3 Oral Absorption: from Physiology to Regulatory

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3.1 SUMMARY

When developing an oral drug, the drug discovery team's goal is to balance the physicochemical and molecular properties that enable a molecule to reach its final target, while maintaining sufficient clinical efficacy and acceptable toxicity.

First, solubility, dissolution rate, lipophilicity, pK_a (negative logarithm of the acid dissociation constant, K_a), and molecular weight (MW) are major physicochemical properties driving the absorption potential of a drug. Indeed, a molecule should be in a solution to permeate the intestinal membranes, and the rate at which the molecule gets into the solution impacts its ability to get absorbed.

Second, after oral administration, a drug encounters physical barriers before it reaches the blood stream. These barriers are the physiology of the gastrointestinal (GI) tract and various GI fluids.

Intestinal absorption consists of the sum of multiple processes. The drug's permeability is a major determinant of its ability to be absorbed in the intestine. Molecules within the "rule of 5" chemical space are usually absorbed through a passive process. However, intestinal transporters could either hinder or promote a molecule's absorption. As an example, the multidrug resistance efflux transporter, MDR1 (P-gp/ABCB1) has been shown to limit oral absorption of low permeable molecules. On the other hand, the peptide transporter PepT1 can promote intestinal absorption or peptidomimetic molecules. Finally, the small and large intestine express metabolic enzymes. Therefore, intestinal metabolism can limit a drugs' oral absorption and lead to drug–drug interactions (DDIs).

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

A multiplicity of tools exists to study intestinal absorption; these range from *in vitro* systems to *in vivo* experimentation. These tools provide data with different degrees of granularity and with distinct human absorption predictability.

Finally, the food and drug administration (FDA) has developed guidelines to assess molecules' properties in terms of intestinal absorption and potential of DDI.

3.2 FUNDAMENTALS OF ORAL BIOAVAILABILITY

In pharmacokinetics, absorption is a process by which a given dose extravascularly (EV) reaches the systemic circulation. The dose could be given orally, subcutaneously, intramuscularly, or any other way different from a direct injection into the vascular system. The term oral "*bioavailability*" (F) is a parameter that is used in pharmacokinetics to quantify the ability of a compound dosed orally to reach the systemic circulation, after surviving any first-pass extraction. Operationally, the systemic F can be determined as described by the following equation: $F = \frac{\mathrm{AUC}_{\mathrm{p.o.}}}{\mathrm{AUC}_{\mathrm{i.v.}}} \cdot \frac{\mathrm{Dose}_{\mathrm{i.v.}}}{\mathrm{Dose}_{\mathrm{p.o.}}}$.

In this equation, $\mathrm{AUC}_{\mathrm{p.o.}}$ refers to the area under the curve (AUC) derived from an oral administration and $\mathrm{AUC}_{\mathrm{i.v.}}$ refers to the intravenous (i.v.) administration. Conceptually, F quantifies the success with which a compound overcomes the potential barriers to reach the systemic circulation. A compound with $F = 1$ (or 100%) indicates that a given oral dose produces an identical systemic exposure to that observed in the corresponding i.v. dose. In this case, the complete dose reaches the systemic circulation, the compound completely overcomes any barriers, and absorption is complete.

Furthermore to On the other hand, $F = 0.5$ (50%) indicates that in transit from the oral administration site to the systemic circulation, half of the compound is lost; in this case, the oral dose to systemic concentration relationship indicates that the oral dose must be twice that of an equivalent i.v. dose to achieve a similar systemic exposure.

Furthermore, two other parameters are often used to describe absorption: $C_{\max}$, which represents the maximal concentration observed following oral dosing and $T_{\max}$, which is the time at which $C_{\max}$ is observed. $C_{\max}$ gives an insight into acute exposure maximums that can be achieved following oral administration, and $T_{\max}$ provides an insight into the timescales of absorption—both of which can be critical for the determination of a safety margin.

Some advantages of the oral (p.o. for per os which means literally "by mouth" in Latin) dosing route are that it is the most convenient, well-tolerated, patient compliant, and cost-effective route of drug administration. A significant disadvantage of p.o. administration includes the multiplicity of complex processes that determine a compound's absorption from the gut into the systemic circulation. As a consequence, the processes governing absorption is a significant source of inter- and intrapatient variability in a compound's pharmacokinetic profile [1].

For oral administration, oral $F(F_{\mathrm{oral}})$ can be defined as follows:

$$F_{\mathrm{oral}} = f_a \cdot F_{\mathrm{g}} \cdot F_{\mathrm{h}} \cdot F_{\mathrm{l}}$$

where f_{a} is the fraction of the dose absorbed from the gut. Physical processes typically determine this process. The terms F_{gut}, F_{h}, and F_{lung} are the bioavailability of the compound in the intestine (g), liver (h), and lung (l), respectively (F_{l} is typically 1,

and) is generally ignored—lung is usually not a barrier to oral administration). In addition to the compound's physicochemical properties, biochemical activities, such as transport and metabolism, hinder a compound's ability to be orally bioavailable. The equation accounts for the various barriers that a compound may encounter en route to the systemic circulation from the gut. It is clearly seen that a lack of f_a or bioavailability in any one of the organs will yield $F_{oral} = 0$ and, subsequently, the absence of systemic exposure.

3.3 ORAL ABSORPTION IN DRUG DISCOVERY

After oral administration, a drug encounters physical barriers before it reaches the blood stream. These barriers are the physiology of the GI tract and various GI fluids. The drug's chemical structure and physicochemical properties determines its ability to penetrate these barriers and reach its final target.

3.3.1 Role of Physicochemical Properties

Multiple processes determine the exposure of a drug after oral administration, and each of these processes is mainly driven by the drug's physicochemical properties.

3.3.1.1 Solubility. Solubility is a critical parameter for absorption because the drug must be in solution to permeate the GI membrane. Solubility is the maximum concentration a solid compound reaches in a solvent matrix at equilibrium and is altered under various conditions. Solubility is mainly driven by physicochemical properties, chemical structure, dissolution rate, and the solvent matrix used. Therefore, in a lead optimization stage, we can improve solubility by modifying the molecule's physicochemical properties and chemical structure.

The most efficient and frequent strategy used by medicinal chemists to increase the solubility is to introduce ionizable groups. Others include reducing lipophilicity, introducing hydrogen bonding and polar groups, reducing MW, and introducing out-of-plane substitutions. Introducing ionizable groups comes at a cost, since this may hinder permeability.

