets, changing them to agents for therapeutic purposes and biomarkers will necessitate intense biological information for drug discovery and in order to outline a clinical path for drug development. Previously, gaining such detailed knowledge from functional, mechanistic and clinical studies has been observed to be unproductive, inept and arbitrary leading to a highly inefficient trail of shifting a genomic discovery to clinic. Our capability to utilise the complete potential of cancer genome to provide individualised therapy will gain from a new concept of transdisciplinary team science that balances the conventional unrestrained exploration in research and greatly synchronised efforts with an aim to convert cancer genomics into verified therapeutic or diagnostic targets.

3.5.3 Immune Therapy

The success achieved by cancer immunotherapy has revealed the strength of the immune system to eliminate tumours, resulting in reinvigorated interest for developing techniques to stimulate anti-tumour immune responses. Natural anti-tumour immune responses can be identified in a few patients with multiple malignant tumours and can also be reactivated by aiming for rate-limiting immunosuppressive mechanisms [9].

Even though radiotherapy and immunotherapy are considered distinct branches of medical oncology, both have progressed rapidly over the past decade. However, biological evidences now suggest that the success of radiation therapy can also be owed to the immune system. Moreover, recent information of tumour models and in patients indicates that treatment responses can be triggered by irradiation. This can be explained by the help of abscopal effect, a phenomenon which witnesses the degeneration of metastases upon exposing the primary tumour to radiation, in spite of the metastasis lying away from the radiation field. This effect is acknowledged to be immune mediated. A possible explanation for this from a molecular perspective involves convergence of radiation therapy and immune therapy on apoptotic pathways, initiating the release of cytochrome c from mitochondria leading to development of the apoptosome in turn activating the caspase network, eventually engendering tumour cell death. The outcomes of Human Genome Project, new developed bioinformatics tools and optimised immunological analytical tools facilitate any protein to be examined for immunogenic epitopes that might be integrated into new upcoming therapies.

3.5.4 Drug Repositioning for Cancer Therapy

Owing to the constant rise in failure rates, unsatisfactory safety profile, high cost and restricted efficiency linked with anti-tumour drug development, the repositioning or repurposing of recognised non-cancer drugs for novel oncology symptoms has developed as an appealing protocol to address the required cancer-related

medical essential [33]. With the quick growth of bioinformatics and its tools, drug repositioning is being facilitated by a significant outflow of in silico protocols.

Owing to the numerous disadvantages of traditional de novo drug development pipeline such as time consuming and expensive, repurposed or repositioned drugs whose preclinical and toxicological studies have been performed in humans manifests a faster and cost-effective protocol. Drug repositioning encompasses a wide range of oncological application, with some drugs exhibiting successful translation to the novel identified anticancer indication after initial failure while others being successful in both original and repositioned application. One example of a repositioned drug is Thalidomide [34] which was introduced in 1957 as an antiemetic in pregnancy. However, owing to its severe teratogenicity, it was later banned. This drug was then used for treatment of leprosy and later in 1990 its inhibitory activity was reported against TNF. Novel repurposes of thalidomide and its analogue lenalidomide observed marked improvement for treatment of multiple myeloma. This drug is thought to work in a mechanism similar to immunomodulatory drugs by inhibiting the production of interleukins, interferons (FN)-g and vascular endothelia growth factor (VEGF).

3.6 Summary

The new era has dawned in the field of oncology drug designing and development. Conventional methods of treatment and post-treatment problems have led to a change in culture of cancer research and treatment. The changes comprise a sturdy and well-communicated relationship among the scientists, other physicians and oncologists in world of academia and those in community hospitals. Training programmes providing knowledge to graduate students in the basic sciences in order to comprehend the issues faced by cancer, criteria for growth and development in academia, assurance of access to the molecular datasets developed with public funding is also needed.

Instead of replacing the conventional treatment methods, a multimodal approach of treatment offers better and more effective results. For instance, a major treatment method called chemotherapy integrates and complements local therapy approaches such as radiotherapy instead of replacing surgery. Immunotherapy in combination with local treatments also promises good results. However, optimisation of interactions in these treatment methods needs large research effort. Novel therapies together with traditional treatment methods seems to be the most effective approach in combating cancer and tackling the problems associated with it.

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Competing Interests The authors declare that they have no competing interests.

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4 Contemporary Approaches for Malaria Drug Discovery

Vijeta Sharma, Sonal Gupta, and Shailja Singh

4.1 Introduction

Malaria is one of the most life-threatening infectious diseases responsible for morbidity and mortality cases worldwide. The World Health Organization (WHO) Malaria Report 2016 estimates 212 million cases of malaria globally with 429,000 deaths [1]. Almost 88% of deaths occur in Africa having the heaviest burden wherein 71% of all deaths occurring in children under the age of 5 years [2]. Although the data shows significant decrease in prevalence of the symptomatic and asymptomatic parasitic infections in sub-Saharan Africa since 2000 [3], stil malaria remains the major cause of death of a child every 2 min particularly in sub-Saharan Africa region. Malaria is caused by an Apicomplexan parasite of genus Plasmodium whereas it is transmitted by infected female mosquito *Anopheles* spp. Human malaria is caused by four main spp. of Plasmodium viz. *P. falciparum*, *P. malariae*, *P. vivax*, and *P. ovale*. Recent human cases due to *Plasmodium knowlesi* were reported. Out of these, *P. falciparum* and *P. vivax* pose the greatest public health

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challenge wherein *P. falciparum* is responsible for the majority of the deaths and its infection predominates in Africa [4]. It is characterized by the intermittent episodes of high fevers and severe anemia and can lead to severe life-threatening neurological manifestations in case of cerebral malaria [5]. Despite many efforts, the development of a vaccine is unsuccessful and chemotherapy is the cure against infections currently [6, 7]. WHO's Strategic Advisory Group of Experts on Immunization (SAGE) and the Malaria Policy Advisory Committee (MPAC) recommended the first malaria vaccine RTS,S/AS01 after receiving positive response from the European Medicines Agency under Article 58 for the chemoprevention in children. Artemisinin combination therapy (ACT) is the gold standard for malaria treatment as the first line of treatment recommended by WHO [8]. Notably, chemoprevention is effective in pregnant women and young children [2]. The use of ACT in malaria-endemic countries resulted in reduction in malaria burden. In ACTs, the functional role of the artemisinin drug is to reduce the parasite load during the first 3 days of treatment, while the partner drug eliminates the remaining parasites [9]. The resistance to ACTs has been detected and spread in many malaria-affected countries [10, 11]. WHO recent report confirmed artemisinin resistance in five countries of the Greater Mekong subregion (GMS): Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand, and Vietnam are malaria-affected countries (WHO/HTM/GMP/2016.11). The recent findings linked mutations in parasite Kelch 13 (K 13) propeller domain with the delayed parasite clearance in vitro and in vivo [12, 13]. With *P. falciparum* becoming resistant to almost all available antimalarial drugs, the risk of multidrug resistance will be soon emerging worldwide. Considering increasing resistance to available drugs, there is an immediate need for the development of a new class of antimalarial drugs with the strategy to control and eliminate all stages of malaria parasite life cycle including blood, liver, and transmission stages [14].

The malaria parasite has a complex life cycle and completes asexual stage of cycle in the human host and sexual stages in mosquito vector. All the signs and symptoms are attributed to the infections in the human host during the asexual blood stage of the parasite life cycle. This is due to repeated invasion and lysis of the erythrocytes by parasite in the blood stage. Owing to this fact, the current therapies aim the blood-stage parasite. Development of new effective antimalarial drugs with the effect against blood-stage, anti-gametocyte for blocking transmission and controlling vector is very much required to eradicate this deadly disease (Fig. 4.1) [15].

Nonetheless, the malaria interventions so far are affordable and highly effective which comprise vector control to block transmission to human host. This includes use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (INR) and larval control along with the chemoprevention of blood-stage infection in humans [16, 17]. In addition, growing funding and awareness will hasten the interventions attributed to the decline in the mortality rate in malaria endemic regions [18].

Better understanding towards the molecular basis of host-pathogen interactions and the mechanisms of the resistance against the drugs would lead to the development of the new drugs to eliminate malaria through and through [19].

Eradication of the malaria would require a perfect medicine SERCaP as described by a global agenda for malaria eradication 2007, where SERCaP stands for the single-exposure redial cure and prophylaxis. A drug targeting multiple stages in parasite life cycle would be an ideal solution to combat the disease, however few compounds with this capability have been identified so far [20, 21]. Therefore the combination of drugs having a two or more class of compounds having different targets would solve the purpose. ACT is the example of such class of drugs, but then the question remains that how to treat it with a single exposure as the latter has a course of continuous exposure of 3 days of doses [22, 23]. Given that the development of a new class of drugs as chemoprevention with a broad range of activity is inevitable.

In this chapter, we discuss the current therapies and new developments in the antimalarial drug discovery, including chemically bioactive compounds and chemically validated targets which would be promising in changing the outlook and development of an effective strategy for the malarial cure. We describe here the discovery of the antimalarial drugs with activity against different stages of the life cycle of the parasite and the methods and techniques available.

4.2 Drug Discovery: An Overview

New technologies and advances have enabled the development of new drugs as antimalarials. Diverse strategies and technologies are being used for the development of novel antimalarial drugs. High-throughput screening and proof-of-principle studies have enabled it to be easier to unravel new drugs with broad activity against different stages of the malaria parasite [24, 25].

Broadly phenotypic screening-based drug discovery and target-based drug discovery remained to be fascinating and advantageous in the identification of a new class of potential compounds which are active against the parasite [26].

4.2.1 Phenotypic Screening-Based Drug Discovery

Phenotypic screening is the traditional method which had always been the most attractive in the development of the antimalarials, as a major leap forward in the drug discovery era has come from this unexpected [27]. Most of the active antiparasitic compounds, currently being used or are under development, are the result of phenotypic screening; however their targets are identified, eventually. It requires the identification of the antiparasitic active compounds by the use of whole cell-based assays in which the effect of the compounds on the growth and proliferation of the parasite is observed after treatment with the library of novel chemical scaffolds.

The assay involves the exposure of the test compound to the parasite culture after a short incubation time and examining for their potency to inhibit the growth by killing the parasite. These types of screening methods are commonly used against the liver stage as well as the blood stages of the parasites as well as transmission

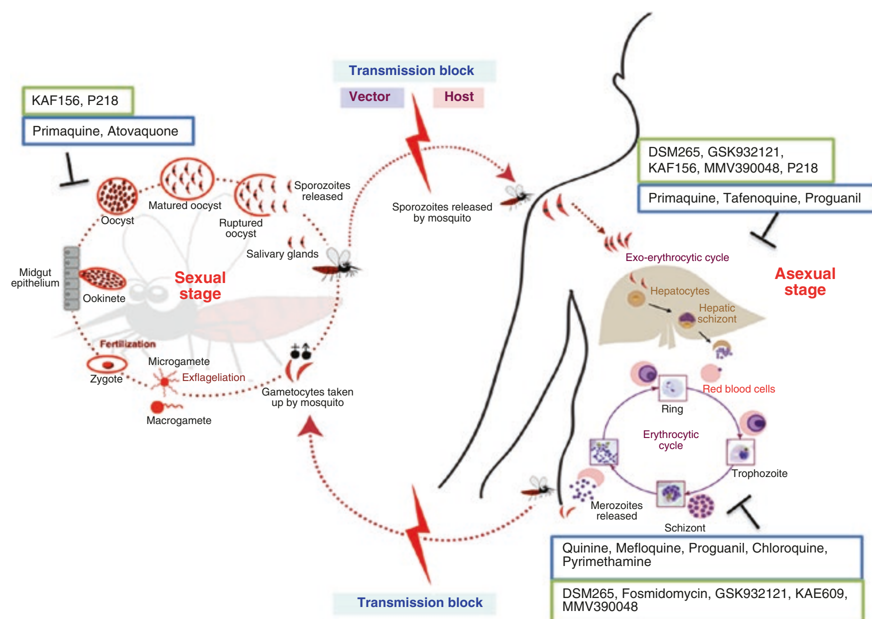

Fig. 4.1 Mode of action of antimalarial drugs at different stages of Plasmodium life cycle: The parasite completes its life cycle: sexual stage in mosquito vector (female Anopheles spp.) and asexual stage in human host. The signs and symptoms are due to the infection during erythrocytic cycle. The transmission of the parasite occurs as gametocytes in red blood cells and from human to mosquito and mosquito to human as sporozoites from salivary glands of mosquito during a blood meal. The closed boxes show site of action of antimalarial drugs (*blue*: conventional antimalarial drugs; *green*: present-day new antimalarial drugs)

block assays to detect the effect on ookinete development and gametocyte formation [28, 29]. The lead screens obtained from screening the libraries of compounds showing best antimalarial activity are assessed for lead optimization, target validation, and identification [30]. In the last few years, the advances in technologies and high-throughput cell-based screening have enabled the screening of almost six million compounds, out of which around 25,000 of these compounds showed good half maxima-inhibitory concentration of 1 μM or lower against *P. falciparum* [31]. Recent advances come up with "genetic validation" strategy, which involves either deletion of gene of interest or conditional knockout of gene at a particular parasite stage [32, 33]. Then phenotypic study of mutant parasite determines the importance of target at different stages of *Plasmodium* life cycle.

4.2.1.1 Pipeline of the Phenotypic Screens

Out of the compounds developed through this approach and are already in use or are under development, the most potent and advanced, is the KAE609 also known as cipagamin or NITD609 [34]. This compound is developed as a result of the combined effort of the global consortium involving collaboration between the Novartis

Institute of Tropical Diseases in Singapore, its Genome Foundation in San Diego, USA, and The Swiss Tropical and Public Health Institute [35, 36]. The novel mechanism of action of the spiroindolone class is identified and hence the target of this drug was discovered later. It has been reported that this drug targets the outer membrane transporter P-type ATPase 4 reported to be important for maintaining the sodium homeostasis in the parasite [37, 38]. KAE609 has cleared the phase I trial and completed human patient trial in Thailand very quickly. It shows seven times more potency and hence kills faster than artesunate and doses used also are relatively low. It is believed to be submitted for the approval in 2017 as reported by Novartis [39].

Another potential candidate is MMV390048 developed by phenotypic screening. It has developed by a team led by the University of Cape Town, South Africa [40]. It is the lead compound obtained from the screening of cluster 4 aminopyridines. Its target was identified to be phosphatidylinositol (PfPI4K) with preclinical development. It has successfully completed the preclinical program and has entered phase I human studies in Cape Town, 2014 [41]. Another class of scaffolds developed by GSK are screened from the database of Neglected tropical disease archive which includes a collection of 13,000 compounds designated as Tres Cantos Antimalarial Set or TCAMS [27]. The scaffolds which showed high potency were narrowed down such as indoline core-containing compounds TCNDC-139046, 5-hydroxytryptamine 2C, and another class of scaffold cyclopropylcarboxamides having lead compound GSK10557714 [42, 43]. The latter showed in vivo activity in *P. falciparum* SCID mouse model [44, 45]. Compounds identified from the liver-stage screens include GNF179, imidazolopiperazine class, but its actual target is believed to be the cyclic amine resistance locus protein PfCarl but it is not proved experimentally [46]. Further lead optimization of this class of scaffolds led to the filtering of KAF156 (GNF156) which is under phase II clinical trials and provides prophylactic protection and shows anti-gametocyte activity [47, 48].

4.2.1.2 Medicinal Chemistry Approach and Other Ventures

Malaria Box, a collection of 400 publicly available compounds distilled from the original 25,000 hits obtained in the high-throughput screening, can be obtained from the Medicines for Malaria Venture (MMV) which is freely available. This enables the researchers in the malaria drug discovery process to identify and collaborate in order to elucidate the mechanism of action of the potential compounds. The artemisinin combination therapies prescribed for the treatment of uncomplicated malaria are artemether–lumefantrine (commercial name: Coartem; partner company: Novartis), modiaquine–artesunate (commercial name: Coarsucam; partner company: Sanofi-Aventis), dihydroartemisinin–piperaquine (commercial name: Eurartesim; partner company: Pfizer), artesunate–pyronaridine (commercial name: Pyramex; partner company: Shin PoongPharamaceuticals), artesunate–mefloquine (commercial name: ASMQ; partner company: Cipla/Cephalon/Mepha), and artemisinin–naphthoquine (commercial name: ARCO; partner company: AMMS, Kunming Pharma Corp) [49].

Here, various medicinal chemistry approaches are adopted to study Structure Activity Relationships (SAR) of the scaffolds through cheminformatic analysis, which are inspired by the chemical structures of the known class of drugs. The SAR studies reveal the properties of the compounds and how they could be modified chemically to improve the properties such as potency, bioavailability, and solubility [50]. Some drugs, which are a result of such approaches, are OZ227, a first-generation synthetic ozonide which is potent as artesunate but showed low efficacy after phase II trial [51, 52]. However, it's been approved for use in India in 2012 by Ranbaxy Laboratories in combination with piperaquine. Other synthetic ozonides are OZ439 also known as artefenomel, which is fast acting; with improved bioavailability it has progressed to phase IIb in combination with piperaquine [53, 54]. RKA182, discovered by University of Liverpool, UK, is undergoing preclinical trials and has high potency [55].

4.2.2 Target-Based Drug Discovery

Another approach, which has been used extensively, is the target-based drug discovery for the identification of the lead compounds. The targets could be proteins having crucial cellular and molecular functions which show druggability and inhibition of which could prove cytocidal. Target-based screening initiatives are there such as Innovative Medicine Initiative's European Lead Factory [56, 57]. The potential targets are mostly channels or enzymes, which are identified using biochemical inhibition studies and essential for the parasite survival. Most common drug targets in *Plasmodium* are as follows: proteases involved in haem polymerization, kinases, transporters, and apicoplast-based fatty acid synthesis pathway enzymes [58–61]. The target selection can be done by chemical validation, which requires specific inhibitor active against particular target of interest [62].

4.2.2.1 Potential Targets and the Pipeline

Various targets are known which are enzymes and proteins chemically validated in *P. falciparum.* The clinically validated targets known in *P. falciparum* are dihydrofolate reductase (DHFR), cytochrome *bc*1, mitochondrial enzyme dihydroorotate dehydrogenase (DHOD), cyclic amine resistance locus (PfCARL), sodium-ATPase4 (PfATP4), phosphotidylinositol-4 kinase, and DXP reductoisomerse (PfDXR) [63–69]. Among them PfATP4 is the target of 7% of the Malaria Box drugs. Intriguingly, new antimalarial targets have been identified in the protein biosynthetic pathway as three tRNA synthetases [70]. The molecules under investigation against these targets are listed in Table 4.1.

The analysis and identification of new novel targets would give new directions in the target-based screening of the drugs. However the elucidation of the targets and the inhibition of those targets in the multiple stages of the parasite life cycle have always been challenging.

Table 4.1 List of validated antimalarial targets and the key molecules under investigation

Molecules (under investigation)	Potential targets (validated)	Chemical class
DSM265	Dihydroorotate dehydrogenase (PfDHODH) (63, 68)	Triazolopyrimidine
Fosmidomycin	DXP reductoisomerase (PfDXR) (65, 67)	Antibiotic
GSK932121 (ELQ300)	Cytochrome bc_1 ($PfCYTbc_1$) (64)	Quinolone-3-diarylether
KAE609 (NITD609)	Sodium-ATPase 4 (PfATP4) (37, 38)	Spiroindolone
KAF156 (GNF156)	Cyclic amine resistance locus (PfCARL) (39, 48)	Imidazolopiperazine
MMV390048	Phosphatidylinositol-4-kinasen (PfPI4K) (41)	Aminopyridine
P218	Dihydrofolate reductase (PfDHFR) (66)	Diaminopyridine

In addition to this, the ancient remedies had been shown to have antimalarial activities. Quinine and artemisinin are the result of such remedies; the latter is derived from the Chinese ancient remedy that was used to treat fever [71, 72]. Others are synthetic modification of the natural products such as atovaquone which is derived from the natural product Lapichol [73]. Other examples of natural products, which have fever-reducing properties, are the bark of *Nauclea pobeguinii*, *Argemone mexicana*, and so on [74, 75]. The compounds obtained from these natural products are under phase clinical trials.

4.3 Conclusion and Future Directions

Malaria poses a serious burden worldwide being the disease affecting billions globally. The mortality and morbidity are high which encourage the development of new strategies to treat the disease. The funding agencies and the initiatives combining with the expertise and technology would make the development of antimalarial drug possible with broad effect against all stages of the parasite. WHO developed the *Global technical strategy for malaria 2016–2030*, in close alignment with Roll Back Malaria Partnership's Action and investment, which was adopted by the World Health Assembly in May 2015 to meet the challenges and defeat malaria for a malaria-free world. This targets the reduction in malaria cases and death toll.

Currently, many groups and ventures are involved with the aim to develop the SERCaPdrug which seems to be happening owing to the efforts and progress made. The main challenge in the antimalarial drug discovery is the investigation of new compounds having potency against all stages and also that should clear the human trials. The drug should be cost effective and transmission blocking, could treat the relapse of the parasite, and also should be refractory towards the resistance spread. The gaps should be filled by the development of a new class of drugs meeting these which has been the most challenging task. The endeavors at the academia-industry

front and the funding agencies made it possible to identify the promising potential drugs that block or suppress the transmission. Nevertheless, more and more efforts are prerequisite using the comprehensive drug discovery approach for the development of a single-cure antimalarial.

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The Story of Kinase Inhibitors Development with Special Reference to Allosteric Site

5

Pabitra Mohan Behera and Anshuman Dixit

5.1 Introduction

Protein kinases are a group of enzymes which play a significant role in every aspect of cellular metabolism. [1] Regulation of kinase activity is crucial in various biological processes like proliferation, apoptosis, cell cycle, differentiation, development, and transcription. [2] Dysregulation of kinase activity has been associated with a variety of diseases including cancer [3–5], diabetes [6], autoimmune, cardiovascular [7], inflammatory [8, 9] and nervous system, etc [10].

The human genome contains 518 (478 typical and 40 atypical) protein kinase genes; they constitute about 2% of all eukaryotic genes. The typical protein kinases are grouped into three major groups (i) protein-serine/threonine kinases (385), (ii) protein-tyrosine kinases (90), and (iii) tyrosine-kinase like proteins (43) based on the phosphorylation of the –OH group of amino acid on target proteins.

The major function of protein kinases is the phosphorylation of proteins that can either stimulate or inhibit the protein functionality. The kinases as mediators of protein phosphorylation are very important in disease pathophysiology (e.g. cancer) by means of mutational activation or by helping the neoplastic growth. Though their activity is tightly regulated in normal cells, they may acquire transforming capabilities due to mutations, overexpression and autocrine-paracrine stimulation. Mutationally activated kinases are in constant state of activation, and with their transforming capability, they become ideal for survival and growth of the cancer cell leading to the dependence of cancer cells (oncogene addiction). It also renders these cancer cells highly susceptible to specific kinase inhibitors [11, 12]. Kinases are considered one of the most important classes of drug targets and design and development of specific kinase inhibitors, has therefore, became a major strategy in drug

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discovery programs. The interest of biomedical research in kinases can be gauzed from the fact that about 5000 crystal structures [13] and 400,000 publications have been reported in PDB and PubMed, respectively, in the last three decades.

5.2 The Structure of Protein Kinase Domain

The elucidation of the crystal structure of PKA in 1991 by Dr. Susan Taylor's group was a significant achievement for understanding the molecular basis for kinase function. Since this discovery the crystal structures of nearly 200 different protein kinases have been determined, amounting to more than 5000 X-ray structures being publicly available.

The protein kinases have a small N-terminal lobe and a large C-terminal lobe in their highly conserved kinase domain. The two lobes are joined by a short hinge region which helps in opening and closing of the kinase structure [14] (Fig. 5.1a). The ATP

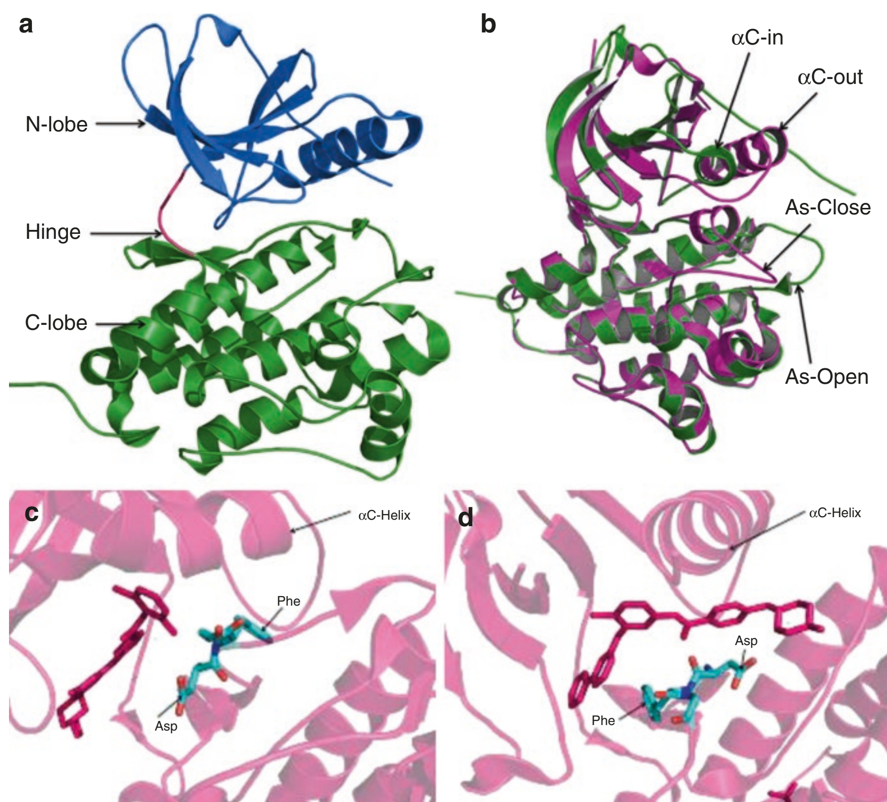

Fig. 5.1 Structural features of protein kinases. (**a**) The 3D structure of a kinase showing N-terminal lobe, hinge region, and C-terminal lobe (**b**) Structures of kinases showing both αC-in and αC-out conformations, activation segment open and close conformations (Alignment of structures PDB ID: 1M17 and PDB ID: 4HJO) (**c**) Kinase structure showing an active DFG-in conformation (PDB ID: 2GQG) (**d**) Kinase structure showing an inactive DFG-out conformation (PDB ID: 3K5V)

Table 5.1 Summary of regulatory features within the kinase domain

Sl. No	Features	Active conformation	Inactive conformation
1	αC-helix	in	out
2	Activation segment (As)	open	close
3	DFG	in	out

binds near hinge region between these two lobes. The small lobe comprises five stranded anti-parallel β-strands (β1–β5) and a αC-helix. The N-terminal has a conserved glycine-rich (GxGxxG) loop in between the β1 and β2 strands. This loop is known as p-loop that helps in positioning β and γ-phosphates of ATP for catalysis. The glycine-rich loop is followed by conserved valine residue which makes hydrophobic contact with adenine of ATP. The third β strand has a sequence of Ala-xxx-Lys, from which the lysine residue couples with α and β phosphates of ATP to αC-helix. The conserved glutamate residue in the center of αC-helix plays an important role in activation and inactivation of the kinases. The formation of a salt bridge between the glutamate of αC-helix and lysine of β3 strand corresponds to αC-in (active) conformation [15], whereas the absence of it leads to αC-out (inactive) conformation of kinases (Fig. 5.1b). The large lobe comprises six α-helical segments (αD–αI) and four short conserved β-strands (β6–β9) [16]. The αE-helix is followed by the β6 strand, the catalytic loop, the β7 strand, β8 strand, and the activation segment containing the β9 strand. The activation segment forms an open structure by extending itself from the catalytic loop and forms the active state of the kinase, thus allowing the binding of substrates. The catalytic loop of kinases has a signature motif of K/E/D/D (Lys/Glu/Asp/Asp) from which the first aspartate residue facilitates the nucleophilic attack of the hydroxyl group onto the γ-phosphorous atom of ATP after withdrawing a proton from –OH group. The second aspartate introduces as the first residue of activation segment and plays an important role in activation and deactivation of protein kinases. In most kinase, the activation segment comprises 20–30 amino acids in length, starts with D/F/G (Asp/Phe/Gly), and ends with A/L/E (Ala/Leu/Glu) signature motifs [17]. The kinases, in general, can interconvert between two conformational states viz. active and inactive. In the active kinase conformation, the aspartate of DFG motif points into the ATP-binding site and coordinates with two Mg^{+2} ions; thus, the activation segment acquires an open and extended conformation (Fig. 5.1c). In contrast, an inactive kinase conformation is characterized by a flipped conformation (180°) of the DFG motif (i.e.) the aspartate of the DFG motif moves away from the ATP binding site (Fig. 5.1d). The structural features signifying the active and inactive kinase conformations are summarized in Table 5.1.

5.3 Protein Kinase Inhibitors

The kinases gained great attention from medicinal chemists for design of their modulators. Since the discovery of Imatinib by Novartis in 2001 many pharmaceutical companies have reported a variety of inhibitors and about 33 kinase inhibitors have been approved by USFDA by March 2017. These inhibitors are classified into different types depending on their binding in the kinase domain. The Table 5.2 enlists all the small molecule kinase inhibitors approved by USFDA.

