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PRECLINICAL DRUG DEVELOPMENT PLANNING

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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.

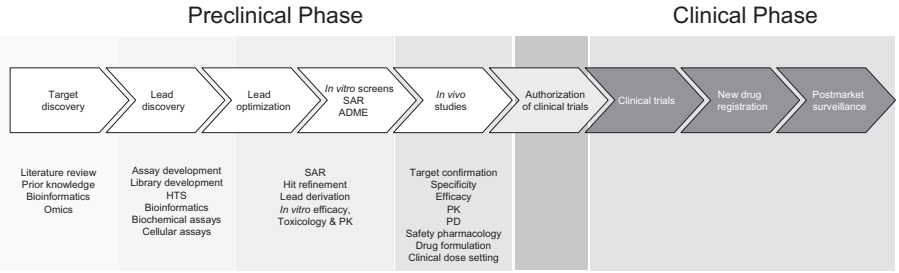

FIGURE 1.1 The key stages of drug discovery and development. A typical series of methods and strategies uses preclinical phases. Note that some of the studies may not be required and the process can be iterative. Refer also to Fig. 1.2 for a more detailed description of toxicity testing planning.

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.

species extrapolation, allometric scaling, and the selection of suitable preclinical models.

Absorption and Distribution For ease of administration, oral delivery is the most favored route of administration. Orally delivered drugs need to possess good gut absorption and clearance, as well as good metabolic stability; this is to ensure adequate systemic exposure. This is expressed as oral bioavailability, which is the fraction of the ingested drug that is available systemically, depends on both absorption and elimination, and which can be estimated from the equation:

$$\text{Oral bioavailability} = \text{Fraction absorbed across the intestinal wall} \times \text{Fraction that is not cleared by the gut} \times \text{Fraction not cleared by the liver} \times \text{Fraction not cleared by the lungs}$$

The fraction absorbed from the gut is dependent on lipophilicity (log *P*), namely, the hydrophobicity, molecular size, and hydrogen bonding potential of the drug, and its permeability, which is dictated by van der Waals forces that are due to nitrogen and oxygen atoms, which influence the polar surface area of a drug molecule. Highly lipophilic drugs are likely to be absorbed directly from the gut into the lacteals and enter the lymphatic system before the general circulation, thus avoiding first-pass metabolism in the liver. On the other hand, small, moderately lipophilic drugs are likely to be passively or actively transported (depending on electrical charge, structure, and intermolecular interactions) across the intestinal barrier into the hepatic portal vein and are destined for the hepatobiliary system. Lipinski's rule-of-five [47] is commonly used to predict the permeability of a compound according to the rule that >5 hydrogen bonds, coupled with a molecular mass of >500 Da, a *c* log *P* > 5, and >10 nitrogen and oxygen atoms are indicators of poor absorption. The full list of criteria for passive absorption through biological membranes is given in Table 1.5.

The biopharmaceutics classification system (BCS) [48] is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability. It takes into account three major determinants of the rate and extent of absorption following the administration of a solid oral dosage—(1) dissolution, (2) solubility, and (3) intestinal permeability—and can be used to avoid some animal studies. According to the BCS, drug substances are classified as follows:

TABLE 1.5 Criteria[a] for Absorption from the Gut for Drug-like Compounds

Rule-of-five:
• MW ≤ 500
• *c* log *P* ≤ 5
• H-bond donors ≤ 5
• H-bond acceptors (N and O atoms) ≤ 10
Additional criteria
• Polar surface area ≤140 Å^2 or Σ H-bond donors and acceptors ≤12
• Rotatable bonds ≤10

[a]These include Lipinski's criteria and experimentally determined criteria for the gut absorption of orally administered drugs.

Class I: High Solubility–High Permeability
Class II: Low Solubility–High Permeability
Class III: High Solubility–Low Permeability
Class IV: Low Solubility–Low Permeability

In the case of Class 2 and 4 drugs, dissolution is the predominant factor, and this can be estimated by using *in vitro* data that correlate well with *in vivo* results. The rate-limiting step for the absorption of a class 1 drug is gastric emptying, whereas permeability is the most important factor in the case of class 3 drugs. On a cautionary note, however, many drug-like compounds are exceptions to this rule, due to active transport mechanisms, the involvement of carriers, and possible biotransformation in the gut, and because the rule only applies to orally administered drugs. The rule is more likely to be applicable where the drug is a mimetic of a natural product. Furthermore, good oral bioavailability is shown by many drugs that are larger than 500 Da, but conformationally constrained, and/or that have reduced polar surfaces. This means that many potentially useful leads are discarded, if predictions are based solely on physicochemical indicators, without additional studies.

Thus, *in vitro* screens for the determination of bioavailability are indispensable during early drug development. *In vitro* systems range from relatively simple subcellular fractions, tissue slices or perfused organ preparations, through primary cultures and cell lines, grown either as mono cultures or cocultures, to three-dimensional organotypic cultures, which include reconstructed tissue models (see Ref. 49 for a review). For instance, *in vitro* approaches to determine permeability include the use of animal tissues or cell lines. Where a drug is absorbed by simple passive diffusion, rat everted intestinal rings [50], single-pass perfused intestinal preparations [51], and the human adenocarcinoma cell line Caco-2 [52] are often used to assess intestinal permeability. The common problem of accounting for differences in biotransformation can be addressed by supplementing these assays with a metabolic component. Everted tissue rings, in particular, can suffer from problems related to tissue viability, while the use of Caco-2 cells suffers from (1) the lack of mucus that coats the luminal surface of intestinal cells *in situ* and (2) the possibility that metabolic properties and other essential properties of the cells are lost during repeated passaging. The need for long culture times has been met by using Madin–Darby canine kidney (MDCK) cells, which, like Caco-2 cells, form a columnar epithelium with tight junctions, but which only require 3 days in culture. MDCK cells express fewer transporters, however, and are thus more suited to situations where there is little indication of the involvement of transporters, where the data from MDCK and Caco-2 cells are comparable [53]. However, where first-pass metabolism occurs, cells expressing CYP enzymes are more useful (see later discussion). A comparison of cell-based and tissue-based permeability data indicates that there are good correlations between data from such studies, at least in terms of their ability to rank drugs according to permeability [54]. However, both methods tend to underestimate the absorption of drugs that are actively transported, and to overestimate the absorption of drugs that are subject to efflux pump transport [55].

Two further methods for the assessment of absorption are (1) the use of immobilized membranes or artificial membrane (IAM) [56] chromatography and

(2) the parallel artificial membrane permeation assay (PAMPA) [57]. IAM and PAMPA are both relatively rapid screens, the latter also being amenable to a microtiter plate format. Hence, both are suitable for screening large libraries of drugs for passive diffusion. Again, these systems are only really suited to absorption studies where there is little indication that a drug might be subject to transporter/efflux protein binding, as determined by direct binding assays. For instance, the drug export pump, P-glycoprotein (P-gp), which protects the brain from toxic substances by transporting them back into the blood, and which is, incidentally, also found in tissues such as the kidney and the liver, has been used as the basis of a model system composed of porcine brain capillary endothelial cells. This system allows the ability of drugs to inhibit the efflux of a fluorescent P-gp substrate out of the cells to be readily monitored in a 96-well format [58]. This model may also be a useful supplement to many of the studies on more complex tissue and cell culture systems, given the key role of efflux proteins in determining internal drug concentrations. Indeed, the internal dose that reaches other tissues may have a crucial bearing on the use of the final marketed drug and the nature of the target patient group. Despite the fact that the placental transfer of drugs and other xenobiotics is known to occur, the process is poorly understood. What is known is that it involves efflux proteins, and that P-gp binding capacity may assist with decisions as to whether reproductive and developmental toxicity testing is likely to be required.

Dermal absorption can be determined by using organotypic skin models comprising stratified layers of epidermal cells, with each layer exhibiting morphological and functional differentiation. This has given rise to several commercially available organotypic and reconstructed human skin *in vitro* culture models, including EPISKIN™ (http://www.loreal.com) and EpiDerm™, and its fibroblast-supported version, Full Thickness EpiDerm™ (http://www.mattek.com). Multilayered models of the tracheobronchial tract are also available and permit squamous metaplasia [59], mucin production, and mucociliary clearance to be analyzed for making respiratory toxicity predictions of inhaled drug preparations [60]. Of particular relevance to the development of many cell-based organotypic models is the use of microporous substrates, which have led to physiologically more relevant culture conditions for studies of transcellular transport and cell–tissue interactions. Also, blood–brain barrier (BBB) function can be monitored by using coculture systems and reconstructed models in which sufficiently tight cell–cell junctions are formed (reviewed in Ref. 61), which might be more appropriate when it is envisaged that a delivery system might be used. For example, in models of the BBB, brain microcapillary endothelial cells can be cultured with astrocytes, glial cells, or neurones [62, 63] and "whole-brain" spheroid culture systems have been used to model absorption in neurotoxicology [64].

The nasal route is also widely used for topical and systemic targeting and has recently been considered as a suitable route for active peptide and protein administration, since the nasal mucosa has a higher permeability than the intestinal epithelial layer, because of its porous and neural pH endothelial basement membrane, and since first-pass hepatic and intestinal metabolism is largely avoided. However, mucociliary clearance is relatively fast, and the dose volume is smaller than that administered by other routes. These factors should be considered during the early development of therapeutics designed for nasal administration. *In vitro* models of human nasal absorption are particularly important, given that there are substantial

differences in nasal structure and function between many laboratory animal species and humans, which complicates data extrapolation [65]. Human nasal epithelial tissues can be obtained noninvasively, but the resulting heterogeneous primary cell population is difficult to culture and maintain. This has prompted the development of a nasal absorption model, based on Calu-3 human lung adenocarcinoma cells, derived from the upper airway of the lung. The cells can be grown at the air–liquid interface, as confluent and polarized sheets, with tight junctions, which secrete mucin, while also possessing cytochrome P450 (CY)PA1 and CYP2B6 activities and transport functions [66].