Beyond the ionization state, other physicochemical parameters, which influence the molecules' solubility, are MW and lipophilicity. Lipophilicity is the tendency of a compound to partition into a nonpolar lipid matrix versus an aqueous matrix. Lipophilicity is commonly determined using log P from octanol/water partitioning. A trend has been shown with lipophilicity where, on average, the higher the log P, the lower the solubility [2]. While neutral molecules have been shown to be more poorly soluble compared to ionizable molecules, when clog $P < 3$, the average solubility of neutral molecules approaches the average solubility of ionizable molecules. The same trend has been shown with MW: as MW increases, solubility decreases [2].

The ionizability of a molecule is driven, in turn, by its pK_a and the pH of the matrix in which the molecule is in solution. For acids, as pH decreases, there is a greater concentration of neutral molecules and lower concentration of anionic acid molecules. The reverse is true for bases (Fig. 3.1). In addition, from a physiological stand point, the luminal pH changes along the GI tract and in the presence of food;

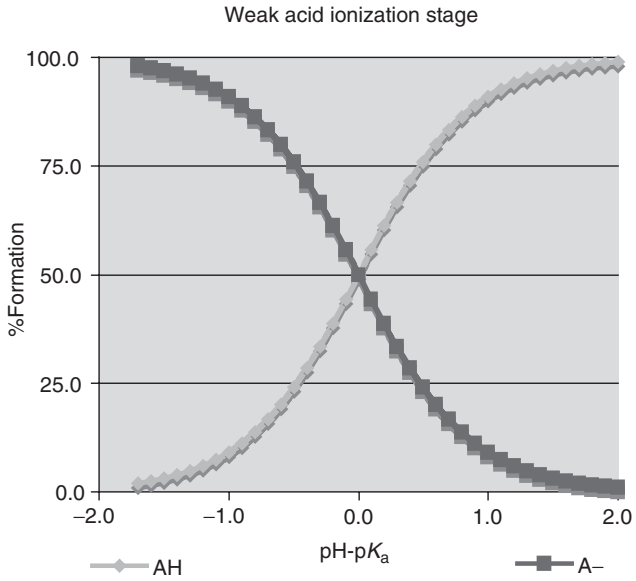

Figure 3.1 Percent formation of the ionized form of a weak acid over a pH range. (See color insert.)

therefore, solubility of acids and bases will vary in an opposite way, if their solubility is mainly driven by their ionization stage.

The drug discovery team will have to determine whether solubility or permeability is the rate-limiting step, and adjust the project strategy accordingly. Indeed, if solubility were the limiting absorption, introducing ionizable moiety would be the strategy of choice.

3.3.1.2 Dissolution Rate. The dissolution rate is the rate at which the molecule dissolves into a solvent from a solid form. Therefore, a molecule with a high dissolution rate will dissolve into solution quickly, leading to a quick absorption phase and increasing its chance to be absorbed within the GI transit time while its solubility remains constant. The dissolution rate depends on the particle size and compound physical and salt form. Reducing the particle size increases the surface area of the solid in contact with the solvent, which increases the dissolution rate.

The most frequent physical form in drug discovery is amorphous. An amorphous solid has no specific organization of molecules, whereas a crystal is a highly organized set of molecules. The amorphous form is often more soluble and less stable than the crystalline form. Therefore, caution is required when dosing poorly soluble amorphous compounds orally since these can precipitate in the GI tract to a more stable and less soluble crystalline solid form, leading to even poorer absorption.

To increase the dissolution rate, a salt form can be developed. Salts can stay in solution in a supersaturated state and delay the compound's precipitation. However, salts of weak acid or base can precipitate because they have converted to the free acid or base, leading to reduced intestinal absorption.

In conclusion, understanding the compound physical form and the size of the particles will help the team to understand if the dissolution rate is limiting molecules'

absorption. Furthermore, the team will determine whether additional formulation work could improve the molecule's dissolution rate.

3.3.1.3 Bioavailability. Lipinski *et al*. showed that particular physicochemical properties are associated with high or low oral bioavailability. They established the famous "rule of 5" that predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the MW is >500 g/mol and the calculated log $P > 5$ [3].

Oral exposure is determined not only by the absorption through membranes of the GI tract but also by the extent to which organs just after absorption are able to extract these orally administered drugs. Therefore, gut and hepatic metabolism are key players in determining drug oral bioavailability. Varma *et al*. analyzed a set of 309 drugs where bioavailability, fraction absorbed (f_a), fraction escaping intestinal extraction (F_g), and fraction escaping hepatic extraction (F_h) were known. They determined which physicochemical property influences these parameters and how medicinal chemists and pharmacokineticists could play with these properties to enhance molecules' bioavailability [4]. They showed that f_a decreases with increasing MW (>500), polarity ($c \log D > -2$), polar surface area (>125 Å^2), total H-bond donors and acceptors (>9), and rotatable bonds (>12). Indeed, such properties limit the capability of small organic molecules to traverse lipid membranes. Molecules with a log P ranging from 1 to 3 are considered to be highly permeable. However, Varma *et al*. noted that high lipophilicity does not necessarily have a detrimental effect on f_a. Their analysis showed that the numbers of free rotatable bonds are negatively related with all three processes leading to a dramatic effect on bioavailability. They also noted that physicochemical properties that favor high f_a tend to be also associated with high rates of metabolism. For example, enough lipophilicity is needed to ensure good membrane penetrability, but too much will cause high hepatic and potentially intestinal extraction owing to metabolism.

This ionization state analysis of the compounds in the database indicated that bases are relatively less bioavailable, although they showed higher f_a than acids and neutrals, evidently because of higher first-pass effect [4]. Indeed, they explained that the higher first-pass effect of basic molecules can be attributed to their affinity for metabolic enzymes and relatively lower protein binding.

3.3.2 Paths to Intestinal Absorption

3.3.2.1 GI Tract Physiology. After oral dosing, the compound first encounters the buccal mucosa, where it can be absorbed. However, buccal or sublingual absorption is generally negligible. The drug is then ingested via the esophagus and arrives at the first part of the GI tract: the stomach. In the stomach, the drug is mixed with gastric acids, pepsinogen, and mucus secretions. Most drugs have limited stomach absorption since the stomach surface area is relatively small (about 1 m [2]), the blood flow perfusion rate is low (150 mL/min) and the gastric emptying time is rapid (0.5–1 h). Therefore, although the stomach's low pH (Table 3.1) favors absorption of acidic compounds, the absorption of acids is, in fact, faster in the small intestine. The small intestine consists of three consecutive sections: duodenum, jejunum, and ileum. The pH of each section increases successively and this progression of pH creates a gradient from the stomach to the ileum (Table 3.1). Absorption in the small intestine is greater because

TABLE 3.1 pH of the Gastrointestinal Tract Under Fed and Fasted State

GI Tract Section	pH, Fed	pH, Fasted
Stomach	3–7	1.4–2.1
Duodenum	5.2–6.2	4.4–6.6
Jejunum	5.2–6.2	4.4–6.6
Ileum	6.8–8	6.8–8
Colon	5.5–7	5.5–7

of the larger surface area (200 m [2]), the high blood flow perfusion rate (1 L/min) and the lengthy transit time (2–4 h). Since absorption is greater in the small intestine, the gastric emptying time can be a controlling step in the speed of drug absorption.