Table 5.2 The FDA-approved kinase inhibitors

Sl. No.	Inhibitors	Company	Year	Targets
1	Sirolimus	Wyeth	1999	FKBP/mTOR
2	Imatinib	Novartis	2001	BCR-Abl, Kit, and PDGFR
3	Erlotinib	Genentech	2004	EGFR
4	Sorafenib	Onyx	2005	B-/C-Raf, mutant B-Raf, Kit, Flt3, RET, VEGFR1/2/3, and PDGFRß
5	Dasatinib	Bristol-Myers Squibb	2006	BCR-Abl, Src, Lck, Yes, Fyn, Kit, EphA2, PDGFRβ
6	Sunitinib	Pfizer	2006	PDGFRα/β, VEGFR1/2/3, Kit, Flt3, CSF-1R, and RET
7	Lapatinib	GSK	2007	EGFR, ErbB2
8	Nilotinib	Novartis	2007	BCR-Abl, PDGFR, DDR1
9	Temsirolimus	Wyeth	2007	FKBP12/mTOR
10	Everolimus	Novartis	2009	FKBP12/mTOR
11	Pazopanib	GSK	2009	VEGFR1/2/3, PDGFRα/β, FGFR1/3, Kit, Lck, Fms, Itk
12	Crizotinib	Pfizer	2011	ALK, c-Met (HGFR), ROS, MST1R
13	Ruxolitinib	Incyte	2011	JAK1/2
14	Vandetanib	Astra-Zeneca	2011	EGFRs, VEGFRs, RET, Brk, Tie2, EphRs, and Src family kinases
15	Vemurafenib	Genentech	2011	A-/B-/C-Raf and B-Raf (V600E)
16	Axitinib	Pfizer	2012	VEGFR1/2/3, PDGFRβ
17	Bosutinib	Pfizer	2012	BCR-Abl, Src, Lyn, and Hck
18	Cabozantinib	Excelixis	2012	RET, MET, VEGFR1/2/3, Kit, TrkB, Flt3, Axl, Tie2
19	Ponatinib	Ariad	2012	BCR-Abl, BCR-Abl T315I, VEGFR, PDGFR, FGFR, EphR, Src family kinases, Kit, RET, Tie2, Flt3
20	Regorafenib	Bayer	2012	VEGFR1/2/3, BCR-Abl, B-Raf, B-Raf (V600E), Kit, PDGFRα/β, RET, FGFR1/2, Tie2, and Eph2A
21	Tofacitinib	Pfizer	2012	JAK3
22	Afatinib	Boehringer Ingelheim	2013	EGFR, ErbB2, ErbB4
23	Dabrafenib	GSK	2013	B-Raf
24	Ibrutinib	Pharma-cyclics and J&J	2013	Bruton’s kinase
25	Trametinib	GSK	2013	MEK1/2
26	Ceritinib	Novartis	2014	ALK, IGF-1R, InsR, ROS1
27	Nintedanib	Boehringer Ingelheim	2014	FGFR1/2/3, PDGFRα/β, VEGFR1/2/3, Flt3
28	Alectinib	Hoffman-LaRoche	2015	ALK and RET
29	Cobimetinib	Genentech	2015	MEK1/2
30	Lenvatinib	Easai	2015	VEGFR1/2/3, PDGFR, FGFR, Kit, RET

Table 5.2 (continued)

Sl. No.	Inhibitors	Company	Year	Targets
31	Osimertinib	AstraZeneca	2015	EGFR T970M
32	Palbociclib	Park Davis	2015	CDK4/6
33	Gefitinib	AstraZeneca	2015	EGFR

http://www.brimr.org/PKI/PKIs.htm

5.4 Classical or ATP-Competitive Inhibitors

The type I inhibitors bind to the active kinase conformation forming H-bonds with the kinase hinge region residues and occupy the adenosine binding pocket. Their binding is independent of the conformation of key structural elements, e.g., helix αC and the DFG. Since the residues at and near the ATP binding site are highly conserved and the unique shape of the adenine site allows for only a little variation in the heterocyclic system, almost all of the inhibitors share only a small number of heterocyclic rings and are typically entropically constrained. Consequently, it is difficult to design type I inhibitors with high selectivity [18].

The type II kinase inhibitors bind to and stabilize the inactive conformation of the kinase thereby preventing the binding of ATP and subsequent activation of the kinase. Similar to type I inhibitors they also occupy adenosine binding pocket. However, they induce a configuration of DFG residues termed DFG-OUT [19]. These inhibitors bind to the ATP binding site and thus are considered as ATP-competitive inhibitors. However, the inhibitors that bind to the inactive conformation face weaker competition from cellular ATP. They may act primarily by shifting the equilibrium between conformational states in a way that prevents kinase activation, rather than by inhibiting kinase activity directly [20]. An advantage of type II inhibitors over type I inhibitors is due to the fact that the amino acids surrounding the newly exposed pocket (due to DFG shift) are less conserved as compared to those in the ATP binding pocket. Thus, it is possible to design inhibitors exploiting this difference to achieve better kinase selectivity [21, 22].

The inhibitors of type-I and type-II are further divided into subtypes A and B depending on their interactions with the residues present in the kinase domain. The type A inhibitors mainly bind to the residues in the front and back cleft, gatekeeper area, and the region separating the small lobe from large lobe. The type B inhibitors bind to the residues present in the front cleft and gatekeeper area only.

5.5 Allosteric or Noncompetitive Inhibitors

Mutations resistant to classical ATP-competitive (Type I/II) inhibitors are quickly emerging and as such often limit the success of targeted cancer therapies. These mutations often result in a steric hindrance obstructing inhibitor binding to the hinge region of the ATP pocket. Some of these mutations may result in increased affinity

for the ATP that shifts balance against a competitive inhibitor. The allosteric inhibitors constitute a group of structurally diverse compounds that bind in sites other than the ATP binding site. These inhibitors (e.g., type III inhibitors) have shown promise toward addressing mutation-dependent drug resistance. They have also been utilized to make more selective inhibitors as they bind in a remote pocket that may not be conserved. Therefore, the identification and development of such inhibitors is the focus of many drug discovery projects.

Type III inhibitors: Certain kinases have an allosteric pocket, adjacent to ATP binding site, where an inhibitor can bind along with ATP. These inhibitors do not interact with hinge residues; they block kinase activity without displacing ATP [23]. We will discuss some of these inhibitors below.

The ERK/MAP kinase cascade dysregulation is implicated in cancer. Mutations in upstream RAS and Raf occur often and contribute to the oncogenic phenotype through activation of MEK and then ERK. The Ras mutations lead to activation of the Raf-MEK-Erk kinases. A lot of research has been done on identification of inhibitors for these kinases. The MEK kinase is one of the targets for which type III inhibitors have been identified. The Parke-Davis identified an inhibitor (PD 098059) that prevented MEK activation of MAPK. This molecule showed a high degree of selectivity over closely related kinases. The co-crystal structure of MEK1 with another compound (PD 318088) showed co-binding of ATP and the inhibitor (PDB ID: 1S9J). This was the first type III inhibitor that binds in the active site without interfering with ATP binding [24, 25]. Another molecule Trametinib is a first US-FDA approved type III inhibitor targeting MEK1 (mitogen-activated kinase-1) for the treatment of B-raf mutated (V600K/E) metastatic melanoma [26, 27, 28]. It binds in the allosteric back pocket region with DFG-in configuration (Fig. 5.2a). The allosteric back pocket refers to a distinctive pocket adjacent to the ATP binding pocket.

Iwatani et al. reported a highly selective series of 1,5-dihydropyrazolo[4,3-c][2, 1]benzothiazines for allosteric inhibition of focal adhesion kinase (FAK) involved in regulation of cellular survival and proliferation (Fig. 5.2b). These compounds were non-ATP compititive inhibitos of FAK [29]. The co-crystal structural analysis revealed that these inhibitors specifically bind to a novel allosteric site within the C-lobe and induce disruption of ATP pocket formation. Interestingly, the phosphorylation of FAK leads to a reduction in allosteric inhibition potency. The structure activity relationship analysis of these compounds indicated that N-substitution of the pyrazole ring is important for achieving allosteric binding and high selectivity among kinases. Tomita et al. employed synthetic medical chemistry approach for the development of potent and selective FAK inhibitors [30]. They used pyrazolo[4,3-c][2, 1]benzothiazines to target the FAK allosteric site. The lead molecule had significant FAK kinase inhibitory activities for cell-free (IC_{50} = 0.64 1M) and cellular assays (IC_{50} = 7.1 1M).

Diarylamine compounds form a major group of allosteric MEK1/2 inhibitors. The molecule cobimetinib has been studied in combination with vemurafenib for the treatment of B-Raf V600E/K mutation-positive advanced melanoma. In 2015, the U.S. Food and Drug Administration (FDA) approved the use of cobimetinib in combination with vemurafenib to treat patients with advanced stages of melanoma [31, 32].

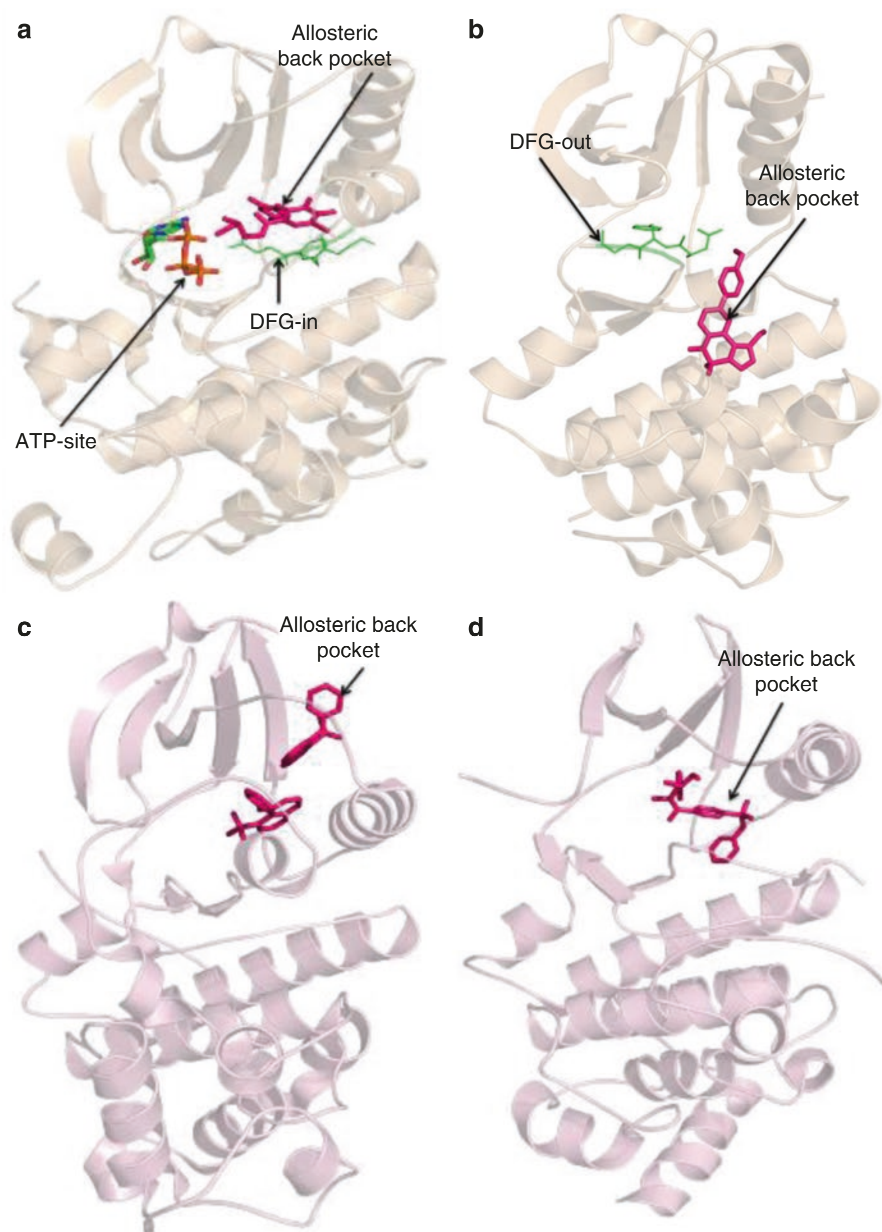

Fig. 5.2 Type III allosteric inhibitors (**a**) Inhibitor with MEK1 (PDB ID: 1S9J) (**b**) Inhibitor with FAK (PDB ID: 4EBW) (**c**) CDK2 kinase (PDB ID: 3PXF) (**d**) LIMK2 kinase (PDB ID: 4TPT)

The compound MK-2206 reported as Akt inhibitor binds to inactive Akt conformation by targeting an allosteric site at the interface of the kinase domain and the pleckstrin homology (PH) domain. It is currently under investigation in clinical studies on breast cancer, NSCLC, nasopharyngeal carcinoma, and other cancers [33].

The displacement of the structural αC helix with type III allosteric inhibitors has recently been exploited in drug discovery. A crystal structure of CDK2 with an open allosteric pocket adjacent to the αC helix has been reported (Fig. 5.2c). Rastelli et al. identified CDK2 allosteric inhibitors with micromolar potency through docking-based virtual screening. These compounds bind into an allosteric pocket of CDK2 formed following displacement of the αC-helix [34]. Godwin et al. reported a novel series of sulfonamides as potent and selective inhibitors of LIM-kinase 2 (LIMK2). The kinetic experiments further revealed that these molecules were non-ATP competitive inhibitors of LIMK2. Structural analysis by X-ray crystallography revealed that these molecules bind in a hydrophobic pocket near the ATP binding pocket with DFG-out orientation (Fig. 5.2d) [35].

Type IV inhibitors: These inhibitors bind at a site remote from the ATP-binding e.g., surface pockets and interfere with binding of key regulators. These sites can be present anywhere in the kinase domain other than the site adjacent to the ATP binding site. The type IV inhibitors are considered as allosteric or noncompetitive inhibitors of ATP as they don't hamper the ATP binding site. Another significant characteristic of type IV inhibitors is that they induce conformational changes which make the kinase inactive [36–38].

A library of highly functionalized pyrazolo[3,4-d]pyrimidines, with a high level of molecular diversity, has been reported by Vignaroli et al. The enzymatic screening of this "privileged scaffold"-based compound collection, showed high activity against Src, Abl wt, and T315I ABL. The study has led to the development of a new allosteric inhibitor of the T315I ABL. The most potent compound showed an IC_{50} value of 3.16 μM against Abl T315I, independent of the concentration of ATP and the peptide substrate [39].

A molecule GNF-2 bind to the myristoyl pocket of the C-lobe of the kinase domain is the first reported type IV inhibitor of Abl. A series of 1,3,4-thiadiazole compounds were reported as promising Abl inhibitors. The lead compound BO1 inhibited T315I ABL in an ATP-independent manner signifying allosteric mechanism of inhibition [40, 41].

Yamada et al. reported ATP noncompetitive WNK1–4 kinase inhibitors as next-generation anti-hypertensive agents. The co-crystallization of the inhibitors with WNK1 revealed an allosteric binding mode consistent with the observed specificity for WNK1–4 kinases. The optimized compound inhibited rubidium uptake by sodium chloride co-transporter 1 (NKCC1) in HT29 cells [42] (Fig. 5.3a, b).

The type V inhibitors: They bind to two different sites on the kinase domain. They are further divided into two categories, i.e., bisubstrate analog inhibitors that target both the ATP and protein substrate binding sites, and bivalent inhibitors that target the ATP binding cleft and other surface (apart from substrate binding site) on the protein kinase. The design of bisubstrate analog inhibitors is based on the fact that different protein kinase group show significant variation in their substrate recognition [43]. Parang et al. designed a bisubstrate analog inhibitor by covalent linking of ATPγS to an analog of the peptide substrate, IRS 727, by a short two carbon spacer with a distance of approximately 5.7 Å between the tyrosine nucleophilic atom of the IRS 727 analog and γ phosphorous of the ATP moiety [44]. The bivalent

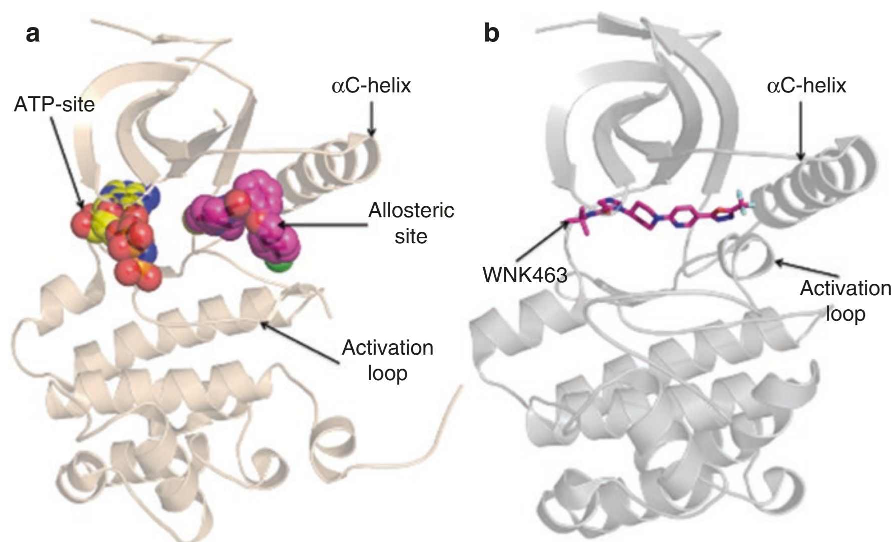

Fig. 5.3 The crystal structures of WNK1. (**a**) Structure representing the binding of inhibitor (magenta) in human WNK1 with AMP-PNP. The allosteric pocket is located near the ATP binding site. (PDB ID: 5TF9) (**b**) Structure representing ATP competitive inhibitor WNK463 in human WNK1 (PDB ID: 5DRB)

inhibitors are designed to target both the catalytic and regulatory domains of the kinases. Profit et al. designed such an inhibitor for tyrosine kinase by tethering of an active site-directed peptide sequence with an SH2 domain recognition sequence through a flexible linker comprising γ-amino butyric acid [45].

The type VI inhibitors: They bind covalently to the kinases by formation of a covalent bond between the alkene portion of the inhibitor and the cysteine residue present within the ATP-binding site in some of the kinases. A beautiful example of selective inhibitor design by exploiting the presence of certain residues at specific positions has been reported by Cohen et al. The authors have noted that threonine, which is a small gatekeeper, provides only a partial discrimination between kinase active sites; therefore, if a second selectivity filter can be applied it may result in a more selective inhibitor. They discovered that there are only three kinases (RSK1, RSK2, and RSK4) which have a highly reactive cysteine on the P-loop (the high reactivity of cysteine owing to its proximity to solvent accessible surface and thus lower p*K*a compared to buried cysteine) and a threonine as gatekeeper. They designed and tested some analogs targeting these two residues and found two selective and irreversible kinase inhibitors [46].

The inhibitors from type-I to type-V are reversible in nature whereas type VI inhibitors are irreversible.

Another example of targeting a surafce exposed Cystein is the design of molecules afatinib and ibrutinib. The alkene part of afatinib makes a covalent interaction with the hinge cystein in the ATP binding pocket. [47, 48].

5.6 Concluding Remarks

The structural and physiological knowledge of protein kinases have improved the understanding of kinase function at molecular level. The drug discovery targeting protein kinases has achieved substantial progress since the discovery of first kinase inhibitor. The success of many kinase inhibitors has propelled it further, and it seems to be the most desirable field in biomedical research after oncological research [49].

In the current chapter, the story of kinase inhibitor design with special reference to allosteric site has been discussed. The review is focused mostly on the allosteric inhibitors, their binding in the kinase domain, and analysis of available crystal structures. Our suggestions for self-motivated researchers in form of future direction of research may include in-depth structural studies of kinases with special reference to DFG-in and DFG-out conformations. Analysis of residue–residue interactions through interaction networks to explore the transformation of signals during binding of different inhibitors and long-term molecular dynamics simulation of reported crystal structures with allosteric inhibitors to decipher the dynamics of the allosteric site(s).

The human kinome is classified into seven major kinase families. However, the discovery of small molecule inhibitors so far is limited to selected group of kinases. The majority of 33 inhibitors approved to date are confined to the tyrosine kinases, serine/threonine kinases, and tyrosine-like kinases family. It indicates that there is a tremendous scope in this field for the future discovery research.

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Recent Advances in the Chemotherapy of Visceral Leishmaniasis

6

Vijay Kumar Prajapati and Rajan Kumar Pandey

6.1 Introduction

Leishmaniases are an infectious disease caused by more than 20 species of the protozoan parasite that have a place to genus *Leishmania* and family trypanosomatidae. It spreads by the bite of a preinfected female sandfly vector belonging to more than 30 species of genus Phlebotomine. Leishmania parasite spends its life cycle in digenetic mode consisting of flagellated and motile promastigote form in the gut of female sandfly vector and when sandfly bites to mammalian host, it converts into aflagellated, nonmotile amastigote form within the phagolysosomal compartment of the macrophage. Leismaniasis manifests mainly three types of clinical spectrum namely cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL). Among them, cutaneous form of leishmaniasis is most common, ranging from self-healing small nodules to giant mucosal tissue damage. It is caused by *Leishmania major, L. maxicana, L. tropica*, *and L. amazonensis*. However, MCL leads to the degeneration of a mucous layer of nasopharyngeal cavity, caused by *L. braziliensis and L. panamensis.* The most severe visceral form of leishmaniasis, also known as kala-azar, black fever, or dumdum fever, causes high fever, weight loss, acute anemia, fatigue, and enlargement of the liver characterized by splenomegaly and hepatomegaly. The immune system of patients gradually decreases and may be lethal, if left untreated. It may be of zoonotic or anthroponotic type based on their transmission characteristics and caused by *L. donovani* and *Leishmania infantum* in Old World and New World, respectively.

Recent epidemiological reports of VL estimate up to 0.4 million cases from 98 countries and 3 territories on 5 continents [1]. Among them, 90% of global VL cases

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present only in six countries namely India, Bangladesh, Nepal, Ethiopia, Sudan, and Brazil [2]. 50% of the visceral leishmaniasis infection burden is present only in India. Rural areas are commonly affected as compared to the urban areas due to the climatic and environmental conditions that play a major role in the growth and development of sandfly vectors. The number of VL patient is increasing continuously due to unavailability of proper treatment options, lack of vaccines, drug resistance into a patient, and uncontrolled increase in sandfly vectors. Over the last few decades, many new drugs and formulations were introduced to treat the VL infection, but most of the drugs have shown toxicity along with resistance condition. Since 1930s, pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) were opted as the first-line treatment option for VL in the endemic countries. But due to extensive drug resistance of more than 60% in few areas of India and Nepal, it was banned since 1990s [3]. In early 1990s, amphotericin B and its liposomal formulation were introduced as a potent antileishmanial drug with high efficacy and negligible resistance. Miltefosine, the first oral therapy for VL, has shown high efficacy of more than 90% with a relapse rate of 20% within 12 months in the clinical trials [4]. Paromomycin is an aminoglycoside antibiotic with antiprotozoal activity. It has shown a cure rate of 94.6% in patients suffering from VL. This drug has made a milestone due to its cost-effectiveness and lack of resistance. The main target of a chemotherapeutic agent against VL is the amastigote form of leishmania, which intracellularly survived and replicated within the macrophages of visceral organ of the vertebrates. This amastigote form exists in the parasitophorous vacuole, which looks like a secondary lysosome with pH ranging between 4.5 and 5.0. The availability of acidic environment has been responsible for the acquisition of nutrient and ion homeostasis in amastigotes of parasite [5]. This process also involves various transporters which are responsible for the drug influx into parasite and they are responsible for the drug susceptibility of the parasite against chemotherapeutic agent. Chemotherapeutic approaches evolve new drugs based on the antileishmanial activity and tissue macrophage target activity systems. A table has been presented here to represent the antileishmanial options for the different faces of leishmaniasis (Fig. 6.1) (Table 6.1).

6.1.1 Diagnosis

In the early twentieth century, classical microbiological diagnostic methods were used after the association of clinical leishmaniasis with Leishmania parasite. Visceral organs like spleen, bone marrow, and lymph nodes are the major shelter for the parasite in the human body. The diagnosis options available for VL are complex as compared to other diseases such as malaria, typhoid, and tuberculosis. Microscopic examination is the gold standard approach to diagnose leishmaniasis apart from biopsy techniques for VL. Splenic aspirate has shown a specificity of >95%, while liver biopsy, bone marrow aspiration, lymph node fine-needle aspiration cytology (FNAC), and blood buffy coat have a sensitivity of 76% only. Lymph gland puncture gives a positive result of 40–50% for kala-azar cases while 58.6% for CL cases. However, the study of a nasopharyngeal swab of visceral leishmaniasis patient

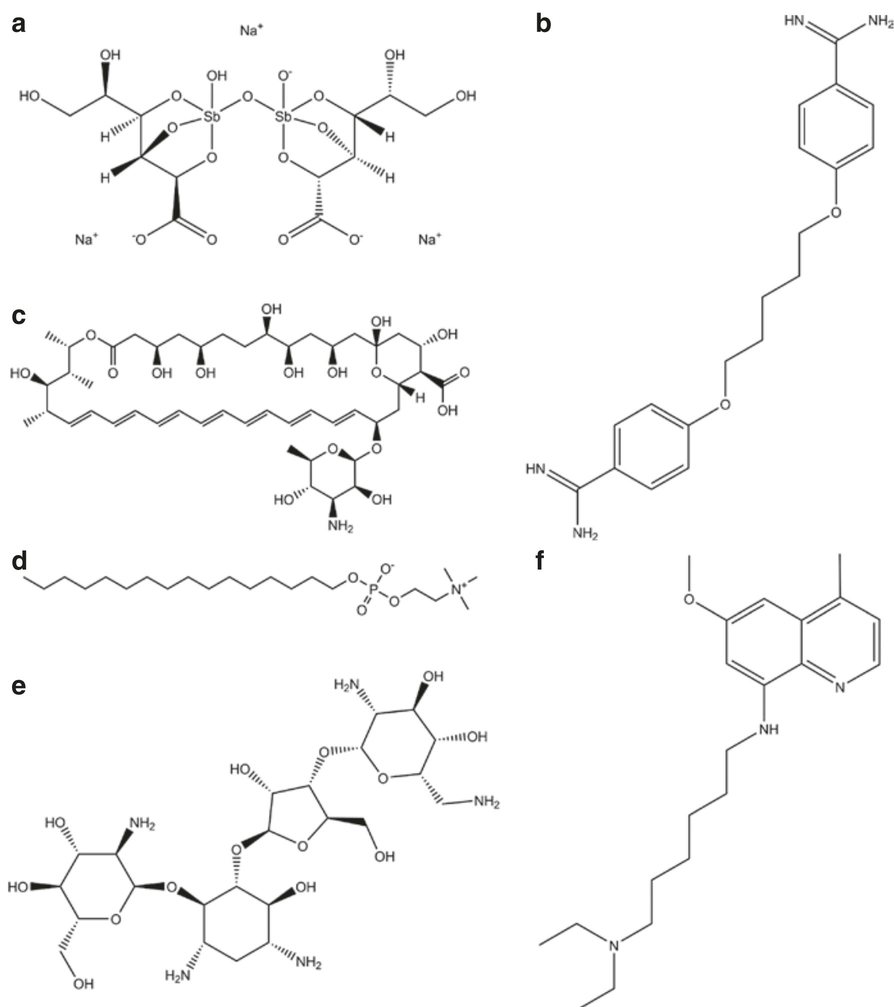

Fig. 6.1 Available chemotherapeutic drugs for the treatment of visceral leishmaniasis. (**a**) Sodium stibogluconate, (**b**) pentamidine, (**c**) amphotericin B, (**d**) miltefosine, (**e**) paromomycin, (**f**) sitamaquine

shows positive result of 28% and 36% with stain and culture, respectively. As compared to aforesaid diagnosis method, immunological approaches such as direct agglutination test, enzyme-linked immunosorbent assay, and rapid antibody detection have shown high sensitivity [6]. According to recent information the molecular diagnostic methods such as polymerase chain reaction-based assays have shown high sensitivity and specificity (up to 100%) for detection of Leishmania parasites. By using these methods we can define the specific features of parasite such as drug resistance or virulence and they provide information to know the disease severity and treatment outcome [7].

Table 6.1 Different clinical forms of leishmaniasis, their geographical distribution, causal leishmania species, vectors, respective reservoirs, and available treatment options

Infection	Geographic distribution	Pathogenic species	Vector	Reservoir	Available treatment
Cutaneous Leishmaniasis (CL)	Semi-deserts in Middle East, North India, Pakistan, North Africa, central Asia	*Leishmania major*	*Phlebotomus papatasi*	Gerbils	Sodium stibogluconate, miltefosine, paromomycin
CL	Sub-Saharan Savanna, Sudan	*L. major*	*P. duboscqi*	Rodents	
CL	Towns in Middle East, Mediterranean Basin, central Asia	*L. tropica*	*P. sergenti*	Humans	
CL	Highlands of Kenya, Ethiopia	*L. aethiopica*	*P. longipes, P. pedifer*	Hyraxes	
CL	Yucatan, Belize, Guatemala	*L. mexicana*	*L. olmeca*	Forest rodents	
CL	Tropical forests of South America	*L. amazonensis*	*L. flaviscutellata*	Forest rodents	
(Mucocutaneous Leishmaniasis) MCL	Tropical forests of South and Central America	*L. braziliensis*	*Lutzomyia spp., L. umbratilis*	Forest rodents, peridomestic animals	Sodium stibogluconate
(Visceral leishmaniasis) VL	North East India, Nepal Bangladesh, Burma	*L. donovani (Asia)*	*Phlebotomus argentipes*	Humans	Sodium stibogluconate, Meglumine antimoniate, Amphotericin B deoxycholate, liposomal amphotericin B, miltefosine (oral), paromomycin, sitamaquine (oral), pentamidine
VL	Mediterranean Basin, Middle East, China, Central Asia	*L. infantum*	*P. perniciosus, P. ariasi*	Dogs, foxes, jackals	
VL	Sudan, Kenya, Horn of Africa	*L. donovani (Africa)*	*P. orientalis, P. martini*	Rodents, canines, humans	
VL	Central America, Northern South America, esp. Brazil, Venezuela	*L. chagasi*	*Lutzomyia longipalpis*	Foxes, dogs, opossums	

6.1.2 Advancement in Visceral Leishmaniasis Chemotherapy

Kala-azar first got attention in 1824 in Jessore, India. However, by 1862 this disease got spread in Burdwan and caused endemic proportions. In 1900s, the agent of Leishmania was firstly isolated by William leishman, who isolated the parasite in a spleen sample of soldiers who died with dum-dum disease, in Calcutta, India. Later on, Upendra Nath Brahmachari, a medical practitioner, firstly discovered urea stibamine, an organic antimonial compound that played a promising role in the treatment of visceral leishamania or kala-azar. Later on, pentavalent antimonials became the traditional treatment option for VL till 1990s. But the extensive resistance of antimonial drugs in endemic areas of India made it banned. Furthermore, amphotericin B, pentamidine, miltefosine, paromomycin, and sitamaquine were introduced.

6.2 Chemotherapies for Visceral Leishmaniasis

Leishmaniasis infection has been included in the list of neglected tropical diseases and has a strong relationship with poverty [8]. At present, there is no vaccine candidate either preventive or prophylactic under the clinical trials for the effective treatment of leishmaniasis diseases. Therefore, the treatment of leishmaniasis only relies on chemotherapy. The first line of the drug used to treat VL was pentavalent antimonials and the second line of drugs are amphotericin B and pentamidine. Miltefosine was introduced as the first oral drug for Leishmania with high efficacy, but the patient has shown resistance in very short time due to point mutation in the genome of leishmanial strain [9]. Paromomycin has also shown high efficacy without any resistance condition. In recent years, many new drugs have been formulated, but no one is considered as an ideal drug for the treatment of VL because of their high toxicity, long duration of the treatment, and development of drug resistance in the patient.

6.2.1 Pentavalent Antimonials

Urea stibamine was the first antimonial introduced in 1930s by U.N. Brahmchari but due to its comparatively high toxicity it was soon after replaced by sodium stibogluconate. Since 1950s, pentavalent antimonials occupied the position of first-line drug for the treatment of visceral leishmaniasis where resistance is not reported. Initialy, antimonials were given in very low dosage of 10 mg/kg/day for only a short period of 6–10 days; later on in 1982 WHO increased the dose quantity to 20 mg/kg/day, but they recommend that total daily quantity of drug should not exceed 850 mg. Antimonials were marketed by two names, generic-form sodium stibogluconate and branded-form meglumine antimoniate. The pentavalent antimonials (Sb^{V}) are basically a prodrug that converts into trivalent antimonite (Sb^{III}), which is an active form of the drug. The reduction of pentavalent antimonials to its trivalent form takes place either in host macrophages or in parasite but it is still a quandary [10]. Apart from its

primary role for the treatment of VL, it was banned in India and Nepal in 2000s due to higher resistance of more than 60% in few districts of Bihar state [11].