As illustrated earlier, because whole perfused organs, tissue slices, tissue isolates, organ fragment cultures, and other organotypic preparations can have limited lifetimes in culture, a wide variety of cell lines are used instead of primary cells and tissues. However, it is important for the cell lines to be thoroughly characterized, since, although use of human tissue and cells is advantageous as it obviates the need for interspecies extrapolation, the more commonly used animal cell lines may also provide data of use during drug development.

Metabolism Metabolism is a major determinant of drug efficacy and toxicity and has a major influence on drug pharmacokinetics. The metabolism of a drug will depend not only on its structure but also on the presence and expression levels of biotransformation enzymes. Metabolism is one of the main factors that determines not only how well a drug is absorbed into the systemic system but also how it is then transported to, and taken up by, specific tissues, as well as how it is eliminated. Susceptibility to biotransformation can dictate the effective dose of a drug at its intended site of action. Hence, predicting the likely routes and consequences of biotransformation is an essential part of early drug screening.

Phase I drug biotransforming enzymes include the CYP enzymes, flavin-containing monooxygenases, alcohol and aldehyde dehydrogenases, aldehyde oxidase, and peroxidase. Phase II pathways involve conjugation reactions, such as glucuronidation, glutathione conjugation, sulfation, methylation, coenzyme A conjugation, and phosphorylation. The enzymes involved are polymorphic and exist as a large number of isozyme forms that have wide substrate specificity and vary in nature and activity according to tissue and species. This is also the case for other metabolic enzymes and, indeed, for individual differences in drug response and metabolism. It is known, for instance, that there are at least 30 variants of the human CYP2D6 [67], which is responsible for the metabolism of almost one-third of all current therapeutic drugs. Phase I biotransformation is the main source of toxic intermediates or active drugs from innocuous parent chemicals (prodrugs). The main redox reaction, catalyzed by CYP-dependent monooxygenases (CYP-DMO), yields more polar and therefore more readily excretable metabolites. These by-products can exhibit biological effects that exceed, or differ from, those elicited by the parent molecule. Hence, a xenobiotic might appear to lack efficacy in an *in vitro* system, due to the absence of a biotransformation pathway necessary for its conversion into an active form.

Most of the systems described so far are not specifically designed with metabolic competence in mind. One way to circumvent problems with metabolic competence is by adding subcellular or cellular metabolizing systems and assessing the production of known metabolites [68]. For instance, by using rat liver microsomes,

it is possible to monitor the biotransformation of small amounts of drugs by using capillary electrophoresis [69]. Metabolic cellular systems can be divided into three main categories: (1) metabolically competent indicator cells (e.g., hepatocytes), (2) coculture systems comprising noncompetent indicator cells (e.g., fibroblasts) mixed with metabolicallycompetent cells, and (3) genetically engineered cell lines that can simultaneously act as indicators of both selected metabolic pathways and toxicity.

Since the liver is the major site of drug metabolism, the use of primary hepatocytes has become well established for studying drug metabolism and drug interactions. Hepatocyte models composed of primary cells assembled into a double collagen layer sandwich provide an *in vivo*-like environment, which can retain some important liver functions long enough for useful data to be obtained [70]. More recently, a mini bioreactor scaffold has been devised for biotransformation studies, comprised of a polycarbonate scaffold in 6-, 24-, and 96-well formats, which supports a gas-permeable PTFE membrane, suitable for medium throughput screening [71]. Animal or human cells can be used, although the latter are often in short supply and rapidly lose their viability and phase I and II biotransforming capacities. However, using animal cells does not overcome potential problems with interspecies differences. HepG2 cells have been used, since they allegedly retain human phase I and II drug-metabolizing activities, as well as bile acid and albumin synthesizing capabilities that are normally lost during hepatocyte culture. In addition, these cells can be transformed with specific genes (e.g., CYP genes), in order to bolster their metabolic competence. Drug interactions with efflux proteins such as P-gp can also be monitored with HepG2 cells, to gauge the extent of drug uptake into the liver [72].

Krebsfaenger et al. [73] constructed a panel of Chinese hamster cell lines that stably expressed variants of the human CYP2D6 gene, or in the case of the genetically engineered and metabolically competent V79 Cell Battery™, tissue-specific human phase I or II metabolic enzymes, including a number of CYP variants, glutathione *S*-transferases, and *N*-acetyltransferases. Since the cell battery also contains cell lines that express equivalent enzymes from other species, there is some scope for resolving species differences in metabolism. This system may not be able to account for the consequences of the overexpression of enzymes, or account for the onslaught of long-range biochemical pathways that may regulate metabolism. Nevertheless, the use of polymorphic cell lines has clear advantages over the use of a genetically homogeneous cell line.

A major problem, particularly when trying to model chronic exposure effects, is simulating *in vivo* perfusion rates by using cell-based systems of biotransformation to (1) ensure the removal of metabolites, and of other products such as reactive oxygen species; and (2) avoid cofactor depletion [74, 75]. Systems based on cell or cell coculture models have been designed to address these problems. Canova et al. [76] have recently described a flat membrane bioreactor, in which primary rat hepatocytes were cultured as collagen sandwiched monolayers on a polycarbonate plate. Since this system permits continuous perfusion, the cells exhibited a high level of metabolic competence and extended viability, as compared to cells grown as adherent monolayers.

Drugs can also inhibit CYP activity without being themselves subject to CYP metabolism, thereby causing the accumulation of toxic substances by modeling the interactions between the drug and the enzyme. Some basic information about the

chemical classes that are subject to biotransformation by these key enzymes is already known and can be used for such predictions. For example, CYP1A1 is very active in metabolizing polycyclic aromatic hydrocarbons, CYP2B1 and CYP1A2 preferentially metabolize aromatic amines, and CYP2E1 metabolizes low molecular weight chemicals. Perhaps the most important enzyme, CYP3A4, metabolizes larger molecules, and only six isoenzymes account for the metabolism of almost half of all the drugs in clinical use, namely, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A3, and CYP3A4 [77, 78]. These enzymes are all polymorphic and (with the notable exception of CYP2D6) are inducibly expressed. Since there are a number of X-ray diffraction structures for CYPs, which can also serve as templates for the homology modeling of other isoenzymes, it is possible to use molecular dynamics simulations and molecule docking to determine whether a novel chemical class of drug candidate is likely to be subjected to CYP metabolism or act as an inhibitor [79]. The advantages of such approaches include the fact that these screens can be used to eliminate poor drug candidates prior to synthesis of the compounds, and undesirable metabolic labile sites can be modified to alter the effective dose and metabolically linked contraindications between medicines can be reduced. Some such *in silico* prediction methods rely on knowledge of the structure and physicochemical properties of the drug in question to serve as alerts. For instance, Singh et al. [80] developed a rapid and semiquantitative model for identifying CYP3A4-labile groups on the basis of exposure and the energetics of hydrogen bonds. This has been used to accurately predict CYP3A metabolism for a number of chemicals [80] (for a review, see also Ref. 81).

The most common technique used to identify metabolic stability, rate of drug biotransformation, and likely metabolic fate of a drug is LC-MS/MS. This technique has recently been automated for high-throughput automated metabolic and protein binding screening [82]. Established cell lines and cell batteries (such as the V79 Cell Battery described earlier) that are genetically engineered to express various phase I and phase II enzymes, either singly or in combination, are particularly important in this respect, since they permit the contributions of specific isozymes to metabolism to be investigated [73] during relatively fast metabolic screening.

1.4 SPECIAL CONSIDERATIONS FOR NOVEL THERAPEUTIC CLASSES

1.4.1 New Classes of Therapeutic Agents: A New Drug Development Strategy?

Over the last decade or so, there has been a dramatic change in the nature of targets, with new protein, nanomedicines, and gene-based and cell-based therapeutics being investigated at an unprecedented rate. These biotechnology-derived pharmaceuticals are mainly used for the diagnosis, prevention, and treatment of serious and chronic diseases and range from blood and blood components and antitoxins to monoclonal antibodies, growth factors, vaccines, gene transfer products, and, potentially, cell-based therapeutics, and they may incorporate nanoparticulate delivery systems. As discussed below, the complex pharmacokinetic behavior of these therapeutics is only just starting to be understood, and attempts to group the preclinical testing of them has been attempted on a very *ad hoc* basis. Nevertheless, there are

some specific considerations for each type of therapeutic that should be borne in mind during early therapeutic development.

1.4.2 Protein-Based Therapeutics

Protein-based therapeutics can be subjected to a broad range of posttranslational modifications, including phosphorylation, glycosylation, and intermolecular and intramolecular bond formation, which can vary, depending on the cell or organism used to generate the product. In the case of replacement therapeutics, it is important to mimic the posttranslational modification seen in humans as precisely as possible. For novel therapeutics, the consequences of using recombinant techniques to produce a biological product, such as a monoclonal antibody, should be evaluated, to ensure that the finished product is sufficiently humanized, has folded correctly; and exhibits the type of posttranslation modification required for its proper function. In all cases, the primary aim is to avoid having the product elicit immunogenic responses that reduce its effectiveness or give rise to adverse effects.

The pharmaceutical industry has developed ways of scaling up the cell-based manufacture of biological products in serum-free and protein-free chemically defined media, in order to minimize the abnormal glycosylation of proteins. This is to comply with a regulatory requirement for the glycosylation profile of a protein to be maintained. This is particularly important, since products such as monoclonal antibodies can elicit antibody-dependent cell cytotoxicity, depending on their glycosylation state [83]. Indeed, protein-based therapeutics are often developed and characterized by mass (e.g., SPR, MS), activity (in cellular or biochemical assays), and immunogenicity assays (e.g., antibody-dependent cell-mediated cytotoxicity and immune cell proliferation assays), more so than in small chemical therapeutics. These measurements are generally obtained much earlier in the drug development process.