In the second part of the duodenum, the sphincter of Oddi allows the gall bladder to secrete bile into the GI tract. Bile is produced by the liver and stored in the gall bladder in humans, monkeys, dogs, and mice. Rats lack a gall bladder and this results in a continuous bile flow into the gut rather than the acute release of bile on gall bladder contraction. Bile, with its detergent-like properties, facilitates the solubilization and chemical breakdown of lipids, which explains why the bile is secreted in the presence of lipids in the duodenum. The presence of peptides and amino acids in the duodenum activates the secretion of pancreatic enzymes: amylases, lipases, and proteases. The pancreatic enzymes can hydrolyze some molecules that contain hydrolysable functional groups, resulting in the deactivation or activation of the drug in the GI tract. In addition, the pancreas secretes bicarbonates ion, which neutralize acid stomach secretions.

At the ileocecal junction, the small intestine connects with the large intestine. Although the large intestine transit time is long (7–20 h), drug absorption is limited mainly because of its small surface area (0.25 m [2]) and its lack of villi.

3.3.2.2 Permeability. Permeability is the rate at which a drug passes through a biological membrane and plays a major role in oral drug's bioavailability. Different mechanisms of membrane permeation exist: passive diffusion, active uptake and efflux, endocytosis, and paracellular flow.

3.3.2.2.1 Passive Diffusion. The predominant mechanism of absorption for commercial drugs is passive diffusion [3]. However, new chemical entities with physicochemical properties outside of the "rule of 5" might alter this trend.

Passive diffusion does not require energy and is driven by concentration gradient. Fick's law of diffusion best describes the parameters driving passive permeability (Eq. 3.1):

$$\frac{\mathrm{d}Q}{\mathrm{d}t} = \frac{DAK}{h}\left(C_{\mathrm{GI}} - C_{\mathrm{p}}\right) \tag{3.1}$$

$\mathrm{d}Q/\mathrm{d}t$ = rate of diffusion
D = diffusion coefficient
K = lipid–water partition coefficient of drug in the biologic membrane
A = surface area of the membrane

h = membrane thickness
C_{GI} = drug concentration in the GI tract
C_p = drug concentration in the plasma

1. $(C_{GI} - C_p)$ represents the difference between free drug concentrations in the lumen and the free drug concentrations in the plasma. Because of the high GI blood flow rate, the drug is rapidly diluted into the blood stream after permeating the GI membranes. The high degree of drug dilution and the relatively high drug dose given orally (in the milligram range) are the bases for a large concentration gradient between the intestinal lumen and the blood stream.
2. K represents the lipid–water partitioning coefficient of a drug across the theoretical GI membrane. The physicochemical properties, such as, log P, of the molecules significantly impact this parameter.
3. A represents the surface area of the GI membrane accessible to the drug. The larger the surface area of the GI tract, the faster the drug permeates. This explains why the duodenum that has the largest surface area is the principal GI region involved in drug permeation.
4. h represents the thickness of the theoretical GI membrane. It is assumed that it is a constant across the different GI regions.
5. D represents the amount of a drug that diffuses across a membrane for a given unit area when the concentration gradient is unity.

K, A, h, and D are constants for a given molecule with a given oral formulation. These parameters define the permeability of a drug and are summarized in one equation (Eq. 3.2). Furthermore, since the free plasma concentration is extremely low, C_p is considered negligible. Therefore, Equation 3.3 can be used as a surrogate for the Flick's law. This equation demonstrates that passive drug permeation through the GI membrane is a first order process.

Permeability equation derived from Equation 3.1:

$$P = \frac{DAK}{h} \tag{3.2}$$

$$\frac{dQ}{dt} = P(C_{GI}) \tag{3.3}$$

In addition to taking the free drug concentration into consideration, the ionization state of the molecules needs to be considered. Since a majority of molecules permeating through the membrane are in a neutral form, the efficacious gradient concentration is based on free neutral compound concentrations. Therefore, pH of the lumen and pK_a of the molecules impact the degree of gradient concentration. As an example, without taking blood flow into account, the amount of neutral form of an acidic compound in the duodenum lumen is much higher than that in the plasma, further driving the gradient concentration in the direction toward greater intestinal drug permeation. However, a formal electrical charge can be highly delocalized and therefore be less of a barrier than believed, especially when lipophilicity is sufficiently high.

3.3.2.2.2 Paracellular Permeation. Paracellular permeation and endocytosis are less frequent mechanisms of intestinal absorption. The paracellular pathway refers to the passage of drugs through the intercellular spaces. The paracellular pathway is governed by tight junctions (TJs). TJs or zonula occludens constitute the major rate-limiting barrier toward the paracellular transport of drugs. The dimensions of the paracellular space are between 10 and 30–50 Å, suggesting that solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route. Transepithelial electrical resistance (TEER), measuring paracellular ion flux *in vitro* and reflecting the tightness of the intercellular junctional complex, are calculated on the basis of the serosal surface area rather than the real surface area of mucosa. As presented in a previous paragraph, the latter can vary dramatically depending on the section of intestine. In the small intestine, mucosal surface area is greatly amplified by villus projections and undulations. When TEER data are corrected for differences in mucosal surface area, the permeability of small intestinal and colonic epithelium is determined to be virtually identical [5,6]. Therefore, paracellular absorption is more likely to occur in the small intestine not because of leakier TJs but because of a larger mucosal surface area.

3.3.2.2.3 Endocytosis. Endocytosis is a constitutive process observed in most mammalian cells for the uptake of macromolecules, for example, lectins. It requires metabolic energy and it is a slow uptake mechanism resulting in a fusion of endocytic vesicles with lysosomes containing high levels of enzymatic activity. Endocytosis may involve specific receptors, for example, vitamin B12 receptor [7]. Endocytosis of compounds is believed to be minimal in the small intestine and is not a quantitatively significant mechanism for drug absorption in the intestine.