If we talk about the entry of antimonial drugs into parasite and macrophage we will get a nonspecific proof, but there may be a possibility that parasitic aquaglyceroporins are responsible for the transport of drugs within the amastigotes. Parasitic phosphate transporter also plays a role in the transport of pentavalent antimonials into amastigotes. The killing effect of antimonials (Sb^{V} and Sb^{III}) against parasite includes DNA fragmentation, inhibition of the glycolytic pathway, apoptosis, inhibition of trypanothione reductase (protects the parasite from the reactive oxygen and nitrogen species of host), β-oxidation of fatty acids, and an increased efflux of thiols.

The responsible factors that evoke to the resistance of antimonial drug in the endemic areas of India include the widespread misuse of the drug due to its easy availability over medical stores and the lack of activation of SbV to SbIII by Leishmania parasite [12]. Resistance to trivalent arsenic was reported in a few districts of Bihar state due to the continuous drinking of arsenic-contaminated water leading to cross-resistance for trivalent antimony. Elevated intracellular trypanothione level also contributes to resistance [13]. However, the increasing level of tryparedoxin peroxidase also contributes to resistance for Sb^{III}. As aquaglyceroporin (AQP1) plays an essential role in influx of drug, modulation of AQP1 expression leads to decrease in the susceptibility of amastigotes for antimonials. Upregulation of various chaperons and stress-related proteins contributes to the repairing of cellular damage induced by antimonials [14].

6.2.2 Pentamidine

After the unveiling of pentavalent antimonials and amphotericin, alternative chemotherapeutic drug pentamidine was introduced in 1980s. Pentamidine is an organic compound of diamine that has gained an attention as a second-line drug against VL with increasing cure rate. Firstly pentamidine showed a cure rate of up to 93%, but after some time cure rate decreased upto 70–80% in some Indian epidemic areas [15]. When pentamidine was combined with antimonials and allopurinol the cure rate increased as compared to single form. After the treatment failure of pentavalent antimonials in Eastern Africa, pentamidine proved as an effective drug.

The mode of action of pentamidine is unknown, but it enters into the promastigotes of *Leishmania donovani* via arginine and polyamine transporter. Pentamidine may be toxic with another disorder like neurotoxicity, hypertension, hypoglycemia, and many others. The drug resistance was also reported, but the exact mechanism is unknown [16].

6.2.3 Amphotericin B

Amphotericin B (AmB) deoxycholate, an antifungal drug, was firstly introduced to treat fungal infection, but later on it was used for the treatment of visceral and mucocutaneous leishmaniasis. The first clinical trials of amophotericin B were

conducted in 1990s for the treatment of visceral leishmaniasis [17]. Amphotericin B deoxycholate has shown high efficacy of 98–100% in those areas where resistance to antimonials has been reported. The initial recommended dose of amphotericin B was 1 mg/kg/day for 20 days leading to a cure rate of 99%, but if the quantity of amphotericin B was changed to 0.5 mg/kg/day for up to 14 days, it also showed very good result. After some years, researchers found that the amount of amphotericin B will be more effective when administered on alternative days as compared to daily doses.

For the effective treatment of leishmaniasis researchers formulated a new alternative drug to increase the efficacy and decrease the toxicity and other side effects and established lipid formulation of amphotericin B. Till date ten forms of liposomal amphotericin B have been tested and various ranges of response were obtained in India [18]. They tested the efficacy at different doses and concludes that the efficacy of liposomal amphotericin B increases along with the increase in the percentage of the dose [19]. The liposomal formulation of amphotericin B proved itself as an efficient drug and increased the cure rate to more than 90%, but the high cost and less availability are wide limitations. The antileishmanial activity of liposomal AmB is due to its activity towards both ergosterol of parasite and cholesterol of infected host macrophage. Amphotericin B makes complexes with cholesterol, therefore inhibiting the binding of promastigotes to the membrane of host cell macrophages. Further higher concentration of AmB makes pore in the parasitic cell membrane leading to increase in osmotic disbalance and free radical formation that ultimately leads to cell lysis. Apart from the good efficacy of amphotericin B, resistance has also been reported from the endemic zone of VL. Other deleterious effects on human body include damaging of kidney tubular cell, an increasing concentration of Ca^{2+} ion, hydrogen ions across the membrane and salt concentration that leads to cell death. The presence of less amount of ergosterol in the cell membrane of leishmania parasite triggers the resistance phenomenon, since ergosterole is the main target of amphotericin B [20].

6.2.4 Paromomycin

Paromomycin was added as an essential medicine in 2007 by WHO. It is an aminoglycosidic antibiotic cultured from *Streptomyces rimosus*, having both antibacterial and antileishmanial activity. Paromomycin effectively cures both visceral and cutaneous leishmaniasis but less availability in endemic areas restricts its use. Paromomycin has been given either alone or in the form of combination with Sb^{v} to treat visceral leishmaniasis. The clinical trials of paromomycin have been done in 1990s in Bihar state of India. The efficacy of paromomycin was found to be excellent as compared with other licensed drugs. This drug could be considered as a cheapest drug for the treatment of visceral leishmaniasis. A patient treated with paromomycin achieved high efficacy of 77–97% as compared with antimonials (66%) along with less toxic effect [21]. The mechanism of action of paromomycin is not clearly known for leishmaniasis while it has been associated with the inhibition of cytochrome C in *Candida krusei*. Recent studies have shown that positively charged

paromomycin is targeted to anionic leishmanial glycocallyx suggesting mitochondria as a crucial target. Paromomycin promotes the association of 50S and 30S ribosomal subunits, thus stopping their recycling process and ultimately inhibiting protein synthesis. This antibiotic also induced respiratory disbalence in the promastigote form of *L. donovani*. Further, it interacts with both 30S and 50S subunits and promotes the association of translation initiation factor 3 (IF3) with the 30S ribosomal subunit [22]. Limited use of paromomycin in the treatment of Leishmania yet does not generate resistance in case of outpatient treatment, but in vitro resistance was reported in the case of *Leishmania donovani* and *Leishamnia tropica.*

6.2.5 Miltefosine

Miltefosine is an alkylphosphocholine, primarily developed as an anticancer drug. This is the first leading oral drug which is used to treat visceral leishmaniasis. Phase I, II, and III clinical trial was conducted and provides a strong evidence of protection against visceral leishmaniasis followed by phase IV trials [23]. The combination therapy of miltefosine and ambisome has been evaluated and has been found to be highly effective with good tolerability, but questions were raised due to the side effect generated due to an extreme combination of these two drugs. Miltefosine has a long half-life of almost 152 h and is therefore responsible for long-term residence and teratogenicity. This situation is responsible for the development of resistance and this abortifacient and teratogenic nature limits its use during pregnancy.

Miltefosine enters into the cell and accumulates to perform its activity. Two signaling transporters namely LdMT and its β-subunit LdRos3, a P-type ATPase, regulated the accumulation of miltefosine within the cell. Both transporters are related to aminophospholipid translocase family. The mode of antileishmanial action is still unclear, but it has been the cause for the apoptosis-like process in the amostigote form of *Leishmania donovani*. Miltefosine reduces the lipid content and enhances phosphatidylethanolamine in promastigote membrane, suggesting a production of phosphatidylethanolamine-*N*-methyl transferase that is responsible for inhibition of leishmanial parasite proliferation process [24]. Miltefosine has yet not shown clinical resistance, but improper use of this oral agent in endemic countries (India) enhances the probability of resistance. The resistance towards miltefosine correlated with the low lipid content in the membrane of Leishmania promastigotes and the amount of phospholipid alkyl chains was lower in miltefosine resistance strains. In India, the efficacy of combination therapy of miltefosine with amphotericin B or paromomycin is very high and it is useful to cure antimony resistance strain of visceral leishmaniasis [25].

6.2.6 Sitamaquine

Sitamaquine is a second oral drug after the discovery of miltefosine for the treatment of visceral leishmaniasis and recently it underwent the clinical trial phase I and II. Sitamaqine is an 8-aminoquinoline that was developed originally with the

collaboration of Walter Reed Army Institute and GlaxoSmithKline [26]. The phase II clinical trial of sitamaquine has shown good efficacy in the Indian subcontinent against visceral leishmaniasis. However, along with its high efficacy, fewer side effects also developed like vomiting, cyanosis, nephritic syndrome, and dyspepsia. The result of Kenyan phase II clinical trial was comparatively different from Indian trials; it showed similar efficacy but somewhat different side effects like abdominal pain, kidney malfunctioning, and headache [27]. Sitamaquine targets succinate dehydrogenase enzyme leading to development of an oxidative stress and ultimately parasitic clearance. The high concentration of sitamaquine affects parasite morphology, motility, and proliferation. Initially, the positively charged sitamaquine interacts with the anionic polar head of phospholipids. After binding, sitamaqine starts to accumulate into cytosolic part of parasite. The drug resistance against sitamaquine is yet not clinically developed, but *Leishmania donovani* promastigote in vitro experiment developed resistance at 160 μL sitamaquine concentration [28].

6.3 Novel Formulation of Anti-leishmanial Drugs

Over the past years, new formulation and alternative drugs of old ones have been available, but at the present time, none of them are ideal drugs due to the very high toxic effect, resistance, long-term treatment, prohibitory price issue, and inadequate mode of insertion not accepted in endemic areas. Therefore, many patients are not capable to complete the whole treatment process due to increasing risk of drug resistance and toxic effect. The combination therapy has shown positive result or short-term solution to delay emerging drug resistance, increasing drug efficacy, and shortening treatment duration [29]. However, this method is limited to the fewest number of FDA-approved drugs and the chances of finding a new mechanism of action compared to parasite are very less.

The discovery of a new formulation of anti-leishmanial drug to treat visceral leishmania should be a very important or long-term objective. In addition, target product profile (TPP) is an important tool for drug discovery management and plays a central role in drug discovery process. Nowadays, the drug discovery process for visceral leishmania mainly follows two approaches, the molecular target-based approaches and the phenotypic target-free approaches [30]. For the target-based approaches, the first step would be the identification and endorsement of potential targets. Recently, many efforts were done to find potential leishmanial targets, initially with the help of TDR database. This database is created on the basis of genetic, biochemical, and pharmacological data related to tropical pathogen additionally associated with computer-based druggability. The phenotypic target-free screening approaches target a particular enzyme or an individual biomolecule, or focuses on a pathway that might be a beneficial solution.

At the present time the combination therapy of anti-leishmanial drug with nanocarriers has been a potential and emerging formulation for the treatment of leishmaniasis. Nanocarriers have a power to penetrate into macrophage cell and are able to release drugs into cell, increasing the local drug concentration and ultimately

arresting the protozoa life cycle. In this strategy nanocarriers target the macrophage cells to treat leishmaniasis infection, which has an ability to overcome all natural biological barriers. Nanocarriers would be less toxic, have high efficacy, enhance selectivity, have high drug solubilization, prevent degradation of the drug, and promote the accurate release of drug at the target site. Nanospheres, polymers, and liposomes have promising nanocarriers for the delivery of anti-leishmanial drug at specific targets. However, this technology is an emerging current treatment option with reduced treatment cost, improved bioviability, and less drug toxicity that definitely enhance the efficacy of the treatment.

Liposomes are small synthetic vesicles in globular shape that can be produced from cholesterol biomolecules and nontoxic phospholipids. Due to small size, hydrophobic and hydrophilic balance, biocompatibility, flexibility, and stability to load various biomolecules as cargo, liposomes are recently used as a mode of drug delivery process. The best example of liposome formulation of amphotericin B is *AmBisome*®; this is discovered in 1981, which has proven to be effective against leishmania parasitic infection.

Functionalized carbon nanotube is a good example of drug carriers as it has been tested as drug transporter against leishmaniasis. In case of *L. donovani*-infected patient, amphotericin B attached with functionalized carbon nanotube has shown high efficacy and good result as compared to conventional amphotericin B [31].

Targeting of the various enzymatic reactions of host and parasite cell pathways, by high-throughput screening, appears to be more beneficial. The use of this phenomenon is a critical step targeted by a specific chemical compound that interferes a specific outcome of the pathway. Recently, these techniques are emerging as tools for the establishment of new drug target against leishmaniasis [32].

Continouosly generating resistance of Leishmania parasitic strain increases the amount of genomic data. Therefore, the genomic sequence provides beneficial information for the discovery of novel vaccine and new chemotherapeutic targets. The development of new molecular tools like microarray and deep sequencing technology and proteomics has an ability to revel these clinical goals. In addition, computational algorithms and emerging bioinformatics with high-throughput virtual screening greatly play a crucial role for identification and formulation of new potent anti-leishmanial drug target. Proteomics and transcriptomics have been used for the identification of stage-specific gene at different stages in Leishmania parasite. These proteomics approaches are capable of finding new chemotherapeutic leishmanial targets and these targets have a potential to control leishmaniasis infection.

6.4 Drug Targets

Several drugs are available to treat VL patients, but the associated toxicity and increase in resistance to kill the Leishmania parasites have urged the need of new compounds to eradicate VL infection. New potential drug targets focus on different enzymes involved in a number of biochemical and metabolic pathways which provides energy for parasitic survival. There are several targets inside the Leishmania

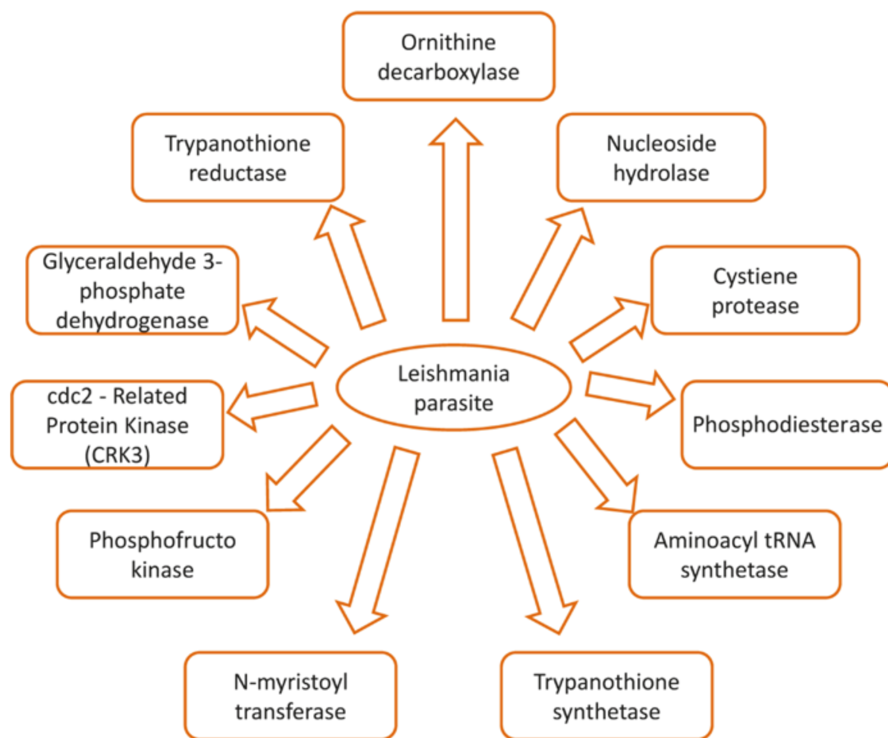

Fig. 6.2 Leishmania drug targets used for screening of anti-leishmanial compounds

parasites, regulating parasite metabolism or redox potential, that can be used to develop new chemically synthesized molecules to kill Leishmania parasites [33] (Fig. 6.2).

6.4.1 Trypanothione Reductase

Trypanothione reductase (TryR) is a flavoenzyme which catalyzes the redox reaction to convert trypanothione (TPT) and NADP into trypanothione disulfide and NADPH, found in the protozoan parasite of genus *Leishmania* and *Trypanosomes*. It is a stable homo-dimer, bounded tightly with each other by FAD molecule. FAD acts as a cofactor and it also requires NADPH as a co-substrate. TryR plays a main role in redox metabolism of parasite; therefore TryR has been regarded as an ideal drug target for leishmania infection. The inhibitions of this enzyme will disrupt the redox balance which may lead to parasite death [34]. The process of selection of drug target depends upon the level of genetic and chemical validation, assay feasibility, toxicity potential, resistance, and drug-ability. TryR has two acive sites; one is found at the FAD-binding site while the second is formed by the interaction of both chains.

6.4.2 Ornithine Decarboxylase

Ornithine decarboxylase (ODC) is found to be involved in polyamine biosynthesis pathway. Polyamines are a component of trypanothione dimer [$T(SH)_2$], useful for *Leishmania* parasite infection. ODC plays a role in housekeeping functions in the amastigote stage of Leishmania parasite. Increased resistance to antimonial drugs has increased the expression of ODC in Leishmania parasites. This enzyme has two active sites and plays a role to increase the virulence property of *L. donovani*. Therefore, it can be taken as a potential drug target for the identification of anti-leishmanial leads [35]. The α-diflu oromethylornithine is an irreversible inhibitor to target ODC while the coenzyme of this reaction is pyridoxal 5′-phosphate. Currently DFMO (α-difluoromethylornithine) is used as a drug for the treatment of patients suffering from protozoan parasitic diseases like visceral leishmaniasis and malaria. DFMO mimics the substrate which binds with the enzyme ODC. The enzyme of ODC with N-terminal extension in *L. donovani* has the future scope to develop the new drugs against leishmaniasis [36].

6.4.3 Nucleoside Hydrolase

The inabilities of protozoan parasites to synthesize purines via de novo pathway make them dependent on salvage pathway for purine synthesis. Nucleoside hydrolase is an enzyme that plays a crucial role in salvage pathway. Leishmania parasites are purine auxotrophs, so for their growth demands purines are necessary. Nucleoside hydrolases have also been identified in other trypanosomatids including *Trypanosoma brucei gambiense*, *L. donovani*, *L. Mexicana*, *L. tropica*, *T. cruzi*, and *T. brucei*. In these protozoan parasites the residues which present in the active site of nucleoside hydrolase are conserved in nature and these conserved residues provide the great promise to design a drug [37]. Another study, on the recombinant enzyme of nucleoside hydrolyses of *L. donovani* (rLdNH), was expressed in bacteria *Escherichia coli* combined with maltose-binding protein (MBP). The recombinant enzyme of rLdNH-MBP showed the capacity to bind with inosine as substrate in in vitro condition [38].

6.4.4 Cysteine Proteases

Cysteine protease is one of the proteases belonging to papain family, present in metazoan and protozoan parasites. As compared to mammalian hosts, leishmania parasites lack redundancy of cysteine protease; it makes them attractive targets for the development of new anti-leishmanial drugs. Cysteine protease plays an important role in infection, metabolism, replication, and development of protozoan parasites. These proteases are essential for survival of Leishmania parasites in macrophage intracellular compartments. These enzymes show virulence along with modulation of host immune responses. Cathapsin-B and cathapsin-L are the

cysteine proteases which are localized in lysosome-endosome compartments, and play a role in intracellular protein degradation [39]. The genome of leishmania parasite consists of multiple copies of cathapsin-L and a single copy of cathapsin-B cysteine proteases [40]. The derivatives of nitric oxide (NO) have the capacity to inhibit the development of parasites; glyceryl trinitrate is one of the releasing drugs of NO, and was used to treat cutaneous leishmaniasis.

The inhibitors of proteases are useful to block the entry of many parasites like *Plasmodium*, *Trypanosoma, Phytomonas serpens*, and *Taxoplasma gondii* into the host [41]. Cysteine protease has also shown its role in the modulation of host immune response, degradation of different host proteins, and autophagy. Lacking of this enzyme in Leishmania parasite or blocking of this enzyme by inhibitors showed reduced virulence activity and infectivity. Therefore, they have identified that the inhibition of this enzyme in different leshmania parasites provides best therapeutic option to combat leishmania parasitic infection [42]. Cystatin is one of the natural inhibitors that have been found to inhibit this enzyme of *L. donovani* [43]. Another natural bioflavonoid has shown anti-leishamnial and anti-proteolysis activity against cysteine protease-B, and the use of this natural inhibitor in specific concentration doesn't give any toxicity towards host cells.

6.4.5 Phosphodiesterases

Phosphodiesterase (PDE) is an enzyme involved in the control of the cellular concentration of secondary messengers like cAMP and cGMP, a key regulator of many physiological processes, and it is ubiquitously expressed [44]. In human beings there are 21 PDE genes, separated into 11 families. These enzymes have been found as therapeutics in many diseases like cancer, pulmonary diseases, and asthma [45]. Leishmania majorly contains five different PDE genes such as LmjPDEA, LmjPDEB1, LmjPDEB2, LmjPDEC, and LmjPDED. Among these five genes LmjPDEB1 and LmjPDEB2 show similarity, but there is a difference among Ala798 and Arg823 of LmjPDEB1 [46]. Etazolate, dipyridamole, and trequinsin are drugs which inhibit the proliferation of *L.major* and *L.infantum* promastigotes with IC_{50} values ranging between 30 and 100 μM [47]. In *L. donovani* promastigotes, flavonoids luteolin and quercetin arrest the cell cycle at G1 phage and eventually lead to increased cell apoptosis, so flavonoids are nonselective inhibitors treated as a specific drug to treat leishmaniasis. Because of the presence of structural similarity between LmjPDEB1 and human PDEs, it provides the chance to design inhibitors to treat leishmaniasis. 3-Isobutyl-1-methylxanthine (IBMX) can bind to the catalytic domain of LmjPDEB1 [48]. Till now, there are so many inhibitors to inhibit PDEs of human beings, but these are not effective to inhibit LmjPDEB1; rolipram is a selective inhibitor of PDE4 with IC_{50} values of 330 μM for the catalytic domain of LmjPDEB1 and also IBMX has shown IC50 value of 580 μM [49]. The other inhibitors like luteolin, a flavonoid, and other nonselective inhibitors of PDEs have shown the inhibition activity of 80% to LmjPDEB1 at 20 μM inhibitor. The structure of PDE provides a template to design drugs to treat leishmaniasis [50]. All three

LmjPDEA, LmjPDEB1, and LmjPDEB2 have the functional complementation; all three are involved in hydrolyzing cAMP. The recombinant of LmjPDEB1 and LmjPDEB2 has been shown to be cAMP specific with a low molar range of Km values. The drugs which are like dipyridamole, trequinsin, and etazolate exhibited the capacity to bind with PDEs; these inhibitors have the capability to inhibit the proliferation of *Leishmania* amastigotes [47].

6.4.6 Aminoacyl-tRNA Synthetase

Aminoacyl-tRNA synthetase (aaRS) is an enzyme, useful in translating the nucleotide-encoded gene sequences into proteins and this enzyme is ubiquitously expressed [51]. This enzyme recognizes a single amino acid and attaches to t-RNA, additionally with the properties of proofreading and editing mechanisms [52]. The charged t-RNA adds further amino acids and it proceeds to elongation process during translation [53]. Mostly eukaryotes carry two genes to each aaRS. They are in the cytoplasmic and mitochondria and are not remunerated to each other if one gene is knocked down. But in trypanosomatids no need of compensation between two genes; only one gene of aaRS per amino acid is enough, except for the amino acids AspRS, LysRS, and TrpRS. The reduction of one gene of aaRS leads to arresting the complete growth of protozoa [54]. These enzymes play a role in cell survival and provide the chance to design drug against them. The *L. major* tRNA synthetase is the first MetRS; its active site is bound with MetAMP and pyrophosphate. The human mitochondrial MetRS sequence shows 30% similarity with LmMetRS catalytic core, but the active site is highly conserved. There are two changes in binding site of anti-codon that is in *L. major* Asn580 and Lys732 whereas in human mitochondrial enzyme Gly and Arg are sufficient to arrive at selective inhibitors to target the enzyme [55].

6.4.7 Trypanothione Synthetase

Trypanothione synthetase is a bifunctional enzyme that catalyzes biosynthesis and involves in hydrolysis of the glutathione-spermidine adduct. Leishmania parasite has shown the unique nature regarding trypanothione, is involved in redox balance, and provides defense against oxidative and chemical stress [56]. This enzyme binds with three substrates such as glutathione, glutathionyl-spermidine, and ATP and then it forms N1, N8-bis (glutathionyl)spermidine, ADP, and phosphate. The main function of this enzyme is to generate the free energy from ATP hydrolysis to conjugate glutathione and spermidine to form glutathionylspermidine intermediate and it leads to form the product of trypanothione. These enzymes are useful to maintain polyamine levels which play a role in cellular proliferation and differentiation. This enzyme consists of C-terminal and N-terminal domains. Among these two, the former is a papain-like cysteine protease useful to catalyze $T(SH)_2$ biosynthesis while the latter is useful to catalyze trypanothione to glutathione [56]. Hecogenin acetate

tubocurarine, tubocurarine chloride, geneticin, dihydrostreptomycin, ribostamycin sulfate, tomatine, beta-carotene, geneticin, dihydrostreptomycin, paromomycin sulfate, capreomycin sulfate, spermine, 18-alpha-glycyrrhetinic acid, and mundulone are in silico anti-lishmanial drugs specifically against *L. major* [57]. The inhibitors of *L. donovani* LdTryS are tomatine, conessine, uvaol, and butelin. Few amino acid sequence alignment of glutathionylspermidine synthase reveals that it is derived from *E. coli* and the enzyme TryS from *Leishmania infantum* [58]. The structural difference between these enzymes provides the opportunity to design novel drugs against leishmaniasis.

The energy metabolism in trypanosomatids only depends on carbon sources of host. Glycolysis pathway is not only to provide ATP but also to produce glycoprotein coats to protect themselves from the immune system. So this pathway is so essential for surviving protozoan parasites. Therefore, this pathway promises the target for drug discovery against Leishmania parasite. In this pathway hexokinase converts glucose into glucose-6-phosphate by the transfer of the γ-phosphate of ATP to glucose. It is present in peroxisome-like organelles called glycosomes while in other organisms these enzymes are cytosolic. There are four types of hexokinases, hexokinase I–IV. Hexokinase IV is called glucokinase; it is present in liver [59]. This enzyme is the first enzyme which is involved in glycolysis; it plays a critical role in the biosynthesis, so it may have the potential to become a drug target. The potent FPPS inhibitors are useful to cure cutaneous as well as visceral leishmaniasis; these inhibitors have shown the ideal activity against *T. cruzi both* in vivo and in vitro. The study related to drugs has been done only on *T. cruzi* and *T. brucei* but not that much on Leishmania strains.

6.4.8 *N*-Myristoyl Transferase

N-Myristoyl transferase (NMT) is an enzyme, useful for the attachment of myristate. It is 14-carbon saturated fatty acid and it is derived from myristic acid (14:0). N-myristylation is a lipid modification and it is also a post co-translational modification. This process plays an important role in protein-protein interaction, lipid-protein interactions, signal transduction pathway, etc. This enzyme has specific functions when it attaches the fatty acid group to specific proteins and NMT is necessary for survival of parasites like *L. donovani, T. brucie,* and *P. falciparum*. So this enzyme has the ability to become a potential drug target to develop and treat various diseases like visceral leishmaniasis and cutaneous leishmaniasis. There is 42% sequence similarity between LdNMT and human NMT (HsNMT); it has provided the opportunity to identify novel inhibitors as therapeutic agents to combat the diseases [60]. Literatures have mentioned that non-hydrolyzable myristoyl-CoA analogue S-(2-oxo) pentadecyl-CoA (NHM) can inhibit visceral leishmaniasis infection. The targeted gene deletion reveals that the single copy of NMT gene from *L. major* is necessary for promastigote viability. As like this, one copy of the NMT gene from *L. donovani* is essential for the requirement of propagation of parasites [61].

6.4.9 Phosphofructokinase

Phosphofructokinase (Pfk) is the enzyme which is involved in the glycolytic pathway and located in glycosomes. The enzymes which are involved in glycolytic pathway are mostly conserved, but Pfk is not that much conserved and it shows the variability in different species. There are two types of Pfks: first one is ATP-dependent while another is PPi-dependent Pfk. PPi dependent undergoes modification, binds with different ligands, and then it provides the chance to know the difference between humans and parasitic enzymes; it gives the information to design a drug based on structure.

Pfk of *L. donovani* and *L. braziliensis* is allosteric in nature, and when it binds with the substrate, it requires AMP for the activation. The inhibition process will be reversed by the activator, AMP [62]. ATP Pfk contains glycine residue at the position of 124 where as in kinetoplastids contains lysine at that position. The substitution of lysine residue by glycine did not show any different effect in enzyme activity in *L. donovani* Pfk, but it shows the effect in *T.brucie*, so they mentioned that the mutation creates effect on the behavior of the enzyme Pfk [63].

6.4.10 cdc2-Related Protein Kinase (CRK3)

The regulation of the leishmania cell cycle occurs by the participation of two molecules which is related to cyclin-dependent kinase (CDK) family. These two molecules belong to cdc2-related serine–threonine protein kinases namely CRK1 and CRK3. CDC-2 and CDC-28 are key regulators of cell cycle regulation and these are ubiquitous in nature. CDK2 and CDK1 are useful to control the progression of G1/S and G2/M, respectively. This process is active in metacyclic promastigotes but not in amastigotes. With IC_{50} value of 100 nM flavopiridol compound is useful to inhibit histidine-tagged CRK3 of *L. maxicana* in vitro. Few inhibitors are not only specific to CDK but they also inhibit other types of kinases. *L. maxicana* CRK3 gene has shown 99% similarity with *L. major* gene. ATP-binding site shows the differences between HsCDK2 and Gln85 and is replaced by alanine in Leishmania CRK3, and His 84 with glutamate [64].

6.4.11 Glyceraldehyde 3-Phosphate Dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an enzyme which is involved in the sixth reaction of the glycolytic pathway. This enzyme is involved in activation of transcription, initiation of apoptosis, vesicle shutting from ER to Golgi, and axoplasmic transport. GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1, 3-bisphosphate. This reaction is in two steps; within these, the first step is favorable and another is unfavorable reaction. The gene encoding this enzyme is considered as housekeeping gene because of its constitutive high expression levels. The inhibitors of this enzyme selectively block

the trypanosomal GAPDH but do not block human GAPDH. In one study, the author revealed that the Golgi GAPDH enzymes consist of sequences with targeting glycosomal signals and this enzyme of Golgi and cytoplasm show 55% identity. The difference between the cytosolic GAPDH enzyme in between *L. donovani* and *L. major* plays a crucial role in survival of *L. donovani* in visceral organs. The deletion of cytosolic GAPDH eventually led to depletion of uptake of glucose. The inhibitors should have the capacity to inhibit both cytosolic and glycosolic enzymes. Then it may be an ideal inhibitor. So this enzyme provides the chance to treat as a drug target. In another study, the authors gave the information that adenosine analogs as tight binding inhibitor with the pocket of enzyme NADPH and the co-substrate id NAD^+ even though adenosine is a very poor inhibitor with IC_{50} value of 50 Mm. When tested against *T. brucei* and *T. cruzi* N^6-(1-naphthalenemethyl)-2′-(3-chlorobenzamido) adenosine inhibited the growth in micro molar range [65].

