PRECLINICAL DEVELOPMENT HANDBOOK

Toxicology

SHAYNE COX GAD, PH.D., D.A.B.T.
Gad Consulting Services
Cary, North Carolina

WILEY-INTERSCIENCE
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CONTENTS

PREFACE

This *Preclinical Development Handbook: Toxicology* focuses on the methods of identifying and understanding the risks that are associated with new potential drugs for both large and small therapeutic molecules. This book continues the objective behind this entire *Handbook* series: an attempt to achieve a through overview of the current and leading-edge nonclinical approaches to evaluating the nonclinical safety of potential new therapeutic entities. Thanks to the persistent efforts of Mindy Myers and Gladys Mok, the 31 chapters cover the full range of approaches to identifying the potential toxicity issues associated with the seemingly unlimited range of new molecules. These evaluations are presented with a thorough discussion of how the approaches fit into the mandated regulatory requirements for safety evaluation as mandated by the U.S. Food and Drug Administration and other regulatory authorities. They range from studies on potential genotoxicity and cardiotoxicity in cultured cells to a two-year study in rats and mice to identify potentially tumorigenic properties.

The volume differs from the others in this series in that although the methods used by the researchers are fixed by regulation at any one time, these methods are increasingly undergoing change as it is sought to become ever more effective at identifying potential safety issues before they appear in patient populations. Although we will never achieve perfection in this area, we continue to investigate new ways of trying to do so.

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PRECLINICAL DRUG DEVELOPMENT PLANNING

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Preclinical Development Handbook: Toxicology, edited by Shayne Cox Gad

1.1 INTRODUCTION

1.1.1 Overview of Objectives

It is well recognized that productivity in drug development has been disappointing over the last decade, despite the steady increase in R&D investment [1] and advances in techniques for producing potentially new candidate molecules. The principal problems appear to be a lack of efficacy and/or unexpected adverse reactions, which account for the majority of drug withdrawals and drugs undergoing clinical testing being abandoned. This high attrition rate could be dramatically reduced by improving the preclinical testing process, particularly by taking account of multidisciplinary approaches involving recent technologies, and by improving the design of preclinical projects to facilitate the collection and interpretation of relevant information from such studies, and its extrapolation to the clinical setting.

The objective of this chapter is to provide an overview of the early drug discovery and development processes. The main focus is the use of *in vitro* and *in silico* methods. This is because these techniques are generally applied during the earliest stages to identify new targets (target discovery) and lead compounds (drug discovery), as well as for subsequent drug development. They are also used to resolve equivocal findings from *in vivo* studies in laboratory animals, to guide selection of the most appropriate preclinical *in vivo* models, and to help define the mechanistic details of drug activity and toxicity. However, the use of animals in preclinical testing is also considered, since animal data form part of new medicine dossiers submitted to regulatory bodies that authorize clinical trials and the marketing of new products. The drug development process that will be considered is shown in Fig. 1.1. Definitions of the terminology and abbreviations/acronyms used in this chapter are listed in Table 1.1.

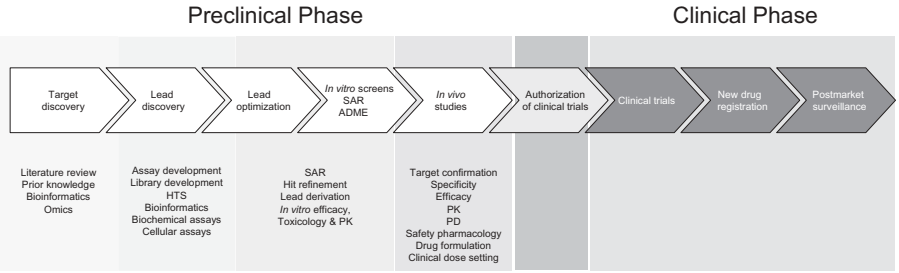

FIGURE 1.1 The key stages of drug discovery and development. A typical series of methods and strategies uses preclinical phases. Note that some of the studies may not be required and the process can be iterative. Refer also to Fig. 1.2 for a more detailed description of toxicity testing planning.

1.1.2 Drug Development Models

An essential part of drug development is the selection of the most appropriate animal, *ex vivo*, *in vitro*, or *in silico* systems, to allow the collection of information that can be interpreted in terms of the effects of a new therapeutic agent in humans or in one or more subpopulations of humans. There are several deciding factors that guide model selection. During early drug discovery screening, the main consideration is whether the chosen model can cope with large libraries of potentially bioactive molecules. It is generally accepted that, while nonanimal models generally lack the sophistication of studies on vertebrate animals and are based on nonclinical endpoints, they are a useful means of filtering out poor candidates during early drug discovery. The possibility of false hits during this stage is accepted as a trade-off, but it is also recognized that data from the use of several techniques and prior information can assist with the weeding out of false hits. The drug development process involves a more extensive evaluation using *in vitro* and *in silico* approaches and preclinical studies in vertebrate animals on a limited number of potential therapeutic agents.

The drive toward the use of systems biology approaches that take into account the roles of multiple biological and physiological body systems earlier in the drug development process has prompted a dramatic change in the way that data from cell-based studies are used. In many instances, data from several tests can be assembled and analyzed by using *in silico* models to gain a systems biology overview of drug ADMET and activity. Advances in comparative genomics have also opened up the scope for using zebra fish (*Brachydanio rerio*) and invertebrate organisms, such as nematode worms (*C. elegans*) and the fruit fly, *Drosophila melanogaster*, during the early stages of drug development. Likewise, advances in information mining, bioinformatics, data interpretation, the omics technologies, cell culture techniques, and molecular biology have the potential to greatly enhance the drug development process. Ironically, up to now, few of these methodologies has been standardized, formally validated, and accepted for regulatory use. Indeed, *in vitro* data are generally considered supplementary to animal data, rather than as an alternative source of information that is useful and applicable in its own right. Nevertheless, *in vitro* approaches provide information about the mechanisms of action