3.3.2.3 Effect of Food. Food can impact the drug intestinal absorption through several mechanisms, such as delay in gastric emptying, stimulation of bile flow, changes in GI pH, alterations in luminal metabolism, or interactions of the drug with food [8]. The extent of the food effect is dependent on the meal characteristics such as calorie content (low vs high calorie meals), nutrient composition (protein, carbohydrate-rich, or high fat meals), volume, temperature of the meal itself, and fluid ingestion.

Food also increases blood flow to the liver; therefore, changes in first-pass extraction may cause differences in bioavailability between the fed and fasted state. For compounds with saturable first-pass extraction, the bioavailability will increase with food intake, whereas the opposite will occur if hepatic enzymes are not saturated during first pass.

In addition to physiologic considerations, factors such as drug and food nonspecific binding, sequestration, or chemical instability can cause drug–food interactions. As an example, if a drug chelates with ions present in the ingested meal, drug dissolution, and/or absorption may be reduced. Furthermore, the meal itself may pose a physical barrier that prevents drug diffusion to the site of absorption resulting in decreased bioavailability. Also, drug instability, as a result of acid degradation, may be exacerbated by prolonged gastric residence after food ingestion.

For highly lipophylic drugs or large MW macromolecules, lymphatic uptake can be increased by the presence of a high fat meal, thereby lowering plasma drug levels.

Since the effects of food depend on the physicochemical properties of the drugs as well as its absorption and metabolism pathway, the extent of food effects are not

always predictable. However, it is well known that food effects on bioavailability are generally the greatest when the drug is administered shortly after a meal is ingested and when meals are high in total calorie and fat content. Hence, the FDA recommends the use of high calorie and high fat meals to study the effect of food on the bioavailability and bioequivalence of drugs. Furthermore, qualitative prediction of food effect is often possible based solely on the Biopharmaceutical Classification System (BCS) class of the drug. Gu *et al*. [9] were able to categorize 80% of a set of 92 drugs as having negative, positive, or no food effect based simply on their dose, solubility, and permeability.

3.3.2.4 Prodrug. A prodrug is any compound that undergoes biotransformation before exhibiting its pharmacological effects. Prodrugs can thus be viewed as drugs containing specialized nontoxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule. The major objectives of prodrug design are to improve chemical stability, solubility, oral absorption, metabolic stability and duration of action, tolerability, and tissue distribution.

A prodrug can be classified as carrier-linked or as bioprecursor. A carrier-linked prodrug contains a temporary linkage of a given active substance with a transient carrier group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic cleavage. A bioprecursor prodrug does not have a linkage to a carrier group, but results from a molecular modification of the active principle itself. This modification generates a new compound, the active principle, which can be transformed metabolically or chemically.

A prodrug activation occurs enzymatically, nonenzymatically, or sequentially (an enzymatic step followed by a nonenzymatic rearrangement). As much as possible, it is desirable to reduce biological variability, hence the particular interest in nonenzymatic reactions such as hydrolysis or intramolecular catalysis.

3.3.3 Intestinal Transporters

The activity of intestinal transporters is now widely accepted as an important determinant of oral drug absorption. Certainly, one major transporter has become a key area of research that continues to grow; the efflux pump ABCB1, also known as *P-glycoprotein* (P-gp) or MDR1. Because MDR1 can play a role in limiting oral absorption of certain drugs [10–12], this transporter has emerged as a potential determinant of drugs' oral bioavailability.

3.3.3.1 Efflux Transporters. Efflux transporters are present on many biological membranes, including the villus tip of the apical brush border membrane of gut enterocytes, and actively cause efflux of drugs from gut epithelial cells back into the intestinal lumen. Following oral administration, intestinal efflux transport processes present a significant barrier toward drug absorption. Efflux transporter proteins are from the ATP-binding cassette (ABC) family. Major transporters that have been shown to play a significant role in drug absorption are MDR1, and breast cancer resistance protein (BCRP; ABCG2), but MDR1 is the most characterized and well known [13].

3.3.3.1.1 P-glycoprotein/MDR1 Overview. Borst *et al*. [14,15] have postulated several likely physiological functions of MDR1. MDR1 protects against the entry of exogenous toxins ingested with food, evidenced by expression in the small intestine,

colon, and blood–tissue barrier sites. It also precludes entry of toxic compounds in the central nervous system and testis [16,17]. Recently, evidence has been reported to show how MDR1-mediated efflux can make intestinal secretion a potential mechanism for drug elimination [18,19]. It is well established that the substrate specificity of MDR1 is quite broad with respect to both chemical structure and size. The structural diversity of MDR1 substrates (and inhibitors) is so broad that it is difficult to define specific structural features that are required for substrates/inhibitors of MDR1. However, some of the properties that are shared by many MDR1 substrates include the presence of a nitrogen group, aromatic moieties, planar domains, molecular size $\geq$ 300 Da, presence of a positive charge at physiological pH, amphipathicity, and lipophilicity [20–24].

MDR1 possesses multiple drug-binding sites [25], and these sites are located in the middle of the lipid bilayer [26]. Since MDR1 removes substrates directly from the membrane, the primary determinant of substrate specificity is the ability of the drug to interact with the plasma membrane and the secondary determinant would be the ability of the drug to interact with one of the binding sites. This could explain the broad substrate specificity of MDR1 as well as why nearly all MDR1 substrates are lipophilic. Shapiro *et al*. [27] demonstrated that MDR1 possesses at least three positively cooperating drug-binding sites, an H site selective for Hoechst 33342 and colchicine, an R site selective for rhodamine 123 and anthracyclines, and another site for progesterone.

The nature of an interaction between two MDR1 substrates or a substrate and inhibitor may be unique. Therefore, caution must be exercised when trying to extrapolate how the substrate/inhibitor may interact with an untested substrate/inhibitor.

3.3.3.1.2 Impact of MDR1 on Absorption. During absorption, MDR1-mediated efflux activity can potentially attenuate the overall bioavailability of its substrates by multiple mechanisms. It can attenuate the rate at which its substrates permeate from gut across intestinal enterocytes (where MDR1 is located on apical membrane) into blood, thus, potentially delaying absorption time, reducing C_{max} and possibly reducing total exposure (AUC). Additionally, MDR1 efflux during intestinal absorption may enhance intestinal metabolic elimination, thus indirectly reducing the amount of compound able to reach the bloodstream [cf. "Intestinal Metabolism" (Section 3.3.4) paragraph below].