Other problems encountered during the development of protein-based therapeutics are caused by the fact that most of the products are human specific, potentially limiting the relevance of preclinical animal-based studies. In the case of the immunomodulatory monoclonal antibody TGN1412, a surrogate anti-rat CD28 antibody (JJ316), specific for the equivalent epitope to that recognized by TGN1412 in humans, was used to probe the mechanism of action of the therapeutic. However, subsequent studies on TGN1412 were conducted on macaques, on the basis that the CD28 antibody in this species possesses an identical TGN1412 recognition epitope to that found on the human protein. None of these preclinical models were able to predict the very serious contraindications that later materialized during Phase I trials [84]. This casts some doubt over the utility of testing surrogate molecules in a surrogate species with regard to the estimation of human safety when applied to humanized products. In the United States, the review of biological products, including monoclonal antibodies and other therapeutic agents with novel mechanisms of action, was until relatively recently conducted by the CBER. However, the CBER recognized that the utility of animal studies in the development of such therapeutic agents is often limited, due to species differences between the molecular targets. As a consequence, the jurisdiction for testing such products has now been transferred to the CDER, and it is almost certain that there will be a requirement that biological products conform to the two species test criteria adopted for other pharmaceuticals.

1.4.3 Gene Therapeutics

Advances in the use of viral vectors (e.g., adenoviruses, lentiviruses, and RNA viruses) and nonviral vectors (e.g., cells, liposomes, and DNA) have resulted in a growing interest in developing gene therapeutics for targeting single gene deficiencies, as well as cancers and neurodegenerative and tissue repair diseases. Nucleotide-based therapeutics will almost certainly be taken up by different cell types in the body via the same mechanisms. However, the key questions are the longevity of these treatments, whether they will result in incorporation into the genetic makeup of specific cell types, and the long-term consequences for the genetic makeup of germline and somatic cells. These products may therefore require early screening for reproductive and developmental toxicity, potentially via the use of fish larval forms and developmental assays with *D. melanogaster or C. elegans*. Genotoxicity screens that involve cell-based screens may also be relevant. Incidentally, the FETAX (frog embryo teratogenesis assay—*Xenopus*) test [85], a 4-day whole embryo developmental toxicity test, has been available for use for several years, but the apparent *status quo* with regard to its evaluation is consistent with concerns over its relevance to humans. The requirement for animal-based developmental, reproductive, or genotoxicity testing can then be assessed on a case-by-case basis, depending on the class of vector, transgene, and delivery method. In the absence of suitable long-term *in vitro* models, chronic toxicity studies in animals might also be justified, in which DNA integration is monitored by sensitive PCR-based techniques, although the two-year rodent assay may not be suitable for this purpose [86].

1.4.4 Nanomedicines

Nanomedicines (and ultrafines) consist of, or contain, organic or inorganic nanomaterials of variable dimensions, which, according to the U.S. Patent Office, are of 100 nm or less in size. The two main classes are carbon-based liposomes, dendrimers, fullerenes, nanotubes, nanowires, nanorods and metal oxide-based quantum dots and PEG-polyester systems. Nanomedicines, such as PEG-modified versions of existing drugs (e.g., PEG-granulocyte colony stimulating factor), are already on the market. Many other nanotechnology-based applications, such as carbon nanotube-based RNA, DNA, protein, and drug delivery systems, are being developed [87]. Indeed, between 2004 and 2005 there was a 60% increase in the number of such products in the development pipeline.

There is currently little regulatory guidance on how nanomedicines should be characterized, and each nanomedicine is generally assessed on an individual basis. It is also clear that information for macroparticle equivalents is often of limited relevance. According to the FDA, toxicity screening of nanomedicines [88] should involve (1) an assessment of physicochemical characteristics, including core and surface chemistry; (2) *in vitro* studies to determine absorption by the intended, and potentially additional, routes of exposure, binding studies, bioaccumulation/cellular uptake studies, and cytotoxicity screening; and (3) *in vivo* understanding of biokinetics and toxicity.

Nanomedicines display physical and chemical properties that are often distinct from their macroparticle counterparts. For instance, the small particle size corresponds with a high surface area: mass ratio means that not only are nanoparticles

likely to be more reactive because of their quantum properties [89], they are also more likely to agglomerate in a way that makes absorption difficult to predict by using biokinetic modeling alone. This would require information about the extent of agglomeration and the proportion of each type of particle in heterogeneous mixtures of particles, as well as a fundamental understanding of how agglomeration affected different reactivities. Agglomeration is dependent on surface chemistry and charge, and this will also have a crucial bearing on uptake into different tissues [90].

Nanoparticles may be able to escape phagocytic activity and may not only have extended biological half-lives but may also elicit immunogenic and inflammatory responses, especially since they may be able to access lung tissues and be able to cross the BBB, the intestinal mucosa, and other physical barriers more readily than macroparticles. Indeed, the size of a nanoparticle is generally that of a typical protein. Note that BBB passage requires nanoparticle dimensions of 20–50 nm, whereas 70-nm particles are able to cross into the pulmonary system, and spherical fullerenes appear to accumulate in the liver [91]. However, at present, there is little information about how nanoparticles are able to interact with the immune system and with specific tissues.

Examining the tendency of nanomedicines to bioaccumulate is an important step in the nanomedicine development strategy, so the need for long-term toxicity testing cannot be overemphasized. There is already some indication as to how readily different types of carbon-based nanomaterials accumulate in different tissues [92], as well as about how physicochemical properties such as size, shape, tendency to agglomerate, surface fictionalization, and chemical composition relate to the absorption, distribution, and biokinetics of nanoparticles (see Ref. 93 for a review).

1.5 *IN VITRO* ASSAYS: APPLICATIONS IN SAFETY PHARMACOLOGY

1.5.1 Background

Many surrogate assays have been developed for the detection of toxicological endpoints. Many of those used specifically for lead development and early ADME characterization have already been considered. Here, we consider *in vitro* assays that are relevant to the early characterization of toxicologically relevant endpoints. A list of methods that are currently available for toxicological endpoints that may be more suited to high-throughput safety pharmacology have been developed and/or validated, some of which have already been afforded OECD Test Guideline status (Table 1.6). These and other such tests may eventually expedite the safety testing of pharmaceutical products. At present, however, they are most commonly used to provide evidence to supplement animal test data. Their potential application to toxicology testing is illustrated in Fig. 1.2. Their validation status will have a bearing on the acceptability of specific tests as part of a medicine's dossier for different regulatory agencies, and there is generally a delay of several years between validation and regulatory acceptance. It should be noted that in the EU, under the terms of *Directive 86/609/EEC*, once an *in vitro* alternative to an animal test has been deemed to be reasonably and practicably available and has been validated for a specific purpose, it must be used instead of the equivalent animal test.

TABLE 1.6 Status of Nonanimal Methods that Are of Relevance to Drug Development[a]

Test Method	Test System	Endpoint	OECD TG Reference and Comments
In Vitro Test Methods for Which There Are OECD Health Effects Test Guidelines (Including Draft Guidelines Under Review for Acceptance) http://www.oecd.org/home/			
Transcutaneous electrical resistance test (TER)	Monitors changes in electrical resistance as a measure of loss of corneum integrity and barrier function; involves skin disks from euthanized rats	Skin corrosion (topical agents)	TG 430
Human skin models (EpiDerm™, EPISKIN™)	Reconstructed human epidermal equivalent (commercial system) used to assess cell viability, involving the MTT reduction test	Skin corrosion (topical agents)	TG 431
3T3 NRU phototoxicity test	BALB/c 3T3 (murine) cell line cytotoxicity based on Neutral Red uptake to measure cell viability; not a direct replacement alternative, as there is no *in vivo* equivalent test	Phototoxicity	TG 432
Corrositex™ membrane barrier test	An artificial barrier system coupled to a pH-based chemical detection system	Skin corrosion (topical agents)	Draft TG 435
Bacterial Reverse Mutation test (Ames)	Revertant bacteria detected by their ability to grow in the absence of the amino acid	Genotoxicity	TG 471
In vitro mammalian chromosome aberration test	Microscopic detection of chromosomal damage to cells in culture	Genotoxicity	TG 473
In vitro mammalian cell gene mutation test	Functional bioassays to monitor mutations in enzyme encoding genes	Genotoxicity	TG 476
Sister chromatid exchange assay	Cells in culture are examined after two rounds of division by metaphase arrest and chromosomal preparation; chromatid exchange is monitored by microscopy	Genotoxicity	TG 479
Gene mutation assay in yeast	*Saccharomyces cerivisiae* exposed to the test substance are grow under different culture conditions used to monitor mutagenic potential (cf. Ames test)	Genotoxicity	TG 480
Mitotic recombination assay in yeast	Crossover or gene conversion following exposure of yeast to the test substance; relies on different growth requirements of mutated and wild-type yeast strains	Genotoxicity	TG 481

Unscheduled DNA synthesis in mammalian cells	Measures the DNA repair synthesis after deletions caused by the test substance; based on the incorporation of radioactive nucleotides into the newly synthesized DNA	Genotoxicity	TG 482
In vitro micronucleus test	Cell-based assay; supplement to TG 474 (*in vivo* micronucleus test); detection of chromosome damage and formation of micronuclei	Genotoxicity	Draft TG 487
Sex-linked recessive lethal test	*Drosophila* are exposed to the test substance. Germline transmission of mutations is monitored through two successive generations	Reproductive toxicity	TG 477
	Validated Methods that Are Yet to Be Introduced into Regulatory Use		
EpiOcular™	Human keratinocyte derived model of the corneal epithelium barrier function	Eye irritation (topical application)	Retrospective (weight-of-evidence) validation (ECVAM)
In vitro micronucleus test	CHL/IU, CHO, SHE, or V79 cell lines are commonly used, with or without metabolic activation, to monitor damage and formation of micronuclei in interphase	Mutagenicity	Retrospective (weight-of-evidence) validation (ECVAM)
Embryonic stem cell test	3T3 cell cytotoxicity and differentiation of embryonic stem murine cell lines used to examine teratogenic potential	Developmental toxicity	Endorsed as screening test (EU)
Postimplantation rat whole embryo test	Morphological assessment of rat embryos	Developmental toxicity	Endorsed as screening test (EU)
Micromass test	Micromass cultures of rat limb are bud monitored for inhibition of cell proliferation and differentiation	Developmental toxicity	Endorsed as screening test (EU)
	Methods Undergoing Validation		
EPISKIN™	Reconstructed human skin system used with MTT assay to monitor barrier function	Skin irritation	Report stage in EU
EpiDerm™	Similar to EPISKIN™	Skin irritation	Report stage in EU