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Strategies for Tackling Drug Resistance in Tuberculosis

7

Laurent Maveyraud

7.1 Introduction

According to the latest Global Tuberculosis Report [1], the number of tuberculosis (TB) deaths was reduced by 22% between 2000 and 2015. Yet, more than ten million new TB cases occurred worldwide in 2015, and, for the second consecutive year, TB ranked first as the deadliest infectious disease, a position formerly awarded to HIV. Indeed, 1.8 million people died from TB in 2015, including 0.4 million HIV-positive patients. The difficulty to cure *Mycobacterium tuberculosis* (*Mtb*) infection finds its root in the specific biology of the bacilli [2]. Once present in the lung, after being inhaled, and if not immediately eliminated by the host innate immune defence, *Mtb* multiplies and spreads into the bloodstream. At this point, a delicate balance installs itself between *Mtb* and the host immune response, the latter being usually able to maintain the bacilli in a latent, nonreplicating, and asymptomatic state. Should the host immune defence weaken, *Mtb* would enter into an active state, leading to bacterial multiplication, tissue damage, and eventually death. In the latent TB state, the bacilli are able to survive for many years into the host, and, as they are in a metabolically inactive state, are highly resistant to drugs.

In the simplest case of drug-sensitive *Mtb*, the elimination of all bacilli from the organism requires a combination of four drugs over a 6-month period [3]: isoniazid and rifampicin are used for over 6 months, in combination with pyrazinamide and ethambutol for the first 2 months. Such a lengthy treatment favors inadequate observance, resulting in the emergence of resistant *Mtb* strains. Multidrug-resistant (MDR) *Mtb* are resistant to at least isoniazid and rifampicin, while extensively

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drug-resistant (XDR) bacilli are also resistant to any fluoroquinolones (such as levofloxacin or moxifloxacin) and to at least a second-line injectable compound (amikacin, kanamycin, or capreomycin). *Mtb* strains resistant to all tested antibiotics were reported [4, 5], although the term totally drug resistant (TDR) has not been approved [6, 7]. In case of resistance, longer treatments with less tolerated and more expensive drugs are required; nevertheless, those treatments fail in about 45% of the estimated 580,000 cases involving MDR *Mtb*, and in about 74% of the 55,000 cases involving XDR *Mtb* [1].

There is therefore an urgent need for new and more efficient compounds that would facilitate patient's adherence (shorter regimen with fewer compounds), be effective against MDR- and XDR-TB as well as against latent *Mtb*, and be compatible with treatments against HIV. This work provides a description of currently used anti-TB drugs, together with their targets and the associated mechanism of resistance. New drugs in the discovery pipeline are also introduced. Then, some of the strategies developed in order to circumvent drug resistance are presented, with an emphasis on the need to develop new molecular scaffolds. Conclusively, some mycobacterial metabolic pathways that are not currently addressed by available drugs are introduced that could provide new targets for future drug development.

7.2 Currently Used Drugs, Associated Target, and Resistance Mechanism

There are currently 28 drugs approved for usage in case of TB (Table 7.1) that are usually used in combination (TBdrugs 1.0 [8]). Prior to May 2016, they were classified into five groups (1–5): drugs in group 1 were first-line anti-TB drugs that were used to treat drug-susceptible TB; those in groups 2, 3, and 4 were to be used in case of drug resistance; and those in group 5 were those for which limited data existed for their efficacy and/or long-term safety, and were to be used in case no other options were available. As of May 2016, the classification evolved in four groups, A to D [9], of drugs to be combined in case of MDR: group A are the fluoroquinolones (levofloxacin, moxifloxacin, and gatifloxacin), group B gathers the second-line injectable agents (amikacin, capreomycin, kanamycin, and streptomycin), group C includes other core second-line agents (ethinoamide or prothionamide, cycloserine or terizodone, linezolid and clofazimine), and members of group D are called "add-on agents" that are not part of core MDR-TB regimen. It is further divided into subgroups D1 (pyrazinamide, ethambutol, and high-dose isoniazid), D2 (bedaquiline and delamanid), and D3 (*p*-aminosalicylic acid, imipenem-cilastatin, meropenem, amoxicillin-clavulanate, and thioacetazon). In case of MDR, cocktails of at least five effective drugs are recommended (one from groups A and B and at least two from group C). Those from group D are to be used in case such cocktail cannot be composed from drugs selected in groups A to C [9].

These drugs target only a few distinct cellular processes (Tables 7.1 and 7.2), among which the DNA-to-protein pathway (replication, transcription, and translation) and cell wall synthesis (arabinogalactan, peptidoglycan, or mycolic acid

Table 7.1 Currently approved anti-TB drugs

Drug	Chemical class	Therapeutic classification	Targeted cellular process	Molecular targets	Genes involved in resistance
Ethambutol	Aminoalcool	Group 1 Group D1	Cell wall synthesis, arabinogalactan	EmbA(Rv3794), EmbB(Rv3795), EmbC(Rv3793) Arabinosyl transferases	*embA, embB, embC, embR, rmlD*
Isoniazid (prodrug, activated by KatG)	Isonicotinic acid	Group 1	Cell wall synthesis, mycolic acids	InhA(Rv1484) NADH-dependent enoyl-ACP reductase	*katG, inhA*
Pyrazinamide (prodrug, activated by PncA)	Nicotinamide analogue	Group 1 Group D1	Debated	Debated	*pncA, rspA, panD*
Rifabutin	Rifamycin	Group 1	Transcription	RpoB(Rv0667) DNA-dependent RNA polymerase β	*rpoB*
Rifampicin/rifampin	Rifamycin	Group 1	Transcription	RpoB(Rv0667) DNA-dependent RNA polymerase β	*rpoB*
Rifapentine	Rifamycin	Group 1	Transcription	RpoB(Rv0667) DNA-dependent RNA polymerase β	*rpoB*
Amikacin	Aminoglycoside	Group 2 Group B	Translation	rrs 16s rRNA	*rrs, eis, whiB7*
Capreomycin	Aminoglycoside	Group 2 Group B	Translation	rrs 16s rRNA	*rrs, tlyA*
Kanamycin	Aminoglycoside	Group 2 Group B	Translation	rrs 16s rRNA	*rrs, eis, whiB7*
Streptomycin	Aminoglycoside	Group 2 Group B	Translation	RpsL(Rv0682) 30S ribosomal protein S12	*rpsL, rrs, gidB*
Gatifloxacin	Fluoroquinolone	Group 3 Group A	Replication, transcription	GyrB(Rv0005), GyrA(Rv0006) DNA gyrase	*gyrA, gyrB*

(continued)

Table 7.1 (continued)

Drug	Chemical class	Therapeutic classification	Targeted cellular process	Molecular targets	Genes involved in resistance
Levofloxacin	Fluoroquinolone	Group 3 Group A	Replication, transcription	GyrB(Rv0005), GyrA(Rv0006) DNA gyrase	*gyrA, gyrB*
Moxifloxacin	Fluoroquinolone	Group 3 Group A	Replication, transcription	GyrB(Rv0005), GyrA(Rv0006) DNA gyrase	*gyrA, gyrB*
Ofloxacin	Fluoroquinolone	Group 3	Replication, transcription	GyrB(Rv0005), GyrA(Rv0006) DNA gyrase	*gyrA, gyrB*
p-Aminosalicylic acid (prodrug activated by DhpS and DhfS)	Aminophenol	Group 4 Group D3	Other	FolA(Rv2763c) Dihydrofolate reductase	*thyA, folC, dfrA, ribD*
D-cycloserine	Oxazolidinone	Group 4 Group C	Cell wall synthesis, peptidoglycan	DdlA(Rv2981c) D-ala-D-ala ligase, AlrA(Rv3423c) Alanine racemase	*alr, ddl, ald, CycA*
Ethionamide (prodrug, activated by EthA)	Thiocarbamide	Group 4 Group C	Cell wall synthesis, mycolic acid	InhA(Rv1484) NADH-dependent enoyl-ACP reductase	*inhA, ethA, ethR*
Prothionamide (prodrug, activated by EthA)	Thiocarbamide	Group 4 Group C	Cell wall synthesis, mycolic acid	InhA(Rv1484) NADH-dependent enoyl-ACP reductase	*inhA, ethA, ethR*
Terizidone	Oxazolidinone	Group 4	Cell wall synthesis, peptidoglycan	DdlA(Rv2981c) D-ala-D-ala ligase, AlrA(Rv3423c) Alanine racemase	Unknown
Amoxicillin/clavulanic acid[a]	β-lactam	Group 5 Group D3	Cell wall synthesis, peptidoglycan	PonA1(Rv0050) Penicillin-binding protein	None reported
Bedaquiline (TMC207, Sirturo)	Diarylquinoline (first-in-class)	Group 5 Group D2, phase 3	Other	AtpE(Rv1305) ATP synthase subunit c	*atpE, mmpR5*
Clarithromycin	Macrolide	Group 5	Translation	RplV(Rv0706) 50S ribosomal protein L22	unknown

Clofazimine, tballiance	Oxazolidinone	Group 5	Unknown	Unknown (possibly electron transport, ATP synthesis)	*mmpR5*
		Group C			
Delamanid (*OPC-67683) (prodrug activated by Ddn)*	Nitroimidazole (first-in-class)	Group 5	Incertain	Unknown, likely inhibits mycolic acid production	*ddn, fdg1, fbiABC*
		Group D2, phase 3			
Imipenem/cilastatin[b]	β-lactam	Group 5	Cell wall synthesis, peptidoglycan	PbpB(Rv2163c) Penicillin-binding protein	Unknown
		Group D3			
Linezolid	Oxazolidinone	Group 5	Translation	RplC(Rv0701) 50S ribosomal protein L3	*rrl* (23S rRNA), *rplC*
		Group C			
Meropenem/clavulanic acid[a]	β-lactam	Group 5	Cell wall synthesis, peptdidoglycan	PbpB(Rv2163c) Penicillin-binding protein	Unknown
		Group D3			
Thioacetazone (prodrug activated by EthA)	Thiosemicarbazone	Group 5	Cell wall synthesis, mycolic acid	CmaA2(Rv0503c) Cyclopropane mycolic acid synthase 2	Unknown
		Group D3			

[a]Clavulanic acid is a β-lactamase inhibitor that protects carbapenems from hydrolysis
[b]Cilastatin is a human dipeptidase inhibitor that protects imipenem from hydrolysis

Table 7.2 Drug targets addressed y currently used anti-TB drugs

Pathway/molecular target	Drugs
Mycolic acid biosynthesis	
InhA	Isoniazid, ethionamid, prothionamid
Cyclopropane mycolic acid synthases (CMASs)	Thioacetazone
Mmpl3	SQ109
Unknown	Delamanid
Peptidoglycan biosynthesis	
D-alanine D-alanine ligase (Ddl)	Cycloserine, terizidone
Translocase I (TL1)	SQ641
ponA1(Rv0050)	Amoxicillin
pbpB(ftsI)(Rv2163c)	Imipenem, meropenem
Arabinogalactan biosynthesis	
Arabinosyl transferases embABC	Ethambutol
DprE1(Rv3790)	PBTZ-169
WecA(rfe)(Rv1302)	CPZEN-45
DNA replication and transcription	
DNA gyrases A, B	Levofloxacin, ofloxacin, moxifloxacin, gatifloxacin, DC-159a
Transcription	
rpoB(Rv0667)	Rifampin, rifapentine, rifabutin
Translation	
rrs(MTB000019) rRNA 16s	Kanamycin, amikacin, capreomycin,
rplV(Rv0706) 50s ribosomal prot L22	Clarithromycin
rpsL(Rv0682) 30s ribosomal prot S12	Streptomycin
rplC(Rv0701) 50s ribosomal prot L3	Linezolid, sutezolid, AZD-5847
rspA() 30S ribosomal protein S1	Pyrazinamide
Nucleic acid biosynthesis	
folA(Rv2763c) dihydrofolate reductase	p-Aminosalicylic acid
Other pathways	
atpE(Rv1305) ATP synthase subunit c	Bedaquiline
qcrB(Rv2196) Ubiquinol-cytochrome c reductase (cytochrome b subunit)	Q203
Membrane disruption	Clofazimine
Cell wall synthesis	Pretomanid, TBA-354, SQ-609
Membrane transport	TBI-166

biosynthesis) are largely predominant, with 13 and 10 drugs, respectively. The recently approved bedaquiline prevents ATP generation through the inhibition of the mycobacterial ATP synthase [10, 11], *p*-aminosalicylic acid is an inhibitor of dihydrofolate reductase [12], and there are three drugs, pyrazinamide, clofazimine, and delamanid, for which the exact mechanism of action still remains elusive.

Most, if not all, anti-TB drugs are facing resistance to some extent [13], to the point that, in the worst case, there are no more therapeutic options available, resulting in patient death [4, 14, 15]. However, since new drugs have entered the therapeutic armamentarium, calling the corresponding TB strains totally drug resistance might not be appropriate [6], although it is now suspected that they display distinctive morphology [5, 16]. Drug resistance arose as the result of spontaneous genomic mutations since horizontal gene transfer does not occur with *Mtb* [17]. Multiple molecular mechanisms are involved in *Mtb* drug resistance, but only those specifically affecting a given drug are mentioned here: modification of the drug target (either the level of expression or the sequence) and inhibition of the enzyme responsible for the activation of the prodrug.

Several anti-TB drugs are prodrugs that require activation by specific mycobacterial enzymes in order to be active (Table 7.1). Isoniazid, the major first-line antitubercular drug, requires activation by the catalase peroxidase KatG before reacting with NADH in order to form the INH-NAD adduct that will bind to and inhibit InhA, a NADH-dependent enoyl-ACP reductase involved in mycolic acid biosynthesis [18]. Two other drugs, ethionamide and prothionamide, target the same enzyme; they also require activation though by another enzyme, the monooxygenase EthA, in order to form an adduct with NADH [19]. Thioacetazone also necessitates prior activation by EthA before covalently modifying the dehydratase HadA, involved in mycolic acid biosynthesis [20]. *p*-Aminosalicylic acid has recently been shown to also be a prodrug that is converted to folate intermediate analogues that ultimately inhibit dihydrofolate reductase [12]. Two enzymes in the folate metabolic pathways, dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS), are likely to be responsible for the activation of *p*-aminosalicylic acid [12], but a functional thymidylate synthase A seems also to be required [21]. Last prodrug of the anti-TB arsenal, pyrazinamide displays quite an intricate mechanism of action. It is converted to pyrazinoic acid, thanks to the pyrazinamidase/esterase PncA, but the possibility that a host-mediated activation might also be involved has been evocated [22]. Furthermore, its precise mechanism of action is still controversial; inhibition of Fas1, the type I fatty acid synthase [23, 24], of the biosynthesis of NAD [25] or disruption of the membrane energetics [26] was proposed.

For prodrugs, mechanisms of resistance are multiple, as resistance-conferring mutations can occur either in the enzyme responsible for drug activation or in the final drug target. This is the case for isoniazid and ethionamide, resistance to which arises from mutations either in the gene encoding the drug-activating enzymes *katG* and *ethA*, respectively, or in *inhA* [27–29]. In isoniazid-resistant *Mtb* strains, mutations of *katG* are the most frequently observed, followed by mutations in the promoter region of *inhA*, resulting in a 20-fold overexpression of InhA. Mutations in the ORF of *inhA* are comparatively rare in isoniazid-resistant strain, whereas they are prevalent in ethionamide-resistant strains [30]. Resistance to pyrazinamide predominantly results from mutation in the *pncA* gene [31], although mutations in *rspA*, encoding the essential ribosomal protein S1 [32] and in *panD* [33], are also suspected to confer pyrazinamide resistance. In the case of *p*-aminosalicylic acid, mutations in the gene *thyA* encoding thymidylate synthase A were reported to

confer resistance [34] as well as mutation in *dhfA*, encoding the dihydrofolate reductase and *ribD*, an enzyme involved in the riboflavin biosynthetic pathway [35].

For drugs that do not require prior activation, the main mechanism of resistance is generally mutation in the gene encoding the drug target. This is what happens for rifamycins (rifampicin, rifabutin, and rifapentin), with mutations occurring in *rpoB*, encoding the β-subunit of the DNA-dependent RNA polymerase [36]; for aminoglycosides (amikacin, kanamycin, and capreomycin), with mutations in *rrs*, encoding the 16S rRNA; for fluoroquinolones (moxifloxacin, gatifloxacin, levofloxacin, and ofloxacin) with mutations in *gyrA* and *gyrB* [37]; for ethambutol with mutations in *embB* [38]; and for streptomycin with mutations in *rrs* an *rpsL* [39].

In all cases, additional mechanism of resistance can occur, such as overexpression of the transcription regulator of the *mmpL5* gene, encoding the efflux pump MmpL5, which causes resistance to bedaquiline and clofazimine [40].

7.3 Drugs in the Pipeline

The front-line drugs used to eradicate drug-susceptible Mtb, isoniazid, rifampicin, pyrazinamide, and ethambutol, were discovered more than 50 years ago, in 1951, 1957, 1952, and 1962, respectively [13]. Development of the second-line drugs is barely more recent, as they were discovered between 1940 (for *p*-aminosalicylic acid) and 1963 (for fluoroquinolones). In 2012, 8 years after being patented, Bedaquilin received conditional approval by the FDA, although phase 3 clinical trials are still under way: it's the first new drug specifically designed for TB to appear in the clinics for nearly 40 years. Delamanid followed 2 years later. The anti-TB drug development pipeline at last irrigates the therapeutic arsenal with new compounds, and there is hope that the flow will not dry out as new compounds are struggling their way forward (Table 7.3). Yet, the recent interruption of the late-phase development of TBA-354, withdrawn from phase 1 in March 2016 [41] and of Posizolid (AZD-5847), interrupted during phase 2 in February 2016 [42], is there to prove that success in drug development is never granted.

There are currently 784 studies registered at the clinicaltrials.gov web site of the US National Institute of Health [43] concerning the topic tuberculosis. About 25% of these studies are observational, and many interventional studies concern evaluation of vaccines, diagnostic procedures, or the use of new drug combinations or modified protocol of administration of already validated drugs. The number of trials involving new anti-*Mtb* drugs is alarmingly small. Table 7.3 gives an overview of new anti-*Mtb* compounds currently being developed, and gathers data from several sources (ClinicalTrials [43], TBdrugs 1.0, NewTBDrugs [44], TBAlliance [45]). It appears that there are 15 compounds that are in clinical development, only 5 of which potentially inaugurate new chemical classes: Q203, PBTZ169, OPC-167832, GSK3036656, and sudoterb (LL3858). Other compounds are either derivatives of existing anti-TB drugs (oxazolidinones, nitroimidazoles …) or trials to reposition molecules (carbapenems, sulfamides, nitrothiazols …). One molecule, FS-1, developed by the Scientific Center for Anti-Infectious Drugs in Kazakhstan, is reported

as being currently in phase 3 of clinical development for treatment of MDR-TB (trial identifier NCT02607449, ClinicalTrials.gov), but no information about its structure or activity could be found. The only other compound in phase 3, pretomanid, also known as PA-824, is a prodrug activated by the nitroreductase (Ddn) in the same chemical class as delamanid, nitroimidazoles. It displays a complex mechanism of action and is active against both replicating and nonreplicating *Mtb* [46]. Five potential first-in-class drugs are in phase 1 or 2; they are (1) GSK3036656, a LeuRS inhibitor developed by GSK; (2) OPC-167832, developed by Otsuka Pharmaceuticals and claimed to display a new mechanism of action; (3) sudoterb (also known as LL3858), registered in phase 2 by Lupin Ltd. in India in 2009, but with no updated data since 2013; (4) Q203, a imidazopyridine amide targeting the cytochrome bc1 complex [47], and (5) PBTZ169, a benzothiazinone discovered at the EPFL in Switzerland, targeting the decaprenylphosphoryl-beta-D-ribose 2-epimerase DprE1 [48]. Given the time necessary to move drugs from phase 2 to approval, and the potential associated attrition, further efforts are required if the global TB epidemic is to be ended at the 2035 horizon [1, 49].

7.4 Designing New Molecular Scaffolds

In the endless fight opposing bacteria and the pharmaceutical industry, bug resistance is most easily addressed by evolving a challenged compound into a usually more complicated new molecule, able to escape, at least temporarily, resistance. This is well exemplified by the successive generations of β-lactams that were developed over the years, in an attempt to face the progression of resistance [50]. Designing new molecular scaffolds, ideally targeting yet unaddressed molecular targets, is likely to provide drugs that should escape current resistance mechanisms. However, designing new “magic bullet” takes about 15 years and requires huge funding [51], yet resistance is likely to arise within a few years. Nevertheless, new classes of compounds are currently being investigated (Table 7.3) and bedaquiline and delamanid were granted conditional approval recently. Bedaquiline (TMC207), the first new drug developed specifically for TB since almost 40 years [3], inhibits the mycobacterial ATP synthase [52]. This diarylquinoline is used since 2011 in more than 50 countries, and cases of resistance were already reported [53]. Delamanid (OPC-67683), a nitroimidazole, has been approved in Europe in 2014. It is believed to interfere with mycolic acid synthesis, although the exact mechanism of action remains elusive [54].

Identifying new active scaffolds usually starts with the identification of a new potential drug target. Deciphering of the *Mtb* genome [55] provided a wealth of information about potential drug targets, and initiated tremendous target-oriented screening effort that did not quite meet their expectations [56–58]. Indeed, an essential target might eventually prove not to be easily druggable and even if active compounds are identified, they will not necessarily translate into efficient drugs in vivo, as the screening procedure overlooks the crucial difficulty of penetrating into an essentially impermeable cell wall. Yet, this target-to-drug approach [57] did allow identifying promising compounds.

Table 7.3 Drugs in the development pipeline

Drug	Chemical class	Status	Molecular target
Posizolid/AZD-5847 (tbdrugs[a])	Oxazolidinone	Discontinued (Feb 2016)	50S ribosomal protein L3
TBA-354 (tballiance)	Nitroimidazole	Discontinued (March 2016)	Unknown
FS-1 NCT02607449[b]	Unknown	Phase 3	Unknown
Pretomanid (PA-824) NCT00567840	Nitroimidazole	Phase 3	Cell wall synthesis
Faropenem NCT02393586	β-lactam	Phase 2	Transpeptidase LdtMt2
delpazolid (LCB01-0371) NCT02836483	Oxazolidinone	Phase 2	Protein synthesis inhibitor
Metronidazole NCT00425113	Nitroimidazole	Phase 2	Unknown
Nitazoxanide NCT02684240	Nitrothiazole	Phase 2	Potentially membrane disruption
SQ109 (newtbdrugs) NCT01218217	Ethylenediamine	Phase 2	Mmpl3(Rv0206c)
Sutezolid/PNU-100480 (newtbdrugs) NCT01225640	Oxazolidinone	Phase 2	50S ribosomal protein L3
Co-trimoxazole (sulfamethoxazole and trimethoprim) NCT01832987		Phase 2	Unknown
Ertapenem NCT01730664	β-lactam	Phase 2	
GSK3036656 NCT03075410	Unknown	Phase 1	Unknown
OPC-167832 (newtbdrugs)	New	Phase 1	
Q203 (newtbdrugs)	Imidazopyridine amide (first-in-class)	Phase 1	QcrB(Rv2196)
LL3858/sudoterb	Pyrole derivative	Phase 1 (?)	Unknown
PBTZ-169 (host activated prodrug) NCT03036163	Benzothiazinone (first-in-class)	Phase 1	DprE1(Rv3790)
BTZ-043 (newtbdrugs)	Benzothiazinone	Preclinical	DrpE1(Rv3790)
CPZEN-45 (tbdrugs)	New	Preclinical	WecA(rfe)(Rv1302)
DC-159a (tbdrugs) PMID: 21282421	Fluoroquinolone	Preclinical	gyrB(Rv0005); gyrA(Rv0006)
GSK-070 (tbdrugs)	New	Preclinical	Unknown
GSK-693		Preclinical	InhA

Table 7.3 (continued)

Drug	Chemical class	Status	Molecular target
SATB-082 (tbdrugs)	Cyclopeptide (firs-in-class)	Preclinical	Unknown
Spectinamide 1599 (tbdrugs)	New	Preclinical	Unknown
SQ609 (tbdrugs)		Preclinical	Inhibition of bacterial cell wall synthesis
SQ641 (tbdrugs)		Preclinical	MraY(Rv2156c)
TBA-7371 (tbdrugs)	New	Preclinical	Unknown
TBI-166 (tbdrugs)		Preclinical	Membrane transport

[a]tbdrugs: http://bic.icmr.org.in/tbdrugs, tballiance: https://www.tballiance.org, newtbdrugs: http://www.newtbdrugs.org
[b]NCTXXXXXX is the reference number of the clinical trial at https://clinicaltrials.gov

7.4.1 Target-to-Drug Approach

One of the best characterized targets of *Mtb* is InhA, an NADH-dependent enoyl-ACP reductase found in the fatty acid synthase of type II, involved in mycolic acid biosynthesis. It catalyzes the reduction of a *trans* double bond conjugated to a carbonyl group of an intermediate covalently linked to an acyl-carrier protein in the FAS-II pathway. As it is the target of the anti-TB prodrugs isoniazid and ethionamide, it is a well-validated drug target that has been studied extensively [59–61]. Most cases of *Mtb* resistance to isoniazid or ethionamide result from mutations of KatG and EthA, the mycobacterial enzymes responsible for isoniazid and ethionamide, respectively, prodrug activation. Designing inhibitors of InhA that would not require preliminary activation is therefore an active area of research. A screening campaign at GlaxoSmithKline identified thiadiazol derivatives as inhibitors of InhA that were also active as *Mtb* growth inhibitors [62, 63]. Further optimization led to compounds with interesting in vivo activity [64] that are currently in the lead optimization phase at GlaxoSmithKline [44].

Ethionamide also targets InhA, and requires activation by the mono-oxygenase EthA, the expression of which is controlled by the repressor EthR. EthA is constitutively expressed at low level, resulting in a low efficiency in ethionamide activation [65] that implies high therapeutic doses. The inhibition of EthR would increase the expression of EthA and subsequently augment ethionamide activity. The crystal structure of EthR [66] was used to design BDM31343, an EthR inhibitor that allowed to substantially reduce ethionamide dosage in a mice model of TB [67]. Further optimization resulted in compounds currently in preclinical development [68, 69].

The glyoxylate shunt is an essential pathway for *Mtb*, especially to allow for persistence into macrophages [70, 71]. As this metabolic pathway is absent in mammals [72], the enzymes involved have been identified as promising drug targets, especially isocitrate lyase (ICL) and malate synthase (MS). ICL resisted many drug discovery efforts for a long time, and it's only recently that salicylanilides were

reported as potent inhibitors of *Mtb* ICL *in vitro*, but also as antimycobacterial agents with comparable potency as isoniazid or *p*-aminosalicylic acid [73]. The case of *Mtb* MS seems more promising, as the substrate binding cavity is larger than in the case of ICL and does not display conformational adaptation upon substrate or product binding [74]. Phenyl-diketo acids were identified as potent inhibitors of MS in vitro, and a structure-based optimization process resulted in compounds combining potency, bioavailability, and low toxicity [75].

Since the seminal work of the group of S. Fesik [76], fragment-based drug design (FBDD) has emerged as a powerful technique for the discovery of new drugs. Indeed, more than 30 currently developed drugs evolved from FBDD programs, among which two drugs, vemurafenib and venetoclax, are approved [77]. FBDD relies on the hypothesis that small molecules, called fragments, with a molecular weight of 150–300 Da are more likely to bind to a protein-active site than larger drug-like compounds, albeit with a lower affinity. Application of FBDD to the field of tuberculosis is maybe not as developed as in other therapeutic area, yet significant efforts are devoted to the identification of new compounds [78, 79]. As the number of validated drug targets increases, the success rate is deemed to improve.

7.4.2 Drug-to-Target Approach

Opposite to the target-to-drug approach, phenotyping screening on whole bacteria is believed to be more effective in delivering active compounds, as this approach identifies compounds that are able to reach their targets in a more physiologically relevant environment [56, 58]. However, this does not provide information about the mechanism of action of the identified compounds, nor does it designate the affected target. This additional information is potentially gained by identifying mutations responsible for generated resistance to the evaluated compounds. Such drug-to-target methodology allowed identifying the F0 subunit of the *Mtb* ATP synthase as the target of the diarylquinoline drug bedaquiline [10, 52]. Recently, a scalable platform was designed, based on whole-cell screening, resistance generation, and whole-genome sequencing [80] and several proteins, not yet targeted by anti-TB drugs, were identified as potential candidates: Pks13, an essential polyketide synthase involved in mycolic acid biosynthesis [81–83]; AspS, an aspartyl-tRNA synthetase; and EccB3, a component of the ESX-3 type VII secretion system [84, 85]. The list of targets identified using whole-genome sequencing coupled to phenotypic screening and resistance generation is growing steadily [57], and includes AtpE, targeted by bedaquiline [10]; Ddn, targeted by delamanid [86] and PA-824 [46]; DrpE1, targeted by BTZ043 [87]; and QrcB, targeted by Q203 [47]. This approach delivers simultaneously compounds active on the bacilli as well as their molecular target, which is an invaluable advantage for the hit-to-lead process, as it allows the use of all tools provided by biophysics, structural biology, and modeling methodologies [88, 89].

Both target-to drug and drug-to-target approaches are susceptible to feed the drug development pipeline on their own, yet it is likely that combining both into a

drug-to-target-to-drug approach might prove to be even more efficient, as it allows overcoming their inherent individual limitations. Phenotyping screening is restricted to compounds included in chemical libraries that are predominantly rule-of-five compliant [90], which limits the explored chemical space. It is noteworthy that 10 out of the 28 anti-TB drugs do not obey rule of five, as being larger than the 500 Da limit or displaying low solubility. Eight additional compounds are smaller than 250 Da, which might be considered as too small to be included in a chemical library. However, identified hits are known to be able to reach their intracellular target. Target-based drug design is susceptible to explore much more efficiently the available chemical space, especially in the case of fragment-based ligand discovery [91] and virtual screening, but translating hit into efficient lead *in vivo* might prove challenging.

7.5 Drug Repositioning

Discovering new drugs is a lengthy and costly process, which is especially problematic in the field of antimicrobials, as the advent of resistance is likely to interfere with their use. As of 2013, it was estimated that the FDA had approved a little more than 1400 new molecular entities [92]. Similarly, the NIH Chemical Genomics Center identified about 4000 molecular entities that were approved for human or veterinary usage worldwide [93], representing a cumulated huge investment of time and money. Drug repositioning, or drug repurposing, is the process of identifying new uses of already approved drugs, in order to reduce the cost and time needed to bring them on the market [94]. There were several reports of successful drug repositioning, as for instance the case of sildenafil [95], a type 5 phosphodiesterase inhibitor initially developed against angina that was first successfully repositioned as a therapy for erectile dysfunction [96] and more recently as a treatment for pulmonary hypertension [97].

It was recently suggested that about one-third of the currently available drugs were susceptible to be repositioned to treat TB [98]. Among the currently approved anti-TB drugs, later generation fluoroquinolones such as moxifloxacin, levofloxacin, and gatifloxacin [99]; the oxazolidinone clofazamine [100]; and carbapenems, imipenem, and meropenem [101], are repositioned antimicrobial agents [3]. Identifying further approved drugs with anti-TB properties would be an efficient means to replenish the drug development pipeline, as such compounds would enter clinical trials directly in phase 2. However, despite appearing appealing, drug repositioning or rescuing, where compounds that failed in phases 2 or 3 for a given indication are redirected towards a new application, is a challenging endeavor that has to face lack of funding and patenting issues [102]. Nevertheless, several pharmaceutical companies are now systematically investigating potential repositioning of their drug portfolio. Additionally, drug repurposing might be more amenable to academia and to small biotech companies than the huge HTS campaigns performed in large pharmaceutical industries [103].

