CHAPTER

1

Introduction

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Drug development is defined as the entire process of bringing a new drug or device to the market. It involves discovery and synthesis, preclinical development (chemical testing, biological testing, pharmacology, toxicology, safety, etc.), clinical development (Phase I–III), regulatory review, marketing approval, market launch and post-marketing development (Figure 1.1).

The process of drug discovery comprises research on 1) target identification, 2) target prioritization/validation, 3) lead identification, and 4) lead optimization.

A range of techniques are used to identify and isolate individual drug targets. The target identification process isolates drugs that have various interactions with the disease targets and might be beneficial in the treatment of a specific disease. This is followed by a target prioritization phase, during which experimental tests are conducted to confirm that interactions with the drug target are associated with the desired change in the behavior of diseased cells. Identification of lead compounds are sometimes developed as collections, or libraries, of individual molecules that possess the properties required in a new drug. Once the lead is identified, experimental testing is then performed on each of the molecules to confirm their effect on the drug target. This progresses further to lead optimization. Lead optimization studies are conducted on *animals* or *in vitro* to compare various lead compounds, to determine how they are metabolized, and what affect they might induce in the body. The information obtained from lead optimization studies helps scientists in the pharmaceutical industry to sort out the compounds with the greatest potential to be developed into a safe and effective drug.

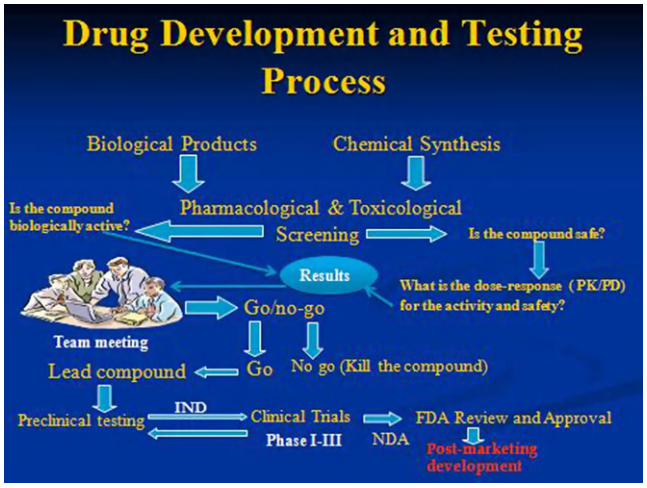

FIGURE 1.1 **The drug development process.**

Toxicology studies in the drug discovery process are conducted to evaluate the safety of potential drug candidates. This is accomplished using relevant animal models and validated procedures. The ultimate goal is to translate the animal responses into an understanding of the risk for human subjects. This demands additional studies and investment earlier in the candidate evaluation, coupled with an arduous selection process for drug candidates and a speedy kill to avoid spending money and time on species that would likely fail in development.

Even after a successful drug candidate for a disease target is identified, drug development still faces enormous challenges; which many drugs fail because of their unacceptable toxicity. Safety issues are the leading cause of attrition at all stages of the drug development process and it is important to understand that the majority of safety-related attrition occurs pre-clinically, suggesting that approaches which could identify 'predictable' preclinical safety liabilities earlier in the drug development process could lead to the design and/or selection of better drug candidates with increased chances of being marketed.

The successful drug candidate undergoes a preclinical safety testing program. Key factors affecting the type of preclinical testing include the chemical structure, nature of the compound (small molecules or biologics),

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proposed human indication, target population, method of administration, and duration of administration (acute, chronic). During preclinical drug testing, the toxicity and pharmacologic effects of the New Chemical Entity (NCE) are evaluated by *in vitro* and *in vivo* laboratory animal testing. Genotoxicity screening is performed, as well as investigations on drug absorption and metabolism, toxicity of the drug's metabolites, and the speed with which the drug and its metabolites are excreted from the body. Likewise, the drug companies will require a pharmacological profile of the product to be developed, including safety pharmacology – the acute toxicity of the drug in at least two species of animals, and short-term toxicity studies ranging from 2 weeks to 3 months must be conducted, depending on the proposed duration of use of the NCE in the proposed clinical studies. Furthermore, preclinical testing may include chronic toxicity, carcinogenicity, developmental and reproductive toxicology testing. All these studies, together with other specialized study types, such as continuous infusion and photoxicity, are discussed in this book.

It is estimated that it takes eight and more years to develop and test a new drug before it can be approved for clinical use. This estimate includes early laboratory and animal testing, as well as later clinical trials using human subjects.

Preclinical safety data are used to select doses in Phase I clinical trial, to provide information on potential side effects, and thus minimize the risk of serious side effects in clinical trials. It also identifies potential target organs and determines toxicity endpoints not amenable to evaluation in clinical trials such as genetic toxicity, developmental toxicity and carcinogenicity.

Toxicology studies traditionally focus on phenotypic changes in an organism that result from exposure to the drug; therefore, efficient and accurate approaches to assess toxicological effects of drugs on living systems are still less developed. Currently, one of the key factors used for a go/no-go decision making relies on the early knowledge of any potential toxic effect. Thus the traditional approach based on the determination of the No-Observed-Adverse-Effect-Level (NOAEL) is far from accurate. One of the limitations of this approach is that it may fail to detect adverse effects that manifest at low frequencies.

Indeed, in the past 20 years new technologies have emerged that have improved current approaches and are leading to novel predictive approaches for studying disease risk. Increased understanding of the mode of action and the use of scientific tools to predict toxicity is expected to reduce the attrition rate of NCE and thus decrease the cost of developing new drugs. In fact, most big pharmaceutics companies are now using improved model systems for predicting potential drug toxicity, both to decrease the rate of drug-related adverse reactions and to reduce attrition rates. A wide range of biological assay platforms, including toxicogenomics and metabolomics employed in constructing predictive toxicity, are included as separate chapters in this book. The discipline of toxicogenomics is defined as the application of global mRNA, protein and metabolite analysis-related technologies to study the effects of hazards on organisms. Examining the patterns of altered molecular expression caused by specific exposures can reveal how toxicants act and cause their effect. Identification of toxicity pathways and development of targeted assays to systematically assess potential mode of actions allow for a more thorough understanding of safety issues. Indeed, there is high expectation that toxicogenomics in drug development will predict/better assess potential drug toxicity, and hence reduce failure rates.

In addition metabolomics, a more recent discipline related to proteomics and genomics, uses metabolic signatures to determine the molecular mechanisms of drug actions and predict physiological toxicity. The technology involves rapid and high throughput characterization of the small molecule metabolites found in an organism, and is increasingly gaining attention in preclinical safety testing.

This book is a comprehensive guide for toxicologists, regulatory scientists and academics hoping to understand safety testing and the drug development process. It provides a snapshot of the complex and highly interrelated activities of preclinical toxicology in small molecules and biologics. The book also highlights several specific areas, including preclinical drug development of oncogenic and non-oncogenic drugs, oligonucleotides, vaccines, ocular drugs, botanics and monoclonal antibodies. In addition, the book has several unique chapters in areas such as imaging, molecular pathology, abuse liability and biostatistics. The final chapter 'Practical aspects of developing in-licensed pharmaceutical products' is intended for small biotech executives with limited funds and resources to advance the drug development process from discovery through to marketing approval. The chapter addresses the chronology of the in-licensing of product candidates.

In closing it must be emphasized that one of the biggest strengths of this book comes from its contributors, who are considered to be authorities in their field. Generally, knowledge of sciences gained through experience in the field shapes personal lives as well as the thinking in the decision making process for day-to-day activities. The experiences of the individual authors currently active in their own specialized areas of interest are carefully crafted in each chapter.

Finally, I would like to thank the contributors for their commitment, and hard work. I also want to express my deep gratitude to Kristine Jones, April Graham, Andy Albrecht and all the production team at Elsevier.