TABLE 1.6 ***Continued***

Test Method	Test System	Endpoint	OECD TG Reference and Comments
	Prevalidated Methods		
SkinEthic eye model	Epithelial corneal cell line used for cytotoxicity testing based on the MTT reduction assay	Eye irritation	Appraisal stage in EU
	Methods Undergoing Development, Prevalidation, or Evaluation		
Tissue culture models	Neutral Red release and silicon microphysiometry or fluorescein leakage bioassays with human keratinocytes and MDCK cells, respectively; red blood cell (RBC) hemolysis test	Eye irritation	Being reviewed by ECVAM for possible retrospective (weight-of-evidence) validation
Organotypic models	Bovine corneal opacity and permeability (BCOP) assay, with postmortem corneas; hen's egg test on the chorioallantoic membrane (HET-CAM assay); isolated rabbit and chicken eye tests (IRE and ICE)	Eye irritation	Being reviewed by ICCVAM for possible retrospective (weight-of-evidence) validation
Cell transformation assay	With SHE and BALB/c 3T3 cell lines	Carcinogenicity	
Modified Leydig cell line	Analysis of progesterone production as a measure of the test substance effects on steroid hormone production	One/two generation study	For use as part of test battery
Testis slices	Assessment of steroid production capacity of the Leydig cells upon exposure of *ex vivo* rat tissue to toxicants	One/two generation study	For use as part of test battery
Human adrenocortical carcinoma cell line	Assay to allow entire steroid pathway effects to be mapped	One/two generation study	For use as part of test battery
Placental microsomal aromatase assay	Monitors the ability of substances to affect steroid production; a subcellular microsomal assay is used industrially	One/two generation study	For use as part of test battery

[a]This is a comprehensive list of methods that have been validated or that are at various stages of development for toxicity testing. More information about these methods and how they can be applied is available from: http://www2.defra.gov.uk/research/project_data/more.asp?!=CB01067&M=KWS&V=reach&scope=0.

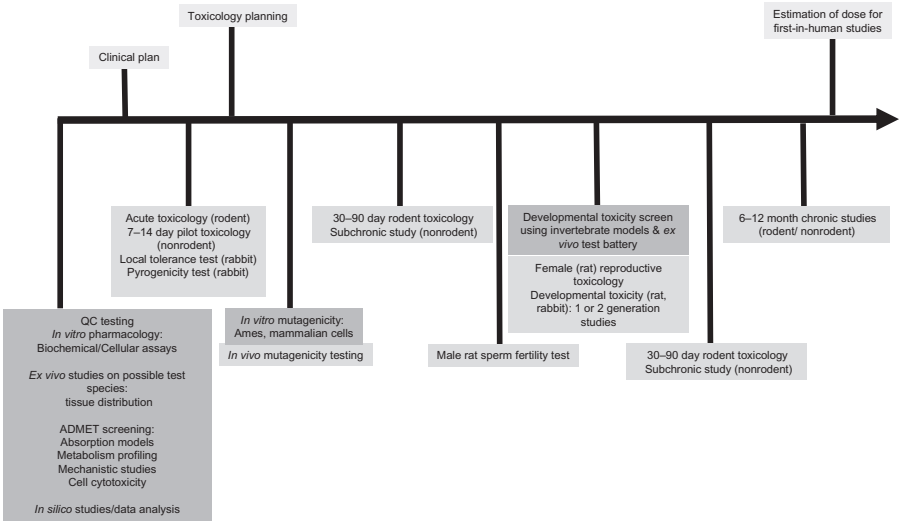

FIGURE 1.2 A typical toxicity plan from formulation of a clinical plan to first-in-human studies. The figure summarizes the general regulatory requirements and the role of *in vitro* and animal studies in preclinical safety pharmacology.

1.5.2 QT Prolongation

The recently developed ICH S7B guideline relates to the prolongation of the QT interval, the time between the start of the Q wave and the end of the T wave in the electrical cycle of the heart. Drugs that prolong the QT interval do so by blocking the activity of the human ether-a-go-go-related gene (hERG) channel found on cardiomyocytes, which plays a role in phase 3 depolarization. This can, but does not always, lead to the potentially fatal, although rare, tachyarrhythmia, Torsades des pointes. Electrophysiological recordings in cells and tissue explants can be used to screen for hERG activity. The most commonly used nonanimal assays involve studies on rabbit left ventricular wedge or perfused heart preparations [94].

Several cell-based and *in silico* prediction tools could also be used effectively for early screening. It is clear that while several classes of drugs, including antihistamines, antiemetics, antibacterials, and neuroleptics, have been associated with QT prolongation [95], not all the drugs of a particular class will cause this effect. Hence, although mutational analysis of hERG and pharmacophore modeling has helped to elucidate how several drugs block channel activity (reviewed in Ref. 96), the prediction of QT prolongation by using *in silico* methods alone is problematical. As a consequence, the first realistic step in screening for hERG-blocking activity generally depends on the use of cell-based studies. It should also be remembered that hERG activity is not the sole determinant of whether a drug is likely to cause cardiac arrhythmias, since the expression and activity of other ion channels can either mitigate or exacerbate the effects of a drug on QT interval prolongation. Metabolism and species differences in ion channel distribution and activity must also be taken into account. Species differences can be avoided by using cell lines such as Chinese hamster ovary or human embryonic kidney cells heterologously expressing hERG, since mammalian cells are the ideal system for studying potassium channel activity at physiologically relevant temperatures (see Ref. 96 for a review). This is the case

even despite the fact that while patch clamping is generally more readily achievable for larger cell types, such as *Xenopus* oocytes expressing hERG, the lower temperature required for their culture can complicate data extrapolation to humans. More recently, human embryonic stem cell (HESC)-derived cell types with primary cardiomyocyte-like properties have become available [97, 98]. However, although these cells act as a suitable surrogate for cardiomyocytes, the use of HESCs raises ethical issues. Adult stem cells derived from tissues such as bone marrow have therefore also been investigated as primary cardiomyocyte surrogates [99].

1.5.3 ICH Guidelines

Pyrogenicity testing forms part of the quality control of pharmaceuticals, particularly of biotechnological products that may be contaminated with gram-negative bacteria. Five *in vitro* methods have been proposed and evaluated as possible alternatives to pyrogenicity testing in animals (usually rabbits). These are based on the use of human whole blood or blood subcellular fractions linked to interleukin-based screens. These methods have been validated under the auspices of ECVAM, and the ECVAM Scientific Advisory Committee has endorsed their use. Draft recommendations regarding the applicability of these methods were published by ICCVAM in December 2006 [100].

The ICH guidelines for the safety testing of pharmaceutical products for human use are listed in Table 1.7. In general, tests are conducted by the same route of exposure as that anticipated for clinical use. Where there is more than one route of exposure, at least two routes should be investigated. Where the intended route of human exposure is impracticable for animal studies, the route of exposure in animal studies will need to be different, although the aim is to establish whether the level of systemic exposure is similar to that via the clinical route. In the case of ocular administration, systemic exposure is not automatically assumed, so some toxicological studies, such as carcinogenicity testing, may not be required, unless there is compelling evidence to suggest otherwise. Carcinogenicity testing is not usually required for peptide and protein replacement therapeutics, unless they are significantly different from their natural counterparts. The dose used is generally derived from the MTD from 3-month toxicity studies, as well as from a consideration of toxicokinetic data and the known or predicted consequences of biotransformation of the chemical. Carcinogenicity testing may also be guided by the outcome of genotoxicity and cell transformation studies, the majority of which involve cell-based screens for gene mutation or chromosomal aberration. The ICH guidelines require three tests for genotoxicity: (1) a test for gene mutation in bacteria, (2) an *in vitro* mammalian cell or mouse lymphoma assay for chromosomal damage, and (3) an *in vivo* test for the detection of chromosomal damage in hematopoietic cells in the bone marrow of rodents.

Determination of the acute toxicity of a single dose of a pharmaceutical no longer requires an LD_{50} determination. Instead, it is most commonly derived from the maximum tolerated dose (MTD). The ICH guidelines recommend that acute toxicity is determined in a rodent and in a nonrodent species. However, in many cases, it may be possible to justify the use of a single species and/or incorporation of the MTD estimation into a dose escalation study from longer term/repeat-dose studies. The justification for conducting repeat-dose studies is partly based on the outcome of single-dose studies. This is especially the case when single-dose studies indicate

TABLE 1.7 ICH Guidelines[a] for Safety Pharmacology

ICH Guideline for Safety Pharmacology http://www.ich.org/	Description of Content
	Acute Toxicity Testing
M3(R1)—Maintenance of the ICH Guideline on Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals	S4—single dose LD_{50} tests for pharmaceuticals have been abandoned; the guideline sets out the duration of acute, repeat dose, and chronic tests in animals, and the criteria for reproductive, developmental, fertility, genotoxicity, and carcinogenicity testing, as well as the times during development that each type of safety test should be performed
	Carcinogenicity Testing
S1A—Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals S1B—Testing for Carcinogenicity of Pharmaceuticals S1C(R1)—Dose Selection for Carcinogenicity Studies of Pharmaceuticals	Long-term studies, with dosing at the MTD level or, in the United States, a dose 100-fold higher than the recommended human daily dose, are conducted in a single rodent species (the rat unless evidence suggests it is not an appropriate species), for a duration of no less than 6 months; additional rodent studies of short-term or medium-term duration may be required (generally in mice/GM mice); a weight-of-evidence approach is used to determine whether there is a likely cause for concern based on genotoxicity tests, the results of cell transformation studies, effects of same-in-class/SAR, preneoplastic lesions in repeat-dose studies, and/or long-term tissue retention of the drug or its metabolites
	Genotoxicity Testing
S2A—Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals S2B—Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals	Provides recommendations as to the bacterial strains and mammalian cell lines or human blood cells that could be used to detect various types of DNA damage; defines the concentrations to be used in each type of test and how to make decisions as to whether to proceed with *in vivo* animal studies
	Toxicokinetic Studies
S3A—Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies S3B—Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies	Toxicokinetic studies are normally integrated with repeat dose/3-month toxicity studies and provide multiple-dose pharmacokinetic data
	Chronic Toxicity Testing
S4—Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing)	Performed for a 6-month duration in rodents and a 9-month duration in nonrodent species