TABLE 1.1 Terminology and Abbreviations

Term	Definition
2D heteronuclear NMR	Two radionuclides are used to construct a two-dimensional map of a binding site by NMR.
Agglomeration	The process of particle attraction and adhesion.
Algorithm	A set of rules to assist with problem solving.
Allometric scaling	The process by which size, blood volume, and anatomical features of an organism are taken into account during extrapolation of information from animals to humans.
Analogue-based minimization	The process of using information about variants of the natural ligand for a target to derive a minimum number of features required of a smaller substance, so that binding affinity, efficacy, and/or specificity for the target in question are retained.
Antisense	A piece of genetic material that is the exact opposite of the natural messenger RNA that encodes a potential protein.
Bioaccumulation	The buildup of a drug or its metabolite(s) in a particular tissue or cell type.
Bioavailability	A measure of the amount of an administered drug that reaches its intended site of action.
Bioinformatics	The management and analysis of information, in order to use computer-based processes to understand biological events.
Biokinetic	Describes the key physiological processes that follow the exposure of an organism to a chemical or drug.
Biomarker	A molecular indicator of a biological event.
Biotechnology product	Replacement therapeutics or recombinant protein or DNA products isolated from or produced by using GM animals, cell cultures, plants, or microorganisms.
Biotransformation	The process by which a substance is chemically or functionally modified within the body, which usually involves the action of specific enzymes.
Combinatorial library	Large libraries of chemicals generated by a combination of acquisition and understanding of the requirements for recognition of a particular target.
Comparative genomics	The study of human genetics by reference to the genetics of other organisms as a means of deciphering human gene organization and function.
Cytotoxicity	A measure of the ability of a substance to damage or kill a cell.
Decision tree	A support tool for selection among competing choices and their possible consequences.
DNAzymes	A DNA-modifying enzyme.
Drug mimetic	A drug or drug-like molecule with a structure or modulatory activity that resembles that of a substance found within the body.
Druggable genome	The sum of the genes, their encoded disease-related proteins, or gene expression regulatory elements, which can functionally be modulated by drugs and drug-like molecules.

Druggable proteins	Proteins that bind drugs with a binding affinity below 10 μM.
Drug discovery	The identification of a potential therapeutic agent.
Drug development	The progress of a lead from drug discovery toward a marketable drug.
Drug-like compound	A compound that has a molecular weight typical of a drug (around 500 daltons) and a structure that indicates it may have pharmaceutical properties.
Efficacy	The capacity of an agent to cause the desired biological effect.
Endpoint	The measurable effect of a substance on a biological system.
Epitope	The recognition site on a molecule for a particular molecule or class of molecules.
Eukaryotic	Describes organisms whose cells possess a nucleus and other membrane-bound vesicles, including fungi, plants, and animals.
Ex vivo	Literally, "out of the living"—used to refer to experiments that are conducted on tissues or cells isolated directly from a living organism.
Gene silencing	The process of preventing a gene from being expressed.
Genome	The entire genetic makeup of an organism.
Genomics	The study of the genetic makeup of an organism.
Genotoxicity	The adverse effects of a substance on the genetic makeup of a cell or organism.
Glucuridonation	The process of conjugating the uronic acid of glucose to substances, to detoxify or inactivate them.
Hapten	A substance that must combine with a carrier, in order to induce specific antibody production.
Hematotoxicity	The adverse effects of a substance on blood cells or on the cells or processes that produce specific types of blood cells.
Hit	The product of the high-throughput screening of large libraries of drug-like compounds, fragments, peptides, or proteins, identified by predominantly one-shot affinity, activity, or *in silico* methods.
Homeobox	DNA sequences found throughout the genome of most organisms that regulate gene expression, particularly during early development.
Homolog	A molecule with corresponding structures or functions in two or more species.
Humanized	The product of a process that is aimed to confer more human-like properties on a molecule, cell, or living organism.
Hydrophobicity	The tendency of a molecule to repel or exclude water molecules. (Means the same as lipophilicity.)
Immunogenicity	The ability of a substance to stimulate an immune response.
Immunohistochemistry	The testing of the ability of a tissue to be stained with an antibody.
Immunoprecipitation	The ability of an antibody–molecule complex to pull a second molecule out of solution as a result of interactions between the antibody recognizing molecule and secondary molecule.
Indels	Insertional or deletion mutations in DNA.
In silico	Using computer-based methods and virtual systems.
In vitro	Literally, "in glass"—used to refer to maintenance of tissues, cells, or cell fractions outside the body from which they were derived.

TABLE 1.1 ***Continued***

Term	Definition
In vivo	Literally, "within the living"—used to refer to experiments conducted on intact living organisms.
Isozyme	Variants of enzymes that catalyze the same reaction(s) but differ from each other in primary structure and/or electrophoretic mobility.
Karyotype	The chromosomal complement of an organism.
Lead compound	A compound identified by hit generation that has suitable physicochemical and functional properties to serve as a starting point for the development of a potentially marketable drug.
Lipophilicity	The affinity of a molecule for a lipophilic environment.
Log *P*	The octan-1-ol/water partition coefficient—used to express lipophilicity.
Macroparticle	Particulate matter of a crystalline nature, generally exceeding a 10 nm diameter.
Margin of safety (MOS)	A ratio of the maximum amount of a substance that causes no effect in animals and the actual exposure (intended or otherwise) of the human population.
Meta-analysis	A statistical process for combining information from different sources.
Metabolic competence	The ability of a system to metabolize.
Metabonomics	The study of metabolic responses to drugs and chemicals.
Microfluidics	Small-scale systems comprised of chambers connected by a fluid matrix.
Molecular dynamics	Computer simulations of the movement of atoms, based on changes in the energy required to maintain certain conformations.
Monte Carlo simulation	A statistical method for studying systems, especially those with large numbers of coupled degrees of freedom.
Nanomedicines	Therapeutic agents based on the use of nanoparticles.
Nanoparticle	A microscopic particle with a unit size not exceeding 100 nm.
Oligonucleotide	A short stretch of synthetic DNA.
Omics	Technologies relating to the study of the genome, proteome, or metabolic responses of cells, tissues, and organisms.
Organotypic	An *in vitro* system designed to preserve or reconstitute the 3D structure of a tissue or organ, to mimic the *in vivo* situation.
Patch clamping	A process for measuring electrical activity across a living membrane by using electrodes.
Permeability	The ability to cross a living membrane.
Phage display	A system whereby a protein is displayed on the surface of a bacterial virus (a bacteriophage).
Phagocytic	Describes the engulfing of a molecule, a microorganism or part of an organism, by leukocytes (a type of white blood cell).
Pharmacokinetic	Describes the uptake, biotransformation, and distribution of a pharmaceutical agent and its metabolites in the tissues, and their subsequent elimination.
Pharmacophore	A collection of electrical and molecular features that define interactions between a molecule and its binding site on its target.