CHAPTER

2

ADME in Drug Discovery

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INTRODUCTION

An Overview of ADME (Absorption, Distribution, Metabolism, Excretion) Science

The scientific discipline of preclinical drug discovery and development can be described as a risk assessment process, whereby data are used to estimate the usefulness of some agent in preventing, curing, or slowing the progression of human disease. The preclinical phase of research allows clinical studies to be initiated and proceed with some knowledge of risk-benefit. It is an iterative process that varies between different programs at any one time. It is also constantly evolving, as new knowledge and technologies are rapidly introduced. The research plan of today has many general similarities and significant differences from 25 years ago. The constants in this process are drug efficacy and drug safety evaluation, which together represent the Science of Pharmacology, the Science of Drugs. The toxicokinetics, pharmacokinetics in a toxicology study, or the study of the relationship of exposure to toxicity, are important for the design of safety studies (toxicology, safety pharmacology, developmental and reproductive toxicology, etc.). These data allow for estimation (calculation) of a safety margin in preclinical studies and ultimately the early estimation of a *Therapeutic Index* in humans. In parallel, the study of absorption, distribution, metabolism and excretion are central to finding new, safe and effective drugs. The central message of this chapter is that early characterization of PK (pharmacokinetic) properties is critical to the development of successful drug discovery programs [2–7].

The ADME scientists have two 'customers' in the preclinical setting: The *drug discovery* scientists, who provide new chemical entities for evaluation in various pharmacology and toxicology screens, and the preclinical *drug development* scientists who provide more refined evaluation of safety and efficacy for preparation of the IND. ADME studies supply the toxicologist with critical measurements of exposure which can be correlated with observed toxicity, which in turn directly relates to

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Therapeutic Index. Early on in the drug discovery and development process, ADME scientists are interested in estimating clearance (CL), bioavailability (F) and pharmacokinetic/pharmacodynamic (PK/PD) data for entry into compound libraries. In addition, ADME scientists are charged with providing to their toxicology colleagues an understanding of exposure and toxicity, the PK/PD (or TK/TD; toxicokinetic/toxicodynamic) relationship and an assessment of the role of metabolism, transporters, drug metabolizing enzymes and drug accumulation in drug safety. This chapter will address ADME in discovery research, or ADME at the interface of drug discovery and drug development, which is commonly now referred to as *early-ADME* (*eADME*). Not all topics will be covered. For example, plasma protein binding (PPB) has been omitted, since it is less important than critical concepts such as stability and clearance [8].

The characterization of ADME properties of compounds early in the drug discovery process has well characterized value for the selection of better drug candidates, and has become more important as technologies impacting this process have developed and matured [9–11]. The cytochrome P450 (CYPs) enzymes are intimately involved in ADME. The catalytic cycle of the P450-dependent monooxygenase system is displayed in Figure 2.1 (showing the second electron insertion step from cytochrome b_5). Over the last 20 years, an understanding of the biochemistry of the Cytochrome P-450 system and the role that CYP inhibition, CYP phenotype and CYP induction plays in the identification of better drug therapies has impacted how preclinical ADME research is conducted [12–14]. Consider that 20 years ago approximately 40% of clinical drug failures could be tied to PK and ADME problems, and today this failure rate is 10% or less for companies with comprehensive, state-of-the-art preclinical discovery/development programs addressing these issues [15]. The drug discovery process continues to evolve and early ADME evaluation has become a routine part of the 'Big Picture' process to examine the utility of drug templates in the discovery of novel therapeutics. At time of writing, the FDA released Guidance for Industry, Drug Interaction Studies, Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, which provide much needed regulatory guidance for many of the ADME investigations discussed in this chapter [16].

Definitions. As already stated, the two constants in the drug discovery process are an assessment of drug efficacy and drug safety. Pharmacology is divided into two distinct domains, the separate but interactive domains of *dynamics* and *kinetics*. *Pharmacodynamics* (*toxicodynamics*) or PD (TD) is the study of the effects of *xenobiotics* (drugs; foreign substances; opposite of *endobiotics*) on the body. *Pharmacokinetics* (*toxicokinetics*) or PK (TK) is the study of the effects of the body on the xenobiotic, or the study of the journey of the drug molecules (the atoms) through and out of the body. Pharmacokinetics, in the broad sense of the term as defined by Leslie Benet [19], includes concentration-time kinetic relationships, chemical reaction kinetics and the formation of new chemical structures (biotransformation; formation of drug metabolites). As stated in *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (2006):

> 'When a drug enters the body, the body begins immediately to work on the drug: absorption, distribution, metabolism (biotransformation), and elimination. These are the processes of *pharmacokinetics*. The drug also acts on the body, an interaction to which the concept of a drug receptor is central, since the receptor

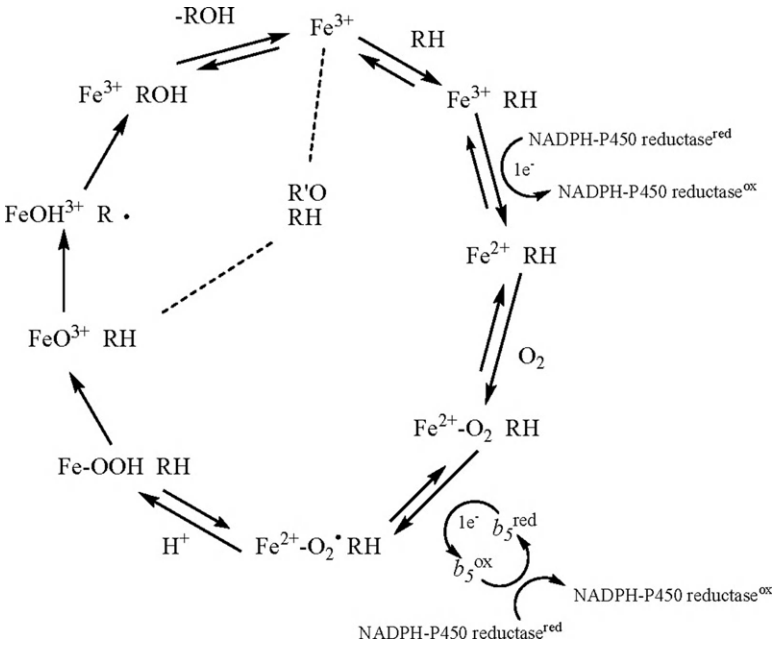

FIGURE 2.1 The catalytic cycle of the P450-dependent monooxygenase system, with the second electron insertion step from cytochrome b_5 (alternatively, NADPH may serve this function).

is responsible for the selectivity of drug action and for the quantitative relationship between drug and effect. The mechanisms of drug action are the processes of *pharmacodynamics'* [17].

It has become common practice to segregate 1) The study of the '*ADME*' of a drug, and in particular the ADME determined by following the distribution of radioactivity, from the narrower definition of 2) PK as the sojourn of the parent drug into, through and out of the blood, and in particular concentration-time plasma/blood data as determined by a selective quantitative method developed for the parent drug, more recently almost exclusively using liquid chromatography-mass spectrometry analysis (LC-MS) for small molecules. Another popular acronym in common usage is DM&PK, i.e., drug metabolism and pharmacokinetics which encompasses the broad definition of PK. Confusing, isn't it? This is why the authors prefer the older, all-encompassing term 'kinetics'/'pharmacokinetics'. ADME is used here by default of common usage. Pharmacokinetics of the parent drug, and active or toxic metabolites is covered in a separate chapter.

Absorption, distribution, metabolism and excretion of a xenobiotic is related to the intrinsic properties of the chemical structure, including its molecular weight, the shape of the molecule ('chemical space'), the ionization properties, the degree of lipophilicity and water solubility of the various forms (charged and uncharged sites), and associations with macromolecules (e.g., a tissue protein binding drug). Some properties are of obvious relevance: compounds that are rapidly metabolized in the liver have poor oral bioavailability. The common barrier to drug distribution is the cell membrane, which is why in the absence of other mechanisms such as active transport (transport of nutrients, for example), substances moving into and out of the cell can pass across the plasma membrane as a result of their lipophilic properties. Other properties determining ADME are not so obvious. For example, redistribution is the mechanism responsible for termination of action of thiopental, a highly lipophilic drug, which rapidly partitions into the brain to act briefly and then redistributes into other tissues, eventually concentrating in adipose tissue [18]. In this example, a physico-chemical property of a drug dramatically effects drug kinetics and therefore dynamics.