7.6 Hitting the Same Target

Identifying the target of new potential drugs is usually a difficult endeavor, although the recent advent of whole-genome sequencing helps reducing the burden of the task. On the other hand, there are already a significant number of targets known to be druggable, as they are addressed by current anti-TB drugs, or currently developed compounds. These are inventoried in Table 7.2. Many efforts are devoted to the identification of new compounds able to inhibit those proteins, using a wide panel of approaches: high-throughput screening, structure-based drug design, fragment-based ligand discovery, or virtual screening.

There are however pitfalls to be expected along this way. The easiest way to address a validated drug target is to evolve an existing compound in order to improve its efficacy or to escape resistance phenomena, as done in the case of fluoroquinolones or aminoglycosides. However, this means targeting a protein for which it is known that the encoding gene is able to evolve and generate resistance. It is therefore quite certain that evolution of resistance will closely follow the chemical evolution of the drug. Yet, meanwhile, the evolved drug might still be a useful addition to the therapeutic arsenal.

InhA is probably the most explored drug target, as exemplified by the more than 80 structures available at the Protein Data Bank (PDB [104]), as of March 2017, corresponding to structures of wild-type InhA or mutants, as well as numerous complexes obtained in the presence of ligands identified with various screening procedures. Surprisingly enough, the structure of the *apo*-enzyme was only elucidated in 2015 [60], while the first structure of InhA, in the presence of its cofactor, was published more than 20 years ago [61]. The most efficient anti-TB drug is isoniazid, which targets InhA, but requires prior activation by KatG in order to be active. MDR- and XDR-TB are resistant to isoniazid, principally as the results form mutation in *katG*, although mutation in *inhA* is also known to occur. Most of the research efforts presently pursued aim at identifying or designing new compounds that would not require activation as a condition for InhA inhibition. These efforts led to the thiadiazole derivatives already mentioned. Additionally, InhA is the target of pyridomycin, a natural product isolated from *Streptomyces albidofuscus* [105]. Pyridomycin was shown to bind to InhA in an unprecedented way, as it simultaneously occupies not only the substrate-binding site, but also the NADH-binding site [106]. Such binding mode is quite promising, as it should delay significantly the arising of resistance. Indeed, any mutation resulting in a modified pyridomycin-binding site is also likely to affect NADH binding, resulting in a less efficient enzyme. Pyridomycin serves also as a good illustration of the potential benefits of screening natural products, which are generally underrepresented in chemical libraries [107], and allow a larger exploration of scaffold diversity [108].

7.7 Emerging Target

To be qualified as a target, several criteria are considered: essentiality, selectivity, and vulnerability [109]. The essentiality of a gene, for survival or growth, should be evaluated in conditions as physiologically relevant as possible. The absence of

homologous genes in the human genome, the selectivity criterion, will ensure that no host pathway is affected by the future drug. A vulnerable target will require low amount of drug to result in cell death. Then remains the question of the druggability of the target: Will it be possible to identify chemical compounds able to inhibit its function? This is quite difficult to assess, and usually relies on previous knowledge of druggability of a related protein, possibly in other organisms.

A survey of recent literature indicates that many new pathways are investigated as part of drug discovery initiatives. Only a few examples will be given here, as this is a topic for which many excellent reviews were recently published [13, 109, 110].

7.7.1 Cell Wall Metabolism

The mycobacterial cell wall attracts lot of attention, as many constituting compounds are characteristic of mycobacteria, even when compared to Gram-positive or Gram-negative bacteria. It is therefore not surprising that many approved drugs target this pathway (Table 7.2), nor that additional drug targets are expected to be found.

Mycolic acids are specific lipids found in Mycobacteria, resulting from the condensation of a C_{24}–C_{26} fatty acid, synthetized by the eukaryotic-like fatty acid synthase FAS-I, with a very long meromycolic acid chain, containing up to 60 carbon atoms, produced by the bacterial like FAS-II [111]. The enzymes involved in FAS-II and in the condensation steps are likely to be potential drug targets. Indeed, Pks13, the polyketide synthase that actually performs the condensation reaction, was shown to be essential [112], as is the case for the enzymes involved in substrate activation FadD32 and AccD4 [83]. In the mycolic acid pathway, several other proteins were reported as essential, as KasA [113]; HadABC, involved in mycolic acid synthesis; the SAM-dependent methyltransferases, responsible for mycolic acid decoration; or the mycolyl transferase [114]. Inhibitor identification or design [115, 116], or at least assay development [117], were reported in most cases, although no drugs are currently developed.

The biosynthesis of peptidoglycan is targeted by multiple antibiotics, such as carbapenems, and those are being repositioned to be used in the treatment of MDR-TB. The mycobacterial peptidoglycan possesses some specific features, not found in other bacteria, such as *N*-glycolylmuramic acids [118], amidation of the carboxylic group of the peptide links [118], and additional glycine or serine residues [119]. Although some inhibitors were reported for the enzymes involved in the synthesis of these compounds [120] or their inclusion in the peptidoglycan [121], no compounds seem to have reached the drug development stage. As a further example of the possibilities offered by the peptidoglycan biosynthesis pathways, the glutamate racemase MurI responsible for the D-glutamate synthesis was recently shown to be essential [122], providing yet another potential drug target in this cellular process.

The mycobacterial cell wall includes a polysaccharide, arabinogalactan, that is covalently linked to the peptidoglycan and onto which mycolic acids are covalently attached. The arabinogalactan synthesis is already a validated target, as ethambutol inhibits the arabinosyl transferases embABC and PBTZ-169, in phase 1 target DprE1, a subunit of the decaprenylphosphoribose 2′-epimerase [87].

7.7.2 Iron Uptake and Storage

As for all organisms, iron is an essential nutrient for *Mtb*. Yet, iron is not easily accessible in human body as it is sequestered by ferritin inside cells and by transferrin in the extracellular environment [123]. In order to have access to iron, *Mtb* synthesizes mycobactins and carboxymycobactins, secretes them, and once loaded with iron uptakes them back [124]. MbtA, involved in mycobactin biosynthesis, was shown to be essential, and inhibitors with potent activity were identified [125]. MmpL3, involved in iron uptake, is the only MmpL protein among 12 that was shown to be essential for *Mtb* [126]. In addition to its role in iron homeostasis, it was shown to take part in the transport of mycolic acids across the membrane [127]. Its role at the intersection of distinct essential pathways is especially relevant in a drug design perpective.

Iron storage in *Mtb* is the role of BfrA, a bacterioferritin, and BfrB, a ferritin-like, both having been shown to be of crucial importance for *Mtb* survival and pathogenicity [128, 129]. The expression of genes involved in iron acquisition and homeostasis is tightly regulated in *Mtb*, the role of IdeR being central in this process [130]. In the presence of Fe, IdeR represses the genes involved in siderophore synthesis and activates those involved in storage, *bfrA* and *bfrB*. Inhibition of IdeR results in unlimited iron uptake and faulty storage.

7.7.3 Carbon Metabolism

Mtb is able to grow and multiply using fatty acids as the principal source of carbon, which fuel the gluconeogenesis and the tricarboxylic acid pathways. The role of the phosphoenolpyruvate carboxykinase PEPCK is central in gluconeogenesis, as it catalyzes the first step in this pathway, the conversion of oxaloacetate and phosphoenolpyruvate. This enzyme was shown to be essential for growth and survival of *Mtb* during infection [131]. One limitation for targeting the central carbon metabolism is the existence of orthologous enzymes in human. There are however sufficient examples of drugs specifically addressing a pathogen target while sparing the human orthologue. Indeed, bedaquilin inhibits mycobacterial ATP synthase 20,000-fold more efficiently than the human mitochondrial protein [132].

When carbon sources are scarce in the environment, the central carbon metabolism pathway fails to provide the necessary carbon for *Mtb* growth. Alternative pathways are therefore activated, such as the glyoxylate shunt or cholesterol catabolism. Enzymes involved in the glyoxylate shunt, and more specifically ICL and MS, were already evocated in this work. In the latent *Mtb* infection state, the bacillus is sequestered in granuloma by the host immune system, which unfortunately protects *Mtb* from drugs. Catabolism of cholesterol was suggested to contribute to the survival of *Mtb* in the macrophage. Cholesterol uptake and degradation depend on three operons: *mec4* is involved in cholesterol uptake, whereas *igr*, the intracellular growth operon, and *hsaACDB* are implicated in cholesterol degradation [133].

As most of the encoded enzymes are absent in the human proteome, targeting them would provide drugs active against latent *Mtb*, provided that the drug can reach its target across the granuloma and the *Mtb* cell wall.

7.7.4 Cutinase-Like Proteases (Clp)

Damaged or partially unfolded proteins are rapidly degraded in the cell, thanks to the presence of Clp. Activity of these proteases is normally strictly regulated, as inappropriate activation would result in anarchical degradation of proteins, whereas an inactivation would lead to the accumulation of misfolded proteins. Dysregulation of this proteolytic activity was shown to be responsible for the antibacterial activity of acyldepsipetides (ADEPs) against *Bacillus subtilis* [134]. Clp is formed by the association of the heptadecameric ClpP1/ClpP2 with the hexameric Clp ATPase. ClpP1 was shown to be essential in *Mtb* [135]. ADEPs where shown to activate ClpP, by allowing its activity even in the absence of the Clp ATPase, inducing non-specific proteolysis of bacterial proteins leading to cell death in several bacterial species [136]. Interestingly, as ClpP was shown to be essential, its inhibition might also be lethal, as is the case in *Caulobacter crescentus* in the presence of cyclic peptides [137].

Conclusion

The field of anti-TB drug discovery has long been neglected, resulting in more than 40 years without new specifically designed compounds brought onto the drug market. This period ended with the recent conditional approvals of bedaquilin and delamanid that inaugurated the use of diarylquinoline and nitroimidazole derivatives, respectively, in the fight against *Mtb*. Such event might hide the fact that the molecules remaining in the drug development pipeline are, with a few exceptions, variations on an existing scaffold. The End TB Strategy, adopted in 2014 by WHO, ambitions to reach a 90% reduction in TB deaths and an 80% reduction in TB incidence by 2030 compared with 2015 [1], and an eradication of TB by 2035 [138]. This ambition calls for a substantial increase in funding TB programs, to encourage the pharmaceutical industry as well as academic research to intensify drug discovery and validation efforts.

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ADMET Properties: Overview and Current Topics

8

Haizhen A. Zhong

8.1 Introduction

Medicinal chemistry is an interdisciplinary research area that deals with the discovery and design of new therapeutic chemicals that have potential applications to useful medicines. A drug molecule, according to IUPAC (The International Union of Pure and Applied Chemistry), is a substance that treats, cures, or prevents diseases in human beings or animals. A drug molecule generally contains one or more functional groups which enable the specific binding between a drug and a target biological macromolecule. The scaffold of a drug should be conformationally constrained such that an array of functional groups can interact with target proteins. A very flexible scaffold would alter the molecular shapes and conformations, resulting in the interactions of this drug with other off-target receptors and thus causing side effects or toxicity.

A drug-like molecule (DLM) possesses the physicochemical properties that might enable it to become a drug should a disease-modifying receptor be identified. A drug-like molecule is not a drug molecule and not all molecules are drug-like molecules. What properties of a molecule make it to be drug-like? Generally speaking, an orally available molecule that satisfies Lipinski's rule (Lipinski-compliant) and shows a balance between lipophilicity and hydrophilicity would qualify it to be a drug-like molecule.

A drug molecule not only is expected to be efficacious, but to have good toxicity profile, which is normally expressed in therapeutic index. Therapeutic index (TI) (also called the margin of safety) is the ratio of the minimum concentration (dose) that produces toxicity divided by the minimum concentration (dose) of a drug which

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produces therapeutic outcomes (Eq. 8.1). The larger the therapeutic index, the safer the drug. It is necessary to be cautious for drugs that are toxic, ineffective, or have a narrow therapeutic window.

$$\text{Therapeutic index} = [\text{minimum toxicity dose}] / [\text{minimum effective dose}] \quad (8.1)$$

More commonly TI can be expressed as Eq. 8.2 because LD_{50} and ED_{50} are easier to measure.

$$\text{Therapeutic index} = [LD_{50}] / [ED_{50}] \quad (8.2)$$

8.2 ADME Properties of a Drug Molecule

8.2.1 Concepts of ADME, Pharmaceutics, Pharmacokinetics, and Pharmacodynamics

The US Food and Drug Administration (FDA) has approved 117 distinct routes of administration for a drug molecule. The common routes are oral, intravenous, intramuscular, topical, inhalational, and intranasal. Among all 117 different routes, oral administration is the most popular one. A patient finds it easier to open the lid of a bottle and take the tablets/capsules rather than taking an injection intravenously. Orally administered drugs are ingested in the mouth, after which it travels through a long journey in the human body until drugs reach the interaction site (if at all) and exerts its pharmacological effect. Pharmaceutics is the discipline of pharmacy that deals with design of an appropriate dosage form such that a drug molecule can be transported safely and effectively to human body. Once a drug enters the body, the body starts to process the four aspects of a drug: absorption, distribution, metabolism (biotransformation), and elimination (commonly called as ADME). Pharmacokinetics is the field that studies these four processes of ADME. The pharmacokinetic process of a drug answers whether a drug is able to get to the site of action. The pharmacodynamic process provides the answer of whether or not a drug is able to produce the required pharmacological effect.

8.2.2 Drug Absorption

When a drug is administered via oral ingestion, it must cross membranes of many cells to reach its site of action. For an oral drug, it must survive the acidic environment of the gastric fluid in the stomach (pH of gastric acid: about 1.4–2.1) and must pass across the plasma membrane, which consists of a lipid bilayer with the hydrophobic tail pointing to the interior while the hydrophilic polar heads are oriented toward aqueous solutions (extracellular or intracellular). Cell membranes are relatively permeable to water. The more hydrophobic the molecule is, the more easily it diffuses across the membrane. Charged or large polar molecules do not diffuse

across the membrane. Drug molecules bound to plasma proteins cannot be carried across the membrane due to the large size of protein–drug molecule complex. Therefore, transmembrane movement of a drug molecule generally is limited to the unbound drug. Embedded in the lipid bilayer of the membrane are carrier proteins (transporters), ion channel proteins, and receptors. Charged or large polar molecules are normally transported by specific carrier proteins.

There are two common types of mechanisms for the transmembrane movement of drugs. The most common is passive membrane transport. In the passive transport process, drug molecules diffuse along the concentration gradient, i.e., moving from high to low concentration by virtue of its solubility in the lipid bilayer. The rate, therefore, is dependent upon both the gradient of the drug concentration on both sides of the membrane, and the lipophilicity of the drugs. High lipophilic drugs tend to have high concentration inside the membrane, leading to larger gradient between the membrane and the cytosolic solution. The large gradient between the membrane and the cytosolic solution causes the fast diffusion from membrane to cytosolic solution. Once the steady state is reached, the concentration of unbound drugs on both side of the membrane should be identical. Passive transport is the major route for uncharged (unionized) and non-polar drugs and requires no energy for the transmembrane movement. Passive diffusion is the dominant means of cross membrane transport for most neutral drugs.

The second type of drug absorption is active transport. Active transport mediated by carrier proteins plays an important role in charged or very polar drugs. Active transport is characterized by a direct requirement of energy as well as high selectivity, saturation, and movement against an electrochemical gradient. When a transporter is saturated with drug molecules, further increasing concentration will not accelerate the rate of cross membrane transport. For instance, Na^+/K^+-ATPase is an enzyme involved in the active transport. Cells contain relatively high concentrations of potassium but low concentrations of sodium ions. Maintenance of such an electrochemical potential is achieved by the Na^+/K^+-ATPase, or the sodium-potassium pump, which pumps sodium out of cells while importing potassium into the cells. Export of sodium provides the driving force for facilitated transport proteins, which import glucose and other nutrients into the cell. The difference between active transport and the facilitated diffusion is that the facilitated diffusion requires no input of energy and is down the electrochemical gradient. For example, the permeation of glucose across a muscle cell membrane is mediated by the glucose transporter protein GLUT4. Active transport can sometimes produce unwanted effects. For example, the P-glycoprotein encoded by the multidrug resistance-1 (MDR1) gene limits the oral absorption of some cancer drugs because it effluxes drug molecules out of cells, reducing the effective concentration of drug molecules.

8.2.3 pH Influence on Drug Absorption

The lipophilicity (hydrophobicity) of a molecule is a measurement of lipid solubility; in other words, the ability of a chemical compound to dissolve in oil, lipid, or

non-polar solvents. It is common to use the partition coefficient (*P*) to measure the lipophilicity. The partition coefficient is calculated by the following equation (Eq. 8.3):

$$P = \frac{[\text{Drug}]_{\text{octanol}}}{[\text{Drug}]_{\text{aqueous}}} \tag{8.3}$$

The greater the partition coefficient (*P*) is, the more hydrophobic the compound is. Most drugs are weak acids or weak bases which exist as a non-ionized form in solution. These molecules are very lipid soluble. The value of *P* is generally in the range of 10^1–10^6. It is more convenient to express the partition coefficient as log *P*, which has a range of 1–6 for most drug molecules.

When a weak acid is dissolved in aqueous solution, the dissociation of the acid gives the proton and the anion (Eq. 8.4). The acid constant is expressed as the concentration of the proton multiplying the concentration of the anion divided by the concentration of the neutral acid (Eq. 8.5). Rearrangement of Eq. 8.5 generates Eq. 8.8, which is the Henderson-Hasselbalch equation. It states the relationship between the pH and the p*K*a of a molecule. According to Eq. 8.8, when the concentration of the ionized form of a drug equals that of the uncharged state, p*K*a = pH. In other words, the p*K*a is the pH at which half the drug is in its ionized form.

Drug absorption across the gastrointestinal lining, a functionally lipid barrier, occurs predominantly via the passive diffusion. Most drugs are either weakly acidic or weakly basic (Table 8.1). Weakly basic drugs are most likely to be absorbed from the gastrointestinal linings via passive diffusion. Weakly acidic drugs tend to be unionized in the relatively strong acidic stomach environment and therefore will be absorbed more in stomach than in the small intestine.

For example, for a weak acid molecule,

$$\text{HB} \underset{}{\overset{H_2O}{\rightleftharpoons}} \text{H}^{\oplus} + \text{B}^{\ominus} \tag{8.4}$$

$$\text{Ka} = \frac{[\text{H}^+][\text{B}^-]}{[\text{HB}]} \tag{8.5}$$

Rearranging the above equation gives

$$[\text{H}^+] = K_a \times \frac{[\text{HB}]}{[\text{B}^-]} \tag{8.6}$$

$$\text{pH} = -\log([\text{H}^+]) = -\log\left(K_a \times \frac{[\text{HB}]}{[\text{B}^-]}\right) = \text{p}K_a - \log\left(\frac{[\text{HB}]}{[\text{B}^-]}\right) \tag{8.7}$$

$$\text{pH} = \text{p}K_a + \log\left(\frac{[\text{B}^-]}{[\text{HB}]}\right) = \text{p}K_a + \log\left(\frac{[\text{base}]}{[\text{acid}]}\right) \tag{8.8}$$

Table 8.1 Ionization constants of oral drugs in the core WHO essential medicine list

Drugs	*C* Log *P*	p*K*a (s)	Therapeutic class
Abacavir sulfate	0.58	5.01	Antiviral
Acetylsalicylic acid	1.02	3.5	NSAID, antithrombolytic
Amoxicillin	−1.87	No Data	Antibacterial
Atenolol	−0.11	9.6	Antihypertensive, antianginal
Atropine sulfate	1.30	4.35	Antispasmodic
Captopril	0.89	3.7, 9.8	Antihypertensive
Carbamazepine	1.98	7.0	Anticonvulsant, antiepileptic
Chlorpheniramine maleate	3.15	9.2	Antihistaminic
Chlorpromazine HCl	5.80	No Data	Antiemetic, antipsychotic
Cimetidine	0.35	6.8	Antiulcerative
Cloxacillin sodium	0.04	2.06	Antibacterial
Diazepam	3.17	3.4	Anxiolytic, muscle relaxant
Erythromycin	1.47	8.8	Antibacterial
Hydralazine HCl	1.02	7.3	Antihypertensive
Ibuprofen	3.68	5.2	NSAID, analgesic, antipyretic
Phenobarbital	1.37	7.3, 11.8	Anticonvulsant, hypnotic
Phenytoin	2.09	8.06–8.33	Anticonvulsant, antiepileptic
Promethazine HCl	4.90	9.1	Antihistaminic, antiemetic
Quinine sulfate	2.79	5.07, 9.7	Antimalarial, muscle relaxant
Theophylline	−0.03	8.8	Bronchodilator
Verapamil HCl	4.47	8.6	Antianginal, antiarrhythmic
Warfarin sodium	2.90	5.0	Anticoagulant

C Log *P* and p*K*a were adapted from Kasim et al. [1]

Generally, the functional groups determine the p*K*a of a drug molecule. Table 8.2 lists the p*K*as for the common functional groups. A molecule may contain multiple functional groups and therefore may exhibit more than one p*K*a. A molecule with more than one functional group may possess both acid and basic properties. For example, ciprofloxacin, a quinolone antibiotic, contains both amines and carboxylic acid. In the stomach fluid, the secondary amine is the strongest base and accepts a proton. In the duodenum, the carboxylic acid is the most acidic functional group and yields a proton, forming a carboxylate anion. A molecule containing both the negative and positive charges is called a zwitterion. Ciprofloxacin in the duodenum is a zwitterion (Fig. 8.1).

It is important to remember that the degree of ionization of a drug in solution depends on two factors, pH and the p*K*a. The lower the p*K*a, the stronger the acid, the weaker the conjugate base. Any unionized functional group that has a p*K*a of <1 or >12 is called "neutral." Such a compound at physiological pH acts neither as an acid nor as a base. Any compound with a p*K*a between 1 and 12 is considered a "weak" acid and is incompletely dissociated.

After absorption, most drugs are transported by the blood. Bioavailability is the fraction of drug dose that reaches the systemic circulation after administration via

Table 8.2 Common organic functional groups and their p*K*a (p*K*a values were taken from evans.rc.fas.harvard.edu/pdf/evans_pKa_table.pdf

Functional groups	p*K*a (s)	Functional groups	p*K*a (s)
Phenol	10	Arylamine (ammonium)	4–5
Sulfonamide	9–10	Aromatic amine (ammonium)	5–6
Imide	8–10	Imine	3–4
Thiophenol	9–10	Alkyamines	10–11; 9–10
Sulfonimide	5–6	Guanidine	13–14
Alkylcarboxylic acid	2–5		
Arylcarboxylic acid	3–4		
Sulfonic acid	0–1		

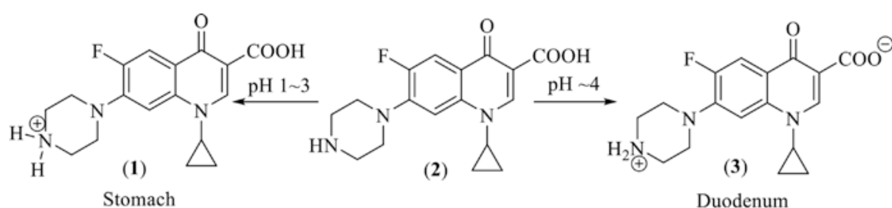

Fig. 8.1 Predominate forms of ciprofloxacin (p*K*a 6.09) [13] at two different locations within the gastrointestinal track

any route of administration. When a drug is administered by IV injection, all doses enter directly into the systemic circulation. For an oral drug, it must be disintegrated from various dosage forms (such as tablets and capsules) to release its ingredients. Rate of dissolution is limited by how fast the solid tablet dissolves and also depends on the surface area of solid and how soluble the solid is. Dissolution of tablets or capsules can vary greatly between the manufacturers of the brand name drugs and the generic drugs, which might result in the difference in bioavailability of a drug. Extra cautions are warranted in prescribing drugs with narrow therapeutic indices. The bioavailability, or effective free drug concentration, can be reduced due to possible metabolism when drugs pass through the liver. The reduced bioavailability due to liver metabolism is called the first pass effect.

For example, the first pass effect of lidocaine is so large that it cannot be administered orally but must be administered via intravenous injection.

8.2.4 Approaches to Improve Solubility

Many rules have been proposed to help develop orally bioavailable drugs, among which the most notable is Lipinski's rule-of-five (RO5). The rule-of-five analysis was proposed by Lipinski et al. in late 1990s and is a very useful guideline for estimating the oral bioavailability of small molecules. Lipinski and coworkers [2] proposed the following rules for compounds with poor absorption or permeation: (1)

the molecular mass is more than 500 Da; (2) the lipophilicity, measured by *C* Log *P* is greater than 5; (3) the number of hydrogen-bond donors is greater than 5; and (4) the sum of nitrogen and oxygen atoms is greater than 10. Alternatively, Ghose et al. [3] found that major compounds in the Comprehensive Medicinal Chemistry (CMC) database have log *P* ranging [−0.4, 5.6], with median of 2.5, and molecular weight, [160, 480] with median of 357; and the molar refractivity (MR) [40, 130] with median of 97. In addition, a GlaxoSmithKline team [4] took into consideration of flexibility and observed that oral bioavailable compounds tend to have ten or fewer rotatable bonds, with polar surface area (PSA) less than 140 $Å^2$. Among these three widely used filters (rules), Lipinski's RO5 is the most popular. The application of these and other rules in the early stage of drug discovery has significantly reduced the attrition rate due to poor bioavailability. However, it needs to point out that there are always some exceptions to the Lipinski's RO5 as quite a few approved drugs violate one or more RO5. For instance, atorvastatin (once a top-sale lipid reducer drug) has MW of 558 and log *P* of 5.8, HIV protease inhibitor darunavir has MW of 548, and antihistamine drug fexofenadine has MW of 503 and log *P* of 7.3 [5]. To reduce the likelihood of filtering out some useful lead compounds, a simple ADMET score was proposed and calculated by using the means and standard deviations of MW and log *P* of available oral drugs, or drugs in the same family (Eq. 8.9). The ADMET scores for atorvastatin, darunavir, and fexofenadine are 3.0, 1.9, and 3.2, respectively. The mean ADMET score for 152 oral drugs is 1.5 [5] and that of 1,791 oral drugs is 1.2 [6].

$$\text{ADMET score} = \frac{\left|\log P_{\text{mean}} - \log P\right|}{\log P_{\text{SD}}} + \frac{\left|\text{MW}_{\text{mean}} - \text{MW}\right|}{\text{MW}_{\text{SD}}} \tag{8.9}$$

In addition to applying filters in early stage of design and virtual screening, other approaches to increasing solubility of oral drugs include the ionizations into different salts: for acidic drugs, sodium, calcium, and potassium salts are introduced, and for basic drugs, hydrochloride, citrate, tartrate, or other acids complexes are used to make salts. The formation of salts normally leads to a better solubility profile. The most commonly used methods for basic drugs is to form salt with HCl (43% top 200 top-sale drugs exist in HCl salts), for acidic drugs is to form sodium salt (11% of top 200 top-sale drugs are in sodium salts) [5]. In addition, cocrystalization of drugs with solvents such as ethanol or water (i.e., hydrates) can also improve water solubility [5].

Another method to change the solubility profile is to apply the concept of prodrugs. Prodrugs are inactive drugs that need to be converted to active drugs via metabolism in vivo. For instance, amprenavir (**4**) is a HIV protease inhibitor with poor solubility that requires 1200 mg, delivered in eight capsules each with 150 mg, or 24 capsules each with 50 mg twice a day. This surely is very burdensome to patients and sometimes very challenging to get patients compliant to taking such a large number of capsules. By introducing a phosphate group fosamprenavir (**5**) shows a much better solubility profile. The sobulibility increases 14-fold from 0.0491mg/mL in amprenavir to 0.685 mg/mL in fosamprenavir [13]. The phosphate

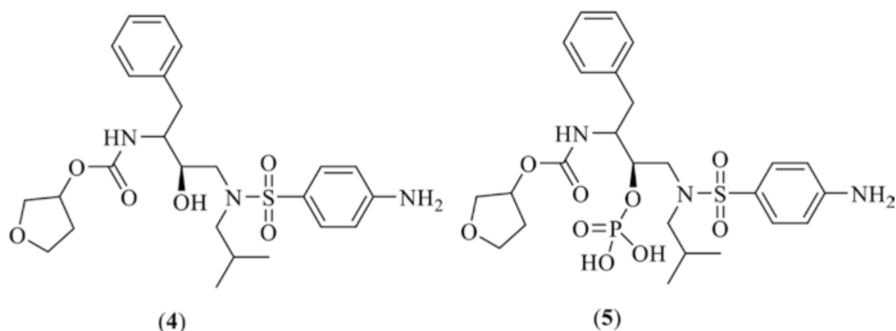

Fig. 8.2 Amprenavir and its prodrug fosamprenavir

ester group in prodrug fosamprenavir can undergo cleavage by phosphatase in vivo to provide the active compound (**4**). The enhanced solubility in (**5**) is attributed to the phosphate group, which is highly ionized at physiological pH (Fig. 8.2).

8.2.5 Drug Distributions

After absorption (i.e., for oral drugs) or systemic administration into the bloodstream (for IV drugs), a drug is distributed to its site of action through the circulatory systems (blood or lymphatic). In the bloodstream, drugs are distributed as either the free form or the plasma protein bound form. However, the plasma-bound drugs have no pharmacological effect. It is the free drug that exerts the therapeutic effect. Albumin is a major carrier for acidic drugs whereas α1-acid glycoprotein is the major for basic drugs. The binding is generally reversible with the exception of very active alkylating agents, which may form covalent bonds with carriers. In addition to blood plasma, many drugs accumulate in tissues as well. Sometimes, the accumulation of a drug in tissues may have a much higher concentration than those in the blood. For example, as much as 70% of the highly lipid-soluble thiopental accumulates in body fat 3 h after administration. Binding of a drug to tissues may serve as a reservoir that prolongs drug action and such a binding is usually reversible. However, such tissue accumulation occasionally can lead to local toxicity. For example, the accumulation of the aminoglycoside antibiotic gentamicin can produce local toxicity in the kidney and vestibular system. Major factors that influence distribution are the physicochemical properties, such as solubility and stability of the individual drugs and the physiological factors of individual patients. A number of physiological factors, such as cardiac output, regional blood flow, capillary permeability, and tissue volume determine the rate and the total amount of drugs being delivered.

Pharmacokinetics studies the relationship between pharmacological effects and the accessible concentration of a drug. Clinical pharmacokinetics attempts to establish a quantitative relationship between the dose (concentration) and effect. The studies on pharmacokinetics can provide guidelines in determining dosage

regiments to improve the therapeutic efficacy and to avoid unwanted side effects. For a drug to be effective, it is necessary to maintain a steady-state concentration of a drug within a therapeutic window. Several factors determine the steady-state concentration of a drug, such as clearance, volume of distribution, half-life, and the bioavailability of a drug.