Plasma clearance rate	The speed at which a substance is removed from the blood.
Polymorphisms	Genetic differences within the population that occur for a given gene at a frequency of 1% or more.
Posttranslational modification	The process by which a protein is altered after it is synthesized, by the additional or removal of specific moieties.
Potency	The comparative ability of a drug to induce the desired effect.
Prokaryote	Cellular organisms that lack a distinct nuclear membrane or membrane-bound organelles (e.g., bacteria).
Proteome	The total protein complement encoded by the genome of an organism.
Proteomics	The study of protein expression patterns in specific cells, tissues, or organisms.
Quantum dot	Nanocrystals comprised of a semiconductor metal core.
Reactive oxygen species	Oxygen radicals or super-radicals that are capable of causing cellular damage.
Recombinant DNA technology	The process of DNA manipulation in an artificial environment.
Redox	The process of loss of oxygen or gain of hydrogen by one molecule accompanied by the gain of oxygen or loss of hydrogen by another molecule.
Reporter gene	A gene that is expressed in response to an upstream biochemical event, which can be used to monitor that event.
Reverse pharmacology	The screening of a library of compounds against one particular target to identify a lead for drug development.
RNA aptamers	RNA-based molecules that bind to enzymes.
RNAi	RNA interference—process of silencing or dampening protein expression.
Signal-to-noise ratio	Measure of the signal strength (change being observed) against the background within an experiment.
Therapeutic agent	A chemical, protein/peptide, DNA, stem cell, natural product, or biotechnology product that forms the active component of a finished pharmaceutical product.
Therapeutic index	The ratio between the toxic dose and the therapeutic dose of a drug, which is related to the MOS.
Three Rs	The principles of *replacement* of animal experiments, *reduction* of the number of animals used in a given study, or *refinement* of the procedures used, in order to minimize suffering and distress.
Toxicogenomics	The use of genomics and bioinformatics to identify and characterize mechanisms of action, based on changes in gene expression as monitored by the production of mRNA transcripts.
Toxicokinetic	Describes the uptake, biotransformation, distribution, and effects of a directly or indirectly toxic substance and its metabolites in the tissues, and their subsequent elimination.
Transcription	The process of messenger RNA production from a gene.
Transgene	A gene or variant of a gene that is inserted into the genetic makeup of an organism.
Vector DNA/RNA	Carrier DNA/RNA that may also facilitate the expression and/or cellular uptake of foreign genetic material by cells and tissues.
Xenobiotic	A chemical or other substance that is not a natural component of the makeup of the organism exposed to it.

TABLE 1.1 *Continued*

Abbreviation/Acronym	Full Name
ADME(T)	absorption, Distribution, Metabolism, Elimination (Toxicity)
ADR	Adverse Drug Reaction
BBB	Blood–Brain Barrier
BCS	Biopharmaceutics Classification System
BRET	Bioluminescent Resonance Energy Transfer
cAMP	Cyclic Adenosine Monophosphate
CASE	Computer Automated Structure Evaluation
CBER	Center for Biologics, Evaluation and Research
CDER	Center for Drugs, Evaluation and Research
CoMFA	Comparative Molecular Field Analysis
COMPACT	Computerized Optimized Parametric Analysis of Chemical Toxicology
CRE	Cyclic-amp Responsive Element
CYP	Cytochrome P450
CYP450-DMO	Cytochrome P450-Dependent Monooxygenase
DEREK	Deduction of Risk from Existing Knowledge
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked Absorbance Assay
EMEA	European Medicines Agency
EPA	Environmental Protection Agency
ERE	Estrogen Responsive Element
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
FRET	Fluorescent Resonance Energy Transfer
GFP	Green Fluorescent Protein
GPCR	G-Protein-Coupled Receptor
HESC	Human Embryonic Stem Cell
HTS	High-Throughput Screening
IAM	Immobilized Artificial Membrane
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Conference for Harmonization
IND	Investigational New Drug
LC-MS/MS	Liquid Chromatography and Tandem Mass Spectrometry

LD_{50}	Lethal Dose that kills 50% of a test group (of animals)
Log P	Octan-1-ol/water partition coefficient
MAP kinase	Mitogen-Activated Protein Kinase
MCASE	Multi-CASE
MDCK	Madin–Darby Canine Kidney
MOS	Margin of Safety
MS	Mass Spectrometry
MTD	Maximum Tolerated Dose
NCE	New Chemical Entity
NCTR	FDA National Center for Toxicological Research
NMR	Nuclear Magnetic Resonance
OECD	Organization for Economic Co-operation and Development
PAMPA	Parallel Artificial Membrane Permeation Assay
PBPK	Physiologically Based Pharmacokinetic
PCR	Polymerase Chain Reaction
PK	Pharmacokinetic
pK_a	The acid-ionization constant
PTFE	Polytetrafluoroethylene
QSAR	Quantitative
QSAR–ES	Quantitative Structure–Activity Relationship—Expert System
QT Interval	The time between the start of the Q wave and the end of the T wave in the heart's electrical cycle
SAR	Structure–activity Relationship
SPA	Scintillation Proximity Assay
SPR	Surface Plasmon Resonance
TOPKAT	The Open Practical Knowledge Acquisition Toolkit
UFAW	Universities Federation for Animal Welfare

of a drug that is vital for the design of *in vivo* animal studies and can add substantial weight to the product dossier submitted to regulatory bodies.

Increasingly, predictions about the ways in which a particular chemical is likely to interact with its desired cellular target are made by undertaking *in silico* modeling. These results are used to filter out poor candidate molecules according to chemical class and structural or functional features during drug discovery. However, filtering of this kind is sometimes impossible, so lead identification still relies to some extent on serendipitous finds from random libraries, rather than on rational lead discovery. For instance, for new chemical entities (NCEs) for which there are no data, i.e., are first-in-class, *in silico* screenings are difficult to handle, particularly where there is also limited knowledge of the structure of the active site of the target. Also, there might be a lack of important information for other compounds. For example, predicting drug effects can be seriously compromised when ADME data on the behavior of a molecule in different tissues and species are lacking. This is confounded by the reality that this kind of information for different individuals will always be limited. Both of the above situations are most evident in the case of large molecules, such as (1) peptides and proteins with complex structures and multiple conformations, (2) humanized products that could be differentially immunogenic in different species, and (3) nanoparticle formulations.

1.1.3 Information Required Prior to Drug Authorization/Approval

Once a new therapeutic candidate has been successfully identified from preclinical studies, the next stage involves the authorization of clinical studies. The information required prior to the authorization of any clinical trial is crucial for the design and execution of preclinical studies, irrespective of whether the aim is to define drug action or provide safety information. Such information includes (1) manufacturing quality, (2) physicochemical properties, (3) efficacy, (4) proposed mechanism of action, (5) selectivity, (6) ADME, and (7) possible adverse effects in humans.

In the United States, the Food and Drug Administration (FDA) handles drug approvals. The FDA has fast tracked this process for treatments for serious diseases where no therapies currently exist [2]. Drug developers are required to submit an Investigational New Drug (IND) Application, in which evidence from preclinical studies is provided for review by the FDA. The FDA decides whether it is reasonably safe for the company to test the drug in humans. Under the FDA's jurisdiction, the Center for Drugs, Evaluation and Research (CDER) and the Center for Biologics, Evaluation and Research (CBER) are responsible for reviewing different types of therapeutic agent applications (Table 1.2). Note that these changes in jurisdiction mean that biological products, the testing of which was at one point based on limited animal tests (because of their poor predictivity), are likely to require more stringent testing under the CDER [3].