Drugs are administered by various routes of administration:

1. Starting outside the body including oral, topical (skin, nasal mucosa, ocular topical),
2. Having an intermediate starting location, such as rectal, vaginal and inhalation,
3. Parenteral routes: intravenous (IV), intramuscular (IM), intraperitoneal (IP), subcutaneous (SC) and depositions (DEPOT).

There are also special parenteral routes, such as intra-articular and various ocular parenteral routes (intravitreal and retrobulbar, for example). The oral route is by far the most important route when discussing ADME and Drug Discovery. We will focus on this route in this chapter, and will not specifically discuss any unique kinetics and ADME associated with other routes of administration.

ADME in Drug Discovery

The drug discovery process is complicated and interdisciplinary. Scientists must work with drug discovery teams for a significant period of time to gain the experience and clarity of scientific vision to lead drug discovery programs. The overall process is usually described as consisting of drug discovery and drug development 'phases', with considerable overlap between these phases [19]. The process (Figure 2.2) can also be described in terms of preclinical and clinical phases; where there is a clear demarcation of activities (the term *commercialization phase* for late stage activities

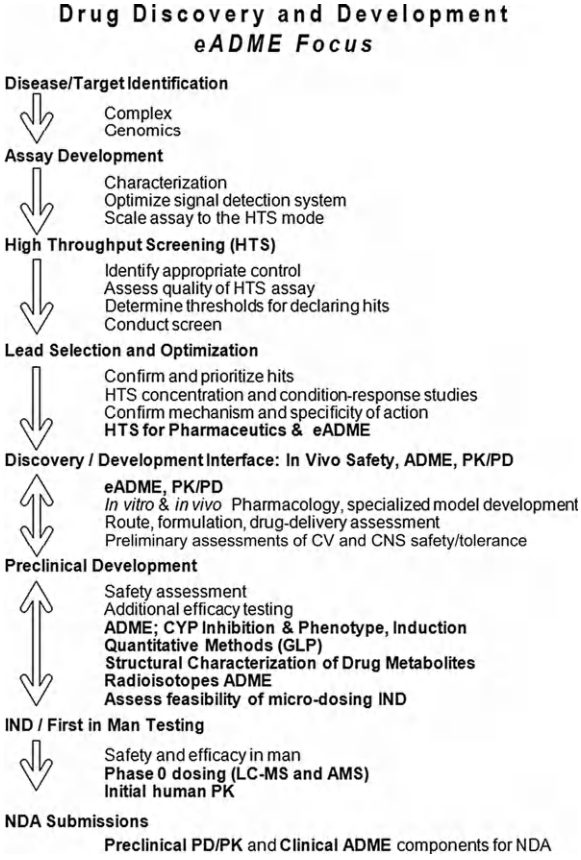

FIGURE 2.2 The *traditional* drug discovery and development process, ADME focus in bold. Different individuals will draw this differently. This is a highly complex and constantly evolving research process.

has also been used). In the modern setting, the pharmacological basis of therapeutics is a highly interactive, dynamic process that includes several iterations of the following processes: The identification of a drug target that will produce the desired effect (decreasing blood pressure, for example); the development of some methodology to evaluate the effect(s) of compounds on this target (*assay* development); the use of this assay to evaluate a large number of compounds (to *screen* a *drug library*); and more refined testing of the pharmacological and toxicological properties of the chemical template and/or lead compounds. The process eventually transitions into a drug development phase, in which a small group of 'lead compounds' are evaluated in a more stringent manner, including *in vivo* testing. When successful, this process leads to selection of a few (1−2) compounds as successful IND candidates and entry into Phase I Clinical Trials [20−25]. The target ID stage has changed with the sequencing of the human genome and the introduction of the *'omics'* technologies of genomics, proteomics and metabolomics. Although the hoped-for revolutionary impact of the '*omics*' and combinatorial chemistry in greatly improving the drug discovery process has not come to fruition, continued technological advances have improved the process of evaluating and testing drug targets. New, safer and more effective drug therapies, both small molecule and large molecule (predominately biologics), will be a part of our future [26−28].

Technological advances impacting the ADME part of pharmacology research include:

1. The ability to follow drug-related material in fluids and tissues without radioactive studies,
2. The early application of PET/SPECT imaging of biologics for early drug disposition studies,
3. The successful identification of 'biomarkers' useful in characterizing PK/PD (TK/TD) relationships,
4. The increased role of *in silico* in making predictions of certain ADME properties for chemical templates and individual compounds.

Technological advances will continue to dramatically impact the *eADME* research process. It is indeed an exciting time for scientists active in the field of drug discovery and development.

One of the most important aspects in determining the 'what and when' for studying ADME properties is cost effectiveness, since cost per compound and the cost of each step increase exponentially at each stage of the drug discovery/development process. The vast majority of compounds do not have the necessary intrinsic properties to constitute effective and safe therapeutics in man, and thus the real job of the drug development scientist is to identify compounds with 'losing' properties, which is a process of elimination, or as drug discovery/development scientists are fond of saying, 'finding and killing the losers'. Thus, the actual job of the drug development scientist is to 'kill' compounds/programs. Those that survive will have a far better chance of success in the clinic. *eADME* is a critical part of this evaluation process.

So, where are 'ADME data' first gathered in the drug discovery process? The answer is that, with the exception of the very earliest stages of new compound characterization, research protocols designed in part to assess ADME properties occur at all stages of the drug discovery/development process, including early studies, as part of the first chemical properties listed in 'Drug Libraries'. For example, an assessment of CYP3A4 inhibition liability (covered below) may be determined along with water solubility and plasma stability and represent one of the early data points determined for new compounds.

The interest in ADME is easy to understand since failure of drugs in the clinic is typically due any of three distinct reasons:

1) Efficacy, 2) Safety and 3) ADME (PK)

Effective ADME programs can greatly impact success in the clinic and early assessment of ADME characteristics has real merit in improving the drug discovery and development process [15]. This chapter has been divided up into:

a. Absorption,
b. Distribution and elimination
c. Metabolism.

Distribution and elimination are considered together, since they are often characterized together (e.g., MS analysis of tissues and excreta) and elimination can be considered to be *distribution out of the body*. Large molecules and biologics will not be considered in this chapter. The chapter 'Use of Imaging for Preclinical Evaluation' (e.g., PET and SPECT) discusse large molecules.

ADME

Absorption

In order for a xenobiotic (drug) to reach the blood, the 'central compartment', when ingested orally (Figure 2.3), it must first pass out of the gastrointestinal tract and be delivered to the liver via the portal vein (the portal vein conducts blood from the digestive system, spleen, pancreas, and gallbladder to the liver). The drug and its metabolites are then available to move into the liver, and from the liver to the blood, where they are then distributed throughout the body by the arterial circulation [29]. There are two major anatomical and

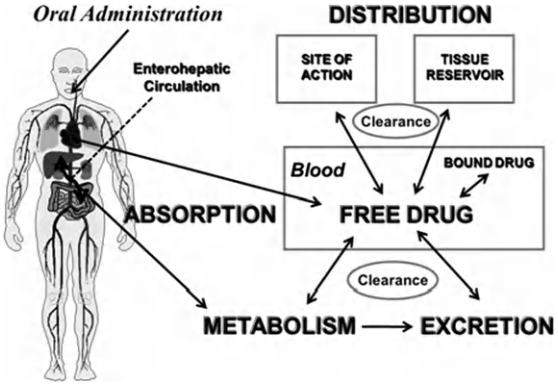

FIGURE 2.3 Scheme for movement of drugs through the body following oral administration.

biochemical barriers to movement of drug from the intestinal lumen to the blood:

1. The tissues between the intestinal lumen and the portal blood and
2. The liver tissues.

The liver is the most important site of the metabolism of xenobiotics, and in this capacity serves as a protection system for the body from chemical insults. Over half of the drugs on the market are primarily cleared by metabolism. It is not surprising that experimental protocols designed to approximate the oral absorption process use tissues and enzymes associated with this process. The important role of GIT transporters and metabolic enzymes in drug absorption is a subject of considerable past and present scientific interest.