Many drugs are eliminated in an exponential decay with the first-order kinetics. After several dosages, a drug can reach a steady-state concentration. Steady-state concentration of a drug is achieved when a drug is administered at a rate that equals the rate of drug elimination. The steady-state concentration fluctuates and is proportionate to dosage interval/half-time. As the rate of absorption increases, the concentrations become larger and as the rate of elimination increases, the concentrations decrease. The average concentration is attained in the steady state and is proportionate to bioavailability (F), and dose/dosage interval (dose), and inversely proportional to clearance (CL) (Eq. 8.10).

$$\overline{C_{\mathrm{ss}}} = \frac{F \times \mathrm{dose}}{\mathrm{CL} \times T} \tag{8.10}$$

Clearance (CL) is defined as the volume of blood in a specific region of the body that is cleared of a drug in a given unit time (Eq. 8.11).

$$\text{Dosing rate} = \mathrm{CL} \cdot C_{\mathrm{ss}} \tag{8.11}$$

where CL is clearance of drug from the systemic circulation and C_{ss} is the steady-state concentration of a drug. For a complete bioavailable drug, the steady-state concentration (C_{ss}) can be achieved when the rate of elimination equals the rate of drug administration.

$$\mathrm{CL} = v_{\mathrm{m}} / \left(K_{\mathrm{m}} + C\right) \tag{8.12}$$

where v_{m} is the maximal rate of elimination (in units of mass/time) and K_{m} is the concentration at which half the maximal rate of elimination is reached. The clearance of a drug is the sum of the clearance of a drug by several organs, such as kidney, liver, and other organs (Eq. 8.13). The clearance can also be calculated from the total area under the curve (AUC) that describes the measured concentration of a drug in a systemic circulation as a function of time (Eq. 8.14).

$$\mathrm{CL} = \mathrm{CL}_{\mathrm{renal}} + \mathrm{CL}_{\mathrm{hepatic}} + \mathrm{CL}_{\mathrm{other}} \tag{8.13}$$

$$\mathrm{CL} = \mathrm{Dose} / \mathrm{AUC} \tag{8.14}$$

The apparent volume of distribution (V_{d}) relates the amount of drug (D, dose) in the body to the concentration of drug (C) in the blood or plasma (Eq. 8.15). Values of V_{d} are usually recorded in units of liters per kg (L/kg).

Note that V_{d} is the apparent volume of distribution, not the volume of the tissue and the circulatory system that are actually perfused by the drug. The range for V_{d} varies from 0.05 to 20.0 L/kg. For drugs that bind to the blood proteins, the concentration of drugs (bound and free) in blood is very high. Consequently, V_{d} according

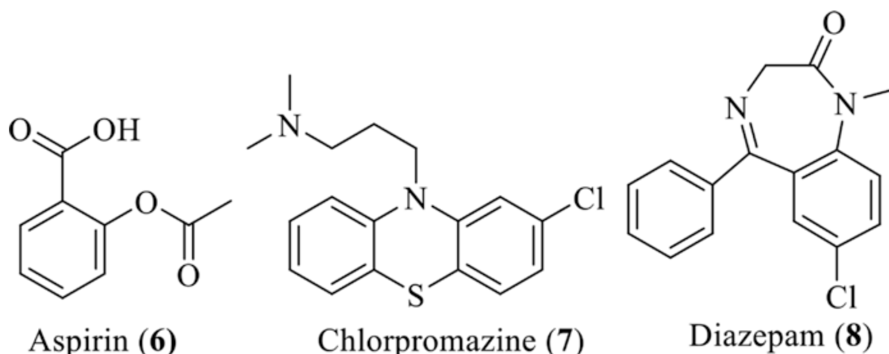

Fig. 8.3 Structures of aspirin, chlorpromazine, and diazepam

to Eq. 8.15 is very small. A value of $V_d < 0.071$ indicates that the drug is mainly distributed within the circulatory system. Values >0.071 indicate that the drug is distributed in both blood and other specific tissues. If a drug binds to tissue proteins, the actual concentration of the drug in the blood is greatly reduced, V_d becomes very large. For example, V_d of aspirin (**6**, V_d: 0.1–0.2 L/kg) indicates that aspirin is mainly distributed via the blood circulatory system. On the other hand, Chlorpromazine (**7**, V_d: 20 L/kg) [13] is highly lipophilic and therefore can accumulate in hydrophobic fatty tissues. Hence, the effective concentration of blood drug is significantly reduced, resulting to a V_d as high as 20 L/kg (Fig. 8.3).

$$V_d = \text{amount of drug in body}\left(D,\text{dose}\right)/\,C \tag{8.15}$$

The half-time ($t_{1/2}$) is the time it takes for the plasma concentration of a drug to drop to half of the original value (Eq. 8.16).

$$t_{1/2} = 0.693 V_{ss} / \mathrm{CL} \tag{8.16}$$

Clearance is the measurement of the body's ability to eliminate a drug. For an elder person, the CL is smaller and therefore the $t_{1/2}$ becomes longer. For example, the half-life of diazepam (**8**) increases with increasing age. A disease process, such as acute viral hepatitis, may alter the clearance and hence the $t_{1/2}$ is different. The unit of clearance is (cm^3/min/kg, or mL/min/kg).

8.2.6 Drug Metabolism

8.2.6.1 Basic Concepts

Drugs are foreign substances to the body and are metabolized by a variety of enzymes into other products called metabolites. Metabolites are generally more polar, more soluble in water, and more readily excreted in the urine. The process of chemically converting a drug to a metabolite is called metabolism or biotransformation. Metabolism can occur before or after drugs reach their sites of action. It can

deactivate a drug or convert a pharmacologically inactive prodrug into an active drug. For chiral drugs, one enantiomer might be metabolized to a greater extent than the other due to the stereoselectivity of chiral metabolizing enzymes.

It is common to classify the drug metabolic reactions into two general phases. Phase I reactions include oxidation, reduction, hydrolysis, and carboxylation. The phase I enzymes introduce the functional group modifications to a drug. Hence, phase I metabolism is often classified as the functionalization phase of drug metabolism. Most of the phase I reactions occur in the liver and deactivate an active drug. In certain instances, metabolism hydrolyzes an ester or amide linkage, leading to an active species. The hydrolysis of an ester or an amide by metabolism takes place in the gut wall. Phase II reactions include most of the conjugation reactions, such as glucuronide, sulfate, and glutathione conjugations. Phase II reactions are catalyzed by phase II enzymes, such as the glutathione-S-transferases (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT), and methyltransferases (MT). Phase II reactions generally produce compounds which are often very water soluble. For example, the highly polar glucuronide moiety usually leads to a rapid excretion of a drug. However, not all phase II reactions generate a more polar metabolite. Methylation and acetylation, for examples, decrease the polarity of the drug.

The phase I and phase II metabolism can be illustrated using morphine as an example. Morphine (**9**) is the principal active ingredient in opium poppy and is a highly potent opiate analgesic psychoactive drug but at the same time has a high potential for addiction. The metabolism occurs predominantly in the liver. Schemes A and B show the phase II metabolism characterized by the glucuronidation at 6-hydroxyl and 3-hydroxyl, respectively, yielding morphine-6-glucuronide (**10**) and morphine-3-glucuronide (**12**), respectively (Fig. 8.4). Morphine-6-glucuronide (**10**) is an active metabolite while morphine-3-glucuronide (**12**) has very low activity and poor distribution properties. Route C shows that morphine can undergo phase I N-demethylation reaction, generating normorphine (**11**), which can be further metabolized by phase II glucuronidation. Normorphine has decreased activity and decreased bioavailability to the central nervous system. Due to the first pass effect, the bioavailability of morphine is pretty low at around 30% [13].

8.2.6.2 Phase I Metabolism

The phase I oxidation reactions are carried out by cytochrome P450 (P450 or CYP), flavin-containing monooxygenases (FMO), and epoxide hydrolases (EH). CYPs are a superfamily of heme containing proteins that catalyze the oxidative reactions of many compounds, including steroids, fatty acids, and xenobiotics. CYPs are highly involved in drug metabolism. Approximately 50% of drug metabolisms are catalyzed by CYPs. CYPs catalyze the C and O oxidation, N-dealkylation, O-dealkylation, aromatic hydroxylation, N-oxidation, S-oxidation, deamination, and dehalogenation. There are approximately 100 CYPs isoforms in humans. The site of action is mainly in the liver. There are 12 CYPs in human that are known to be important for metabolism of xenobiotics. The most active CYPs for drug metabolism are CYP2C, CYP2D, and CYP3A subfamilies. CYP3A4 is the most abundantly expressed and involved in the

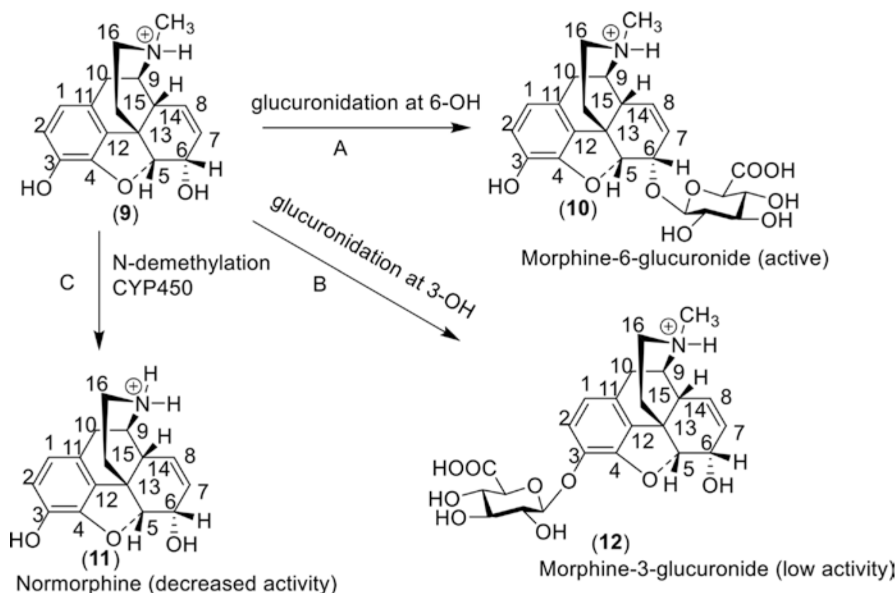

Fig. 8.4 Metabolism of morphine

metabolism of approximately 50% of clinical drugs [5]. Lipophilicity is important for the binding of a substrate to cytochrome P450. Increasing lipophilicity leads to an increase in metabolic clearance of a drug. There are large differences in levels of expression of each CYP (alleles of CYPs) between individuals. This large difference is due to the presence of genetic polymorphisms and differences in gene regulation. In other words, different versions of the same gene products can be observed in different people. This has a significant effect on different drug metabolism among different individuals, people from different age groups, different sex, and different ethnic groups.

For example, the water-insoluble (S)-phenytoin (**13**), an anticonvulsant used in the treatment of epilepsy, is metabolized by the phase I CYPs to 4-hydroxyphenytoin (**14**), followed by phase II glucuronization by the uridine diphosphate-glucuronosyltransferase (UGT), to generate a highly water-soluble 4-hydroxy-phenytoin-β-D-glucuronide (**15**, Fig. 8.5). This process converts a very hydrophobic molecule to a very hydrophilic metabolite that is readily eliminated via the bile. The first step of phenytoin metabolism involves aromatic hydroxylation. In human, *para*-hydroxylation is the major route of metabolism for many phenyl-containing drugs.

An effective method to slow down metabolism of aromatic drugs is to introduce a substituent group on the para-position of the phenyl ring. For example, the anti-inflammatory drug diclofenac (**16**) is metabolized much faster than fenclofenac (**17**, Fig. 8.6). Diclofenac is readily oxidized to 4-hydroxydiclofenac with a half-life of 2 hours [13]. Fenclofenac, on the other hand, has a half-life of more than 20 hours.

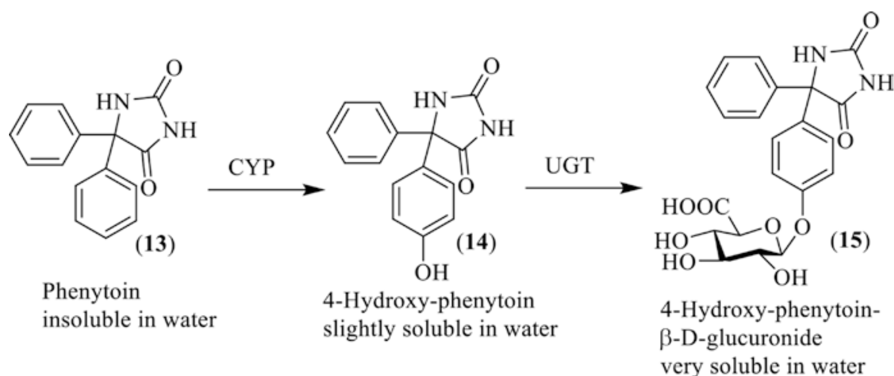

Fig. 8.5 Metabolism of phenytoin by phase I CYP and phase II UGT

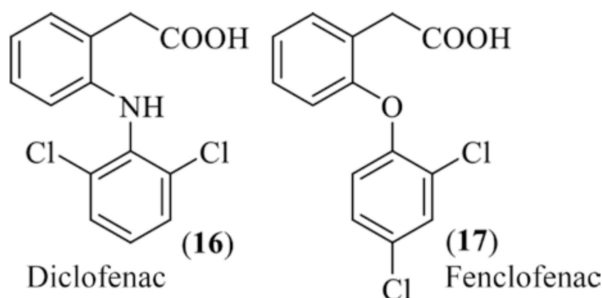

Fig. 8.6 Structures of diclofenac and fenclofenac

Please note that a drug can undergo one or more metabolic pathways. For example, amphetamine (**18**) can be oxidized to the nitro compound (**19**), or a ketone (**20**) via common intermediates such as the hydroxylamine and the oxime (Fig. 8.7).

Similarly, the secondary amine β-blocker propranolol (**21**) can be metabolized to an amine compound (**22**) via N-dealkylation or to a carboxylic acid metabolite (**23**) via oxidative deamination (Fig. 8.8).

The analgesic agent acetaminophen is relatively nontoxic at therapeutic doses. Acetaminophen has enjoyed its reputation for safety for generations and is currently used in more than 300 over-the-counter and prescription products, from headache and cold remedies, cough suppressant syrup to sleep aids. However, it should be noted that the margin for acetaminophen between a safe dose and a potentially lethal one is small and that people may not realize that they take more than the recommended daily dose before it is too late. Overdose of acetaminophen can lead to acute liver failure and even death. Recently, an FDA panel recommended lowering the maximum daily dose to 2,600 mg from 4,000 mg and limiting the amount in a single over-the-counter pill to 325 mg, instead of the current 500 mg (extra strength). Adding acetaminophen to opioids in prescription pain relievers may aggravate the acetaminophen overdose problem. The combination of acetaminophen and hydrocodone in Abbott Laboratories' Vicodin contains 5 mg of the opioid and 500 mg or

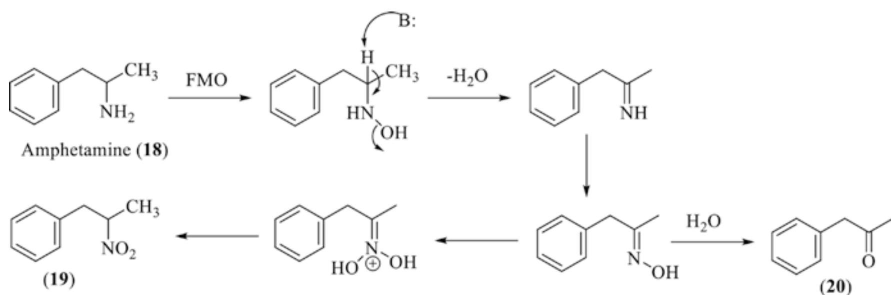

Fig. 8.7 N-Oxidation pathways of amphetamine

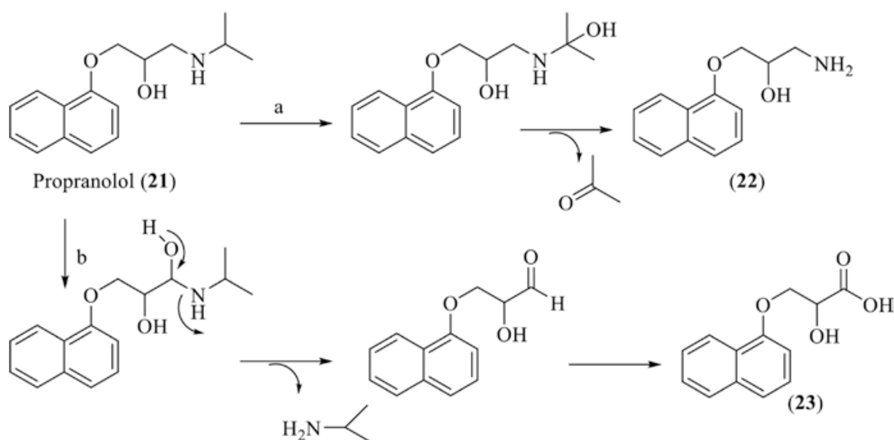

Fig. 8.8 Oxidative metabolism of propranolol

750 mg of acetaminophen. Patients taking Vicodin and an acetaminophen-containing sleep pill can end up taking a large dose of acetaminophen without realizing it.

The toxicity of an overdosed acetaminophen (**24**) to both the liver and kidney is due to the depletion of liver or renal glutathione levels. Three mechanisms have been proposed to explain the acetaminophen-induced toxicity. The common intermediate of these three pathways is the electrophilic metabolite (**25**, Fig. 8.9D). The nucleophilic residues on the glutathione (GSH) attacks metabolite D, leading to a conjugated compound F. When the overdose of acetaminophen depletes 80% or more glutathione, macromolecules in liver and kidney react with the electrophilic metabolite D, leading to the observed liver or renal necrosis. The first mechanism (pathway a) involves an epoxidation reaction. If the pathway were correct, incubation of acetaminophen in the presence of $^{18}O_2$ should result in a metabolite with ^{18}O in the epoxide ring, which was not observed. Pathway b involves N-oxidation of the amide bond. However, the *N*-hydroxyacetaminophen was not found either. The third mechanism is a hydrogen atom abstraction reaction via an acetaminophen radical. It was found that adding radical scavengers protected the animals, supporting the free radical mechanism. The free radical intermediate formation in liver is CYP450

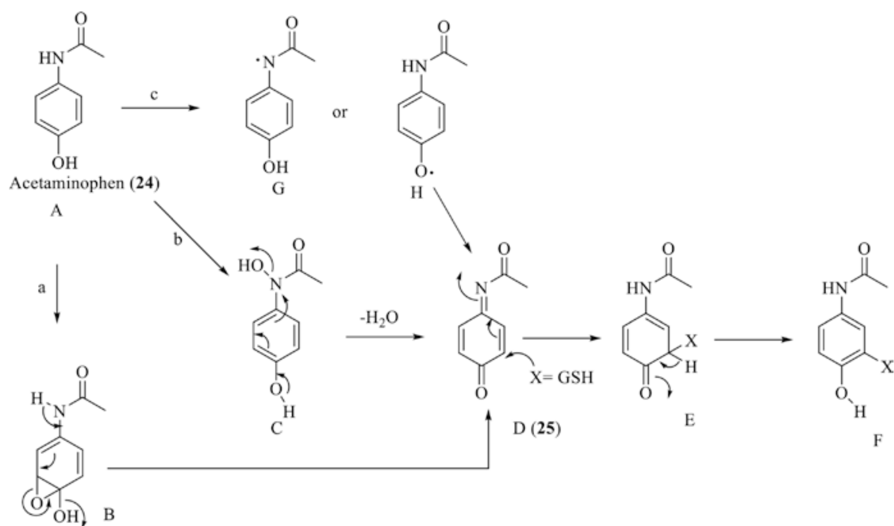

Fig. 8.9 Possible mechanisms for acetaminophen caused toxicity

dependent. Ethanol can induce an isozyme of CYP450 that could be responsible for the formation of the free radical intermediate G or H. Therefore, drinking alcohol while taking acetaminophen can cause much severe liver damage. Taking radical scavenger such as Vitamin C may help alleviate the damage. In the kidney, the CYP450 activity is low. The formation of the benzoquinone D is catalyzed by the prostaglandin G_2 (PGG_2).

8.2.7 Excretion of Drug Molecules

Excretion or elimination is the collective term used for irreversibly removing a drug from the body. This process reduces the concentration of a drug at its site of action. A slow elimination process helps build-up of the required drug concentration to maintain the therapeutics effects. Drugs are eliminated from the body either in unchanged form or in structurally different metabolites. The kidney is the most important organ for excreting drugs and their metabolites through the urine. Some drugs, however, are excreted via the bowel in the feces or in the biliary excretion.

8.3 Drug–Drug Interactions

When two or more drugs are administered together and metabolized by the same enzymes, drug–drug interactions occur. The drug interactions may be pharmacokinetic (i.e., the delivery of a drug to its site of action) or pharmacodynamic (the response of the drug target).

8.3.1 Pharmacokinetic Interactions

A number of mechanisms may affect drug delivery to the site of action. Changes in gastrointestinal absorption and metabolism in the liver are important factors to consider. Coadministration of tetracycline antibiotics with aluminum ions in certain antacids or ferrous ions in oral iron supplements prevents the absorption of tetracyclines due to the insoluble chelates formed between tetracyclines and ions.

Most drug–drug interactions involve the cytochrome P450 enzymes (CYPs). CYPs play a key role in the metabolism of a large number of drugs. The transcription of CYP450 mRNA and synthesis of xenobiotic-metabolizing enzymes can be promoted (induced) or inhibited by drugs. Drugs inducing CYP expression are called CYP inducers. This phenomenon is called enzyme induction, which means that the rate of synthesis of an enzyme is increased due to the usage of a drug. Examples of drugs stimulating the activity of the CYP450 isoforms include antibiotics, anticonvulsants, non-nucleoside reverse transcriptase inhibitors, and herbal drugs and those listed in Table 8.3. Drugs metabolized predominantly by these enzymes generally have low plasma drug concentration. Loss of efficacy is the unfortunate consequence of these drug interactions. The induced CYPs, however, may not catalyze the metabolisms of the inducers. For example, phenytoin induces CYP3A4, but is hydroxylated by CYP2C9. Patients taking oral contraceptives while taking St. John's Wort may have an increased risk for pregnancy. St. John's Wort induces CYP3A4, which increases the oxidative metabolism of the oral contraceptives.

Cigarette smoking has been shown to increase the levels of CYP1A1 and CYP1A2 isoforms. Cigarette smokers have lower plasma levels of imipramine, estradiol, and pentazocine. On the other hand, chronic alcoholics show an increased level of CYP2E1 enzyme and metabolize phenobarbital, tolbutamide, and phenytoin more rapidly than nonalcoholics.

Table 8.3 Inhibitors and inducers of CYP450 subfamilies

Inducers	Inhibitors
CYP3A	CYP3A
Phenobarbital (and CYP2B, CYP2C)	Imidazole antifungals
Phenytoin, primidone	Quinidine, tamoxifen, rivastatin
Erythromycin, St. John Wort	Atorovastatin, felodipine, nifedipine
Carbamazepine	CYP2D6
Rifampicin, rifabutin	Quinidine, acebutol, betaxolol
CYP2E	Chlorpromazine, desipramine
Ethanol	Other
CYP2D6	Chloramphenicol (CYP2B, CYP2C)
Ritonavir, phenobarbital, phenytoin	Cimetidine (nonselective)
CYP1A	Spironolactone
Smoking, omeprazole, phenobarbital	Flurbiprofen (CYP2C9)

On the contrary, a metabolizing enzyme whose activity is inhibited by a drug prolongs the half-life of another molecule and leads to accumulation during treatment, sometimes causing severe adverse effects. There are three mechanisms for CYP450 inhibition: reversible inhibition, metabolic intermediate complexation of CYP450, and mechanism-based inactivation of CYP450.

Drugs able to interact strongly with the lipophilic sites on the CYP450 apoprotein and the heme-iron active center can reversibly inhibit CYP450. These drugs include the fluoroquinolone, antimicrobials, cimetidine, the azole antifungals, quinidine, and diltiazem. For example, cimetidine inhibits the oxidation of diazepam, warfarin, ibuprofen, tolbutamide, chlordiazepoxide (via inhibition of CYP2C), carbamazepine, and 6-hydroxylation of steroids (CYP3A).

Noninhibitory alkylamine drugs, after CYP450-mediated oxidation, generate a metabolite, nitrosoalkane, which have a high affinity to form a stable complex with CYP. This phenomenon is called metabolite intermediate complexation. This complexation makes the CYP450 unavailable for oxidation of other drugs. Drugs demonstrating this type of inhibition include orphenadrine (anti-Parkinson's drug) and mifepristone.

Mechanism-based inhibition involves drugs that are noninhibitory of CYP450 and that their metabolites generated from the CYP450 oxidation bind irreversibly to CYP450. This process is also called "suicide inhibition." Drugs containing alkene and alkyne function groups belong to this category. For examples, the 17-α-acetylenic estrogen, 17-α-ethynylestradiol, cyclophosphamide, and spironolactone are drugs that are able to alkylate the CYP450 after being metabolized.

For example, many drugs are metabolized predominantly by CYP3A isozymes. These drugs include immunosuppresants (e.g., cyclosporine), HMG-CoA reductase inhibitors (e.g., lovastatin, simvastatin, and atorvastatin), HIV protease inhibitors (e.g., ritonavir, amprinavia, and indinavir), benzodiazepines (triazolam), lidocaine, and Ca^{2+} channel antagonists (diltiazem, felodpine, and nisoldipine). Antifungal agents ketoconazole and itraconazole are potent CYP3A4 inhibitors. When ketoconazole or itraconazole is co-administered with an anti-HIV protease inhibitor (ritonavir), the clearance of the protease inhibitor is reduced due to the fact that both drugs compete with CYP3A4 for metabolism. The plasma concentration of the protease inhibitor thus is increased and the therapeutic effect is prolonged. This enhances the risk of toxicity.

Drug transporters such as P-glycoprotein are important for drugs that are dependent upon P-glycoprotein-mediated transport. Digoxin is mainly dependent on P-gp transport for elimination. Inhibitors of P-gp (e.g., verapamil, diltiazem, quinidine, ketoconazole, itraconazole, and erythromycin) may elevate plasma digoxin concentrations to the toxic state.

Drug metabolism can also be influenced by diet. It is generally not recommended to take medication with grapefruit juice because components found in grapefruit juice include potent CYP3A4 inhibitors, which could increase the bioavailability of a drug. Drugs dependent upon CYP3A4 for metabolism, such as HMG-CoA reductase inhibitors (e.g., statins), and Ca^{2+} channel blockers (e.g., Nifedipine, Amlodipine,

Nisoldipine, and Pranidipine) demonstrate higher oral bioavailability due to the presystemic CYP3A4 inhibition caused by ingestion of one glass of grapefruit juice. The reduction in intestinal CYP3A4 concentration is fast up to 47% decreased can be observed within 4 h of grapefruit consumption. The active components found in grapefruit that are responsible for CYP3A4 inhibition include flavonoids (e.g., naringenin, **26**) and furanocoumarins (e.g., bergamottin, **27**, and 6′,7′-dihydroxybergamottin, **28**) (Fig. 8.10) (Table 8.4).

Please note that drugs with different stereoisomers may have different metabolic profiles. Different enantiomers might be catalyzed by different CYP subfamily proteins and therefore might exhibit different clearance (CL). For example, the S-(-)-enantiomer (**29**) of Warfarin is 3–5 fold more potent than the R-(+) isomer (**30**). The clearance of the R-enantiomer of warfarin is about 70% of that of the S-isomer. The S-enantiomer is metabolically cleared by CYP2C9, yielding aromatic hydroxylation metabolite (**31**), while the R-isomer is metabolized by CYP1H2, CYP2C19, and CYP3A4 to aliphatic hydroxylation metabolites (**32** and **33**) (Fig. 8.11).

8.3.2 Pharmacodynamic Interactions

Combinations of drugs are employed due to the additive or synergistic beneficial effects. Nitroglycerin, a nitric oxide donor, produces vasodilation by NO-dependent elevation of cyclic GMP in vascular smooth muscle. Sildenafil, tadalafil, and vardenafil exert their pharmacological effects by inhibiting the type 5 isoform of phosphodiesterase that inactivates cyclic GMP in the vasculature. Co-administration of Sildenafil and nitroglycerin can cause catastrophic hypotension.

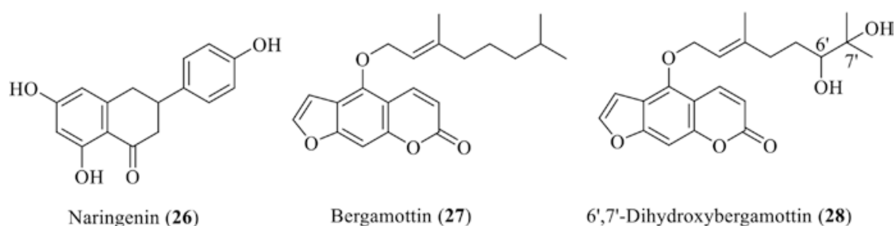

Fig. 8.10 Active components in grapefruit

Table 8.4 Some substrates and reaction types for human CYP3A4 isoform

Reaction types	Drugs
N-demethylation	Imipramine, tamoxifen, cocaine, dextromethorphan
N-deethylation	Lidocaine, diltiazem
N-oxidation	Dapsone, quinidine
6β-Hydroxylation	Testosterone, progesterone, hydrocortisone, prednisolone

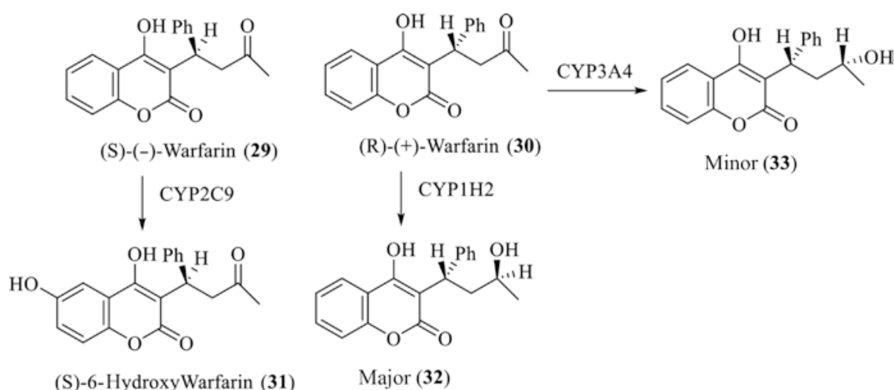

Fig. 8.11 Metabolic pathways of warfarin enantiomers

8.4 Current Topics in ADME Research

To help expedite the drug discovery process and to assist in predicting the ADMET properties of compounds, many databases have been developed.

ChEMBL (http://www.ebi.ac.uk/chembl) is an open database containing more than 1.6 million unique compound structures and 14 million bioactivity data points with more than 11,200 protein targets. Users can search compounds by structures and can find on the current ChEMBL website regarding information on Lipinski's RO5, compound chirality, binding affinity, toxicity, and ADME information.