The FDA has exclusive executive control over decisions regarding drug approvals in the United States. However, in Europe, it is possible to have a drug approved by a number of different routes. This is because companies can apply either via the EMEA (European Medicines Agency) for pan-European approval or via one or more national agencies. However, since November 2005, all new drugs for the major diseases, including AIDS, cancer, diabetes, and neurodegenerative disorders, and

TABLE 1.2 CDER and CBER:[a] Review of New Therapeutic Agent Applications

CDER

- Traditional small molecule therapeutics
- Growth hormone, insulin, and other endocrine peptide therapeutics
- Monoclonal antibodies
- Proteins (e.g., cytokines, enzymes, and other novel proteins), except those specifically assigned to the CBER, namely, vaccines and blood products that are assigned to CBER
- Immunomodulatory agents (but not vaccines)
- Growth factors intended to modulate hematopoiesis *in vivo*
- Combination products where the primary mode of action is that of an agent assigned to the CDER

CBER

- Products composed of human, bacterial, or animal cells or fragments of cells, for use as preventative or therapeutic vaccines
- Gene therapy products
- Vaccines
- Allergenic extracts used for the diagnosis and treatment of allergic diseases
- Antitoxins, antivenins, and venoms
- Blood and blood products from humans or animals
- Combination products where the primary mode of action is that of an agent assigned to the CBER

[a]The CDER and CBER are afforded jurisdiction by the U.S. FDA.

medicinal products developed by means of biotechnological processes must be approved via the EMEA.

With the globalization of the pharmaceutical industry, the International Conference on Harmonization (ICH) guidelines have, since 1990, set out to standardize drug applications in terms of their content and format. Japan, the United States and the European Union (EU) comply with these requirements for the quality, safety, and efficacy assessment of new drugs. These guidelines operate alongside national requirements. Quality assessment guidelines are provided to standardize the assessment of drug stability (shelf-life), and the management of risks due to impurities, such as residual solvents and infectious agents, such as viruses (which can be present when a drug is isolated from plants, animals, humans, or cell lines). The guidelines also require the standardization of cell lines, test procedures, acceptance criteria, and procedures for formulation and development. Efficacy guidelines are also provided, to standardize the conduct, interpretation, and reporting of clinical trials.

There are some important practical considerations that should be borne in mind when conducting preclinical studies. The most comprehensive guidelines are those provided for drug safety testing, which cover a number of toxicological endpoints, including carcinogenicity, genotoxicity, reproductive and developmental toxicity, and immunotoxicity. Some of the guidelines apply generically to all new drugs, while others focus on specific types of therapeutic agents, such as biotechnology products. These guidelines are essential reading for researchers engaged in drug development and are considered in more detail throughout the remainder of this chapter.

Another important source of reference is the Organization for Economic Co-operation and Development (OECD). By ratifying the convention of the OECD, many European countries, Australia, Japan, New Zealand and the United States have agreed to abide by a set of test guidelines for assessing the human health effects of chemicals [4], which apply equally to the testing of therapeutic agents. Later, we refer to a number of nonanimal methods and refinements of animal procedures accepted by the member countries of the OECD.

1.2 FINDING NEW DRUG TARGETS

1.2.1 Background

Until relatively recently, drug development focused on a limited number of targets, against which NCEs with a desired effect could be selected. These "druggable" targets were once most extensively investigated by using animal models. However, greater access to recombinant DNA technology means that most early screens are now conducted primarily by using different genetically engineered cell lines expressing putative targets that can be arrayed into high density plastic plate formats suitable for interactions between the targets and potential lead chemicals (for methods, see later discussion).

Overington et al. [5] derived a consensus figure for the number of therapeutic drug targets for the FDA-approved drugs that were available in 2005. They identified 324 drug targets for all classes of approved therapeutic agents, which were targeted by in excess of 1357 drugs, of which 1204 were small molecules and 166 were biologicals. Cell surface receptors and channels represented the targets for >50% of all the FDA-approved drugs. A further 10% of the drugs, including monoclonal antibodies, also target other cell surface proteins. Most of the remaining targets were enzymes, nuclear receptors, DNA, or ribosomes. These targets represent a minute fraction of the genome, and a mere 3% (266 proteins) of the predicted proteome.

According to this survey, on average 5.3 new druggable targets are discovered each year. This means that many more potential drug targets remain to be discovered. Whether a potential drug target will be a good therapeutic target, however, depends on whether (1) it plays a key role in gene regulation, (2) it is selectively expressed in certain disease states or tissues, and (3) it has a definable and unique binding site.

Often, a further important piece of information is the nature or identity of the endogenous modulator. For example, >1000 G-protein-coupled receptors (GPCRs) have been cloned from various species, including 160 distinct human subtypes with known ligands, although these represent only a limited set of targets for current therapeutic agents. A further 100 or so are orphan receptors, for which there is currently no known natural ligand. In such cases, the starting point is the gene, from which the protein receptor can be expressed and used to screen large combinatorial libraries of chemicals in the search for a modulator. Such a reverse pharmacology strategy uses the orphan receptor as a "hook" for screening libraries and hit generation, where little is known about the natural ligand. In many cases, receptor models use the crystal structure of rhodopsin as a template, as this is the only GPCR whose structure has been resolved. The importance of GPCRs is emphasized by the fact that, although >20% of the top 200 current best-selling drugs interact with these cell

surface receptors, they generate worldwide sales of drugs such as cimetidine, losartan, and ropinerole of over $20 billion (U.S.) [6].

1.2.2 Impact of New Technologies on Target Discovery

Comparative genetics can provide much relevant information, particularly with regard to the role of human-specific genes and the suitability of animal models for drug development. The application of microarray techniques, standards, and resources that permit the comparison of gene expression patterns across species and between cell types and tissues has started to provide some insight into the metabolic and biochemical differences between health and disease states. A good example of this is the Cancer Genome Anatomy Project (www.ncbi.nlm.nih.gov/CGAP) [7], in which mutational sites in cancer cells have been identified.

A cursory examination of the 373 completed genome sequences for archeal, prokaryote, and eukaryote [8] species suggests that, although genome size increases from archea through prokaryotes to eukaryotes, genome size is not directly linked to the number of genes within the functional genomes, nor with evolutionary status. It is, however, clear that, as the complexity of organisms increases, so does the complexity of gene regulation and the level of genetic redundancy—the ability of several genes to rescue loss-of-function of another gene. Nevertheless, for highly conserved genes, such as those that are involved in early development, and homeobox genes, studies on early life stages of species such as zebra fish and invertebrate models can indicate the roles of genes. However, in general, such studies are more relevant to safety pharmacology than to mechanistic and efficacy studies. It is worth bearing in mind that computational predictions and statistical analyses have suggested that the bacterial *Escherichia coli* and human genomes account for 35 common metabolic pathways, namely, those that are important in biosynthesis and in degradation and respiratory processes [9], and that, possibly as a result of bacterial infection, a number of bacterial genes have become permanently integrated in the human genome [9, 10]. This opens up the possibility of using bacterial studies to decipher a limited number of biochemical pathways affected by drugs, as well as for genotoxicity testing.