The above points concerning movement of drug from the GIT to the blood are very important, since most drugs are administered orally (PO). Physico-chemical properties (e.g., solubility), cell membrane permeabilities, specificities for transporters and drug metabolizing enzyme substrate specificities are important in oral absorption, and thus also in the characterization of compounds under evaluation.

Physico-Chemical Properties and Permeability

Scientists experienced with the drug discovery and development have coined the phrase 'does it look like a drug' – by which they mean do the physico-chemical properties of the drug candidate fit the drug profile (fall within some characteristic range; small molecules). One of the more useful observations concerning physico-chemical properties is the 'Lipinski rule of 5' which states that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight (MW) is greater than 500 and the calculated Log P (CLogP) is greater than 5 [30–31]. Small molecule compounds (drug candidates) with atypically large molecular weights and a large number of heteroatoms do not 'look' like orally available drugs. One good example of a drug which successfully entered clinical development and which does not 'look' like it would exhibit significant oral bioavailability (F) is tirilazad (Freedox®). This drug must be administered intravenously (IV), and has a nominal MW of 624, a CLogP of 5.02, two carbonyl oxygen atoms and 6 basic nitrogen atoms (Figure 2.4). The alicyclic tertiary amines represent good candidates for CYP metabolism. It is not surprising that the oral bioavailability (F) for tirilazad is zero to extremely low.

In Silico. The use of software to predict chemical, pharmaceutical and biological properties of compounds from chemical structures is an area of intense interest. This subject lies outside the scope of this chapter and will only be mentioned briefly. Several recent overviews have been published [32–36]. *In silico* prediction of physico-chemical properties has developed to the point of being relatively useful for Log P, Log D, pKa and lipophilicity, but prediction of water solubilities has proved to be far more difficult. One reason for this is that predicting the various *forms* that a solid can take (such as crystalline vs. amorphous solid) is difficult for novel compounds. Prediction of ADME properties by *in silico* methods is highly variable and is less effective for novel compound templates.

Physico-chemical properties (water solubility, Log D, CHI, stability). Physico-chemical properties of compounds, such as molecular weight, charge state, water solubility and lipophilicity, in part result in the observed *in vivo* ADME properties. As for their influence on what is called the '*drug-ability*' of compounds (a slang term referring to certain properties of a compound or template as relative to overall 'ideal' drug properties), exhibiting *poor* physico-chemical properties (pharmaceutical properties) is not always

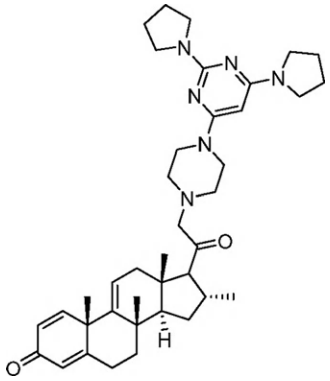

FIGURE 2.4 The structure of tirilazad, Freedox®. This compound is a good example of a drug that does not follow *Lipinski's Rule of 5*.

a show stopper, but can make drug development very difficult. Water solubility and lipophilicity influence the dissolution of drugs in the GIT and the ultimate free drug concentration, since they determine the ability of the drug to dissolve in and move through cell membranes and distribute throughout the body. Since water solubility, lipophilicity and permeability are important parameters in estimating drug absorption properties *in vivo*, they are discussed in this section.

The solubility of a compound in water is measured at thermodynamic equilibrium in a saturated solution. The concentration at saturation is determined by LC-UV (LC-ultraviolet) or another appropriate analytical procedure. This is usually done in both water and/or in phosphate buffered saline, pH 7.4, and at physiological osmolality. Water solubility is also estimated in a high-throughput screening (HTS) setup by adding the compound dissolved in DMSO into buffer or water at a wide final concentration range and noting the turbidity of the solution (if cloudy, then the drug is assumed not to be completely in solution).

Log D. A partition coefficient is the ratio of the amount of compound existing in a non-ionized state in two immiscible solvents; usually n-octanol and water. The pH is adjusted such that the predominant form is the non-ionized form. This is expressed as Log P:

$$\text{Log P}_{(\text{octanol/water})} = \log\left([\text{unionized solute}]_{\text{octanol}} / [\text{unionized solute}]_{\text{neutral water}}\right).$$

A more physiologically relevant measure is Log D, which is the ratio of non-ionized form in octanol to the non-ionized plus ionized forms in water:

$$\text{Log D}_{(\text{octanol/water})} = \text{Log}\left\{[\text{unionized solute}]_{\text{octanol}} / \left([\text{unionized solute}]_{\text{water}} + [\text{ionized solute}]_{\text{water}}\right)\right\}$$

For drug research, these values are typically measured at pH 7.4, with the aqueous phase being buffered such that the drug does not alter the pH.

The chromatographic hydrophobicity index (*CHI*) [37–38]. As with Log D, the chromatographic hydrophobicity index (CHI) is a measurement of the lipophilicity of a drug. The elution properties of compounds are evaluated using a rapid gradient reversed-phase liquid chromatography (RP-LC), typically with UV or MS detection. The analysis is carried out under acidic, neutral and basic conditions (pH = 2.0, 7.4, and 10.5). CHI was originally calculated by first determining the isocratic retention factor (log k') at various acetonitrile concentrations and plotting log k' as a function of that concentration. From this relationship, the slope (S) and the intercept (log k'(w)) values were obtained, and the hydrophobicity phi(0) calculated as −log k'(w)/S. There is a linear correlation between the gradient retention time values, t(R) and the isocratically determined phi(0) values. In practice, a plot of CHI vs. retention times for standards is used to determine CHI for the test compound.

Parallel Artificial Membrane Permeability Assay (PAMPA). PAMPA is a screening technique to estimate passive diffusion permeability (transcellular permeation). PAMPA estimates passive diffusion alone with no consideration of active transport. It is desirable to consider a large pH range when considering absorption from the GIT. The apparatus consists of a donor compartment and an acceptor compartment. The movement from donor to acceptor compartments through an artificial membrane containing lipid is determined. Multi-well plate 'sandwiches' have been devised for high-throughput operation. Data obtained in this manner correlates well with Caco2 (a cell line used to study drug transport) data, passive movement from the GIT, movement through the skin and distribution into the brain. Caco2 and MDCK permeability are discussed in the next section.

Membrane Bound Drug Transporters

It has become clear that drug transporters play a key role in the absorption and elimination of drugs into and out of organisms, including man. Recognition of this fact is critical in the discovery and development of new therapeutic agents. This section will focus on those transporters which have been well characterized, and for which *in vitro* methods exist that can be used as screening tools for the rank-ordering of drug candidates in the lead optimization activities leading up to selection of a lead candidate(s) for further development. Because this is an active area of research and an area where regulatory guidance is still being formulated, this section is expanded somewhat relative to other topics. Figure 2.5 was taken directly from the recent CDER (Center for Drug Evaluation and Research) Guidance, and shows the locations of some of the transporters discussed below [16]. The expression of transporters in the GIT, the liver and in renal tubules is displayed. Drug transporters are membrane bound, or in most cases, trans-membrane, proteins that are present in all organisms. These proteins act to pump a myriad of nutrients and ions into the cell and mediate the efflux of cellular waste, environmental toxins, and xenobiotics out of the cell. The activity of these membrane transport proteins may be passive, acting to facilitate the passage of molecules down their concentration gradients into or out of the cell via a process not requiring energy (ATP or reducing equivalents). Conversely, many transporters actively pump molecules and ions against their

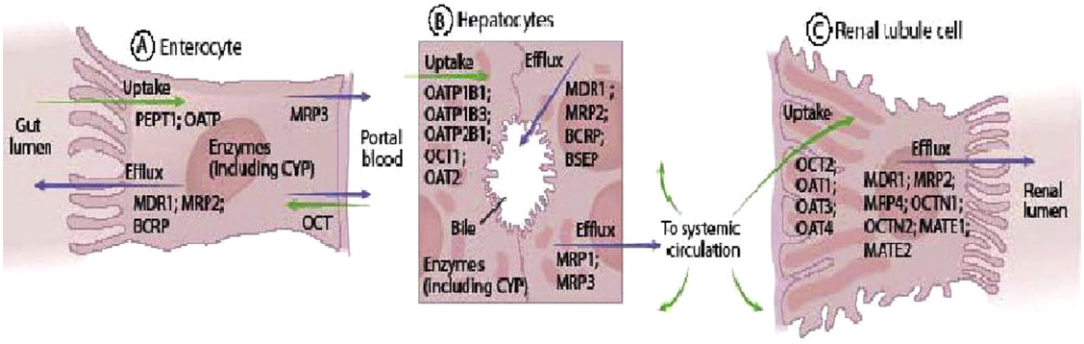

FIGURE 2.5 Figure showing location of efflux and uptake transporters in the GIT, liver and kidney thought to be important in drug ADME. Abbreviations: MRP: multidrug resistance associated protein; PEPT1, peptide transporter 1; OATP: 368 organic anion transporting polypeptide; OAT: organic anion transporter; OCT: organic cation transporter; 369 BCRP: breast cancer resistance protein; MDR1: multidrug resistance 1(P-glycoprotein (P-gp)); MATE: 370 multidrug and toxic compound extrusion protein.

concentration gradient in an active transport process that requires energy [39–41].