BindingDB (www.bindingdb.org) is a publicly accessible database of protein-ligand complexes containing information such as K_i, IC_{50}, EC_{50}, and K_D. Currently, BindingDB contains approximately 7,000 protein targets and 450,000 small molecules. BindingDB also allows users to perform virtual screening studies. DrugBank (www.drugbank.ca) [13] is a drug database that hosts 8,200 drug entries including more than 2,000 FDA-approved small molecule drugs. It contains many ADMET properties such as bioavailability, clearance, half-time, dosage, solubility, and drug metabolism and available metabolite structures.

A new ADMET database (admetSAR, http://lmmd.ecust.edu.cn:8000/) is available to search the ADMET data for more than 96,000,000 unique compounds with 45 kinds of ADMET-associated properties. This database can be searched by CAS registry number, common name, IUPAC name, or SMILE name. This database contains information regarding human intestinal absorption, blood–brain barrier penetration, Caco-2 permeability, CYP450 information (substrates and/or inhibitors of CYP1A2, 2C9, 2D6, and 3A4), and hERG inhibition [7].

Another ADMET database (a PharmacoKinetics Knowledge Base, PKKB) was compiled with more than 10,000 experimental ADMET measurements of 1,685 drugs. Collected properties included log P, solubility, volume of distribution, intestinal absorption, Caco-2 permeability, bioavailability, metabolism, half-life, clearance, toxicity, half lethal dose in rat or mouse and so on [8].

While admetSAR and PKKB database contain compounds with reported ADMET properties, pkCSM (http://structure.bioc.cam.ac.uk/pkcsm), a new web-based program has been developed to predict the ADMET properties of a given molecule. The prediction of pkCSM was based on graph-based signatures. The results on reported training set and test set regarding the prediction of Caco2 permeability and water solubility were impressive [9].

The human ether-a-go-go related gene (hERG) protein is a potassium channel whose blockage is considered to cause sudden death due to drug-induced QT syndrome. Drugs inhibiting hERG thus would pose potential toxicity liability. Methods able to predict hERG inhibition are thus in high demand. Some reports use ligand-based pharmacophore models to predict the hERG inhibition behavior. For instance, Ekins et al. in 2002 [10] reported a first pharmacophore model consisted of four hydrophobic and one positively charged points, while Cavalli et al. [11] developed a pharmacophore model with a positively charged tertiary amine and three aromatic or hydrophobic centers. Among more than 15 physicochemical parameters, hydrophobicity parameter A log P, solubility logs, and molecular weights (MW) appear to be most distinguishing between hERG active and inactive compounds; for the 369 hERG inactive compounds and 344 active compounds, log P for the active versus inactive was 2.416 vs. 1.334, log S of −6.53 vs. −4.01, and MW of 405 vs. 313, respectively [12].

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9 Cheminformatics Approaches in Modern Drug Discovery

Salma Jamal and Abhinav Grover

9.1 Traditional Drug Discovery and Bottlenecks

Drug discovery is the process by which novel candidate chemical compounds are identified and validated as medications for the treatment of specific disorders and diseases. Traditionally, drugs were discovered through identification of active components from traditional therapies without prior knowledge of the biological target. This was later replaced by chemical repositories of natural compounds or synthetic small molecule compounds which were screened against cell lines or organisms for the identification of compounds having desirable therapeutic properties. The traditional drug discovery process includes various steps: selection of disease, identification of target and its validation, searching lead compound effective against target and its synthesis, preclinical testing in animal models and human clinical trials [1] as is evident from Fig. 9.1. However, these steps are associated with serious bottlenecks which include the large amount of time and costs involved in making and testing of the novel candidate entities identified. This led to the need of newer technologies that could automate the drug discovery process such as high throughput screening (HTS) where millions of chemical compounds could be screened per drug target per year [2]. In order to meet the increased requirement of novel chemical compounds, combinatorial chemistry technologies, which made it possible to make millions of chemical compounds in one go, were started to be used [3]. Conversely, it resulted in disappointing results as this process could not yield significant drug

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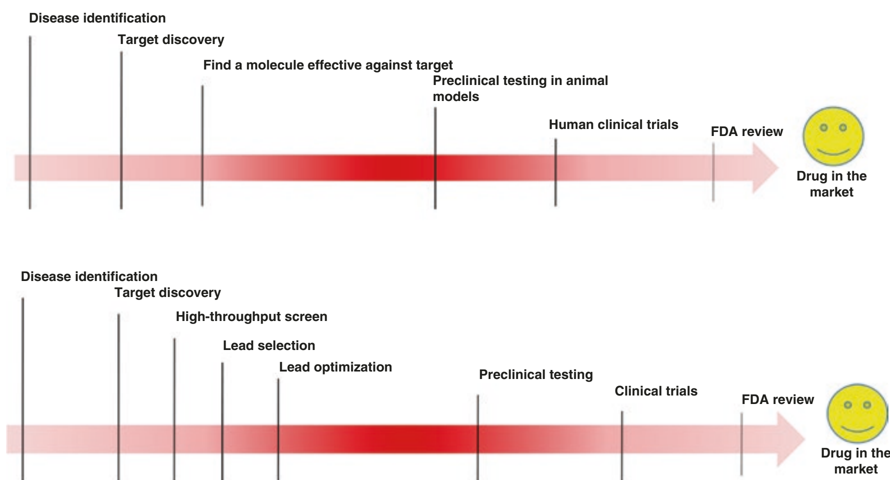

Fig. 9.1 Shows the traditional drug discovery process and modern process of drug discovery and development

candidates due to lack of chemical diversity as well as drug-like properties leading to wastage of large number of compounds. Another problem to be addressed was the identification of candidate molecules with desirable therapeutic properties from the large pool of compounds. In order to overcome these drawbacks, a method was needed which could predict drug-like compounds as well as absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the screening compounds and thus various cheminformatics, sometimes referred to as chemoinformatics or chemical informatics, approaches were introduced. Cheminformatics approaches are widely used by scientists, researchers and pharmaceutical as well as other chemical and related industries in the drug discovery process in addition to a plethora of other applications which would be discussed further.

9.2 An Introduction to Cheminformatics

F.K. Brown defines chemoinformatics as

"the mixing of those information resources to transform data into information and information into knowledge for the intended purpose of making better decisions faster in the area of drug lead identification and optimization" [4] as is shown in Fig. 9.2.

As quoted by W. Warr at http://www.warr.com/warrzone2000.html,

"Chem(o)informatics is a generic term that encompasses the design, creation, organization, management, retrieval, analysis, dissemination, visualization, and use of chemical information" by G. Paris (August 1999 Meeting of the American Chemical Society) [5].

Various other definitions have been given for cheminformatics that include "Chemoinformatics – A new name for an old problem" by M. Hann and R. Green

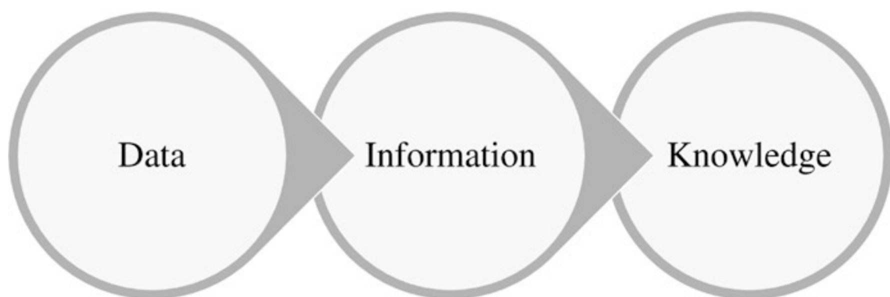

Fig. 9.2 Shows the flow of information in cheminformatics

[6] and "The application of informatics methods to solve chemical problems" by J. Gasteiger and T. Engel [7]. Cheminformatics can be broadly defined as use of information technologies and computers to solve chemical problems such as data mining, information retrieval and extraction, topology of chemical compounds and various others. Cheminformatics has been known to mainly deal with small molecules which involves analysis of scientific data and extraction of information to assist in development of new compounds, where bioinformatics, in addition to large chemical compounds, deals with genes and proteins. However, both the approaches, cheminformatics and bioinformatics, are adjunct to each other in providing us deeper insights for biomolecular processes such as prediction of structure and function of proteins, ligand binding to active sites and enzyme catalysis. A perfect example of this can be seen in drug design process where bioinformatics methods are used for identification of targets for novel drug candidates and cheminformatics methods are used for finding these new small molecule drug candidates [5].

9.3 Cheminformatics, Its Importance and Various Aspects

The major role of cheminformatics is to store, search and handle enormous amounts of chemical data which comprises ever increasing number of millions of chemical compounds generated every year. Furthermore, it also includes extraction of information and knowledge from this chemical data which could be used to model the relationships between chemical structures and biological activities and predict the bioactivities of other chemical compounds from their structures. The four important problems solved by cheminformatics include storing a molecule, searching the exact molecule in a database, substructure searching and similarity search. Based on these, cheminformatics has three major aspects which are: information acquisition, information management and information use (Fig. 9.3).

Information acquisition deals with the generation and collection of experimental and theoretical data. Recent years have seen advancements in high throughput screen technologies and combinatorial synthesis which has enabled generation and analysis of large number of chemical compounds which could be tens or hundreds or thousands or even millions of molecules in a very short time period. Since such a

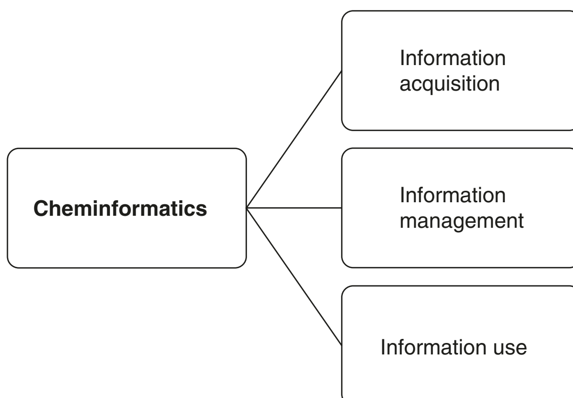

Fig. 9.3 Shows the various aspects of cheminformatics

huge amount of information is being generated, a novel technique is needed which could store and analyse this information for an effective purpose and the answer is Cheminformatics [8].

Information management deals with the storage and retrieval of the chemical information. Various methods which could result in two- and three-dimensional representation of conformations of chemical structures and similarity searching have been discovered. Also, this information is now been incorporated into chemical information storage systems using which molecules with high percentage of features matching to target the molecule can be searched and retrieved [8].

Information use includes analysis of data as well as its application to various biochemical problems. Using the chemical informatics tools and techniques, the raw data collected can now be successfully accessed and used to obtain valuable results such as prediction of unknown properties of chemical compounds consequently leading to development of novel candidate drug-like compounds [8].

9.4 Cheminformatics Approaches

Various approaches have been followed for the effective implementation of cheminformatics which include chemical structure representation, selection of descriptors, modelling of properties, classification and pattern recognition, virtual screening and structure- and ligand-based drug design.

9.5 Chemical Structure Representation Via Descriptors or Features

One major challenge for cheminformatics is the correlation of chemical information with biological data for which appropriate description of chemical structures is very important. Descriptors or features are the properties of an object which can be measured and if that object is a molecule, descriptors may be defined as the

mathematical representation of the chemical information encoded within the molecule. Todeschini and Consonni defined molecular descriptors as, “The molecular descriptor is the final result of a logic and mathematical procedure which transforms chemical information encoded within a symbolic representation of a molecule into a useful number or the result of some standardized experiment” [9]. Various features of the chemical compounds can be quantified which include theoretical properties such as number of various atoms such as hydrogen, oxygen, etc., donors and acceptors, number of rotatable bonds, etc., as well as experimental properties like log P, polarizability, and many more. Molecular descriptors can be computed using a number of commercial and free molecular descriptor generation software which include ADAPT [10], DRAGON [Talete, Milano, Italy], ADMET Predictor [Simulations Plus Inc., Lancaster, CA], JOELib (JOELib/JOELib2 cheminformatics library), Molecular Operating Environment (MOE, Chemical Computing Group) (Chemical Computing Group Inc. 2015), MARVIN beans [ChemAxon], PowerMV [11], PaDEL [12] and many more.

9.6 Descriptor Selection for Dimension Reduction

Not all the descriptors chosen to represent the molecule are relevant for predicting the biological activity of the compounds which on the other hand increase the dimension of the matrix, say for a library of n compounds, m descriptors are chosen where m could be any number, the resulting matrix would be a large $n*m$ dimensional matrix [1]. Also the accuracy and robustness of the models generated to predict the bioactivity of compounds depend on the descriptors chosen to represent the molecules [13]. Thus in order to reduce the dimensions and noise as well as to identify significant descriptors, descriptor selection is needed. Another problem is the repetition of the descriptors for the entire library of the compounds, say a feature m has same values for all the n compounds of a library. Thus this feature is not providing any significant information for the prediction task and could be removed without having any impact on the accuracy of prediction [14]. Descriptor or feature selection is a technique to find an ideal subset of features, from the set of original features, which may be less in number but are not redundant, exceedingly important and have most contribution towards the prediction [15]. The remaining features are considered irrelevant and are discarded. An optimum subset of features comprises descriptors which are related to the bioactivity of the compounds, are highly informative, are independent of each other, and are easy to understand and noise free [16].

9.7 Classification Using Machine Learning Methods

Machine learning methods are very popular in cheminformatics and have been widely used for classification of active compounds and prediction of unknown properties of compounds. Machine learning is based on learning from a set of training

instances with known properties and then predicting values for unknown properties of test instances. Tom M. Mitchell's defined machine learning as "A computer program is said to learn from experience E with respect to some class of tasks T and performance measure P, if its performance at tasks in T, as measured by P, improves with experience E" [17]. As quoted from Arthur Samuel's definition of machine learning in 1959, machine learning is a "field of study that gives computers the ability to learn without being explicitly programmed" [18]. Various methods/algorithms have been known to be used for the learning purposes such as artificial neural networks, decision-tree based learning, Bayesian models, support vector machines and k-nearest neighbours and many more. We have discussed the aforementioned machine learning algorithms in the following section.

9.8 Artificial Neural Network

Artificial neural networks (ANN), also known as neural networks, are a computational approach which mimic the biological neural network of the brain and work the way human brain solves the problems with large clusters of neurons connected with axons. The term "network" in ANN refers to the interconnected neurons in different layers of the system. The system works using three layers, input layer which is the first layer consisting of input neurons and sends data, via synapses¸ to the second layer which is a hidden layer and this hidden layer further sends the data to the third and the last output layer. The number of layers may increase with the complexity of the systems. The synapses which connect these layers are associated with weights that are manipulated during the learning phase. Basically, three parameters are used to define ANN which are: the pattern that interconnects the different layers, the weights connected to these layers which are fluctuated during learning and the activation function responsible for the conversion of weighted input neurons to outputs [19]. ANN have been widely used for a range of cheminformatics applications such as in the work on steroids by So et al. [20], study of oestrogen receptor agonists by Li et al. [21], and also by Briem et al. [22] to identify possible kinase inhibitors and many more to count.

9.9 Random Forest

Random forest (RF) is a decision-tree based technique which uses an ensemble of trees for the classification task. The training data consisting of multiple features for each instance is used to build the decision trees. A multitude of decision trees are constructed during the training phase and the output class is the mode of the classes output of the individual trees. The trees consist of nodes, branches and edges which correspond to the features, values and classes, respectively. One feature is selected randomly at each node which separates the objects/instances to classes with maximum information gain. There is no pruning of the trees and each tree is grown to the largest possible extent. The tree is terminated when each of the features has

been considered at least once or if the feature gives same value for all the training objects. The trained forest of trees is then used for the prediction of future unseen data [23]. RF method has been successfully used in cheminformatics for investigation into mutagenicity data [22], development of hERG blocker classifiers [24], quantitative-structure activity relationships models (QSAR) [25] and skin sensitization data [26].

9.10 Naive Bayes

The naïve bayes (NB) classifier is a probabilistic classifier which uses Bayes theorem and classifies the test instances to the class with highest probability. The algorithm assumes that all the features are conditionally independent of each other and thus have impartial contribution towards the task of prediction irrespective of any correlation among the features [27]. The advantage of NB classifiers over other classification algorithms is its simplicity and that it's extremely fast. A NB model is easy to generate and does not involve any complex parameter tuning which makes it highly efficient for handling large datasets. NB classifiers have performed quite well and are frequently used in cheminformatics, for target prediction [28], classification of drug-like molecules based on their biological activities [29], for toxicity prediction [30] and various other studies.

9.11 Support Vector Machines

Support vector machine (SVM) is a non-probabilistic binary linear classifier that separates the instances belonging to two classes using a gap which is as wide as possible. This gap is defined by a separating hyperplane which is the output of the SVM algorithm and categorizes new instances to the class depending on which side of the hyperplane these instances fall. SVM can also efficiently perform non-linear classification using kernel functions. Some common kernel functions include polynomial, Gaussian radial basis function, and hyperbolic tangent [31]. SVMs are one of most widely used methods in cheminformatics and have been used for prediction of toxicity [30], classification of kinase inhibitors [22], mutagenic toxicity prediction [32] and prediction of biological activities of small molecules and drug-repurposing [33].

9.12 K-Nearest Neighbours

The k-nearest neighbour algorithm (kNN) is based on the principle that the categorization of an instance to a particular class depends on the majority of votes of its neighbours. For example, to predict the class of an instance, *x*, the classifier will look for k-nearest neighbours of instance *x* and assign it the class to which most of the k-nearest neighbours belong. K-nearest neighbours are selected on

the basis of the distance between the test instance and training instances in the feature space. Euclidean distance is usually used for this purpose; however, other metrics like Jaccard distance could also be used [34]. Many studies have used kNN classification algorithm for the prediction of anticancer drugs [35], psychoactivity of cannabinoid compounds [36], and mutagenicity of chemicals [37] and several others.

9.13 Applications of Cheminformatics

There is a wide range of applications of cheminformatics starting from storage and retrieval of chemical compounds to prediction of their properties, QSAR, virtual screening and drug design and various others like textile industry, molecular, material and food science and combinatorial organic synthesis [38]. We have discussed some of the typical applications of cheminformatics.

9.14 Storage and Retrieval of Chemical Compounds

This is the primary application of cheminformatics which involves storing chemical compounds information generated through experiments and retrieval of information and structures from chemical databases [39]. Table 9.1 provides the list of the databases for the storage of information on chemical compounds.

Table 9.1 Provides the list of the databases for the storage of information on chemical compounds

PRIVATE Database	Supplier
ACD (Available Chemicals Directory)	MDL Information Systems Inc.
ASINEX	AsInEx Ltd.
CDD (Collaborative Drug Discovery)	Collaborative Drug Discovery, Inc.
ChEMBL	European Bioinformatics Institute (EBI)
ChemIDplus	U.S. National Library of Medicine, National Institutes of Health
ChemSpider	Royal Society of Chemistry
CSD (Cambridge Structural Database)	Cambridge Crystallographic Data Centre
InterBioScreen	InterBioscreen Ltd.
Maybridge	Thermo Fisher Scientific Inc.
MedChem	Daylight Chemical Information Systems Inc.
NCI96 (National Cancer Institute data)	Daylight Chemical Information Systems Inc.
PubChem	National Centre of Biotechnology Information
Spresi95	InfoChem GmbH
TSCA93	Daylight Chemical Information Systems Inc.
WDI (World Drug Index)	Derwent Publication
ZINC database	University of California San Francisco

9.15 Prediction of Properties of Chemical Compounds

One of the most important tasks for chemists is to generate compounds with desirable properties. A large number of compounds generated through HTS and CC technologies had to be discarded since these compounds did not have drug-like properties. This wastage could be minimized if we had a technique to predict physical, chemical or biological properties of the compounds and synthesize compounds with properties which could result in novel drug-like candidates [5].

9.16 QSAR

QSAR method involves the prediction of properties of chemical compounds from their structures. QSAR models comprise predictors which consist of physiochemical properties or theoretical molecular descriptors of chemical compounds and predict the biological activities of the chemicals. The models first establish a relationship between structures of chemical compounds and biological activities and based on that predict the activities of novel chemical compounds [40].

9.17 Virtual Screening

Virtual screening has become one of the important tools for identifying drug-leads. The technique involves computational screening of large in silico libraries of compounds to identify compounds with desirable properties such as having activity against a biological target and filtering unwanted compounds [41]. Various virtual screening methods are applied that include docking if the target structure is known [42], similarity approaches if the target structure is unknown but the ligands are known [43] and structure–activity relationship approaches if neither the structure of the target nor the ligands structure is known [44].

9.18 Cheminformatics and Modern Drug Discovery

Recent years have seen large amount of chemical data generated through integration of CC and HTS technologies for the purpose of drug discovery. However, this data can be effectively utilized only if we have techniques to store, handle, analyse and apply it in the drug discovery process. The traditional drug discovery process involved identification of disease and drug target followed by synthesis of molecule effective against that disease. This molecule would further be investigated for pharmacodynamics and pharmacokinetic properties and toxicity and then taken up for clinical trials. This is a costly and lengthy process with the risk of the lead molecules being failed in the clinical trials leading to wastage of time, money and efforts. This highlights the need for the technique which could identify the problematic lead compounds and predict the biological activities and ADMET properties of the

chemical compounds before the preclinical testing thus reducing the rate of failures and speeding up the process of drug discovery and development. Cheminformatics is one such technique which plays a major role in identification of drug target and lead compounds active against the drug target and prediction of their ADMET properties. The modern drug discovery process involves four steps: identification of target and its validation, lead identification, lead optimization followed by preclinical trials (Fig. 9.1).

Once the target is identified, the lead compounds with desirable properties active against that target could be screened out from the enormous number of diverse chemical compounds available through cell-based assay compounds or various databases of small molecules owing to the HTS technique. Various cheminformatics approaches like machine learning could be used to generate computational models which could identify novel drug candidates from lead compounds. Further, the selected candidate compounds could be docked to the protein target to find out the compounds having affinity towards the target. When the drug-like compounds have been identified, these could be taken forward to evaluate ADMET characteristics using computational models thus eliminating the undesirable compounds at earlier stages of drug development and cutting down the costs and time involved. Various other cheminformatics techniques like similarity searching and substructure searching could be applied for the identification of novel scaffolds from large chemical compounds repositories.

9.19 Tools and Techniques Used in Cheminformatics

Various cheminformatics toolkits that can be used by cheminformaticians for chemical data mining, virtual screening and structure–activity relationship studies have been developed. We have listed some of the tools below:

- ChemDraw [45] is a Macintosh and Microsoft windows program first developed by David A. Evans and Stewart Rubenstein in 1985 and later by the cheminformatics company CambridgeSoft. This is a molecular editor tool which, along with Chem3D and ChemFinder, is part of the ChemOffice suite of programs.
- ChemReader is a fully automated tool that extracts chemical structures information from images in research articles and translates that information into standard chemical formats that can be searched and analysed [46].
- ChemSketch is a molecular modelling program that allows drawing and modification of structures of chemical compounds and structural analysis that includes understanding of chemical bonds and functional groups [47].
- ChemWindow is a program developed by Bio-Rad Laboratories, Inc. that allows drawing chemical structures, 3D visualization and database searching.
- Chemistry Development Kit (CDK) is a JAVA software for use in bioinformatics and cheminformatics available for Windows, Linux and Macintosh. The program allows 2D molecular generation, 3D geometry generation, descriptors and fingerprints calculation and supports various chemical structure formats [48].

- ChemmineR is a R language cheminformatics program for analysing small molecule drug-like compounds data and enables similarity searching, clustering and classification of chemical compounds using a wide range of algorithms [49].
- JME molecular editor is a JAVA applet that allows to create and modify chemical compounds and reactions and can display molecules within an HTML page [50].
- Molecular Operating Environment is a scientific vector language based software program the applications of which include structure- and fragment-based design, pharmacophore synthesis, protein and molecular modelling and simulations in addition to cheminformatics and QSAR.
- Open Babel is a software which is used for the interconversion of chemical file formats. It also allows substructure searching as well as fingerprints calculation [51]. It is available for Windows, Linux and Macintosh.
- OpenEye is a drug discovery and design software kit and its areas of application include generation of chemical structures, docking, shape comparison, cheminformatics and visualization. OpenEye toolkits are available in multiple programming languages that are C++, JAVA and python.
- Chemaxon provides various cheminformatics software programs, applications and services for drawing structures of chemical compounds and their visualization, searching and management of chemical databases, clustering of chemical compounds and drug discovery and design.
- PubChem is a large repository of chemical compounds and their biological activities obtained through biological assays. The database is maintained by National Centre for Biotechnology Information and is freely accessible [52].

Various other tools such as PowerMV, PaDEL, CDD (Collaborative Drug Discovery), RDKit, 3D-e-chem, ADMET Predictor, MedChem Studio, MedChem Designer, Mol2Mol, Chimera, VMD, ArgusLab, ChemTK, Premier Biosoft and many others are also widely used for cheminformatics applications. Table 9.2 lists the companies that provide cheminformatics software and tools.

Table 9.2 Lists the companies that provide cheminformatics software and tools

Accelrys, USA	CambridgeSoft, USA
Advanced Chemistry Development, Canada	Bioreason, USA
Agilent Technologies, USA	Aventis Pharma (France, Germany, USA)
Bayer (Germany, USA)	Novartis Pharma, USA
Chemical Diversity Labs, USA	Molsoft, USA
Daylight USA	ACD/Labs (Advanced Chemistry Development, Inc.)
Golden Helix Inc., USA	Schrödinger, USA
ID Business Solutions Ltd., United Kingdom	OpenEye Scientific Software, USA
Modgraph Consultants Ltd., United Kingdom	Molinspiration, Slovak Republic
Molecular Networks, Germany	Eidogen-Sertanty, USA
PharmaDM, Belgium	SciTegic, USA
Rosetta Biosoftware	MolMine Bioinformatics Software Solutions

Conclusion

The large amount of HTS data generated in recent years has triggered the need for developed cheminformatics systems. Cheminformatics methods enable us to manage and understand increased amount of chemical data, and analyse and exploit the results obtained from experiments. In addition to chemical information, cheminformatics methods also allow to retrieve information about physical properties, spectroscopic data, crystallographic and 3D molecular structures, chemical reaction pathways, functional groups, docking and various other parameters. Cheminformatics has made the process of drug discovery and development very easy and fast. In this chapter, we have discussed about cheminformatics and its various aspects and approaches, traditional drug discovery process and role of cheminformatics in modern drug discovery system. Cheminformatics has already been successfully integrated into various fields such as chemistry, bioinformatics and many more; however, there still is a need for developed and advanced cheminformatics systems for solutions to unsolved problems.

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Pharmacogenetics and Personalized Medicine

10

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Abbreviations

3′-UTR	3′-Untranslated region
5-FU	5-Fluoruracil
ABCB1	ATP-binding cassette transporter family member B1
AIFA	Italian Medicine Agency
AML	Acute myeloid leukemia
BCR-Abl	Breakpoint cluster region-Abelson
CML	Chronic myeloid leukemia
CRC	Colorectal cancer
CYP2D6	Cytochrome P450 isoform 2D6
CYP3A4	Cytochrome P450 isoform 3A4
CYP450	Cytochrome P450
ddPCR	Digital droplet PCR
DNMT	DNA methyl transferase
*dpyd*DPD	Dihydropyrimidine dehydrogenase
EGFR	Epithelial growth factor receptor
ELN	European Leukemia Network
FDA	Food and Drug Administration
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HMGCoA	Hydroxymethyl-glutaryl coenzyme A
hOCT1	Human organic cation transporter family member 1
lncRNA	Long-noncoding RNA
miRNA	microRNA

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NAT	N-acetyl transferase
ncRNA	Noncoding RNA
NGS	Next-generation sequencing
NSAID	Nonsteroidal anti-inflammatory drug
NSCLC	Non-small-cell lung cancer
OS	Overall survival
PCR	Polymerase chain reaction
PFS	Progression-free survival
RR	Response rate
SLCO1B1	Solute carrier organic anion transporter family member 1B1
SLCO1B3	Solute carrier organic anion transporter family member 1B3
SNP	Single-nucleotide polymorphism
TYMS	Thymidylate synthase
WGAS	Genome-wide association study

10.1 Introduction

The study of human genome and its regulation is one of the most exciting fields of modern research, because many efforts have been and are still addressed to the investigation of prognostic biomarkers of disease. Through those markers, it may be possible to achieve an early diagnosis and to anticipate the evolution of a specific disease. At the same time, when the diagnosis has been obtained and the stage defined, every possible marker predictive of drug efficacy and/or tolerability may give exceptional advantages to the patients, their caregivers, and relatives. Indeed, while approaching the treatment of a disease, the knowledge about the probability of therapeutic benefits (from disease stabilization up to the cure) or the risk of toxicities (which ultimately could require treatment delay or discontinuation) becomes critical for patients' management.

The search for those biomarkers has come to impressive advances thanks to three fundamental factors, the first of which may be considered the improved knowledge about the structure, function, and controls of nucleic acids. Mutations and genetic polymorphisms have an impact on the expression, structure, and function of the corresponding coded protein, thus having the possibility to influence both the occurrence/severity of a disease and the effects of the drugs. However, those investigations are not completely able to reduce in a variable degree the uncertainty that may characterize the biomarker/s. For that reason, as explained in the following paragraphs, other genetic and nongenetic factors are included in the analysis (i.e., epigenetic mechanisms of gene expression control, clinical characteristics of the patients). This means that the appropriate statistical analyses should be adopted.

Second, the growing mess of findings is possible thanks to the methodological advances that are increasing the sensitivity and the specificity of techniques, such as the ddPCR that allows the amplification of single-nucleotide sequences at very low concentrations. At the same time, a broader range of applications is available for the research and its translation into clinical settings. Nowadays, it is possible to go beyond the investigation of candidate genes (and polymorphisms) through the sequencing of

Table 10.1 Screening methods to detect changes in nucleotide sequence currently available for pharmacogenetic studies

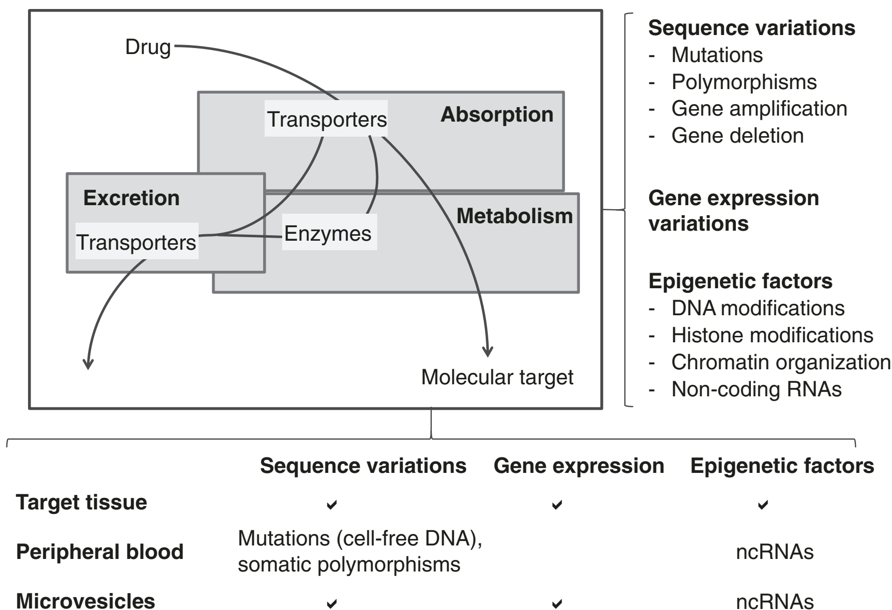
Screening	Methods
Direct	• Direct sequencing
	• Pyrosequencing
	• Amplification refractory mutation system (ARMS)
	• Combined real-time PCR and ARMS
	• Reverse dot blot
Indirect	• High-resolution melting (HRM)
	• Single-strand conformation polymorphism (SSCP)
	• Restriction fragment length polymorphism (RFLP)
High sensitivity	• Fluorescent amplicon generation (FLAG)
	• Mutant-enriched PCR
	• Real-time PCR analysis (allelic discrimination)
	• PCR-RFLP analysis
	• GWAS (genome-wide association study)
	• NGS (next-generation sequencing)

a complete genome, in a manner that is faster and cheaper than what we were expecting just few years ago (Table 10.1). Indeed, the development of techniques and methods to investigate the mutational/polymorphic status of genes is rapidly growing, and commercial kits are now available at reduced costs. The majority of those techniques are based on real-time PCR, have a greater sensitivity, and are faster than the PCR/sequencing techniques. Finally, some of those techniques can detect a variation in the nucleotide sequence (a mutation or a polymorphism) in the most accurate way (direct screening methods) or they just detect the variation without giving information on its nature. It is worth noting that those molecular techniques may generate a huge mass of data, whose handling and analysis need the availability of ad hoc software.