TABLE 1.7 ***Continued***

ICH Guideline for Safety Pharmacology http://www.ich.org/	Description of Content
	Reproductive Toxicity Testing
S5(R2)—Detection of Toxicity to Reproduction for Medicinal Products Toxicity to Male Fertility: An Addendum to the ICH Tripartite Guideline on Detection of Toxicity to Reproduction for Medicinal Products	one- or two-generation studies (generally in rodents) may be needed, depending on the intended use, form, and route of administration of a drug; incorporates embryotoxicity studies (usually on rabbits/rodents), but also considers the merits of other test systems, such as tissue, organ, and organism cultures
	Biotechnological Products
S6—Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals	Pharmaceutical products from cell cultures and transgenic plants/animals, such as proteins, hormones, antibodies, and proteins extracted from human tissues, are primarily subject to these guidelines; normally, two species are used, but in some cases only a single species is relevant; if no species is relevant, the creation of a GM model is considered
	Miscellaneous Safety Pharmacology
S7A—Safety Pharmacology Studies for Human Pharmaceuticals S7B—The Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) By Human Pharmaceuticals	Defines the way in which toxicity of the major organ systems can be monitored by using animal and *in vitro* methods; one of the criteria for determining whether safety pharmacology needs to be performed is whether a drug is able to enter into the systemic system, so many topically administered drugs are excluded from full safety evaluation QT prolongation: criteria are given for *in vitro* and *in vivo* animal tests
	Immunotoxicity Testing
S8—Immunotoxicology Studies for Human Pharmaceuticals	Addresses the use of local tolerance/allergy tests at the site of injection (generally in rabbits); and the use of assays that monitor humoral and cell-mediated immunity and immunomodulation; the requirement for immunotoxicity testing is based on a weight-of-evidence approach

[a]The ICH website has additional information about the testing criteria and study design for each endpoint. Guidelines for quality assessment are also available from this site.

that the half-life of the drug (and/or its metabolites) in organs or tissues significantly exceeds the apparent half-life of the elimination phase in plasma, and when the steady-state levels of a drug are higher in repeat-dose studies than in single-dose studies. Predicted tissue-specific retention that leads to tissue-specific toxicity is also a justification for repeat-dose studies. Pharmaceuticals developed for site-specific delivery might also require repeat-dose studies.

Determining the acute dose toxicity of biotechnology products in animals is likely to be of limited relevance to safety assessment. This is because such products (e.g., recombinant proteins) are highly human specific and are likely to give rise to immunogenicity. However, the latter effect can be investigated by using the murine local lymph node assay (LLNA). This test has been accepted in the United States and the European Union as an alternative to the guinea pig maximization test for assessing allergic contact dermatitis caused by pharmaceuticals, and is now part of the OECD Health Effects Test Guidelines (TG 429).

The possibility of using *in vitro* systems to permit the high-throughput assessment of chronic toxicity is currently very limited, predominantly due to the problems inherent in undertaking repeat exposures and maintaining long-term cultures. However, one system developed from stem cells consists of a neurosphere with an outer layer of cells surrounding a growing core [64] that can be maintained in culture for up to a year. This system might be amenable for development into a long-term assay for assessing neurotoxicological endpoints (see Section 1.3.2).

1.6 SYSTEMS BIOLOGY

Many of the screens and assays described thus far are focused on measuring a single parameter. However, although they provide information relevant to the *in vivo* assessment of new drugs, they lack some of the complexities of animal studies. Nevertheless, this can sometimes be an advantage, particularly as the effects observed during *in vivo* studies can be due to a multitude of unknown mechanisms, and this can confound data interpretation and extrapolation. As illustrated next, recent developments in the omics-based technologies and microfluidics may provide a way of addressing this caveat against the use of existing cell-based and tissue-based screens.

1.6.1 Omics-Based Technologies

Biomarkers Biomarkers discovered from, and subsequently used in, nonclinical studies can play several useful roles in drug development. During lead discovery, a biomarker can assist with the screening of chemicals, based on the ability of the candidate molecule to modulate the activity of a specific process, such as a biochemical pathway. In some cases, these biomarkers can be used to develop reporter gene screens, such as the CRE and ERE activation-linked expression of GFP or enzymes. In this way, the application of biomarkers can provide information about target interactions in support of the feasibility of a given therapeutic approach, as well as to assist in the development of high-throughput screening systems to expedite drug discovery.

Biomarkers are also relevant for selecting the most appropriate test species, since the absence of a significant marker is likely to be indicative of differences in the

activities of a drug in humans and the test species. The use of biomarkers as surrogate endpoints can identify the commonality between *in vitro* and *in vivo* effects, leading to dramatic improvements in the power of meta-analysis and of other approaches, where the goal is to assemble and evaluate value of information from various sources, such as animal experiments, human studies, and *in vitro* studies. This is, of course, subject to the very careful selection of the biomarkers to be used. One consideration is a need to readily detect changes in levels of biomarkers and to be able to relate such changes to drug-related efficacy or toxicity. In the interests of animal welfare and experimental convenience, metabolites and other small molecules that can be detected noninvasively in blood, stool, hair, or urine samples are preferred. This is because such biomarkers can be continually monitored during an experiment, and large numbers of samples can be collected, thereby allowing statistically reliable data to be generated from a small number of animals. However, most importantly, such biomarkers can also be readily analyzed in body fluid and stool samples taken from participants in clinical trials and can be used in the conduct of postmarket surveillance of large cohorts of patients.

Omics Toward Biomarker Discovery *Genomics* can be used to identify new targets, since it is now possible to analyze changes in the transcription of >20,000 genes in various cell types and tissues in multiparameter experiments. There are two key approaches: (1) the detection of cDNAs that correspond to the entire protein-encoding gene; and (2) the use of RNAi to determine the relevance of mRNA levels in terms of protein expression and gene function. Recently, these approaches have been used to assess the influence of noncoding RNAs on specific biochemical pathways in mammalian cells [101]. The application of genomics has already proved useful in fundamental research and for gaining a mechanistic understanding of drug activity. However, so far, it has made only limited contributions to drug discovery [1]. Similarly, *toxicogenomic* approaches, in which global changes in gene expression resulting from exposure of specific cells or tissues to a test substance are identified, have been of limited use up to now. Nevertheless, the microarray or serial analysis of gene expression, by using commercially derived or custom-made gene chips, such as those from Affymetrix (www.affymetrix.com/), is a technique that is already proving useful for the identification of markers of hematotoxicity and carcinogenicity [102, 103].

Proteomics is an approach that involves measuring total cellular protein and determining the posttranslational modification and fate of proteins. The most applicable techniques involve spotting cell lysates onto arrayed antibody wells to search for potential biomarkers and to profile molecular pathways. In this way, much useful information on specific protein–protein interactions has been gained by using yeast-2-hybrid systems, and coimmunoprecipitation or other affinity-based methodologies. The antibodies required for this purpose are being generated and characterized by the Human Proteome Organization (HUPO) [104]. The reason why data from proteomics can be more relevant than the information from genomic analysis is that changes in mRNA levels do not necessarily correlate with changes in protein expression. Furthermore, it is clear that a drug might alter the protein recycling/degradation/synthesis rates and/or the extent, as well as the nature, of posttranslational modification. However, proteomics is limited by the fact that some classes of proteins are difficult to resolve (e.g., membrane proteins) and it is currently not amenable to high-throughput formats.

Metabonomics is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" [105]. It has traditionally required the use of analytical methods such as MS and NMR, in conjunction with separation techniques such as gas or liquid chromatography, which are primarily suited to the resolution of low molecular weight metabolites. Although NMR is generally less sensitive than MS-based methods, newer techniques such as magic angle spinning NMR cater for the direct metabolic profiling of as little as 20 mg of tissue or biofluids [106, 107]. Typically, drug testing by metabolic profiling involves the collection of body fluids from animals or human volunteers and is conducted alongside drug metabolism and PK studies.

In the case of animal experiments, many variables have complicated the interpretation of data from metabonomic studies in animals. Such variables include the species and strain of animal, gender, age, and the influence of diurnal variations and environmental factors and stressors. Other factors include the choice of dosing vehicle and the acclimatization period prior to the conduct of studies in metabolism cages. Nevertheless, the development of increasingly sophisticated analytical techniques (see later discussion) has permitted the use of smaller group sizes and reductions in the amounts of body fluids required, and reductions in the need for invasive studies, particularly where metabolic profiling is conducted on urine samples.

The main advantage of metabonomics over proteomics and genomics is that there is a much stronger link to a tangible physiological response to a drug, because it is not reliant on potentially delayed effects on protein expression, but rather on the measurement of biochemical changes that result from the modulation of specific pathways.

1.6.2 Microfluidics Systems

A microscale cell culture system that consists of a series of chambers each containing mammalian cells connected by a fluid network can be used to assess drug dynamics as a quasi *in vivo* surrogate system [108]. The development of robotic DNA printing systems [109] that allow cells to be transformed *in situ*, and adaptations that facilitate the growth and maintenance of such microfluidics systems, without the need for plating, can, as a result, be used for longer term as well as real-time studies [110]. The use of human cells instead of animal cells greatly increases the relevance of such systems to human safety.