In considering the transport of drugs in the discovery and development process, greatest attention has been focused on transporters from two major superfamilies due to their roles in the uptake into and elimination of drugs out of the cell, respectively. By virtue of these activities, these membrane transport proteins can give rise to drug resistance and significant drug-drug interactions. As a comprehensive review of this area is beyond the scope of this chapter, we will focus on the most well characterized transporters from the two major genetic superfamilies; the ABC (*A*TP *b*inding *c*assette) transporter family and the SLC (solute carrier) transporter family.

Most ABC proteins are active transporters that hydrolyze ATP to actively pump their substrates across membranes. There are 49 known genes for ABC proteins, which can be grouped into seven subclasses or families (ABCA to ABCG) [39]. The most studied transporters in the ABC superfamily are P-glycoprotein (P-gp, *MDR1*) and the cystic fibrosis transmembrane regulator (CFTR).

The SLC superfamily includes facilitated transporters and ion-coupled secondary active transporters that reside in various cell membranes. Forty-three SLC families with approximately 300 transporters have been identified in the human genome [40–42]. In view of the fact that membrane drug transporter activity can have a major influence on the pharmacokinetic, safety and efficacy profiles of drugs, several key questions become critically important for drug development. These questions include which transporters are of clinical importance in drug absorption and disposition, and what *in vitro* methods exist that represent viable methods for screening development candidates for interactions with these transporters. These and other important factors in the discovery and development process are discussed below.

ATP BINDING CASSETTE (ABC) TRANSPORT PROTEINS: P-GLYCOPROTEIN (P-GP, MDR1, ABCB1)

P-gp (MDR1, ABCB1) mediates the ATP-dependent export of drugs from cells. As with all ABC-transport proteins, the ABC region of P-gp binds and hydrolyzes ATP, and the protein uses the energy for transport of its substrates across the membrane. It is expressed in the luminal membrane of the brush-border cells in the small intestine, in the epithelial and other cells which comprise the blood-brain barrier, in the apical membranes of hepatocytes and in kidney proximal tubular epithelia.

P-gp plays an important role in the intestinal absorption and in the biliary and urinary excretion of drugs, while in the cells of the blood-brain barrier it has a role in limiting the entry of various drugs into the central nervous system. The level of expression and functionality of P-gp can be modulated by inhibition and induction, which can affect the pharmacokinetics, efficacy, safety or tissue levels of P-gp substrates [43–45]. Initially discovered as a result of its interaction with multiple anticancer drugs, P-gp is responsible for the efflux across biological membranes of a broad range of therapeutic drugs. P-gp substrates tend to share a hydrophobic planar structure with positively charged or neutral moieties. These include structurally and pharmacologically unrelated compounds, many of which are also substrates for CYP3A4, a major drug-metabolizing enzyme in the human liver and GI tract. Alteration of MDR1 activity by inhibitors (drug-drug interactions) affects oral absorption and renal clearance. Drugs with narrow therapeutic windows (such as the cardiac glycoside digoxin and the immunosuppressants cyclosporine and *tacrolimus*) should be used with great care if MDR1-based drug-drug interactions are likely.

Cell lines that express P-gp, as well as polarized, inside-out membrane vesicles prepared from these cell

lines, can be used to determine whether a drug is a P-gp substrate or inhibitor. In these polarized cell monolayer preparations, P-gp is located in the apical plasma membrane. When efflux across the cell membrane is measured in these cell monolayers, the ratio of basal-to-apical to apical-to-basal flux is used to evaluate whether P-gp could play a significant role in transporting drugs across these cell monolayers. Transport across cells is not always related to excretion; P-gp may also have a role in drug penetration into the central nervous system [46–48]. Likewise, a high efflux ratio does not always translate into poor oral absorption. The involvement of P-gp in absorption of a drug is more pronounced in cases in which there is an apparent balance between metabolism and efflux.

BCRP (MXR, ABCG2)

The human membrane transport protein known as the Breast Cancer Resistance Protein (BCRP) has been shown to be responsible for resistance to a number of therapeutics. The BCRP transporter is encoded by the ABCG2 gene. As with other members of the ABC superfamily of transporters, BCRP uses energy derived from ATP hydrolysis to pump drugs and xenobiotics across the plasma membrane. It serves to limit the absorption of substrates, prevent them from entering the brain and also to mediate their hepatic elimination. The drugs to which BCRP can confer resistance in tumor cell lines include mitoxantrone, methotrexate, topotecan derivatives, bisantrene, etoposide, SN-38 and flavopiridol [49–51].

BCRP is present in many normal tissues, for instance, in the apical membrane of placental cells, in the bile canalicular membrane of hepatocytes, in the luminal membranes of brush border epithelial cells in the small intestine and colon and in the venous and capillary endothelial cells of almost all tissues [52]. The localization of BCRP in those tissues with barrier or elimination functions results in the BCRP transporter having a significant pharmacological role in the disposition of drugs and xenobiotics.

BSEP (SPGP, ABCB11)

The ABC superfamily transport protein known as the Bile Salt Export Pump (BSEP) is encoded by the ABCB11 gene. BSEP is expressed in liver hepatocytes on the apical side of the bile canalicular membrane. It serves to pump bile salts from the liver into bile and as such is the predominant facilitator of bile acid efflux in hepatocytes.

BSEP activity in the liver canalicular membrane is inhibited by a number of drugs or drug metabolites. This is potentially a significant mechanism for drug-induced cholestasis. Dysfunction of individual bile salt transporters such as BSEP is an important cause of cholestatic liver disease. This can occur due to genetic mutation, suppression of gene expression, disturbed signaling, or steric inhibition.

In addition to bile salts, BSEP mRNA has been shown to be induced by classical liver enzyme inducers. There is, however, a limited amount of information on whether atypical BSEP inducers such as 3-methylcholanthrene (3MC) are also substrates of the export pump. BSEP mediates the transport of taurocholic acid (TC) very efficiently. The rate and amount of transport into polarized membrane vesicles can be quantified using methods such as LC/MS/MS, and also by labeling with fluorescent or radioactive (^{3}H-TC) tags. Compounds that interact with the transporter can modulate the rate of TC transport. If a substance is a transported substrate, it might compete with TC, thus reducing the rate of TC transport. If a compound is an inhibitor of the transporter, it will block the transport of TC into polarized membrane vesicles. Some compounds can be co-transported with TC, increasing its rate of transport compared to the control level [39–40].

SOLUTE CARRIER (SLC) TRANSPORT PROTEINS: ORGANIC ACID TRANSPORT PROTEINS (OATPs)

The organic anion transporting proteins (OATPs) belong to the *SLC* gene superfamily of transporters and are twelve trans-membrane domain glycoproteins expressed in various epithelial cells. Some OATPs are expressed in a single organ, while others occur ubiquitously. The functionally characterized members of the OATPs mediate sodium-independent transport of a variety of structurally independent, mainly amphipathic organic compounds, including bile salts, hormones and their conjugates, toxins, and various drugs. Uptake transporters (OATPs, NTCP, OCT1, and OAT2) are localized in the basolateral membrane. These transporters mediate the uptake of substrates into the liver from the circulation. OATP1B1 and OATP1B3 are liver specific and show broad substrate specificity (statins, rifampicin, and telmisartan). Inhibition of OATP-mediated uptake of several statins by cyclosporin A and rifampicin causes clinically significant DDIs [39–40,53–55].