The third factor that has fuelled the search for biomarkers (especially those with a predictive role) is the arising of targeted therapies. From a pure pharmacological point of view, every drug has its molecular target (and therefore it is a targeted therapy), but more specifically the term targeted therapy refers to the drug that is developed on the basis of a well-known molecular target, and imatinib is the paradigmatic example of such a generation of new drugs. Therefore, the occurrence and severity of a disease depend on the expression and/or activity of a specific protein against which a drug is developed. On these premises, it is clear that the analysis of the molecular target within the pathological tissue (i.e., the mRNA levels or mutations) or in the whole body (i.e., a somatic polymorphism) may be useful to anticipate the therapeutic activity of a certain pharmacological regimen. If healthy tissues also express the target that is essential for cell growth and proliferation then the analysis of the biomarker may anticipate the tolerability, but the biomarkers predictive of efficacy and tolerability could not be coincident, as in the case of 5-FU (*tyms* and *dpyd*) or statins (HMGCoA reductase and SLCO1B3). Other factors are still contributing to pharmacogenetic studies, as well as ad hoc study designs that will be discussed in the following paragraphs.

10.2 Pharmacogenetics: Areas of Investigation

What makes the modern pharmacological therapy an advanced management of patients and their diseases is the possibility to choose drugs and adjust doses on an individual basis. However, this is not completely true, because if we look at the possibility to optimize the treatments to obtain the best clinical outcomes then we may see that the response to therapies is more or less variable, depending on the intrinsic characteristics of the target (be it a cell, a tissue, or a patient), the drug (its therapeutic index, for example), and other endogenous and environmental factors. Because the mechanism of drug action is known at therapeutic doses, changes in the efficacy and tolerability may depend on the different expression or altered structure of the molecular target. This possibility is accompanied by similar changes in other proteins that are involved in the pharmacokinetics (for example, transmembrane transporters or enzymes for drug metabolism) or in secondary mechanisms of action (as well as the induction of prolonged QT interval and the use of some antidepressants) [1]. Therefore, the variability in drug response may be a common feature and it is heritable, so that these factors may be investigated through the evaluation of patient's genome and its variations in nucleotide sequence and gene expression (Fig. 10.1).



	Sequence variations	Gene expression	Epigenetic factors
Target tissue	✔	✔	✔
Peripheral blood	Mutations (cell-free DNA), somatic polymorphisms		ncRNAs
Microvesicles	✔	✔	ncRNAs

Fig. 10.1 Several pharmacogenetic factors may influence the efficacy and tolerability of pharmacological treatments. Variations in nucleotide sequences and gene expression together with epigenetics may be investigated in target tissues, peripheral blood, or microvesicles to discover biomarkers

10.2.1 Gene Expression

The variable presence of a target protein within the tissues may influence the activity of a drug in a clinically significant manner. It is conceivable that differences in gene transcription could be responsible for the different levels of protein concentrations within a cell or a tissue [2], despite the fact that several issues may impede the demonstration of such correlation [3].

The analysis of gene expression finds another interesting area of application in deciphering the biochemical and genetic effect of a drug on cell metabolism, proliferation, and activities. Indeed, the exposure of cells to a specific drug could influence the gene expression of several target genes, which are directly involved in drug disposition and/or pharmacodynamics. For example, when patients affected by CML are exposed to imatinib the gene expression profile of some genes may change, and those changes are significantly associated with a different outcome of chemotherapy [4]. In a similar manner, the exposure to carbamazepine and phenytoin may cause the overexpression of some genes possible related to the risk of developing idiosyncratic reactions [5]. Again, the exposure of human pancreatic cancer cell lines to tolfenamic acid, a NSAID, is able to modulate several genes that control cell proliferation and survival probably through the involvement of the SP1 transcription factor [6].

The areas of investigation presented above may take some advantages through the adoption of the newest technical platforms such as expression microarrays and GWAS. Those techniques make feasible the analysis of gene expression in the largest scale. Results from those approaches are genetic “signature” that may be finalized to several purposes, such as new pathological classifications of the disease on the basis of different biochemical pathways [7]. From another side, the findings of those analyses may identify those patients with the highest (or lowest) probability of response to pharmacological treatments. For example, a GWAS found that common genetic variants were able to influence gemcitabine and cytarabine sensitivity in in vitro experiments [8], and a following clinical trial performed in 937 patients confirmed that SNPs in the *chd4* gene were predictive of gemcitabine outcome in terms of OS [9].

With the increased spreading of those techniques, a great number of bioinformatic tools has been produced and validated, being necessary the correct handling, quality control, and analysis of such an amount of raw data [10]. More intriguingly, the analysis is not fully based on genetic data, but also considers possible gene-gene interactions (the so-called epistatic interactions) [11], because they may explain that part of drug response that a single-locus analysis cannot do. Indeed, the epistatic interaction of somatic mutations in *egfr* (i.e., the T790M) and the amplification of the met *gene* may explain the resistance against gefitinib in NSCLC patients [12].

Noteworthy, the investigation of gene expression in target and nontarget tissues is a time-dependent feature that could be influenced by several factors. For example, it has been demonstrated that the time elapsed from surgical excision and tissue fixation or freezing to preserve the integrity of nucleic acids may significantly influence the levels of cytosolic mRNA: the longer the period of warm ischemia before

fixation, the more variable the levels of gene expression [13, 14]. Therefore, those evidences strengthen the necessity of careful handling of tissue specimens, especially when a gene expression profiling will be performed on those samples.

Another important characteristic is the heterogeneity of gene expression levels within the different areas of the same tissue, an issue particularly relevant in tumor biology [15]. Indeed, the presence of different subclones within the same tumor mass explains the risk of treatment failure due to the emergence of resistant clones. Moreover, a candidate-gene analysis may hide further putative druggable mechanisms responsible for tumor growth and proliferation. The adoption of GWAS or microarray strategies may help in overcoming these limits [16]. Furthermore, the inclusion of normal stroma within a tumor sample could weaken a possible gene-phenotype relationship; hence laser microdissection allows the collection of tissue of interest or cells [17].

10.2.2 Mutations and Polymorphisms

The relationship between occasional mutations or polymorphic alleles and characteristics of a disease or its response to therapy warrants the detection of these changes in nucleotide sequence. Almost all of the areas of diseases therapy can benefit from those genetic analyses, as in the case of ABCB1 polymorphisms and drug-resistant epilepsies [18], the SNPs of hOCT1 and imatinib pharmacokinetics and pharmacodynamics [19–21], the variable response to opioids related to several SNPs [22], the different biotransformation of tacrolimus caused by polymorphic alleles of the CYP3A4 isoform [23], or, more recently, the severe toxicities induced by statins and transmembrane transporter SLCO1B1 variant alleles [24].

One of the milestones in cancer genetics was represented by the evaluation of mutated genes as possible causes of cancers, as in the case of mutated *ras* or *tp53* genes [25, 26]. Their presence within a tumor mass was associated with a worse prognosis because of the increased aggressiveness of the neoplasm [26]. In the following years those mutations were found to be predictive of chemotherapy response [27, 28], and included in the list of analyses for the pharmacological management of patients. As discussed above, the dissection of biochemical pathways offered other targets to the investigation. For example, besides the analysis of mutational status of *k-ras* gene in colorectal cancers, the evaluation of *egfr* mutations has become a standard analysis, because some of those mutations have a significant influence on cetuximab activity [29, 30]. In agreement with those findings, different variant alleles of *egfr* were associated with response to small molecules like gefitinib and erlotinib in the treatment of NSCLC [31].

Although spontaneous mutations and polymorphisms could have the same impact on cell phenotype, they have striking differences. The mutations occur in a relatively low frequency within a portion of a tissue and represent a clonal characteristic of that tissue. For example, the occurrence of the point mutation T315I during treatment confers resistance to several inhibitors of the BCR-Abl tyrosine kinase activity in CML patients [32, 33], and it represents the emergence of a

drug-resistant clone. On the contrary, polymorphisms are changes in nucleotide sequence that occur in at least 1–3% of population and are present within the DNA of each single cell of the body. Therefore, polymorphisms are variant alleles that could be easily investigated by extracting DNA from the target tissue or, when it becomes difficult, from a surrogate tissue such as blood or other matrices, as well as saliva (i.e., buccal swabs). Indeed, the presence of epithelial cells within the saliva ensures the possibility to obtain DNA in such an amount that a great number of analyses are available [34].

The different position of the SNP along the gene is responsible for the different effects of those polymorphisms on protein synthesis. For example, SNPs within the 5′ promoter region, introns, or the 3′-UTR may affect mRNA transcription and translation, through the variable levels of mRNA synthesis or changes in the cytosolic half-life of mRNA molecules. On the contrary, SNPs within the open reading frame affect the function and the structure of the coded protein and, as a consequence, that of the corresponding phenotype. As a technical note, some of the polymorphisms are synonyms, meaning that the changes in nucleotide sequence do not parallel that in amino acids. Nevertheless, synonymous polymorphisms may alter the function of the spliceosome complex during RNA processing, binding of miRNAs, and mRNA degradation [35]. Furthermore, the initiation, elongation, and folding of the coded protein may be disrupted. For example, the synonymous SNP c.3435C>T of the *abcb1* gene has an effect on the timing of cotranslational folding that results in changes of substrate specificity [36].

Finally, when several polymorphic loci along the same gene are analyzed at the same time, it is useful to investigate whether their alleles may be inherited together (so that they are forming a haplotype). The linkage disequilibrium of those loci depends on being in close proximity, so that chromosomal rearrangements are not frequent. The haplotypes resulting from the variable combination of alleles may give their contribution to the understanding of the efficacy of pharmacological therapies. For example, the *abcb1* haplotype containing only polymorphic alleles at loci c.1236, c.2677 and c.3435 (see below) was predictive of both nephro- and hepatotoxicity induced by chemotherapy in AML patients [37]. Interestingly, from the analysis of complex haplotypes some "tag SNPs" may be identified. These polymorphisms are representatives of the polymorphisms within the chromosomal region of interest, hence the evaluation of their genotype could be able to predict the haplotype-associated phenotype. For *abcb1*, the most common SNPs investigated in several studies are those located in the exons 12 (c.1236C>T), 21 (c.2677G>T/A), and 26 (c.3435C>T), and they are in strong linkage disequilibrium. However, that chromosomal region is characterized by the presence of several polymorphisms, and one interesting study has described the presence of six haplotype-tagging SNPs representative of the polymorphisms surrounding the c.3435C>T SNP [38].

A more complex approach to the investigation of polymorphisms and mutations is represented by NGS, which can handle thousand or more polymorphisms at the same time [16, 39]. NGS offers a sequence analysis that may be carried out at different levels of precision, being the target sequence (or the whole genome) sequenced more times: the highest the number of replications that cover the region of interest

(i.e., the magnitude of coverage rate, 5×, 10×, 20×, etc.), the highest the robustness of the data and the probability to discover very rare changes in nucleotide sequence. As discussed for GWAS and microarrays, NGS studies allow the investigation of complex phenomena and the identification of genetic signatures that could have a prognostic or a predictive value.

10.2.3 Epigenetics

The study of epigenetic factors possibly associated with drug efficacy and tolerability started with the collection of evidences regarding the correlation among histone acetylation, DNA methylation, and drug effects. Those two modifications depend on the presence of specific enzymes, namely DNMT, HAT, and HDAC [40–42], whose activities have opposite effects on gene transcription: the highest the DNA methylation, the lowest the gene expression, because the transcription machinery has no access to the DNA. On the contrary, the acetylation of histones allows the unfolding of double-strand DNA from nucleosomes and the reading of the genetic code. More recently, several studies have demonstrated that histone modifications are numerous, also including crotonylation, sumoylation, etc. [43, 44]. In order to investigate the effect of histone modifications on gene expression levels, and the corresponding phenotype, the histone-associated DNA may be collected by immunoprecipitation (ChIP) and followed by microarray analysis (ChIP-chip), sequencing (ChIP-seq), or serial analysis of gene expression (Chip-SAGE) [45]. The reason why histone studies warrant efforts is related to the fact that endogen and environmental factors (among which drugs) may influence the activity of HATs and HDACs, thus influencing gene expression of a variable number of genes. For example, the loss of HATs and HDAC regulation in B- and T-lymphocytes may drive the genesis of lymphoma and leukemia malignancies because these enzymes control cell proliferation, survival, and functions of mature cell elements [42]. Those evidences are accompanied by the possibility to obtain a therapeutic benefit from the inhibition of HDAC, as demonstrated by butyrate and valproic acid, or more recently by vorinostat, belinostat, and panobinostat [42, 46, 47].

DNA methylation is catalyzed by several DNMT isoforms that are overexpressed in solid neoplasms and hematological malignancies [48]. The result of their increased activity is partially associated with the methylation of CpG islands in promoter regions of tumor-suppressor genes, and with an aggressive phenotype of the cancer cells. These characteristics make DNMTs putative targets of anticancer agents [49]. Interestingly, several techniques are available for the analysis of DNA methylation (see for review Kurdyukov and Bullock, 2016) [50], both at the candidate gene and at the genome level, and they can be applied to the discovery of predictive biomarkers. For example, the analysis of methylation profile in CRC patients demonstrated that alterations in gene methylation (both hypo- and hypermethylated) affected the apoptotic, signaling, and proliferation pathways, and ultimately those DNA modifications were significantly associated with tumor recurrence [51].

However, other epigenetic processes contribute to the control of gene expression and translation, as well as the structural organization of chromatin through the recruitment of chromatin-remodeling enzymes [52]. The ncRNAs, which include miRNA and lncRNAs, may regulate the transcription of several target genes, resulting in possible drug-resistance phenomena in human cancers [53, 54]. The miRNAs are short nucleotide sequences (approximately 22 bases) that bind complementary sequences in the 3′-UTR of target mRNAs [55], which in turn lose their function and are degraded. The lncRNAs have regulatory functions in several biological processes, due to their specificity to interact with both DNAs and proteins [56–58], and this confers them both a prognostic and a predictive role in several diseases, as well as tumors, and cardiac and metabolic diseases [59–61].

It is worth noting that ncRNas may be released within the circulation where they can be isolated and used as biomarkers. A paradigmatic example is the analysis of circulating miRNAs in patients who received heart transplantation, because some of those miRNAs were associated with graft rejection [62], and similar findings were obtained in patients who received antiplatelet drugs [63]. It is worth noting that the extraction of microvesicles from plasma ensures the analysis of what happens in target neoplastic tissues. Indeed, the microvesicles released from the cells are carrying mRNAs, miRNAs, and proteins, as they may recapitulate the genetic and biochemical characteristics of the parental cell [64]. For example, in multiple myeloma and in other hematological diseases, the investigation of bone marrow niche may help in identifying the mechanisms responsible for drug resistance and possibly leading to treatment optimization. However, the investigation of pathogenic mechanisms of disease and the monitoring of the drug sensitivity through bone marrow biopsies can be difficult for several reasons, whereas circulating microvesicles may represent an optimal source of information. Tumor cells uses exosomes, a small kind of microvesicles (30–150 nm), to vehicle cell surface signalling proteins and nucleic acids that can reprogram the stroma [65]. This phenomena favor tissue invasion, metastatization [66], and alteration of the bone marrow niche that can host cancer cells [67].

10.2.4 Gene and Chromosomal Duplication/Deletion

The metabolic rate of drugs in the liver is so important for the efficacy (and tolerability) of pharmacological treatments that predicting patients' phenotype (i.e., poor, normal, or rapid metabolizers) has become an intensive area of research and clinical application. Since the fast and rapid metabolizer phenotype with respect to acetylation of some drugs by NAT enzymes [68], the interest has been addressed to the allelic variants of CYP450 isoforms [69, 70]. The numerous CYP450 isoforms harbor several polymorphisms that are responsible for the variable biotransformation of drugs [71], even if the phenotype corresponding to the ultrarapid metabolizer may depend on gene duplication and variable copy number [72, 73]. Indeed, the most rapid transformation of codeine into morphine (with the following increased risk of adverse reactions) depends on the duplication of CYP2D6 gene [74, 75], as

well as the higher doses of several tricyclic antidepressants [76–78]. In cancer chemotherapy, gene amplification is a common acquired mechanism that may cause drug resistance during pharmacological treatments. Indeed, studies described the amplification of *tyms* and BCR-Abl in response to the administration of 5-FU and imatinib [79, 80].

On the contrary, the deletion of chromosomal regions leads to the loss of genes coding for enzymes, receptors, or structural proteins, with changes in phenotype and drug sensitivity. This is the case of the deletion of the chromosomal region 8p21, which is a probable pathogenetic cause of multiple myeloma [81] and at the same time responsible for the poor RR (approximately 50%) to bortezomib [82]. Indeed, the deletion was associated with the loss of several genes involved in the apoptotic pathway as well as *bcl2*, *tp53*, and *trail-r4*.

For both amplification and deletion, the exact localization of the chromosomal region involved in those phenomena is of paramount importance, because it may help in deciphering the subsequent phenotypes and their translation into biomarkers for the identification of possible therapeutic strategies.

10.3 Personalized Medicine or Patients' Stratification?

From previous paragraphs it appears that a more or less straight pathway can lead to the discovery of prognostic and predictive biomarkers. As stated in the Introduction section, that pathway has become possible thanks to the availability of improved technical platforms for genome analysis, the better knowledge of molecular and biochemical processes, and the development of targeted therapies. The ultimate goal of those research activities is the development of biomarkers that could be useful to better characterize the disease (through a genetic signature), to anticipate the patient's prognosis, to choose the right pharmacological regimen, and to predict drug efficacy and/or tolerability. The technique may investigate few candidate genes, more complex panels, or the entire genome, at different levels: gene expression, and changes in gene sequence both at the nucleotide (i.e., mutations and polymorphisms) and chromosomal level (i.e., amplifications and deletions). Otherwise, focusing on epigenetic factors may add further opportunities to achieve the goals listed above.

Beyond those opportunities, the application of a predictive biomarker in real world is deeply influenced by some issues, the first of them being the reliability of the marker itself. All of those biomarkers that have been introduced in clinical routine are capable to predict with higher sensitivity and specificity treatment efficacy or tolerability. A sound example is the mutational analysis of *bcr-abl* fusion gene by NGS [83–85], because the occurrence of the T315I mutation confers resistance to all tyrosine-kinase inhibitors used for CML therapy with the exception of ponatinib [86]. Analogously, the *egfr* and eml-alk4 mutational analysis and monitoring represent optimal markers for the choice of the drugs for the treatment of lung cancers [87, 88]. On the other side, the SNP c.1905+1G>A (rs3918290) that causes the skipping of the exon 14 in the mRNA of *dpyd* gene (the variant allele DPYD*2A) is

predictive of severe or lethal toxicities from fluoropyrimidines [89]. However, not always the findings of pharmacogenetic analyses can help in attaining those goals, especially when a variable degree of that uncertainty still remains unexplained. For example, even in the absence of the DPYD*2A allele, some patients experience severe toxicities, because of the presence of other polymorphisms along the gene [90]. In that case, a possible (and partial) solution is to perform the genetic analysis on a panel of polymorphisms rather than on the *2A only [91], or to include other known factors (even if nongenetic) to predict treatment tolerability [92]. The aim of these strategies is to attain patients' stratification according to the probability of benefitting from the pharmacological treatment or to the risk of drug-induced toxicities.

Therefore, some of those markers really help physicians to "diagnose, plan treatment, find out how well treatment is working, or make a prognosis" [93], which is a definition of *personalized medicine*. The uncertainty about patient's survival and quality of life, treatment efficacy, and tolerability is significantly reduced, but not completely abated because some molecular processes in the target tissues may be influenced by the drugs themselves, as discussed above. Therefore, rather than belonging to the concept of personalized medicine, these approaches are part of the *stratified medicine* that "reflects the realistic effects of medicines at population level" [94]. Groups of patients belonging to the same heterogeneous population are identified and addressed to different treatments according to their probability/risk of response/toxicity. In that phase, the optimization of pharmacological treatments in terms of drugs and doses is enough broad to compensate for the large interindividual variability. For example, the recommended doses of fluoropyrimidines on the basis of *dpyd* genotyping translated into a DPD activity score are 100, 75, 50%, etc. [95]. However, the fine-tuning of the dose can be attained through other approaches, such as that of the therapeutic drug monitoring of 5-FU plasma concentrations [96].

10.4 Transfer and Application of Pharmacogenetic Markers

One of the most important challenges of pharmacogenetics is the translation of biomarkers into the clinics, making them available for patients' stratification, or, when possible, for treatment individualization. To accomplish this aim, there are several strategies that may be adopted, but for all of them the validation of the biomarkers in prospective studies is the definitive proof.

As anticipated in the previous paragraphs, the development of a biomarker may take place in a simultaneous or sequential manner with respect to drug development. The studies have investigated the majority of biomarkers after drug registration in order to improve efficacy and ameliorate tolerability. The advent of targeted therapies has changed that process. Indeed, the discovery of Philadelphia chromosome and the presence of the BCR-Abl fusion protein with its tyrosine-kinase catalytic site brought to the development of imatinib contemporary to the need of monitoring the response to the drug. Since that former example, several ones have followed, and now the presence of the molecular target should be confirmed because it

represents an absolute requirement for the use of the drug. Therefore, the development of the pharmacological agent along clinical trials may sometimes parallel that of the corresponding companion diagnostic [97]. That assay is specifically developed for and often co-marketed with the drug, and the draft guidance documents released by the FDA in 2015 and 2016 about companion diagnostics and their co-development with a therapeutic product witness the importance of these tests [98, 99]. Complementary diagnostics are similar to companion diagnostics but they are linked to a class of drugs rather than a specific agent, and their use is not strictly labelled [97].

Beyond these definitions, the link between the drug and its biomarker, its discovery, and validation is the main point of discussion (Fig. 10.2). During that process, the design of the study takes on great importance because it may accelerate the biomarker discovery and validation. Many studies are just exploratory trials, lacking from an adequate number of patients and appropriate methodologies to transfer the validation of the marker to future studies. That situation stimulates the proposal of methodological guidelines for conducting trials aimed at biomarker identification and validation [100].

When a general study design is adopted, all of the patients have the same probability to receive an interventional/experimental treatment regardless of their genetic status. Then, findings are collected and stratified according to biomarker characteristics and appropriate statistical analyses are performed. On the contrary, in a biomarker-guided design patients' stratification occurs before treatment, so that the effect of the combination drug-biomarker is emphasized. There are several schemes available for these purposes [101, 102]. For example, the enrichment design is based on the enrolment of patients positive for the biomarker to compare the efficacy of a standard drug with respect to the targeted therapy. Otherwise, the study may be

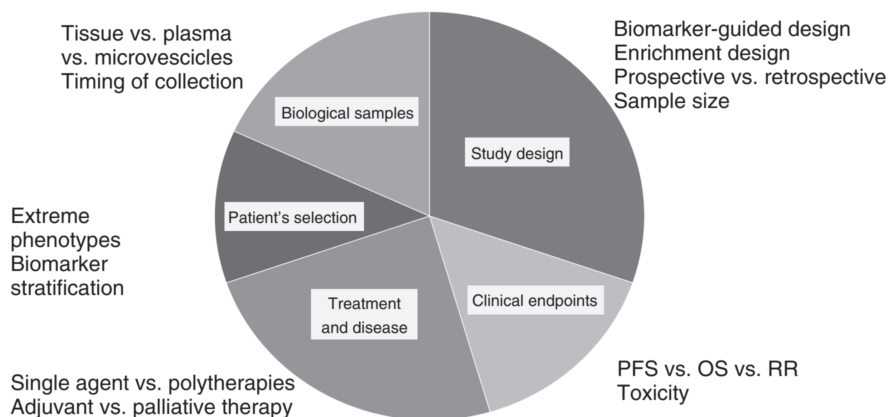


Fig. 10.2 The design of clinical trials aimed at the investigation of pharmacogenetic biomarkers should choose appropriate experimental design, clinical endpoints, and enrolment criteria to attain their objectives. Furthermore, treatment schedules (i.e., single agent vs. polytherapies) according to staging of the disease require careful evaluation of study characteristics

based on the enrolment of patients who have extreme phenotypes, for example a striking difference in an endpoint as PFS or tolerability. The comparison of the two groups for a biomarker, a panel, or wider dataset may result useful to interpret the findings. However, choosing the tails of the population distribution means that the study could require a huge enrolment. In other designs, biomarker-negative individuals may be enrolled in the control arm.

The choice of the clinical endpoint represents a critical step [100]. Indeed, some endpoints seem better than other: for example, in oncology, PFS and response rate may be quickly monitored; they depend on the active treatment and are not influenced by following therapies. The overall survival may need longer times of observation, and the findings could depend on the clinical history of the patients, which includes all of the therapies administered up to the exitus. Prospective trials should be preferred, because retrospective trials may harvest limited databases and the registration of variables and clinical notes could be incomplete. However, in some cases retrospective studies warrant attention, because they may achieve their goals more rapidly. Finally, the discovery of biomarkers predictive of combination therapies could be more difficult than single-agent treatments, but, at present, several schedules include more than one drug, especially in onco-hematology. In this setting, the use of high-throughput techniques may be an advantage [16].

Whatever the design could be, the evaluation of the biomarker is accelerated only when some criteria are fulfilled. The prevalence of the marker within the population should be high enough to allow the implementation of a powered and feasible study. Indeed, too few patients carrying the biomarker impede the enrolment for the experimental arm that may not be completed, or the expected sample size is too large. As discussed above, the biomarker should be accurate with high sensitivity and specificity [101, 102]; otherwise the uncertainty will be a critical issue. From patients' perspective, suboptimal sensitivity and specificity could mean that a beneficial drug for someone may be prescribed to other people who will not experience any therapeutic advantage. A close monitoring of treatment efficacy over time and the evaluation of other markers could help in overcoming that problem, even if the patients could still be reluctant to adhere to stratified medicine protocols. Overall, the validation of biomarkers in prospective clinical trials is the most critical phase, but to achieve reliable and robust findings all of the trial characteristics have to be defined, from the aims and endpoints up to the enrolment criteria and, as stressed before, the sample size.

Finally, the adoption of biomarkers may bring important therapeutic advantages for patients and caregivers, and also the national health systems may expect advantages by adopting patients' stratification, which warrants the reduction of direct and indirect costs. Indeed, the selection of patients according to their possible outcomes after therapies does allow an appropriate allocation of resources, the reduction of adverse events, a decrease in hospitalization and supportive therapies, etc. The registries set up by AIFA are a real-world application of pharmacogenetics to control the prescription of new drugs [103]. Indeed, the evaluation of a biomarker is often required and the genotype has to be confirmed before the patient may receive the treatment. For example, vemurafenib may be administered to melanoma patients if

they are carrying a *braf* gene mutated at codon 600; otherwise the prescription of the drug is halted. For ponatinib, the prescription is limited to CML patients with leukemic cells harboring the T315I mutation of BCR-Abl. Moreover, the monitoring of drug efficacy should be performed after 1 and 3 months of therapy according to the ELN guidelines [32] through the evaluation of the hematological, cytogenetic, and molecular responses.

10.5 Discussion and Conclusions

The combination of improved knowledge, better techniques, and newest targeted drugs has fuelled the pharmacogenetic field in recent years. Indeed, pharmacogenetic studies have achieved important findings in deciphering the relationships among drug treatments, efficacy, and tolerability through the discovery of biomarkers and their testing in patients' population.

However, some points of discussion emerge from the previous paragraphs, as which characteristics the ideal biomarker should have. The optimal biomarker has proven specificity and sensitivity, and is reliable and robust, and its evaluation may be performed in the target cells or, in some cases, in a surrogate tissue. Many of these ideal characteristics are true for the majority of biomarkers. The presence of a predictive genotype may be investigated with instruments that allow the highest levels of sensitivity (i.e., ddPCR) even in the presence of low amounts of nucleic acids extracted from liquid biopsies. Furthermore, the increase in coverage rate (i.e., 10× or more) for NGS may ensure the detection of those polymorphisms and mutations that occur at very low frequencies. The absolute specificity depends on the marker/s (for example, DNA and histone modifications), their number (one candidate gene or genetic signatures from microarray, GWAS and NGS), the platform used (i.e., the increased coverage for NGS), and the clinical endpoint. Laser microdissection does focus the evaluation only on target cells, while pharmacogenetic analyses may be performed on cell-free nucleic acids isolated from peripheral blood or RNA from plasma microvesicles that are surrogate tissues. As presented above, that approach increases the possibility to monitor the disease over time and during the treatment. Furthermore, the use of unsupervised techniques for biomarker analysis (i.e., GWAS and NGS) may unravel new pathogenic mechanisms or processes responsible for drug resistance that may become actionable targets.

The ideal pharmacogenetic biomarker has further characteristics: it is widely available, hence ensuring that the maximum number of patients could receive a benefit from its application. The costs for the acquisition of modern instrumentation, sequencing services, and facilities further increase the possibilities to perform biomarker-driven studies and the following adoption in routine activities. In parallel with the diffusion of techniques, their costs have dropped off in the last years, and the full-genome sequencing costs less than 1000 USD, a cost hard to believe at the beginning of 2000.

Some issues may flaw the findings of pharmacogenetic studies and the development of a genetic biomarker: an inappropriate design and underpowered studies, poor definition of clinical endpoints and selection of technical platform for the analysis, and inadequate informatic tools to handle the large amount of data generated by high-throughput techniques are possible drawbacks that limit the reliability and robustness of pharmacogenetic biomarkers.

In conclusion, the discovery of possible pharmacogenetic biomarkers is living great opportunities under new technical, methodological, and pharmacological perspectives. In some cases, those three characteristics are present at the same time, but even their partial absence may expedite the search for new markers and their validation. It is important to remind that the possibility of optimizing therapeutic regimens, through patients' stratification or personalized medicine, represents a true advantage for the patient, his/her relatives, and the caregivers. For someone, that approach could spare resources; for others it may protect or increase the quality of life and the health status.

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