Unicellular eukaryotes, such as yeast, share remarkable genetic and functional similarities with multicellular eukaryotes. The most useful yeast strain in terms of dissecting protein and gene interactions is *Saccharomyces cerevisiae*. At 12,100 kilobases, the *S. cerevisiae* genome is much smaller than the human genome. However, because its gene density is 50 times greater than that of the human genome, genes found in the *S. cerevisiae* genome resemble around 30% of the genes associated with diseases in humans [11]. Since the entire genome of *S. cerevisiae* encodes no more than 6000 proteins, it is relatively straightforward to investigate gene function in yeast and make genome-wide microarray measurements. Such data, together with information from other sources, have made it possible to identify a number of putative drug targets [12] and protein–protein interactions [13], thereby facilitating the development of extensive maps of protein and gene interactions. Such studies in *S. cerevisiae* have been particularly useful in neurodegenerative and ageing research and in studies on diseases that arise as a consequence of mitochondrial DNA damage. One example is the observation that yeast mutants for α-synuclein result in a large change in yeast sexual reproduction, as well as causing cytotoxicity,

both endpoints of which are suited to high-throughput screening assays for new treatments for Parkinson disease [14].

Subsequent studies on yeast-based models of Parkinson disease have suggested that there is substantial scope for using yeast for the high-throughput screening of chemicals for drug discovery [15]. For example, *S. cerevisiae* possesses three distinct G-protein-coupled receptors (GPCRs), which are involved in pheromone (Ste2 and Ste3 receptors) and glucose sensing (Gpr1) [16]. These receptors are related, albeit to a limited extent, to the vastly expanded human GPCR repertoire. By coupling heterologously expressed human GPCRs to the yeast MAP kinase pathway (associated with yeast mating and growth arrest), in yeasts where the MAP kinase pathway is linked to reporter gene expression [17], it is possible to monitor receptor recognition and activation by simple growth or colorimetric reporter assays.

Caenorhabditis elegans is another organism that can be used in early drug discovery. This nematode worm is transparent, has a short life span, is a mere 1 mm in length and 80 μM in diameter, reproduces every 3 days by self-fertilization to produce over 300 offspring, and is a multicellular organism composed of exactly 959 somatic cells. It displays many of the basic features of higher eukaryotes, including the possession of muscle, excretory cells, and neural cells, and has been extensively used to increase understanding of the mechanisms of gene regulation and gene function. Antisense knock-out or knock-down of gene expression can be achieved simply by feeding the worm with *E. coli* bacteria transformed with plasmid DNA containing antisense DNA. More recently, RNA interference (RNAi) has been used to manipulate the genomes of organisms such as *C. elegans*, although the possibility of transmission of RNA silencing to subsequent generations can occur [18]. Like all multicellular organisms, *C. elegans* exhibits programmed cell death (apoptosis) [19], in a way that is very similar to that seen in higher organisms as part of ageing and disease processes. Similarities between the signaling pathways involved in the regulation of cell proliferation in *C. elegans* and humans suggest that this organism might provide information on the regulation of cell proliferation, which will be of relevance to cancer therapeutics. The entire 302-cell nervous system of this worm has been mapped by electron microscopy, and although the average human possesses somewhere in the order of 100 billion neurons, it seems that neurotransmission is similar in the two species. Thus, *C. elegans* possesses the major classes of ion channels, receptors, transporters, and neurotransmitters that make it a suitable candidate for some forms of drug screening, such as the discovery of new dopaminergic drugs. Similarly, *D. melanogaster* shares much of its basic neurobiology with higher organisms, including humans. It possesses the same neurodegenerative states, neurotransmission mechanisms, and receptor homolog that are found in humans as key targets for neurally active therapeutic agents, making studies with these organisms useful for the development of treatments for conditions such as Parkinson's disease [20].

1.2.3 Data Mining

Novel drug targets can also be found in other ways, including data mining. This involves analyzing the literature, to determine the biochemistry underlying particular human diseases, and human physiology. In addition, human population genetics studies can be undertaken, to determine the roles of human genes, how they interact, the consequences of population differences at the gene level, and, ultimately, the complete physiology of the human body. In the last-named case, since the

possibilities for human studies are limited, most of the information gathered comes from fundamental research that examines modes of interaction of specific substances with any given novel targets, and the modulation of their physiological roles, by combining several approaches, including *in vivo* studies.

The next step is to define whether a newly discovered potential drug target is a feasible target, by identifying the binding site of the proposed molecular target. In this respect, the potential for data mining has been greatly enhanced by the recent development of a druggable-protein database. This can provide information that is useful for deriving rules for the computational identification of drug binding sites. Indeed, there are now algorithms designed specifically for this purpose [21]. Some analyses relate to the identification of pockets within the binding site that serve as potential specific drug targets. However, this approach can be complicated, since the binding pocket that is targeted by an endogenous or natural modulator of target function might include only part of the binding site, or might lack it altogether. A recently described approach to this problem, in which 2D heteronuclear NMR is used to screen drug-like and fragment libraries for interactions with proteins, generates additional reliable data than is obtainable from conventional high-throughput screens. While such information can be used for computational application, including the refinement of protein models, it is limited by the number of protein structures that are currently available. An exception to this are quantitative structure–activity relationships (QSARs) generated by computational techniques such as CoMFA, which rely on molecular descriptors for molecules that are specific for a target, in order to generate a set of conformers that can be used to predict the ability to bind to a protein.

1.3 TRADITIONAL APPROACHES TO DRUG DISCOVERY AND DEVELOPMENT

1.3.1 Hit to Lead

The current attrition rate for NCEs can be gauged from the fact that, on average, for every 7 million molecules screened, only one product is marketed [22]. These odds have resulted in the concentration by pharmaceutical companies on refining, rather than expanding, their chemical libraries and methods. A further important factor that determines the success of early drug screening is the choice of methodologies used to identify hits and to screen potential leads and their derivatives. In this section, we describe the key stages and methodologies used for hit generation, hit confirmation, lead, identification and lead characterization (Table 1.3).