OTC1

For the elimination of environmental toxins and metabolic waste products, the body is equipped with a range of broad-specificity transporters that are present in the liver, kidney, and intestine. The polyspecific organic cation transporters OCT1, 2, and 3 (SLC22A1–3) mediate the facilitated transport of a variety of structurally diverse organic cations, including many drugs, toxins, and endogenous compounds. OCT1 and OCT2 are found in the basolateral membrane of hepatocytes, enterocytes, and renal proximal tubular cells. OCT3

has a more widespread tissue distribution and is considered to be the major component of the extra-neuronal monoamine transport system (or uptake-2), which is responsible for the peripheral elimination of monoamine neurotransmitters. Studies with knockout mouse models have directly demonstrated that these transporters can have a major impact on the pharmacological behavior of various substrate organic cations. The recent identification of polymorphic genetic variants of human OCT1 and OCT2 that severely affect transport activity thus suggests that some of the inter-patient differences in response and sensitivity to cationic drugs may be caused by variable activity of these transporters [39–40].

SLC TRANSPORT PROTEINS

Among the SLC superfamily, two families (SLC21 and 22) with polyspecific members have been identified, which together mediate the transport of a variety of structurally diverse organic anions, cations, and uncharged compounds. The SLC21 family of organic anion transporting polypeptides is currently known to consist of nine members in humans, transporting a range of relatively large (usually >450 Da), mostly anionic amphipathic compounds, including bile salts, eicosanoids, steroid hormones, and their conjugates. The SLC22 family currently consists of 12 members in humans and rats, encompassing organic cation transporters (OCTs), the carnitine transporter (OCTN2/SLC22A5) the urate anion-exchanger (URAT1/SLC22A12) and several organic anion transporters [39–40].

THE ROLE OF MEMBRANE TRANSPORTERS ON ADME CHARACTERISTICS OF DRUGS

The body is continuously exposed to a variety of environmental toxins and metabolic waste products. To rid itself of these compounds, it is equipped with various detoxification mechanisms such as metabolizing enzymes and transport proteins mediating their inactivation and excretion. For excretion, a plethora of transmembrane transport proteins is present in the major excretory organs (liver, kidney, and intestine). The solute carrier (SLC) superfamily is by far the largest superfamily of transporters, consisting of about 225 members in humans.

Whereas most of these transporters are highly specialized, mediating facilitated transport of essential nutrients (e.g., glucose, amino acids, nucleosides, and fatty acids), some members are more generalized. Due to their broad substrate specificity, the latter are also termed polyspecific transporters. They play a major role in the elimination of, and protection against, noxious compounds.

P-gp can export an astonishing variety (chemically diverse) of amphipathic drugs, natural products, and peptides from mammalian cells, powered by the energy of ATP hydrolysis. The transporter consists of two homologous halves, each with 6 membrane-spanning helices and a cytosolic nucleotide binding domain. Pgp has been purified and studied extensively, but its mechanism of action is still not well understood. X-ray crystal structures of P-gp bound to two cyclic peptide substrates has shown that the protein has a large, flexible, drug-binding cavity located within the membrane-bound domain. Drugs can bind to several sub-sites within this pocket, via different sets of interactions, helping to explain the unusual poly-specificity of the transporter. P-gp substrates are generally lipid-soluble, and interact with the protein within the membrane before being either expelled into the extracellular aqueous phase or moved to the extracellular of the membrane.

P-gp substrates include many drugs that are used clinically, and the protein plays an important role in drug absorption and disposition *in vivo*. It is a key determinant in the pharmacokinetic profile of many drugs, and, ultimately, the clinical response. The protein is located at the luminal surface of the intestine, and limits absorption of drugs from the gut. Its presence in the luminal membrane of brain capillary endothelial cells also makes a major contribution to the blood-brain barrier, and strongly reduces accumulation of many different drugs in the brain. The physiological role of P-gp is thought to involve protection against toxic xenobiotics and endogenous metabolites by efflux or secretion of these compounds following absorption by other mechanisms. The transporter also plays an important role in the multidrug resistance (MDR) displayed by many human tumors, and it is an important factor in predicting the outcome of chemotherapy treatment [39–40,43–45].

If a drug interacts strongly with P-gp, the compound will likely have reduced absorption in the gut, very limited entry into the brain, and be unable to enter drug-resistant tumors. Screening drugs for their ability to compete with P-gp-mediated transport of a probe compound can give quantitative information on their affinity for the transporter, and provide an indicator of their behavior *in vivo*. The availability of this type of information for a specific drug can be useful in anticipating potential problems with its use in a clinical setting.

TRANSPORTER MEDIATED DRUG-DRUG INTERACTIONS: P-GLYCOPROTEIN

Drug-drug interactions involving membrane transport can be classified into two categories. One is caused by competition for the substrate binding sites of the transporters, and the other by a change in the expression level of the transporters. As mentioned

previously, P-gp has a very broad range of substrate specificity; hence drug-drug interactions involving it are very likely. P-gp inhibitors, such as quinidine and verapamil, are known to increase plasma concentrations of digoxin, a cardiac glycoside, because they block its biliary and/or urinary excretion via P-gp inhibition. Since the therapeutic range of digoxin is small, changes in its plasma concentration are potentially very serious.

ORGANIC ANION (OATs) AND ORGANIC CATION TRANSPORTERS (OCTs)

OCTs transport a number of drugs including cimetidine, metformin, procainamide, and triamterene from the plasma into hepatocytes and renal tubular cells. As with the Cytochromes P450, a variety of different OAT and OCT transporters exist. It is well known that probenecid inhibits the renal secretion of many anionic drugs via organic anion transport systems. The renal clearance of furosemide, ciprofloxacin and benzylpenicillin is reduced by co-administration of probenecid. OAT1 is a candidate for the transporter responsible for these interactions on the renal basolateral membrane because probenecid has been found to be able to inhibit OAT1 [39–40,53–55].

Metformin's uptake into the liver, where it exerts its pharmacologic effect, is mediated by OCT1, while its elimination via the kidney is primarily due to OCT2 activity. The capacity of OCT2 to transport metformin is at least 10 times greater than OCT1. Thus, OTC2 in combination with the renal elimination of metformin are primarily responsible for its pharmacological properties. Cimetidine is also known to be a substrate for OCT and can compete with metformin for both OCT1 and OCT2. Because OCT2 is primarily responsible for metformin's elimination, competition from cimetidine will result in reduced renal clearance of metformin and elevated plasma concentrations. Procainamide is another known OCT substrate. Its renal clearance has been reduced following co-administration with several drugs, including amiodarone, levofloxacin, and cimetidine [53–55].

The clinical outcome of drug-drug interactions based on OAT or OCT inhibition will depend on the pharmacological properties of the drug in question. For example, inhibiting the hepatic uptake of a drug may reduce its metabolism, leading to higher plasma concentrations. If the site of action of the drug is intrahepatic, however, a reduction in the desired pharmacological effect also may occur, despite increased plasma concentrations. Nevertheless, the resulting increase in the drug's plasma concentration may lead to an increase in side effects unrelated to the drug's therapeutic effect. An example would be that patients taking statins might have an increased risk of myopathy, whereas those on metformin could have a greater risk of developing lactic acidosis. The effect of inhibited renal clearance will depend on the percent of drug eliminated via the kidney and its therapeutic window. In general, clinically significant effects will occur with drugs having at least 50% of their elimination via renal secretion and which also have a narrow therapeutic window [39–40,53–55].