It has been shown through studies with mdr1a $(-/-)$ mice and in clinical trials, that the mean absorption time, AUC and C_{max} following oral administration of MDR1 substrates is affected by directed efflux activity of MDR1 in the intestine [28–30]. For example, the plasma AUC values determined for a 10 mg/kg dose of paclitaxel observed in mdr1a$(-/-)$ mice were indeed several times higher following oral administrations compared to values obtained in wild-type mice [31]. Oral bioavailability of MDR1 substrates such as tacrolimus, cyclosporin, and talinolol are known to be incomplete and variable in the clinic, as these are regulated by intestinal MDR1 and modulated by coadministered drugs, genetic polymorphisms, and disease states. In humans, duodenal MDR1 mRNA content was significantly correlated with the AUC and C_{max} of oral talinolol [32]. Also, the mRNA levels of MDR1, but not CYP3A4, correlated well with the concentration/oral dose ratio and the oral dosage of tacrolimus [33]. Long-term St John's wort treatment decreased talinolol AUC with a corresponding increase in intestinal MDR1 expression, suggesting that St John's wort has a major inductive effect on intestinal MDR1 [34].

MDR1-mediated efflux activity is an important determinant of digoxin oral absorption and this has been observed from DDI between digoxin and quinidine, and digoxin and rifampicin. Digoxin absorption is not influenced by first-pass metabolism and any changes to digoxin absorption are thought to be due to changes in the actions of MDR1. The interaction between orally administered quinidine and digoxin results in a dramatic enhancement in digoxin C_{max} and AUC [35–38]. Conversely, treatment with the MDR1 inducer rifampicin, has been shown to decrease digoxin C_{max} and AUC in humans [39]. In fact, it was shown that intestinally expressed MDR1 (induced by treatment with rifampicin) closely correlated with digoxin AUC in a negative fashion.

However, it has been shown that the *in vivo* intestinal absorption of highly soluble and highly permeable MDR1 substrates is dominated by their high permeability, and MDR1 plays a minimal role in the intestinal absorption of these compounds [40]. Cao *et al*. [40] reported that Verapamil intestinal permeability did not correlate with any gene expression patterns in the intestine, and suggested that MDR1 plays a minimal role in the *in vivo* intestinal absorption because of Verapamil's high permeability.

Therefore, for highly permeable compounds, a relatively high fraction absorbed can be expected when dissolution in the GI tract is not the rate-limiting step. On the other hand, for highly soluble and low permeable MDR1 substrates, MDR1 limits intestinal absorption in the distal segments of the small intestine but plays a minimal role in the proximal intestinal segments because of significant lower MDR1 expression levels in this region [41]. In conclusion, it is important to note that MDR1 efflux activity does not always predict a compound's absorption profile. Absorption is a highly complex multifactorial process in which MDR1 can play a part. The magnitude of the effect of MDR1 efflux activity on a compound's absorption profile, ultimately, depends on the MDR1 activity in combination with other critical factors such as solubility, permeability, and metabolism.

3.3.3.1.3 Other Efflux Transporters. In addition to MDR1, MDR related protein (MRP), also plays an important role in multidrug resistance of cancer therapy, and in affecting the behavior of other drug substrates. The MRP proteins are a relatively large protein family consisting of at least nine members. The amino acid sequences for MDR1 and MRP2 show only 15% similarity [42]. Other differences in their protein structure include different numbers of transmembrane domains (12 for MDR1 and 17 for MRP) and different orientation of their N-termini.

MRP2, previously referred to as *canalicular multispecific organic anion transporter* (cMOAT), is an important ATP-binding cassette transporter and consists of 1541 amino acid residues, encoded by ABCC2 gene. This apical membrane bound transport protein is highly expressed in the canalicular membrane, and is also present in the intestine, kidney, and the blood–brain barrier (BBB) [43–47].

BCRP, also known as *mitoxantrone resistance protein* (MXR), is the product of the ABC half-transporter gene ABCG2. BCRP expression is high in placenta and haematopoietic stem cells, while low in small intestine, colon, hepatic canalicular membrane, breast, venous, and capillary endothelium [48,49]. The physiological functions of BCRP include the extrusion of porphyrins from haematopoietic cells and hepatocytes, as well as the secretion of vitamin B2 (riboflavin) and possibly other vitamins (such as biotin and vitamin K) into breast milk [50]. BCRP shares some substrates as well as inhibitors with MDR1 and MRPs. BCRP has been reported to affect oral absorption, distribution across BBB and blood–placenta barrier, and hepatobiliary

elimination as well as milk secretion (see chapter titled Predicting Human Biotransformation Pathways of this volume and summary by Xia *et al*. [51]). Sulfasalazine low absorption from the small intestine is mainly due to MRP2 and BCRP but not MDR1. Although these efflux transporters hinder sulfasalazine intestinal absorption they enable its colonic targeting and therapeutic action [52]. Dahan *et al*. [53] have also shown that the combined effect of MDR1 and MRP2, but not BCRP, dominates colchicine transepithelial transport, leading to complete coverage of the entire small intestine and makes the efflux transport dominate the intestinal permeability process.

3.3.3.2 Uptake Transporters. Solute carrier (SLC) transporters are localized in the GI barriers. SLC transporters do not require ATP and transport the drugs according to their concentration gradient, thereby improving the intestinal absorption of a wide range of drugs [54]. Drug transporter-relevant SLC members in the intestine at the apical surface of epithelial cells include SLC organic anion transporter families (OATP subfamilies; gene SLCO), SLC peptide transporter family (PepT1; gene SLC15A1) and organic zwitterion/cation transporters (OCTNs; gene SLC22) [55]. While the impact of PepT1 on drug absorption is well established, the clinical significance of intestinal SLCOs and OCTNs in drug absorption needs to be confirmed and established. Therefore, we will focus this paragraph on PepT1 transporter.

3.3.3.2.1 PepT1 Overview. The expression of PepT1 was found primarily in the small intestine, particularly in the duodenum [56,57]. The physiological substrates for proton-coupled peptide transporters are mainly cationic, anionic, or zwitterionic di- and tripeptides. Free amino acids and larger peptides are excluded. A peptide bond in the narrower sense is not an essential structural requirement for a substrate [58].

The uptake of PepT1 substrates is mediated by a proton gradient at the apical surface of epithelial cells, the system is known as a *proton-dependent cotransport system* [59]. Indeed, an inward proton gradient is established at the brush border membrane by the Na^+ /H^+ exchanger, and then the influx of protons back into the epithelial cells is coupled by PepT1 to transport its substrates [60]. PepT1 has generally been characterized as a low affinity/high capacity transporter with a wide variety of compounds as substrates. Drug molecules transported by PepT1 include β-lactam antibiotics such as penicillins and cephalosporins [61,62], the anticancer agent bestatin [63], angiotensin-converting enzyme (ACE) inhibitors such as captopril, and the ester prodrugs enalapril and fosinopril [64]. Prodrugs of acyclovir and L-dopa can also be recognized and transported by PepT1 [65,66].