Before 1980, nearly all drugs were small molecules of around 50 to 1000 times smaller than the size of a typical protein at around 500 daltons, or smaller. Extensive combinatorial libraries of small molecules are generated in-house by all large pharmaceutical companies, often by diversity-oriented synthesis, in which small molecular building blocks are randomly combined in all possible spatial orientations. Screening libraries can consist of thousands of chemicals and rely on an appropriate hit generation and lead characterization strategy. The chemicals concerned must meet certain purity, molecular weight, lipophilicity (log P), and functional conformer criteria.

Schreiber [23] first used diversity-oriented synthesis to generate bead-attached libraries of target-oriented and diversity-oriented chemicals. This approach involves

TABLE 1.3 Key Methods Used During Hit Generation and Lead Optimization[a]

Methods	Assay Principles	Advantages	Limitations
Affinity-based biophysical methods			
Mass spectrometry	Relies on the affinity of a compound for a protein to cause mass/charge shifts.	Can handle large drug-like/ fragment mixtures.	Not truly an HTS platform; poor at resolving mixtures; false hits.
NMR	Monitors the location of radionuclides in the target–ligand complex and is used to probe the active site of folded/*in situ* proteins/DNA. A number of new higher resolution techniques (e.g., magic angle spinning NMR) do not require high purity target proteins.	Provides structural information for *in silico* platforms; suited to screening large fragment libraries.	Does not provide SAR data; false hits; weakly potent fragment hits are poorly detected.
X-ray crystallography	X-ray diffraction by crystallized protein/ protein–ligand complexes.	Provides structural information; HTS platform.	Weakly potent fragment hits are poorly detected; erroneous assumption about structural similarity can lead to some compounds being discarded; there are not crystal structures available for all target proteins.
Biochemical screens			
Scintillation proximity assay	Monitors energy transfer changes as an indicator of binding interactions.	Provides kinetic data	High background; limited plate format; not easily correlated to physiological effect.
Radiometric binding assays	Uses radioactive tracing of target–tracer/molecule interactions.	Direct measurement of binding interactions; adaptable for a wide range of possible target-based screens.	Relatively expensive to generate suitable tracer; health and safety considerations; not real-time measurements.

TABLE 1.3 ***Continued***

Methods	Assay Principles	Advantages	Limitations
SPR	Commonly based on the target being immobilized on a chip and the compound mixture being passed over it. Interactions are monitored as an electrical readout.	Permits kinetic measurements; can be used to identify hits from complex mixtures.	Chip preparation and availability; requires relatively large amounts of materials; more suited to detailed mechanistic studies than HTS.
Nonradioactive assays	Includes colorimetric/absorbance-based assays (such as ELISA), luminescence-based assays, and fluorescence-based assays (e.g., FRET, real-time fluorimetry, fluorescence correlation spectroscopy), as generally used in conjunction with cell-based assays (see below).	Generate quantitative data suited to SAR; can give real-time data; can provide mechanistic information; suitable to HTS formats.	Often more suited to later stages of lead discovery.
Cell-based assays			
Reporter gene assays	Involves the use of genes such as those encoding GFP, luciferase, and β-galactosidase coupled to a biochemical pathway modulated by a substance to monitor the extent or modulation.	Generates quantitative data suited to SAR; minimum resources needed.	Not truly HTS; can give equivocal data; false hits; not well suited for fragment screens.
FRET	Monitors energy transfer between a fluorescent energy donor and acceptor as a measure of the proximity between the two groups, commonly found on the target and a tracer.	Suitable for high density formats; provides mechanistic information; broad range of applications; real-time monitoring of interactions.	High incidence of false hits; prone to fluorescence quenching.

TABLE 1.3 ***Continued***

Methods	Assay Principles	Advantages	Limitations
BRET	Similar principles to FRET.	Suitable for medium density formats; provides mechanistic information; suitable for monitoring protein–protein interactions; real-time monitoring of interactions.	Some limitations on application; involves protein engineering of the target.
Reporter gene	Based on recombinant protein engineering and expression technology to couple an endogenous pathway to the expression and/or activity of a protein from a transgene in response to drug modulation of a target.	Several commercially available plasmids (e.g., with cAMP, calcium, and estrogen responsive elements); sensitive high throughput assay formats.	High incidence of false hits; long incubation times; indirect correlation with target modulation.
Electrical readout	Includes biosensor-based methods and patch clamping.	Suitable for monitoring channel activity.	Not truly suited to HTS; limited utility
Second messenger assays	Based on a direct measurement of one of more downstream changes in signal mediators in response to drug modulation of a target. Includes assays such as those that measure changes in intracellular calcium (FLIPR/ Aequroscreen), cAMP, and many more.	Direct measurement of the effects of a substance.	Only suited to some types of targets (e.g., receptor, channels, enzymes); time consuming.

TABLE 1.3 ***Continued***

Methods	Assay Principles	Advantages	Limitations
Fluorophore and chromophore-based methods	Rely on the use of an ion-sensitive dye to detect intracellular changes in ion content.	Suitable for monitoring increases in intracellular calcium, potassium, and sodium ions.	Sensitivity dependent on dye chemistry.
Cell proliferation assays	Includes methods such as dye or radioisotope uptake, protein estimations, cell counting, and oxygen sensor measurements to monitor the competence, viability, and growth rate of cells.	Minimum resources needed; generic application; quantitative data can be obtained.	Difficulty equating to physiological endpoint.
In silico methods			
Protein modeling	*Ab initio* or homology-based protein structure modeling based on amino acid sequence analysis and biophysical/biochemical data.	Binding site identification and pharmacophore modeling.	Need experimental confirmation of findings.
Molecular docking/SAR/combinatorial chemistry	Molecular dynamics simulations and energetic calculations.	Virtual screening prior to chemical synthesis.	Need experimental confirmation of findings.
PBPK modeling	Mathematical prediction of the fate of a drug.	Can be used to identify the sites of action of a drug and to estimate likely internal dose.	Reliant on large amounts of data; can involve considerable mathematical expertise.

[a]A number of different approaches are used during drug discovery and development. Here, a list of methods applicable to hit generation and lead development are listed alongside the main advantages and limitations of each method or group of methods. HTS, high-throughput screen.

the use of fragments—small chemicals—of around 120–250 kDa. Generally, these fragments display lower (10 μM to millimolar) affinities for a target than do more complex, drug-sized chemicals (affinities within the nanomolar range). It is therefore necessary to complement fragment screens by using sensitive analytical techniques, such as protein-detected or ligand-detected NMR [24], MS [25], X-ray crystallography [26], and SPR [27] (although the last named is generally more applicable for hit confirmation; see later discussion). These techniques are preferable to

bioassays, such as cell-based binding or functional assays, or to the step-wise combination of hit fragments either by chemical synthesis or by combining pharmacophores [28]. Despite the fact that the method used to screen fragments affects the success of such screens, the hit rate for fragment-based lead discovery is substantially higher than that for drug-like screens, there being an apparent inverse relationship between chemical complexity and target complementarity. Indeed, a screen of <1000 fragments might identify several useful hits for lead development.