1.6.3 Enabling Technologies for Multiparameter Studies

All the approaches discussed generate such a large amount of information that data analysis and interpretation can be very complicated, so complex analytical methods are needed to integrate and analyze the results. One of the most useful of these methods is pattern recognition. Pattern recognition algorithms differ widely in the ways in which data are analyzed and used to develop new hypotheses. The further development and application of these methods (reviewed in Ref. 111) could transform the ways in which systems biology approaches will facilitate the use of information from omic-based studies, data mining, pathway informatics, and other types of study, in order to gain an overview of drug action. At present, more than 150 databases, data analysis tools, and software suites are already available for this purpose [112]. These bioinformatics platforms mainly comprise commercially available clustering techniques that enable the extraction of exposure-related infor-

TABLE 1.8 Pathway Signal Databases and Generation Tools for Systems-Based Drug Development[a]

Pathway Tools
MetaCore http://metacore.com/web/index.php
A pathway modeling database
Pathway Studio www.ariadnegenomics.com
Assists with the interpretation of omics data and the generation of pathways that can be updated with information from other sources
Pathway Analyst http://path-a.cs.ualberta.ca/
Predicts pathways in which a target protein may be involved
Gepasi http://www.gepasi.org/
Software for modeling biochemical pathways; incorporates biokinetic simulation tools
Jdesigner http://sbw.kgi.edu/software/jdesigner.htm
A system that allows biochemical networks to be drawn
Whole cell biochemical pathways
Genomatica http://www.genomatica.com/index.shtml
A collection of models for whole cells and organisms that displays gene, protein, and biochemical networks
E-Cell http://www.e-cell.org/software
A simulation, modeling, and analysis tool for application to complex biological systems
Whole tissue/organ pathway tools
Human Physiome Project https://www.bioeng.auckland.ac.nz/physiome/physiome_project.php
A computational database that aims to build integrative models of biological systems, and whole organ/tissue structure and functions
Clinical data
Entelos http://www.entelos.com/
For virtual patient simulations based on normal and disease physiology, to assist in deciphering disease-related pathways

[a]The rapidly growing number of resources is impossible to list in full. However, these are some key resources that can be used during drug discovery and development.

mation on changes in proteins, in metabolites, or in gene expression, and can overlay information from different sources to find common hotspots or to identify biochemical networks. The length of the branch corresponds to the similarities between datasets. To further reduce the number of false-positive biomarkers, a common problem with all omics-based technologies, condition-specific algorithms, modeling approaches, and data-mining software packages have recently been developed (reviewed in Ref. 113; Table 1.8). There are also several tools that assist with the weighting of information from these varied approaches (Table 1.8).

1.7 COMPUTATIONAL METHODOLOGY USED IN EARLY PRECLINICAL DRUG DEVELOPMENT

1.7.1 Combinatorial Chemistry and SAR

By avoiding the incorporation of particular chemical groups into a drug, the likelihood of toxicity can be greatly reduced. Computational methods are used at the

very early stages of drug development to analyze the behavior of virtual chemical libraries, in terms of target specificity and affinity, or in terms of transport across physiological barriers, metabolism, or the causation of off-target modulation [114]. These predictions can rely on either a local set of data for a limited number of structurally related chemicals or global models. The latter require a large training set for refining a computational predictive tool and are generally able to identify alerts that may result in toxicity with greater success than locally trained tools (i.e., those based on a small set of known compounds). Affinity receptor binding data are very useful for the development of predictive computational models of closely related analogues of a drug molecule, as is information on the natural ligand derived from *in vitro* target–ligand binding studies.

An ability to generate an accurate model of the target protein is also very useful. Such models can be created *ab initio* or by homology-based modeling. In fact, the protein target models themselves are generated by using several approaches, the most popular of which combines primary sequence analysis for structural signatures that indicate probable protein folding with X-ray diffraction and crystallographic studies on a protein of the same class. A variety of molecular docking programs can be used, and most algorithms are based on an assumption that the target (generally a protein) is a rigid structure, while the ligand is flexible. The notable exceptions are force-field-based methods, such as molecular dynamics and Monte Carlo simulations, which allow for flexibility in both the target and the ligand. This approach accommodates the proposed "induced fit" model for most peptide and protein ligands. Fragment-based incremental methods are similarly suitable for assembling ligands from fragments that are incrementally docked into the target.

QSAR modeling is useful when each member of the same class of chemicals (sharing key structural elements and physicochemical properties) acts by a common mechanism. It involves generating rules from a training set of molecules based on their structures and known biological activities. These rules are then used to predict the biological activity of a novel candidate molecule with structural features that fall within the applicability domain of the model. For each docking application, a scoring system to identify virtual hits and a rule base that can accommodate experimental SAR data, are essential. The reader is referred to an excellent review on this topic [115] (see also Table 1.9).

1.7.2 *In Silico* Prediction of ADME and Toxicity

Adverse drug reactions (ADRs) remain one of the major reasons why a drug that has passed through preclinical testing will eventually be dropped following clinical trials. Efforts have recently been made to develop computer-based prediction tools and other such expert systems, for use in assessing the ADR potentials of drugs. Although there are four general types of expert systems, all of them are built up from experimental toxicity data. Hence, the early prediction of whether a drug is likely to have adverse effects can be used to refine compound libraries prior to high-throughput hit generation and lead development. DEREK (Deduction of Risk from Existing Knowledge) is a knowledge-based system, which focuses on molecular substructures (or "alerts") associated with known toxicological endpoints [116, 117]. It is able to predict three different endpoints, including mutagenicity, for which it was found to be 84% correctly predictive for a set of 226 chemicals. CASE

TABLE 1.9 Examples of QSARs that Can Be Used During Lead Discovery and Optimization[a]

Database	Type of Information
ChemTree http://www.goldenhelix.com/chemtreesoftware.html	Statistical analysis QSAR for chemical library refinement
QikProp http://www.schrodinger.com	ADME prediction QSAR for lead generation and optimization
Catalyst http://www.accelrys.com/products/catalyst/	3D QSAR and query-based database management tool
CoMFA (Comparative Molecular Field Analysis) http://www.chem.ac.ru/Chemistry/Soft/COMFA.en.html	3D QSAR technique based on data from known active molecules; applicable when the structure of the target protein is unknown
VolSurf http://www.moldiscovery.com/soft_volsurf.php	Produces 2D molecular descriptors from 3D molecular interaction energy grid maps, for the optimization of *in silico* PK properties (eADME or IS-DMPK)

[a]More information about these methods and how they can be applied is available from http://www2.defra.gov.uk/research/project_data/more.asp?!=CB01067&M=KWS&V=reach&scope=0. Website links accessed 30 January 2007.

(Computer Automated Structure Evaluation) is an automated rule induction system [118]. It generates its own structural alerts by breaking down each chemical into all its possible fragments that are then classified as biophores (associated with toxicity) or biophobes (not associated with toxicity). CASE has been adapted by the FDA to provide MCASE QSAR-ES, which is able to predict carcinogenicity with an accuracy of 75% [119]. TOPKAT is an automated QSAR system [120] that uses large amounts of data for diverse compounds to build a set of descriptors. Decision tree-based methods are typified by OncoLogic [121], which, as its name suggests, is also used to predict carcinogenicity. The toxicological endpoints predicted by these and other such computer-based tools are summarized in Table 1.10. However, it should be noted that the predictivity of these tools is strictly determined by the quality of the molecular descriptors and the training set used to generate the prediction algorithm. In many cases, it may be possible to increase the predictive power of these and other such expert systems, by using more than one expert system for each endpoint. For instance, when 14 carcinogens were submitted to COMPACT and HazardExpert, used separately they predicted 10 and 8 actual carcinogens as carcinogenic, respectively, but when the two methods were used together, all 14 carcinogens were identified [122]. Tools such as INVDOCK, which combine molecular docking with structural alert profiling, may also be useful tools [123].

1.7.3 Prediction of Tissue-Specific Exposure: PBPK Modeling

A fundamental problem when using hazard data for risk assessment is the need to relate the effects detected at the dose level administered to a test system (the external dose) with the effects that would be caused by the dose that actually reaches the target in humans (the internal dose). The internal target organ dose can be predicted by undertaking toxicokinetic studies. Physiologically based biokinetic

TABLE 1.10 Examples of Computer-Based Expert Systems Available for ADME and Toxicity Predictions

Name	Type of Expert System	Website	Some of the Endpoints Predicted[a]	
CASE/ MCASE/ CASETOX	Automated rule based	www.multicase.com	• Carcinogenicity • Teratogenicity • Mutagenicity	• Acute toxicity • Chronic toxicity
DEREK	Knowledge based	www.chem.leeds.ac.uk/luk	• Teratogenicity • Mutagenicity • Respiratory sensitization	• Carcinogenicity • Skin irritation • Skin sensitization
HazardExpert	Knowledge based	www.compudrug.com	• Oncogenicity • Mutagenicity • Teratogenicity • Immunotoxicity • Neurotoxicity	• Membrane irritancy/ sensitivity • Bioavailability • Bioaccumulation
TOPKAT	Automated rule-based QSAR	www.accelrys.com	• Carcinogenicity • Mutagenicity • Developmental toxicity • Skin sensitization	• Eye irritancy • Biodegradability • Acute toxicity • Chronic toxicity
OncoLogic®	Decision tree approach	http://www.epa.gov/oppt/cahp/actlocal/can.html	• Carcinogenicity	
COMPACT	Knowledge-based QSAR	www.surrey.ac.uk/SBMS	• Carcinogenicity via CYP1A-related and CYP2E-related metabolic activation	

[a]The endpoints that can be predicted using each expert system are based on the current status of each system. More information about these methods and how they can be applied is available from http://www2.defra.gov.uk/research/project_data/more.asp?!=CB01067&M=KWS&V=reach&scope=0. Website links accessed 30 January 2007.