3.3.3.2.2 Impact of PepT1 on Absorption. It has been well established in recent years that PepT1 is a major player in the intestinal absorption of β-lactam antibiotics allowing their oral administration [60,67]. The transport protein accepts many β-lactam antibiotics as substrates because of its steric resemblance to the backbone of physiologically occurring tripeptides.

After oral administration, most angiotensin-converting enzyme (ACE) inhibitors such as captopril, enalapril, and lisinopril exhibit 30–100% absorption, likely because these molecules sterically resemble di- or tripeptide structures, thereby sharing an intestinal transport pathway [68,69]. However, Brandsch *et al*. [58], applying the two-electrode voltage clamp technique to Xenopus laevis oocytes expressing PepT1, concluded that the oral availability of ACE inhibitors that generate tiny inward currents

and display $K_i > 15$ mM cannot be explained by their interaction with the intestinal peptide transporter, especially at pharmacological doses, indicating other routes for absorption.

The human intestinal peptide transporter appears to be an effective target for increasing intestinal absorption of some small molecules. It is attractive as a prodrug delivery target because of its high capacity, broad substrate specificity, high expression in the intestinal epithelium, and low occurrence of functional polymorphisms [70,71]. Therefore, by using enzymatically hydrolyzable bonds in the preparation of PepT1-targeted prodrugs, it is possible to dramatically improve the systemic availability of poorly absorbed drugs, with limited systemic exposure to the intact prodrug. Han and Amidon described this general strategy as "peptide transport associated prodrug therapy" [72]. However, there are still relatively few examples of PepT1-targeted prodrugs in literature, with valacyclovir being the most widely studied [65]. Perkins and Abraham, at Eli Lilly, put in practice this approach and delivered the prodrug LY544344, demonstrating the utility of PepT1-targeted nonester prodrugs to overcome poor permeability and low bioavailability. This compound exhibits near-ideal prodrug properties, with good solubility and chemical stability, extensive and reproducible absorption across species, low concentrations of circulating nontoxic prodrug, and pharmacokinetic linearity across a wide dose range [73].

3.3.4 Intestinal Metabolism

The small intestine is the first site capable of metabolism of orally ingested xenobiotics. Therefore, it can play an important role in the first-pass metabolism of chemical compounds because both phase I and phase II metabolic enzymes are expressed [74,75]. Indeed, a number of therapeutic drugs such as cyclosporine [76], verapamil [77], and midazolam [78,79] undergo extensive intestinal first-pass metabolism.

Using Western blot analysis on microsomes prepared from mucosal scrapings from the duodenal/jejunal portion of 31 human donor small intestines, Paine *et al*. [80] demonstrated that CYP3A and CYP2C9 represent most intestinal cytochrome P450 enzymes, accounting for 80% and 15% of the total immunoquantified P450s, respectively. They also found that the expression of CYP3A4 varies along the length of the small intestine, decreasing from the duodenum to the distal ileum. The CYP3A4 content in the intestine is estimated to be $<1\%$ of that in the liver [80,81]. Furthermore, the greater overall weight of the human liver (1.5 kg) relative to that of the small intestine (0.7 kg), which when combined with the P450 concentrations and the microsomal protein contents provides for a greater overall metabolic capacity for the liver.

Interindividual variability in expression of phase I and II metabolic enzymes has been observed more frequently in small intestine compared to the same enzymes in the liver [82,83]. The mechanisms for the small intestinal variability in expression are not well known. One possibility is that the intestinal P450s are differentially induced by dietary and environmental factors [75].

As in liver, selective inhibition or induction of intestinal metabolizing enzymes either by dietary or environmental xenobiotics or by coadministered drugs has been identified as an important source of drug interactions and a contributor to variability in oral drug bioavailability [51,84,85].

Recent evidence has shown that MDR1 can play a role in determining the oxidative metabolism of its substrates that are also substrates of CYP3A. Indeed, several factors

have led to the observation that MDR1 and CYP3A4 may act in concert to determine the oral absorption and bioavailability of drugs [86–89]. In addition, MDR1 and CYP3A4 can be induced by many of the same compounds although it has recently been shown that these proteins are not coregulated [90]. It is well known that there exists a large degree of overlap between the broad substrate specificities of MDR1 and CYP3A4 [86], and it seems reasonable that the combined actions of MDR1 and CYP3A4 could contribute to the low oral bioavailability determined for many of these dual substrates. Studies involving cyclosporin A transport across Caco-2 cell monolayers have shown how the actions of MDR1 and CYP3A4 may act coordinately to enhance the attenuation of apical to basolateral transport of this drug. It was observed that cyclosporin A metabolism was much greater when the compound was transported in the apical to basolateral (absorptive) direction, than in the basolateral to apical (secretory) direction [91]. It was postulated that the reduction in the apical to basolateral flux of cyclosporin A, caused by apically directed MDR1 efflux, enhanced the exposure of the compound to CYP3A4, and thus a greater amount of metabolism was achieved [91].

In contrast to these findings, several pharmacokinetic models have been published that fail to predict an increase in CYP3A metabolism because of MDR1 efflux [92–95]. Recently, Badhan *et al*. [96] used a physiologically based model to confirm this hypothesis, demonstrating that intestinal drug metabolism is influenced directly by drug concentrations within the enterocyte, which is governed by the degree of MDR1-mediated drug efflux and drug permeability through the enterocytes. For drugs possessing apparent permeabilities in excess of 15×10^{-6} cm/s, increasing efflux ratio was predicted to result in an increase in CYP3A drug clearance by up to 12-fold in the distal intestine.

3.4 TOOLS AND METHODS TO ASSESS DRUG INTESTINAL ABSORPTION

A multiplicity of *in vitro* and *in vivo* tools are used to study intestinal absorption, providing data with different degrees of granularity and with distinct human absorption predictability.

3.4.1 *In Vitro* Tools

3.4.1.1 Permeability. One of the key parameters in predicting and understanding both GI absorption and the ability of a compound to cross cell membranes in general is permeability. For drug dosed orally, the membranes of most interest to the pharmaceutical industry are those in the GI tract, and for central nervous system (CNS) compound the, BBB. It is difficult to perform measurements on these complex membranes directly so a variety of *in vitro* models have been utilized over the years to gain an understanding and estimation of how a compound may cross biological membranes *in vivo* [97].