A "library in tube" method is being developed for large mixtures of chemicals, which has been adapted from a concept put forward by Brenner and Lerner in 1992 [29]. This technique involves coding each chemical with a DNA tag, in order to identify the attached chemical by PCR, such that mixtures of chemicals can be panned against a target. This approach has much potential for diversity-oriented hit generation (see Ref. 30 for a review).

Biochemical screening can be performed by using several types of readout, including those reviewed in Ref. 31. Whatever the assay used, it should display good signal-to-noise ratios and should also be reproducible. The two most commonly used screening formats are radiometric and nonradiometric assays, both of which are suitable for intact cell or tissue-based studies. Radiometric assays include filtration-based methods, where the unbound radioactive probe (generally the radioligand specific for the target) competes for ligand binding with the unlabeled screen compound, after which it is removed in readiness for scintillation counting, or for scintillation proximity assays (SPAs), where β-particle emissions from isotopes with short β-particle path lengths (namely, ^{3}H and ^{125}I) are measured *in situ* by using scintillant-impregnated microspheres. The amount of reduction of the radiolabel signal intensity due to competition is measured. The use of the former isotope renders the method amenable to a 384-plate format, while the latter is generally more suited to a 96-well format.

Nonradiometric assays include those based on colorimetric, fluorescent, luminescent, or electrical changes. Commonly used methods include proximity-based fluorescent resonance energy transfer (FRET), which can be used to monitor interactions between a fluorescent donor and an acceptor on the target, and to screen chemicals. This technique is suited for both monitoring a wide range of molecular interactions and to 1536-well formats. One example of how FRET may be useful is in the screening of enzyme inhibitors [32]. The drawbacks of this method are the high incidence of false positives and problems with fluorescent quenching. Bioluminescent resonance energy transfer (BRET) is another proximity-based screen. This method, while being prone to quenching, requires the use of proteins such as *renilla reinformis* luciferase donor and green fluorescent protein (GFP) acceptor, in the presence of coelenterazine a (luciferase substrate). BRET is generally more useful for screening interactions between large molecules, such as proteins, due to the bulky nature of acceptor and donor groups, luciferase, and GFP. Nevertheless, it can also be used to screen for chemicals that perturb such interactions, and indeed, BRET has been proposed as a screen for HIV-1 protease inhibitors [33]. The sensitivity of both FRET and BRET is dramatically improved when there is a large difference between the emission spectra prior to and following energy transfer from the donor to the acceptor group.

Other commonly used screens rely on the expression of a reporter gene (e.g., β-galactosidase or luciferase) in response to the activation of a specific pathway.

However, many more screening techniques are specific for the targets in question, as is the case for GPCRs [34] and HIV-1 [35]. An example of the usefulness of electrical readouts is the examination of the interaction between DNA and metallocompounds. In this case, the DNA is immobilized on electrodes, and interactions with the drug can alter the electrical output [36]. Generally, these functional assays (with the notable exception of SPA) can provide a mechanistic overview of drug action. However, further insight can be gained by using surface plasmon resonance (SPR). SPR is a real-time monitoring system based on change in mass, in which microgram amounts of the target are immobilized on a chip and exposed to the test chemical. The flow rate and wash rate can be varied, such that not only can the individual chemicals in a mixture be resolved according to rank order of affinity, but also the on–off rates of binding can be monitored. Membrane protein targets, however, are difficult to isolate and refold into the chip matrix, so SPR is far more useful for the screening of drugs that target soluble proteins and DNA [37].

As a typical screen of 1 million chemicals can take 6 months to complete, there is interest in expediting hit generation by using higher density plate formats or by chemical pooling. Increasing the assay density by increasing the well density is feasible, but is highly dependent on the nature of the screen. Chemical pooling involves placing multiple chemicals into each well of a plate, with a single chemical overlap between two wells. This can reduce the screening time to a matter of weeks. However, factors such as the possibility that two of the compounds in the same well will cancel the effects of each other or will act synergistically, can result in false negatives and positives, respectively. It is also general practice to include pairs of structurally related chemicals in each screen.

A new drug can also be developed as a result of rational drug design, particularly when there is extensive knowledge of the structure and function of the target protein, as well as available computer models and the capability to dock virtual compounds into the active site. In many cases, however, the original first-in-class compound was designed by modification of the endogenous ligand for the target. The classical example of this is the design of small nonpeptide antagonists that target neuropeptide receptors (e.g., neurokinin receptors) by gradual structural minimization and constraint of the natural endogenous receptor ligands [28]. In general, the design of these smaller nonpeptide ligands, based on knowledge of the natural ligand, requires extensive peptide analogue generation and screening for efficacy and activity, so as to identify the key interactions and functional groups on the peptide that determine specificity and activity. In the above example of neurokinin receptor binding, the key interactions were identified as being with the terminal Phe-X-Gly-Leu-Met-NH_2 motif. Indeed, all ligands that retain neurokinin receptor affinity contain aromatic rings and amine groups that fit into the receptor pocket.

The latter analogue-based minimization of the natural ligand for a target protein is particularly relevant, given that larger molecules such as peptides and proteins are increasingly being investigated as clinical agents. Currently, more than 40 peptides are marketed worldwide, with some 700 more at various stages of development as drug leads. Similarly, there are some 120 antibody-, hormone-, and enzyme-based therapeutics currently on the global market. Many of these therapeutics are more specific and more active than their small molecule counterparts, and they accumulate less readily in tissues, with generally lower oral bioavailability and less stability.

They are all potentially immunogenic and are relatively expensive to manufacture. These molecules are also not generally amenable to rational design strategies and are often developed by *de novo* routes with limited *in silico* approaches, in view of the difficulties associated with docking flexible peptides and proteins into the target protein.

Screening for peptide, polypeptide, and protein therapeutic leads presents a problem, in that large libraries are generally not amenable to chemical synthesis. One solution to this problem is to use systems in which the peptide is linked to the DNA that encodes it. Phage display, for instance, is a technique that allows one or more genes encoding any number of protein variants to be expressed in an anchored form amenable to affinity probing. The genes of interest are inserted into the genome of a nonlytic phage, which is introduced into bacteria. The proteins encoded by the genes are expressed (displayed) on a defined coat protein of the respective phage. Phage display libraries of over a billion different peptide or protein sequences can be prepared, the only limitation being the efficiency with which the bacteria are infected. By using the molecular target as a probe to isolate hits from this library, it is possible to undertake successive rounds of optimization until the most specific hits are identified. Phage display, and the similar, more recent ribosomal display systems [38], can be used to screen for protein and hapten hits for drug development and have proved particularly useful with respect to the development of specific antibodies [39]. However, the need for folded proteins has led to the development of a yeast-display technology, whereby proteins are presented in their folded form on the yeast cell wall. These anchored systems all facilitate miniaturized screening and, in the case of the yeast-display libraries, FACS [40].