(PBPK) modeling is a way of predicting ADME *in vivo* by combining results from the literature and from computational techniques [124], and by extrapolating data from *in vitro* studies between species. Some key PBPK methods are listed in Table 1.11. Plasma protein binding can have a crucial bearing on the internal dose, since it determines the free (unbound) concentration (this also has a bearing on the composition of cell culture media) [125]. Differential equations can be derived, which, when solved, provide information of relevance to humans. A better, biologically based, dose–response model of *in vivo* toxicity can then be developed from external dose data.

Significant advances are currently being made in biokinetic modeling, including the development of software programs and databases for the rapid generation of new models (http://www.hsl.gov.uk/capabilities/pbpk-jip.htm). These will improve the usefulness of this approach for evaluating large numbers of chemicals and will assist with the interpretation of *in vitro* hazard predictions for risk assessment purposes.

TABLE 1.11 Examples of Programs for the Prediction of Biokinetic Properties

Name	Supplier	Website	Properties Predicted[a]
Cloe PK®	Cyprotex	www.cyprotex.com	• Potential exposure • Absorption from GI tract • Plasma, tissue, and organ concentrations • Renal excretion • Hepatic metabolism
iDEA pkEXPRESS™	LION Bioscience	www.lionbioscience.com	• Absorption from GI tract • Systemic circulation • Bioavailability • Plasma concentration • Elimination
Megen100	Health & Safety Lab	www.hsl.gov.uk/capabilities/pbpk.htm	• Oral and intravenous absorption • Concentration–time profiles for plasma and major organs and tissues • Hepatic metabolism
PK-Sim®	Bayer Technology Services	www.bayertechnology.com	• Oral absorption • Concentration–time profiles for plasma and major organs and tissues • Bioavailability • Renal and billiary excretion

[a]More information about these methods and how they can be applied is available from http://www2.defra.gov.uk/research/project_data/more.asp?!=CB01067&M=KWS&V=reach&scope=0.

One key issue is the role of transporter proteins in the absorption and uptake of a drug or its metabolites, as this will determine the internal dose to which any particular organ is exposed. Problems with crystallizing membrane proteins have made it inherently difficult to generate 3D models of many important transporter proteins. Homology and comparative modeling can, however, be used to generate models by reference to experimental data, the structure of related proteins, and more fundamental predictions of protein folding and tertiary structure, made from the primary amino acid sequences of relevant proteins. For instance, three ATP-binding cassette (ABC) transporters of bacterial origin have been crystallized, and from these structures, comparative modeling has been used to generate models of ABC transporters that play a key role in drug efflux in humans. Another approach is to generate a pharmacophore from experimental data for known transporter binding molecules, as in the case for monoamine transporters, then to use QSAR systems to make

predictions as to whether a novel drug is likely to be a transported. These approaches are discussed in more detail in Ref. 126.

1.8 ANIMAL MODELS USED IN PRECLINICAL TESTING OF PHARMACEUTICALS

1.8.1 Selection of a Suitable Test Species

Although not an absolute regulatory requirement, it is still widely accepted that drug development should involve initial studies conducted in a rodent species, followed by studies in a nonrodent species. In addition, further studies may be conducted in other species, such as the rabbit (e.g., for local tolerance and for pyrogenicity). There is often flexibility in the nature of the tests required by most regulators for a new medicine's dossier. For instance, where only a single species can be shown to be relevant, regulators may be willing to consider information from nonanimal studies to decide whether the two-species testing requirement can be waived.

The Universities Federation for Animal Welfare (UFAW) has published a handbook that lists the major characteristics of laboratory species, to which the reader is referred [127]. Perhaps of more general importance is the use of existing preclinical, clinical, and basic research data in the species selection process. Information from mechanistic and early *in vitro* studies is particularly important, as is understanding differences between species in the spatial and temporal expression of the drug target and in target modulation and activity. The starting point is understanding any significant differences between the pharmacology of the human protein and the possible test species. Sequence homology and structural homology do not guarantee the functional equivalence of species homologs. Once functional equivalence has been established by using cell expression systems, the next stage is to understand the tissue distribution of the target in the selected test species. This can involve *in vivo* studies with target-specific probes such as fluorescent or radiolabeled antibodies or ligands. In general, however, this information is more readily attainable from tissue distribution studies, including immunohistochemistry. A good illustration of how this can be important was made in a recent paper, which highlighted differences between the human and rat versus the mouse in the distribution and pharmacology of serotonin receptors, which showed that the mouse is a poor preclinical model for some classes of antipsychotic agents [128]. The rat was a more suitable rodent model, although the mouse has already been used in some preclinical studies for CNS-active serotoninergic drugs. With the greater availability of genomic information, species selection (or, indeed, the selection or generation of an appropriate GM animal model) can be made on the basis of amino acid sequence, as is the case when developing humanized antibodies. In some cases, this may obviate the need to conduct extensive studies in higher order vertebrates, such as primates.

In addition to differences in target expression, differences in plasma clearance rate and routes of excretion can also have a crucial bearing on whether a particular species or strain is used [129]. Differences between anatomical and functional properties associated with the intended route of administration (as in the case of nasal absorption, see Section 1.3.2), functional differences between species homologs of

efflux and transporter proteins such as P-gp [130], and differences between the distribution and activities of key metabolic enzymes predicted to be involved in the biotransformation of a drug may limit the choice of test species.

Where more than one species is likely to be used for preclinical studies, the choice of species may also involve a consideration of the need for allometric scaling. This allows the prediction of human pharmacokinetics from the pharmacokinetics of a drug in a test species, by taking body weight into account. A recent study [131] indicates that although allometric scaling is most reliable when PK parameters are available for five species, certain three-species combinations (such as mouse/rat, monkey, and dog) are adequate, whereas other three-species combinations (such as rabbit, monkey, and dog) are significantly worse. In general, two-species combinations are poorer for making allometric predictions, and certain combinations, such as mouse and rat, rabbit and monkey, and dog and monkey, are particularly poor. However, in practice, two-species combinations are commonly used, which usually involve a rodent and a nonrodent species.

Species selection is also dependent on the feasibility of a study and on the availability and cost of acquiring and caring for the animals concerned. Developmental toxicity, for instance, could not be reasonably or ethically conducted on primates or some other vertebrate species and is generally conducted in rodents, because of their small size, shorter life spans, and the larger litter sizes produced at each generation. Preliminary studies may be conducted with fish larval forms and invertebrates.

1.8.2 Experimental Design

The ICH guidelines advocate the principles of the three Rs. That is, prior to the use of vertebrate animals, scientists are required to consider: (1) whether there are alternatives to using animals that can provide information that is as valid and acceptable to regulators as animal data; (2) where there are no replacement alternatives, how prior information and information from nonanimal experiments could be used to reduce the number of animals used; and (3) how animal experiments could be refined in order to reduce suffering (e.g., by using more humane endpoints).

The first step in designing an animal experiment is to clearly define its objectives in terms of the nature of information sought or the hypothesis to be tested. The correct grouping of animals according to their treatment (nature and frequency of intervention, etc.) and husbandry regime (light–dark cycle, handling, monitoring, etc.) reduces variation in experimental data. Standard operating procedures should be developed that take into account the scientific objectives and animal welfare issues associated with each experiment. For instance, all the animals (control, vehicle control, and test animals) should be handled with the same frequency, for the same periods of time and by the same technician, and subjected to identical procedures. Other causes of variation stem from inadvertent infections, which can be minimized by good laboratory practice, routine health surveillance, and using suitably ventilated cages. Whether food and fluid control (i.e., limiting daily supply), timed feeding and water supply, or *ad libitum* feeding and drinking are appropriate can also be an important consideration. The effects of weight, age, sex, and/or strain on the experimental outcome can have important implications for experimental design and can be accounted for in various ways, for example, by appropriate grouping of animals

(some examples are given in http://oslovet.veths.no/compendia/LAS/KAP28.pdf). This is because different strains of rats can vary dramatically in their clinical responses to a drug, so selecting the most appropriate isogenic strain can be difficult and it might sometimes be appropriate to use more than one strain in each experiment. There are a number of useful resources on this topic (e.g., Ref. 132).

The number of possible variables highlights the importance of a pilot study. Pilot studies that are designed on the basis of existing information can be used to identify logistical, animal welfare, and scientific problems and to address specific scientific questions, prior to the conduct of larger animal studies [133]. Whether an experiment entails multiple endpoints, different sexes, and/or different strains, the interpretation of the data might be compromised without prior and careful statistical planning, or in the worst case scenario, be impossible, resulting in the need to repeat the experiment.

The ICH guidelines recommend carcinogenicity testing, where the weight of evidence suggests it may be required or where there is insufficient evidence to rule out the possibility of carcinogenic hazard. Such testing normally should be conducted only in rats, as there is evidence to suggest that the rat bioassay is more predictive of human risk Cancer-associated biomarkers have an enormous potential to streamline preclinical drug development and can also be used to devlop humane endpoints and facilitate temporal studies in small numbers of animals [134].

1.9 USE OF PRIOR INFORMATION

1.9.1 Sources of Prior Knowledge

Information retrieval is an important aspect of drug development, and a list of databases that provide information that can be used is given in Table 1.12. The tremendous rate at which information is increasing in volume and is diversifying requires that computational tools are developed to support the extraction and ranking of information according to its relevance and reliability. In addition, models of how different elements of a biochemical pathway interact can be derived mathematically, and such models have been used to successfully construct biochemical networks of relevance to drug discovery [135].

Some special sources of prior information include data suites that collate information about specific biochemical pathways, specific diseases, human genetics, and human sub-populations. Because most diseases have complex etiologies, particularly diseases such as cancer, heart disease, stroke, and diabetes, which arise from a combination of lifestyle, environmental, and genetic factors, several large-scale population studies are being undertaken (some examples are given in Table 1.12). These studies may contribute to a greater understanding of the complex basis of such diseases, leading to improvements in drug discovery and target selection where patterns that link genetic differences to drug effects or disease can be established.