The most simplistic measure of permeability is lipophilicity. The general trend indicates that highly lipophilic molecules are likely to be more permeable. However, the scale of measurable lipophilicity is relatively compressed, which results in very little resolution of permeability for compounds that are structurally similar, and the process of crossing a membrane is too complex to be described by lipophilicity alone [98]. Thus, in an attempt to provide a more refined measure of permeability, cell monolayers

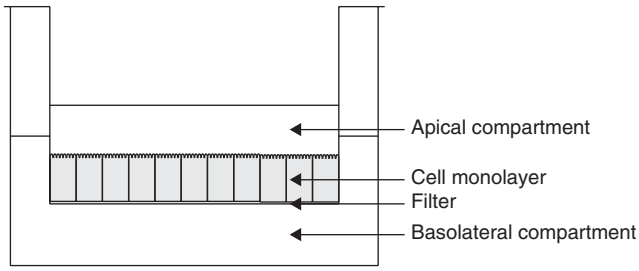

Figure 3.2 *In vitro* system used to determine drug permeability through a cell monolayer. (See color insert.)

grown on membrane supports were introduced in the 1990s [99]. The most common cell monolayer is the Caco-2 cell line, which is a polarized human colonic cancer cell line that forms integral monolayers and TJs when grown for ~20 days. These cells can be grown in 6-, 12-, 24-, and 96-well plate formats, which lend themselves well to robotic automation for both seeding and feeding, and permeability measurements [100,101]. Transcellular permeability is the movement of the compound through the cell itself and is often referred to as *passive permeability*; it is the parameter of most interest at the early screening stage. The typical experimental setup for a Caco-2 permeability experiment is to measure the apparent rate of permeability (P_{app} in 10^{-6} cm/s) of the compound in both an apical to basolateral (A to B) and a basolateral to apical (B to A) direction after incubation at 37°C for a given period of time (Fig. 3.2). The relative rates of permeability in the A to B and B to A directions indicate the compound's permeability and subsequent influx or efflux ratio (B to A/A to B). The advantage of Caco-2 is that it is a well-established technique in academia and industry for high quality permeability measurements, but the culture of the cell line is expensive and manually intensive when used as a screen for permeability.

MDCK (Madine-Darby Canine Kidney) is a dog kidney cell line that has also been applied to permeability measurements [102–104]. Although this cell line requires 3 days of culture compared to 15–21 days for most Caco-2 models, it is still relatively expensive and requires cell culture expertise. It is also less physiologically relevant, as this is a canine kidney cell line and generally the permeability measured is used to estimate intestinal absorption in humans.

The realization that the pharmaceutical industry required a cheaper but higher throughput measure of permeability resulted in the introduction of the parallel artificial membrane permeability assay (PAMPA). PAMPA utilizes artificial membranes designed to "mimic" biological membranes such as the GI tract and the BBB [105–108]. Extracted or synthetic lipids are solubilized in a solvent and dispensed onto a membrane support to form a lipid membrane. The amount of compound that moves from the donor chamber to the acceptor chamber after a period of incubation is measured, along with the amount left in the donor chamber.

These measurements and other parameters characteristic of the experimental setup are used to determine the effective permeability, P_e, and the retention factor, R_f [107]. PAMPA is a cost-effective screen because it is a physicochemical measure of permeability that is performed in a 96-well plate and is very amenable for automation. Despite the fact that PAMPA is another step further away from a physiologically relevant measurement for *in vivo* permeability or absorption, it can be used in the pharmaceutical

industry as a primary screen for measuring the intrinsic permeability of thousands of compounds per week [105,108]. PAMPA does not provide information about whether a compound is a substrate for active influx or efflux transporters that could be determined using the Caco-2 cell line. However, if this type of information is important then it can be determined at a later stage when the compound shows potential as a new drug candidate. PAMPA does aid the chemist, as the output can be a simple measure of permeability that allows for clearer structure – activity relationships (SARs) and easier interpretation when attempting to design a more permeable series.

3.4.1.2 Efflux Transporters. Some *in vitro* systems of note that are commonly used to both identify transporter substrates and study their effects on cellular disposition include the Caco-2 cell model and transfected MDCK cells [109–114]. The efflux transporters show a polarized expression in each model (localization to either apical or basolateral membrane) [115,116]. Taipalensuu *et al*. investigated the expression and interindividual variability in transcript levels of multiple drug efflux systems in the human jejunum and compared the expression profiles in these cells with that of Caco-2 cells. They showed that the transcript levels of 9 out of 10 ABC efflux transporters correlated well between jejunum and Caco-2 cells but BCRP exhibited a 100-fold lower transcript level in Caco-2 cells compared with jejunum. They concluded that Caco-2 cell line is a useful model of jejunal drug efflux, if the low expression of BCRP is taken into account [117].

MDR1 is a common efflux transporter expressed naturally in Caco-2 cells. However, the MDR1 levels are different in Caco-2 cells from lab to lab and in the gut wall of patients. These differences make it difficult to extrapolate effects seen *in vitro* to those *in vivo* when considering permeability. Shirasaka *et al*. [118] developed a method to estimate the permeability of MDR1 substrate drugs in human intestine from the expression level of MDR1 in cell lines, and thus the possibility to predict oral absorption of those drugs. Although Caco-2 cells express major intestinal ABC transporters, the high cost of sustaining these in culture for two to three weeks versus the three-day requirement for MDCK, make the latter an attractive model to assess compounds' affinity to MDR1 or any other efflux transporter.

3.4.2 *In Situ* and *In Vivo*

Although *in situ* models are seldom used in drug discovery setting, these models, for example, *in situ* single-pass perfusion of the rat intestine, can provide useful mechanistic information. The drug concentration in the intestine is known and controlled and the subsequent barriers a compound has to cross to reach the portal blood circulation are identical in the *in situ* and *in vivo* situation, which definitely contributes to the reliability of the *in situ* model. However, these are labor intensive and low throughput systems. Therefore, the drug discovery team relies on *in vivo* pharmacokinetic studies to give a sense of the absorption profile of their molecules. Rat is normally used to determine molecules' bioavailability and the fraction absorbed. Comparing data on 98 drugs, Zhao *et al*. [119] showed that rat absorption is similar to human absorption in terms of percentage of dose absorbed. Therefore, evaluation of *in vivo* absorption in rats could be used as an alternative method to predict the extent of intestinal absorption in humans following oral administration. To explain the good correlation between human and rat absorption data further, Cao *et al*. [120] determined the expression of

all known transporters and metabolizing enzymes in both human and rat duodenum and colon. They found a moderate correlation for the expression levels of transporters in the duodenum of human and rat and no correlation for the expressions of metabolizing enzymes. These findings provide the molecular mechanisms for the similarity and correlation of drug absorption and explain the lack of correlation in bioavailability between rat and human [120].