TRANSPORTER MEDIATED DRUG RESISTANCE

The multidrug-resistance protein (MRP) has been recognized as being correlated with drug resistance in cancer chemotherapy for some time. MRP is a transmembrane protein which is, in part, responsible for the resistance of human tumor cells to cytotoxic drugs. Stably transfected, MRP-overexpressing cells have been shown to be resistant to doxorubicin, daunorubicin, vincristine, VP-16, colchicine, and rhodamine 123, but not to 4′-(9-acridinylamino) methanesulfon-m-anisidide or taxol. Intracellular accumulation of anti-neoplastic drugs (daunorubicin, vincristine, and VP-16) is decreased and the efflux of drug (daunorubicin) is increased in these cells [39–40]. Accumulation of daunorubicin has been shown to be reversed when the plasma membrane of these cells is permeabilized using non-ionic detergent. This would seem to demonstrate conclusively that MRP lowers the intracellular daunorubicin level by pumping the drug out of the cells against a concentration gradient, thereby identifying it as a transmembrane efflux pump [56].

Pancreatic cancers are among the tumor types which have proven to be most chemoresistant to a variety of chemotherapy agents. Chemoresistance of this nature can be mediated by various cellular mechanisms, including a reduced uptake of the drugs into the target cells; alterations within the cells, such as changes in the metabolism of the drugs; changes in the cellular capacity for DNA repair; and an increased efflux of the drugs from the cells. In studies of human pancreatic carcinoma cells, Hagmann et al. have shown that in cells stably transfected with human transporter cDNAs, or in cells in which a specific transporter was knocked down by RNA interference, 5-fluorouracil treatment affects the expression profile of relevant cellular transporters including multidrug resistance proteins (MRPs), and that MRP5 (ABCC5) influences the chemoresistance of these tumor cells [57]. Similarly, cell treatment with the nucleoside drug gemcitabine or a combination of chemotherapeutic drugs can variably influence the expression pattern and relative amount of uptake and export transporters in pancreatic carcinoma cells. In addition, cytotoxicity studies with MRP5-overexpressing or MRP5-silenced cells additionally demonstrated a contribution of MRP5 to gemcitabine resistance [57].

Chemotherapy is a major form of treatment for cancers. Unfortunately, the majority of cancers are either resistant to chemotherapy or acquire resistance during treatment. One of the mechanisms by which human cancers develop multidrug resistance is the overexpression of efflux transport proteins on the plasma membrane of cancer cells. P-glycoprotein (P-gp) and the multidrug resistance protein 1 (MRP1) have been shown to confer resistance to a broad spectrum of chemotherapeutic agents. Several other human ATP binding cassette (ABC) transporters with a potential role in drug resistance have been described as having a role in multidrug resistance. Among them, a novel protein, now known as the breast cancer resistance protein (BCRP) or mitoxantrone-resistance protein (MXR) [58] or placenta-specific ABC protein (ABCP), were shown to be present in the plasma membrane of the drug-resistant cells overexpressing the transporter [59]. Such studies provide strong evidence that BCRP is a cause of drug resistance for certain types of chemotherapeutic agents, including mitoxantrone and topotecan, in tissue culture models. BCRP is prominently expressed in organs important for absorption (the small intestine), distribution (the placenta and blood-brain barrier), and elimination (the liver and small intestine) of drugs, and an increasing amount of evidence is now emerging to support the conclusion that BCRP also plays an important role in drug disposition [39–40].

METHODOLOGIES FOR EVALUATING DRUG INTERACTIONS WITH TRANSPORTERS

Drug-drug interaction involving hepatic membrane transporters can occur by competition for the same substrate-binding site of the transporter, very tight binding, by binding which interferes with the transporter allosterically leading to inhibition of transporter activity, or by change in expression level of transporters. This has the potential to alter the blood concentration time profiles of drugs, leading to elevated levels of a co-administered compound. Evaluating the substrate potential of a drug candidate for the hepatic uptake transporters *in vitro* is particularly beneficial when the liver is the drug target. For example, the hepatitis C drugs alpha-interferon and S-acyl-2-thioethyl esters or the HMGCoA inhibitors (statins) must achieve adequate concentrations in the liver for pharmacological activity.

In addition to drug-drug interactions, hepatic transporters also play a role in toxicities including cholestasis and hyperbilirubinemia. Drug-induced hepatotoxicity is a major problem in drug development and there is growing evidence that inhibition of bile acid transporters is a contributing mechanism.

Hepatocytes in suspension, attached to tissue culture dishes, or in primary sandwich-culture are all good models of hepatic transport. The contribution of transporter mediated uptake to hepatic clearance (CLH) was recognized when CLH was consistently under-predicted for many series of chemotypes using just metabolic stability for the calculations. Factoring in transporter-mediated hepatic uptake, along with metabolic clearance using hepatocytes in suspension, improved these predictions [39–40].

ABC transporter assays including epithelial cell barrier systems using Caco-2 or LLC-PK1 cells are widely accepted *in vitro* models which are used to rank the absorption of drug candidates. In addition to these standard models, one may specifically measure human P-gp-mediated drug transport using cDNA transfected LLCPK porcine cell lines. These human P-gp expressing cell lines allow the study of this important efflux transporter without interference from other expressed transporters.

Alternatively, a less specific but faster ATPase assay in membranes or membrane vesicles allows determination of whether the compounds of interest interact with ABC transporters. ATP hydrolysis is required for *in vivo* drug efflux by ABC transporters. The membrane ATPase assay measures the phosphate liberated from drug-stimulated ATP hydrolysis in ABC transporter membranes [42–47].

Caco-2 cells are the most popular cellular model in studies on passage and transport. They were derived from a human colorectal adenocarcinoma. In culture, they differentiate spontaneously into polarized intestinal cells possessing an apical brush border and tight junctions between adjacent cells, and they express hydrolases and typical microvillar transporters. This cell line was first used as a model for studying differentiation in the intestinal epithelium, and later for estimating the relative contributions of paracellular and transcellular passage in drug absorption.

Caco-2 cells, despite their colonic origin, express in culture the majority of the morphological and functional characteristics of small intestinal absorptive cells, including phase I and phase II enzymes, which can be detected either by measurement of their activities toward specific substrates, or by immunological techniques. CYP3A, which is present in almost all intestinal cells, is very weakly expressed in Caco-2 cells, but expression levels can be increased by treatment with 1α,25-dihydroxyvitamin D3, an inducer of CYP3A4, or transfection of CYP3A4 cDNA. The resulting expression levels do not reach the levels observed *in vivo*, however. With regard to phase II enzymes, Caco-2 cells do express *N*-acetyl transferase and glutathione transferase. In summary, the Caco-2 cell transport assay seems a good and predictive approach to understanding transport across the intestinal absorptive barrier [39–40].

Drug transport assays in polarized cell monolayers can be used to screen for P-gp involvement in transport.

P-gp, encoded by MDR1, is expressed in the human intestine, liver, brain, and other tissues. Localized to the cell membrane, P-gp functions as an ATP-dependent efflux pump, capable of transporting many structurally unrelated xenobiotics out of cells. Intestinal expression of P-gp may affect the oral bioavailability of drug molecules that are substrates for this transporter. P-gp substrates can be identified by a direct measure of transport across polarized cell monolayers. Bidirectional transport (apical to basolateral and basolateral to apical) is measured in Caco-2 cells, or in LLC-PK1 cells expressing P-gp cDNA and corresponding control cells. Quantitation of the rate of transport and total mass transported can be achieved by a variety of methods including HPLC/MS/MS, fluorescence or by using a radio-labeled substrate [39–40,47]. Evaluation of drug candidates as a substrate and inhibitor of P-gp should be performed according to FDA guidance [16].

As a means of studying the transport of drugs and xenobiotics into and out of the brain, capillaries can be isolated from brain, and digested to separate out brain capillary endothelial cells for growth in cell culture. The endothelial monolayer can be grown on porous membranes, which can be placed in side-by-side diffusion chambers for measurement of drug transport across the monolayer *in vitro*. The problem with this approach is that blood-brain barrier (BBB)-specific gene expression is severely down-regulated *in vitro*. For example, the expression of the Glut1 glucose transporter or the LAT1 large neutral amino acid transporter is down-regulated >100-fold in cultured endothelium compared to freshly isolated brain capillaries. For example, L-DOPA for Parkinson's disease is effective, because this drug crosses the BBB on the LAT1 endogenous transporter [39–40].