Perhaps the most directly relevant information is that available from preclinical and clinical studies on existing therapeutic products. Usually, this information is not publicly available, but some confidential information is being incorporated into many databases. PharmaPendium™ [136] is an example of a recent public resource that includes drug safety data (preclinical and clinical) for FDA-approved drugs. Such resources are likely to prove invaluable for researchers and regulators alike.

TABLE 1.12 Information Resources[a]

Omics Databases and Resources	
SRS http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+srsq2+-noSession	A gene/protein sequence retrieval system that can be used to browse various biological sequence and literature databases
ToxExpress http://www.genelogic.com/genomics/toxexpress/	A toxicogenomic profiling suite that can be used in biomarker discovery
MIAME http://www.mged.org/Workgroups/MIAME/miame.html	Minimum Information About a Microarray Experiment: criteria needed to permit the interpretation of the results of the experiment unambiguously and, potentially, to reproduce the experiment
Array Track http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/	Developed by FDA National Center for Toxicological Research (NCTR); an integrated suite designed to manage, analyze, and interpret microarray data
KEGG http://www.genome.jp/kegg/	Kyoto Encyclopedia of Genes and Genomes provides a complete computer representation of the cell, the organism, and the biosphere
Unigene http://www.unigene.com	Provides an organized view of the transcriptome
SNP http://www.ncbi.nlm.nih.gov/projects/SNP/	The Single Nucleotide Polymorphism database
SPAD http://www.grt.kyushu-u.ac.jp/spad/	The Signaling PAthway Database (SPAD): an integrated database for genetic information and signal transduction systems
Pharmacology, Toxicology, and Biological Systems	
BIOPRINT http://www.cerep.fr/cerep/users/pages/Collaborations/bioprint.asp	A pharmacology and ADME database that contains *in vitro* pharmacology profiles, ADR, PK and clinical data for over 2500 marketed drugs, failed drugs, and reference compounds
BioRS http://biors.gsf.de:8111/searchtool/searchtool.cgi	A biological data retrieval system
CEBS http://www.niehs.nih.gov/cebs-df/index.cfm	Chemical Effects in Biological Systems: a knowledge base for information and resource exchange
BIND http://bond.unleashedinformatics.com	Biomolecular Interaction Network Database: designed to store full descriptions of interactions, molecular complexes and pathways
BioCarta http://www.biocarta.com/	Provides interactive graphic models of molecular and cellular pathways
BRENDA www.brenda.uni-koeln.de	A collection of enzyme functional data
CSNDB http://geo.nihs.go.jp/csndb/	Cell Signaling Networks Database: a database and knowledge base for signaling pathways of human cells
SwissProt http://expasy.org/sprot/	A protein sequence database with descriptions of the function of proteins, protein structure, posttranslational modifications, variants, etc.
TransPath http://www.biobase-international.com/pages/index.php?id=transpath	Provides information about (mostly mammalian) signal transduction molecules and reactions, focusing on signaling cascades that change the activities of transcription factors and thus alter the gene expression profiles of a cells

TABLE 1.12 ***Continued***

PathArt http://jubilantbiosys.com/ppa.htm	A database of biomolecular interactions with tools for searching, analysis and visualization of data
DSSTox http://www.epa.gov/ncct/dsstox/index.html	The EPA's Distributed Structure-Searchable Toxicity Database for improved structure–activity and predictive toxicology capabilities
TOXNET http://toxnet.nlm.nih.gov/	A database on toxicology, hazardous chemicals, environmental health, and toxic releases
PharmGKB http://www.pharmgkb.org/	A database on relationships among drugs, diseases, and genes
	Literature Database
PubMed www.pubmed.com	A database that includes over 16 million citations from MEDLINE and other life science journals for biomedical articles back to the 1950s; PubMed includes links to full text articles and other related resources
Bio-Frontier P450/CYP http://www.fqs.pl/	A database for testing CYP interactions
	Human Population Genetics and Toxicity Databases and Resources
The Collaborative on Health and the Environment http://database.healthandenvironment.org/	A searchable database of links between chemical contaminants and human diseases
The Personalized Medicine Research Project (Marshfield Project) http://www.marshfieldclinic.org/chg/pages/default.aspx	A human population genetic database to understand the interplay of human genetics, diseases, and environmental factors
Medgene^{SM} database http://hipseq.med.harvard.edu/MEDGENE/login.jsp	A database of disease-associated genes
CARTaGENE http://www.cartagene.qc.ca/	A source of information on the genetic variation of a large population
Latvian Genome Project http://bmc.biomed.lu.lv/gene/	Large-scale human population genetic project to discover disease linkages
Estonian Genome Project http://www.geenivaramu.ee/	A source of information on the genetic variation of a large population
The United Kingdom Biobank (UK Biobank) http://www.ukbiobank.ac.uk/	Genetic and medical information is being collected for 500,000 UK volunteers
Translational Genomic Research in the African Diaspora (TgRIAD) http://www.genomecenter.howard.edu/TGRIAD.htm	A database to understand disease, genetics, and environmental factor linkage in people of African descent
Obesity gene map database http://obesitygene.pbrc.edu/	A database of genetic markers associated with obesity
COGENE the Craniofacial and Oral Gene Expression Network http://hg.wustl.edu/cogene/	A consortium that looks at the genetics of early development, in particular, craniofacial disorders

TABLE 1.12 *Continued*

Human genome variation database http://hgvbase.cgb.ki.se/	Contains links to a number of single nucleotide polymorphism databases for particular diseases
The International HapMap Project http://www.hapmap.org/	A consortia aimed at finding genes associated with human disease and response to pharmaceuticals
GenomEUtwin http://www.genomeutwin.org/	A database of human population genetics aimed specifically at finding genetic and lifestyle linkages to disease that involves studies on twins
Public Population Project in Genomics (P3G) http://www.p3gconsortium.org/	A consortium that aims to develop a human population genetics database

[a]This is a list of resources that are applicable to understanding the output of omics-based studies and compiling a systems biology view of diseases and drug effects. Websites accessed 30 January 2007.

Other databases include the Adverse Drug Effects database [137], which stores information on approved drugs, including the severity and incidence of adverse effects, which is relevant to the discovery and design of new clinical products.

1.9.2 Standardization of Data Collection and Meta-Analysis

The quality and completeness of the available toxicological data will significantly affect the level of confidence in the preclinical data. The application of Good Laboratory Practice (GLP) should increasingly help to standardize the way in which experiments are designed, conducted, and reported, thereby improving the quality of the information available for guiding subsequent studies.

Meta-analysis is a statistical approach, which is used to combine data from different sources, but it needs to be applied with great caution. It is particularly difficult to use meta-analysis when different datasets contradict one another. Nevertheless, the use of surrogate endpoints, such as biomarkers, may dramatically improve the power of meta-analysis, since appropriate biomarkers can be used to increase the credibility of animal and human cell-based and tissue-based preclinical studies, and to facilitate extrapolation between such *in vitro* studies and preclinical *in vivo* studies, in animals and in humans.

Several potential biomarkers of exposure and toxicity can be considered. For example, such a scheme was originally proposed by Sobels [138] for the extrapolation of data on genetic damage from animals to humans, and was subsequently modified by Sutter [139] to permit *in vitro–in vivo* extrapolation. In some cases, threshold doses can be set, solely on the basis of *in vitro* tests (e.g., for some genotoxins). This parallelogram approach can then be used to extrapolate preclinical data to effects on humans, according to the paradigm:

$$\text{Human } (\textit{in vivo}) \text{ toxicity} = \frac{[\text{Rodent } (\textit{in vivo}) \text{ toxicity} \times \text{Human } (\textit{in vitro}) \text{ toxicity}]}{\text{Rodent } (\textit{in vitro}) \text{ toxicity}}$$

The concept assumes that the ratio of *in vitro* toxicity to *in vivo* toxicity for any particular endpoint is broadly comparable across species. Up to now, this approach has been used for extrapolating data on genetic damage but has proved to be less useful for extrapolating other forms of toxicity data to humans, because of the relatively complex mechanisms of toxicity that are involved. Nevertheless, in a recent paper [140], the concept was applied to a comparison of rat and human skin penetration rates *in vitro* and to predicting the *in vivo* effects of topically applied substances. As key biomarkers for drug effect and toxicological endpoints become available, the applicability of this approach is likely to expand to other areas of drug development and safety pharmacology.

1.10 CONCLUSIONS

Despite decades of research and development, the issue of adverse drug reactions that result in drug withdrawals remains a significant problem. This problem is confounded by the fact that information from clinical studies on human volunteers and patients is often kept from public scrutiny. Indeed, only a small number of pharmaceutical companies post their clinical trials information on publicly available registers. A recent study indicates that target organ toxicities in humans are not always predicted reliably by preclinical tests in animals. The predictivity of cardiovascular, hematopoietic, and gastrointestinal toxicity is around 80% but is lower for toxicity to the liver, skin, and nervous system [141]. Hence, there is an urgent need for a new approach to drug development, which involves the targeted use of new and advanced technologies that are based on defined cell systems, either as standalone alternatives to animal studies or as tools to assist with the extrapolation of animal data to humans.

Indeed, in 2004, the FDA produced a report that suggested that the fall in drug development returns was due largely to the failure to use the new technologies such as genomics, proteomics, and bioinformatics platforms to detect safety problems that cannot be identified in the more traditional animal-based methods. These newer, and often systems biology-based, approaches hold enormous potential in this respect but are very much in their infancy. One of the most significant problems is the difficulty in standardizing and validating these new technologies, in order to ensure that the quality of data and the quality of data analysis form a suitable basis for safety assessments. These systems are being developed at an unprecedented rate. For example, a consortium of global pharmaceutical giants has been assembled to put forward biomarkers and screening assays for consideration by the FDA. It remains to be seen whether this initiative will reduce the current drug attrition rate. Preclinical planning must look at both the existing regulatory requirements and the scope for cost- and time-effective studies that make the maximum use of the new and exciting technologies.

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