The techniques used for developing genetically based therapeutics share some similarities with more traditional drug discovery approaches. Genetically based therapeutics include plasmids containing transgenes for gene therapy, oligonucleotides for antisense applications, DNAzymes, RNA aptamers, and small interfering RNAs for RNAi [41]. So far, two such products have been approved for clinical use and many more are in the course of development, so this important group of therapeutics requires specific consideration in the context of preclinical planning. Very little is currently understood about the suitability of many genetically-based therapeutics. It is known, however, that the design of the vector crucially determines delivery and nuclear uptake, and also that the promoter used will determine the expression levels of the transgene and the efficiency of gene silencing (reviewed in Ref. 41). Since uptake is a key determinant of efficacy, the development of these therapeutic agents must be used together with an evaluation of DNA delivery techniques, such as microinjection, electroporation, viral delivery systems, and carrier molecules that either promote cellular endocytosis (e.g., cationic lipids or amines) or facilitate uptake (e.g., carbon nanotubes) (see Ref. 42 for a review and Section 1.4.4). Equally, the expression of the encoded DNA is reliant on the precise nucleotide sequence, with codon use often resulting in changes in the expression of the encoded protein product and, in some cases, to its cellular fate.

Whatever the discovery route for a lead compound from drug-like libraries or fragment libraries, it is clear that most of the drugs that are currently marketed are highly similar to the leads from which they were derived [43]. This makes lead discovery a crucial step in the drug discovery process. The most widely used approach to confirming leads is affinity-based screening [44], where qualitative (e.g., rank

order) or quantitative (K_d, IC_{50}) measurements are used to monitor interactions between compound libraries and protein, RNA, or DNA targets, by using approaches such as standard binding assays, NMR, SPR, or X-ray crystallography. Other approaches involve the use of changes in biochemical events that have been identified from target modulation or predicted by *in silico* screening. A combination of all three approaches has the advantage over using biochemical techniques alone, of reducing the number of false hits while allowing higher screening throughputs. For instance, experimentally based screening may result in false hits, because of (1) nonspecific interactions (predominantly hydrophobic in nature), (2) aggregation or poor solubility of the drug, and (3) purities, reactive groups, or chemical stability that are not readily discernible from *in silico* predictions. MS-based methods result in fewer false positives because of nonspecific hydrophobic interactions, poor solubility, impurities, and reactive functional groups. In practice, however, the method used for hit generation is dependent on the resources available.

In the case of *in vitro* biochemical and cellular assays, miniaturized formats can be used to screen around 1 million drug-like molecules, by using 1–50 μM concentrations and a 30–50% activity cutoff between potential hits and failures [45]. Where fragment libraries are used, activity might only be detectable at substantially higher concentrations, and by using more-sensitive techniques. As a result of these selection criteria, the rate of false hits (and failures) is also relatively high.

Hit confirmation generally involves biochemical assays to confirm that the observed activity is linked to the desired mechanism of action. The choice of methodologies is important, since it is at this stage that eliminating false-positive hits becomes most important and depends on the necessary properties of the final drug. It is also at this stage that hits begin to be ranked according to specificity, activity, and suitability to be used for lead development. Indeed, data from hit confirmation studies are often amenable to structure–function analysis by using *in silico* methods that may ultimately guide decisions as to the most favorable leads.

This process is developed further during the hit-to-lead stage, in which potency is no longer considered to be the deciding factor, but selectivity, the feasibility of chemical synthesis and modification, the mechanisms of target interaction and modulation, pharmacokinetics, and patentability of the final drug have become increasingly important. Many of these issues are considered later. It is important to note, however, that determining whether individual fragment hits fulfill these criteria is much more problematical. The ability to chemically modify a hit lends itself to the three main ways of generating a lead compound from initial promising hits and subsequently derivatizing and modifying the lead to give the final drug, namely, by using biophysical or biochemical methods, cell-based screens, or *in silico* predictions.

It is at the above stage of development that the possible risks associated with a new drug candidate begin to be addressed. The affinity and specificity of the drug candidate for the desired target can often dictate whether it will be discarded at an early stage. For instance, if there is a difference of several orders of magnitude in affinities for selected targets and off-targets, the drug is less likely to have predictable side effects. That is, it is possible that a drug may have a desirable effect within one concentration range, above which it causes toxicity. The relationship between the desired therapeutic effects of a drug and its adverse effects is expressed as a margin of safety (MOS; also referred to as therapeutic index)—being the difference between the effective dose and that which gives rise to toxicity.

Two important sources of information can contribute to a widening of the MOS during lead optimization. The first is a fundamental understanding of the mechanisms of interaction with the desired target and off-targets. The second is information from combinatorial chemistry and rapid *in vitro* screens to determine the relationship between structure and activity, which can then be applied to developing computational analysis techniques. This is a fundamental principle of rational drug design, where the original lead is often structurally related to the endogenous substance that modulates target activity. On a final note, however, rational drug design is not applicable in all circumstances, and a great deal of drug discovery still relies heavily on the serendipitous discovery of new drugs by empirical screening of various chemical classes.

1.3.2 Pharmacokinetics

Introduction Lead derivation and optimization are guided by three predominant factors: efficacy, specificity, and pharmacokinetics. Pharmacokinetics is the study of the time course of drug absorption, distribution, metabolism, and excretion (ADME), and how ADME relates to the therapeutic and toxic effects of a drug. The key parameters and methods used in ADME studies are listed in Table 1.4. During the 1990s, it was noticed that many drug candidates were abandoned during clinical trials due to poor pharmacokinetics [46]. This, in part, reflects problems with

TABLE 1.4 Key ADME Parameters and Methodologies[a] for Early Studies

Physicochemical properties
Chemical stability and degradation
Solubility
pK_a
Lipophilicity (log P)
Binding target screens
Plasma protein binding
Nonspecific interactions/binding studies
Absorption and distribution
Passive transport into the systemic circulation system—Caco-2 MDCK cells
P-gp substrate/transporter assays
Absorption screening—models of the blood–brain, placental/reproductive, epithelial, and, corneal barriers
PBPK modeling
Metabolism and excretion
CYP metabolism
CYP inhibition/induction
Glucuronidation
Nuclear receptor activation
Regulation of lipid and cholesterol metabolism
Aromatase inhibition
Metabolite stability
Kidney cells and tissue preparations

[a]These approaches are increasingly being used by pharmaceutical companies in an attempt to reduce drug attrition rates.