Alternatively, these transport systems can be studied *in vivo*. Drug transport from blood to cerebrospinal fluid (CSF) is a function of drug transport across the choroid plexus epithelium, which forms the blood-CSF barrier *in vivo*. This epithelial barrier is anatomically separate from the BBB, which limits drug transport from brain into brain interstitial fluid (ISF) across the capillary endothelium. The capillary endothelium (the BBB) and the choroid plexus (the blood-CSF barrier) have different transporter gene expression profiles. Drugs may readily enter CSF, owing to rapid transport across the choroid plexus, but *not* undergo significant transport into brain tissue, due to limited BBB transport. This is illustrated with azidothymidine (AZT), a treatment for neuro-AIDS. AZT is readily transported into CSF, but is not transported across the BBB.

BBB active efflux transporters (AET) such as p-glycoprotein actively export drugs from brain to blood. There are many other BBB efflux systems for both small and large molecules. The efflux transporters can be measured using the brain efflux index (BEI) method, which involves the direct injection of the drug into the brain under stereotaxic guidance. The kinetics of drug loss from the brain compartment (which is a function only of BBB efflux transport) can then be quantified [39–40].

Metabolism in the GIT and Liver: Stability Testing

Clearly, metabolism is important in the absorption of drugs from the GIT into the blood stream. The absorption and distribution of a drug following PO administration leading to some desired pharmacological effect occurring at a target organ, such as the brain, requires that the metabolism of the drug is not extensive, either in the gut or the liver, or '*first pass metabolism*'.

STABILITY TESTING: PLASMA AND MICROSOMAL STABILITY

Several simple stability tests allow the assessment of the '*drug-ability*' of a compound or chemical template. For stability testing, typically a one data point determination (with a zero time point control) is made, in which the time and other parameters (such as protein concentration) are such that a certain percentage loss of compound can be used as a screening data point (information to keep or eliminate a compound, or information to consider in the context of additional compound data) [1,2,4,5].

PLASMA STABILITY

Drugs must have sufficient stability in the body to exert a pharmacological effect over a reasonable period of time. A wide variety of compounds are unstable (are degraded) when incubated in blood or plasma at rates that are inconsistent with the PK properties necessary for drug therapeutics. Plasma (blood) stability determination is a widely used, simple test that can eliminate compounds in drug discovery screens. Typically, a compound is incubated in plasma (blood) at approximately 10 μM at 37°C for 30 to 60 minutes and its stability determined by an appropriate analytical method, such as LC-UV or LC-MS. Viable drug candidates should be relatively stable under these conditions.

MICROSOMAL STABILITY

Microsomes are artificial structures derived from pieces of endoplasmic reticulum (ER) formed during tissue homogenization. They are prepared by differential centrifugation at 10,000 and 100,000 × g and contain cytochrome P450 enzymes (CYPs), but do not contain soluble enzymes. The preparation of S9 fraction, soluble fraction and microsomes is displayed in Figure 5.6. The family of CYP enzymes contained in microsomes are responsible for Phase I biotransformations of xenobiotics, and incubation of test material with hepatic

microsomal preparations in various species is the primary means by which the Phase I biotransformations of xenobiotics (drugs) are determined. For microsomal stability determination, the compound is typically incubated in approximately 1.0 mg/ml microsomal protein, phosphate buffer, pH 7.4 at 37°C for 30 minutes. Its stability is then determined by an appropriate analytical method such as LC-UV or LC-MS. Viable drug candidates should have a species specific, pre-determined percentage remaining under these conditions. Alternatively, microsomal stability testing can be undertaken in an HTS format using rodents [60].

Distribution and Excretion

For simplicity, we have combined drug distribution and drug excretion in this section. Modern eADME studies that evaluate drug-related material distribution by MS usually analyze specific tissues, blood/plasma and excreta. For example, when a drug is administered by IV or PO, samples of blood (plasma), tissues (brain, for example) and excreta (urine and feces) are taken at specific time intervals and these samples are analyzed for drug-related material by LC-MS, giving both distribution and excretion data. The evolving technologies surrounding the use of radioactive tags to study the disposition of biologics early in drug discovery using PET and SPECT are discussed in a separate chapter.

The study of the distribution of a xenobiotic is the study of its movement into, through and out of body compartments. Data are expressed in terms of concentration-time for the parent drug and its metabolites. Kinetic analysis can afford insight into drug properties and PK/PD relationships. A concept central to any discussion of distribution is the *volume of distribution.* The (apparent) volume of distribution (V_D) is a pharmacokinetic *'parameter'* used to quantify the distribution of a medication between plasma and the rest of the body. Certain changes in physiological function(s) and certain disease states may alter V_D. This is discussed in detail in the chapter on PK (Chapter 3) to which reader is referred.

KINETICS OF METABOLISM IN MICROSOMES, HEPATOCYTE S9 FRACTION AND HEPATOCYTES

Over the last 25 years or so there has been an increase in the use *of in vitro* systems as models used to estimate the *in vivo* ADME properties of drugs and chemical templates under evaluation for *drug-ability* [61–62]. For example, the rate of metabolism (and metabolic clearance) observed *in vitro* for hepatic preparations can be used as an estimate of what to expect *in vivo*. To some extent, the popularity of *in vitro* systems has been driven by their use by numerous research groups and the eventual commercialization of products that are economical and effective. However, there is a lot of 'bang for the buck' in using *in vitro* systems in drug discovery research. *In vitro* systems include intact cells such as perfused liver preparations, liver slices, freshly prepared or frozen hepatocytes and other cell lines. Cell fractions include S9 fraction, soluble fraction (cytosol) and microsomes (Figure 2.6).

THE RATE OF DRUG DISAPPEARANCE IN LIVER MICROSOMES OR HEPATOCYTES [63]

A typical experiment for *stability screening* purposes using microsomes involves incubation of drug at approximately 3 μM. Typical conditions would be incubation of the drug at 37°C with 100 mM phosphate buffer pH = 7.4, 1.0 mM NADPH and approximately 1.0 mg/ml microsomal protein. Reaction starts with the addition of NADPH (time zero). Typically, 5–7 time points are taken (0, 2.5, 5, 10 20, 30 and 60 minutes, for example) and the reaction is stopped by the addition of methanol or acetonitrile. The drug is analyzed using LC-MS and the kinetics of disappearance data are entered into drug (chemical) libraries. The rate of disappearance of drug using plated hepatocytes and hepatocytes in suspension has become more popular recently; a more expensive experiment. One problem with this approach to kinetic analysis is that the kinetics of movement of drug across membranes becomes a complicating factor, although it can be argued that this is a situation closer to that encountered *in vivo*.

In Vivo *eADME Disposition and Balance Studies*

The impact of new analytical techniques and instrumentation, and improvements in existing technologies on the way we conduct biochemical studies, cannot be overstated [64]. For example, the ability of mass spectrometry to obtain high quality spectra or quantify molecules in

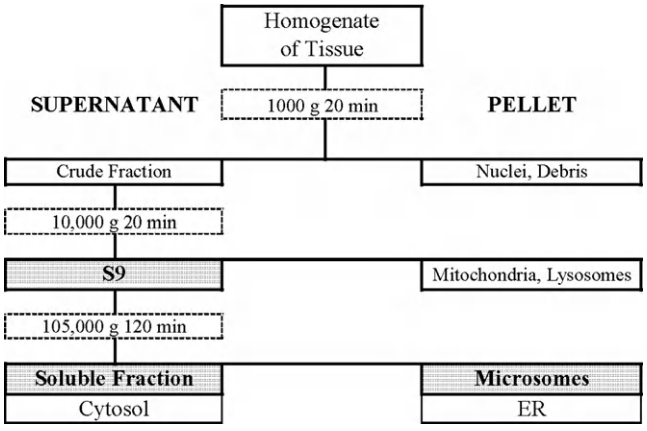

FIGURE 2.6 Preparation of microsomal, S9 and soluble fractions commonly used in drug metabolism studies.