Chiou *et al*. [121] conducted a retrospective evaluation of using dog as an animal model to study the fraction of oral dose absorbed of 43 drugs in humans. The correlation between mean fraction of oral dose of drugs absorbed in humans and dogs is relatively poor; absorption is generally better in the dog. Although the dog is commonly employed as the nonrodent preclinical species for studying oral absorption in drug discovery and development, one should exercise caution in the interpretation of data obtained [121].

3.5 FDA AND INTESTINAL ABSORPTION

3.5.1 BCS Classification

The Biopharmaceutics Classification System (BCS) is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability (Amidon *et al*.) [122]. BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate release solid oral dosage forms: dissolution, solubility, and intestinal permeability. According to the BCS classification, drug substances are classified as follows:

Class 1: High Solubility – High Permeability
Class 2: Low Solubility – High Permeability
Class 3: High Solubility – Low Permeability
Class 4: Low Solubility – Low Permeability.

A drug substance is considered highly soluble when the highest dose strength is soluble in 250 mL or less of aqueous media over the pH range of 1–7.5.

The permeability class boundary is based indirectly on the extent of absorption, that is, the fraction of dose absorbed, of a drug substance in humans and directly on measurements of the rate of mass transfer across human intestinal membrane. Alternatively, nonhuman systems capable of predicting the extent of drug absorption in humans can be used (e.g., *in vitro* epithelial cell culture methods). In the absence of evidence suggesting instability in the GI tract, a drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose.

The BCS classification can be used to request a waiver from the FDA for *in vivo* bioavailability and/or bioequivalence studies for immediate release solid oral dosage forms.

3.5.2 FDA View on MDR1

Having developed guidelines for using *in vitro* metabolism studies to assess drug metabolism (specifically cytochrome P450)-mediated potential DDI, the FDA has initiated an effort to develop similar guidelines for transporter-mediated DDI (http://www.

fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064982.htm). Reflecting on the availability of extensive literature and industry data on the role of MDR1 in DDI, the FDA has developed a more comprehensive guideline on MDR1-mediated DDI. The FDA acknowledged the role of other transporters in inhibiting or inducing drugs disposition but without proposing guidelines on how to study these transporters. The International Transporter Consortium (ITC) recently published a paper addressing this specific gap [123].

For details of the guideline and decision trees developed by the FDA, the reader is referred to the website: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064982.htm.

The guideline discusses design of *in vivo* DDI studies including the choices of substrate and interacting drugs, labeling implications, decision trees for substrates and inhibition data, and how the *in vitro* assays should be run. To identify MDR1 substrates for *in vivo* drug interactions studies, the guideline proposes the following process (i) examining bidirectional transport of the test compounds in Caco-2 or MDR1-MDCK cell monolayers, (ii) selecting likely substrates based on an efflux ratio >2, (iii) confirming MDR1 substrate activity by showing that specific inhibitors of MDR1 decrease the efflux ratio, and (iv) selecting compounds for *in vivo* MDR1-related interactions studies if transport of a test compound with an efflux ratio of >2 in these test systems is inhibited by MDR1 inhibitors.

To identify MDR1 inhibitors for *in vivo* drug interactions, the guideline proposes the following process (i) examining bidirectional transport of MDR1 probe substrates across Caco-2 or MDR1-MDCK cell monolayers, (ii) determining the ability of the test compound to decrease net flux ratio of a MDR1 probe substrate, (ii) determining K_i or $[I]/IC_{50}$ of the test compounds, and (iv) selecting compounds with K_i or $[I]/IC_{50} > 0.1$ for *in vivo* drug interaction studies with a MDR1 substrate such as digoxin.

3.5.3 Recommendations from the International Transporter Consortium

The ITC comprises industrial, regulatory, and academic scientists with expertise in drug metabolism, transport, and pharmacokinetics [123]. The consortium was formed to address transporter-related questions from pharmaceutical scientists involved in drug development. They identified which transporters, based on current knowledge, are well-established determinants of pharmacokinetics; discussed methodologies to characterize drug–transporter interactions using *in vitro* and *in vivo* studies; and proposed recommendations that are important for drug development scientists in guiding preclinical and clinical studies of transporter-mediated drug interactions [123].

The consortium focused its work on a set of seven transporter proteins based on compelling evidence that they are involved in drug absorption, disposition, and/or drug drug interaction (DDI): OATP1B1, OATP1B3, OCT2, OAT1, OAT3, MDR1, and BCRP. MDR1 and BCRP are transporters proteins playing a key role in the absorption of new chemical entities (NCEs). For details of the ITC recommendations and decision trees, the reader is referred to the ITC paper [123].

In summary, to identify MDR1 and BCRP substrates, the ITC broadened the FDA drug interaction guidance and decision tree to include both MDR1 and BCRP. The ITC also recommends that when an NCE is identified as MDR1 or BCRP substrates, preclinical and clinical information should be assessed to determine whether a clinical

in vivo DDI study is warranted. In particular, the relative contribution of the transporter-mediated pathway to the overall clearance of the drug is the primary determinant of whether an inhibitor will have a major effect on the disposition of the NCE.

A NCE is considered to be a potential MDR1 and/or BCRP inhibitor if the net flux ratio of a MDR1 (or BCRP) probe substrate is decreased in the presence of the NCE using a bidirectional transport assay. Zhang *et al*. [124] proposed that drugs exhibiting an $[I]_2/IC_{50} \geq 10$ should be evaluated to determine whether intestinal MDR1 or BCRP inhibition occurs *in vivo*. In that publication, $[I]_2$ is the theoretical maximal GI NCE concentration after oral administration and calculated as the highest clinical dose (mg) in a volume of 250 mL. Using this approach, a drug with MW of 500 Da and an *in vitro* IC_{50} value of 10 μM will have a $[I]_2/IC_{50}$ value >10 at a dose of ~12 mg or greater. Recently, using data from 26 digoxin DDI clinical trial and applying statistical analyses of binary classification and receiver operating characteristic (ROC), Cook *et al*. [125] revised cutoff values and ratio of $[I]_2/IC_{50} > 5$ were identified to minimize the error rate, a reduction of false negative rate from 36% to 9%.

The ITC discussed the characteristics of an NCE that would trigger an *in vitro* interaction study with a specific transporter: the physicochemical properties of the drug and the organ(s) involved in its clearance. In addition to *in vitro* data, human pharmacokinetic property of an NCE is also important in assessing the likelihood of a clinical transporter-related DDI.

ACKNOWLEDGMENTS

The author thanks Matt Troutman (Pfizer) for helpful discussions while planning this manuscript.

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