

## Human Intestinal Cellular Characteristics and Drug Permeability

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James E. Polli, Anand Balakrishnan, and Paul R. Seo

### 1. Introduction

Oral drug administration is the most convenient method to administer drugs, particularly in the treatment of chronic diseases. This preference is generally the case, even when the site of drug action is beyond the gastrointestinal tract (GI), requiring the drug to be systemically available. For systemic drug availability, drug dosing via the oral route requires the drug to exhibit sufficient gastrointestinal absorption. Two components of drug absorption from solid oral-dosage forms (e.g., tablets, capsules) are: (1) drug dissolution from the dosage form, which results in the drug being in solution within the gut lumen, and (2) drug permeation across the GI wall. In this chapter, elements of GI drug permeability are discussed, with special emphasis on the enterocyte and the *in vitro* Caco-2 cell model. Included is a discussion of the lipid plasma membrane, *in vitro* drug permeability and its relation to *in vivo* permeability, and a discussion of future research directions.

### 2. Methods to Study Intestinal Drug Absorption

Prior to discussing the *in vitro* Caco-2 monolayer model as a model for intestinal drug permeability, it should be recognized that numerous methods are available to study intestinal drug absorption. In Table 1, methods can be categorized as *in vivo*, *in situ*, and *in vitro*. Selection of any method will depend on several considerations, including the question to be answered and the availability of resources. Methods range in degree of integration and complexity.

#### 2.1. *In Vivo* Methods

*In vivo* systems, such as whole animal or human studies, to measure oral drug bioavailability can be considered the most complex experimental systems, because all potential barriers (e.g., dissolution, intestinal permeability, metabolism) are present to limit drug absorption. Consequently, mechanistic interpretations from such complex systems is often limited, if not impossible. Rather, the main advantage of *in vivo* studies is their ability to answer specific, often practical questions. *In vivo* studies are used to measure absolute oral drug bioavailability, determine bioequivalence of one product to another, and assess pharmacokinetic impact of a formulation change.

**Table 1**  
**Hierarchy of Methods to Study Intestinal Drug Permeability**

<i>In vivo</i>	Bioavailability; human bioequivalence; scintigraphy study; human intestinal perfusion; fistulated animals
<i>In situ</i>	Intestinal perfusion (various formats); chronically isolated intestinal loop
<i>In vitro</i>	Cell cultures (e.g., cell monolayers; cell suspensions), including transfected cells; excised tissue (e.g., tissue flap, everted sacs, intestinal rings); artificial lipid membranes (e.g., parallel artificial membrane, black lipid membrane); membrane vesicles (e.g., brush-border membrane vesicles, basolateral membrane vesicles).

*In vitro* systems, such as the Caco-2 cell monolayer system, benefit from relative simplicity in design, allowing for mechanistic interpretation. However, this lack of integration with other physiologic and biochemical components that can influence overall drug oral bioavailability, and naturally limits the scope of interpretation for such simple systems. A panel of multiple *in vitro* tests is typically required to estimate oral drug bioavailability, or delineate the major mechanism of absorption.

These *in vivo* designs can be supplemented with scintigraphy, to allow for imaging of the dosage form, in order to relate observed pharmacokinetics with the disposition of the dosage form during intestinal transit. For example, scintigraphy elucidated the role of small intestinal residence time as a contributing factor in oral saquinavir bioavailability (1). In an *in vivo* study of saquinavir capsules in humans, saquinavir bioavailability was greatly enhanced when the drug was co-administered with food, relative to the drug without food. Scintigraphy data allowed for the relating of saquinavir pharmacokinetic profiles to the temporal disposition of the drug in the stomach and small intestine. Data indicated that that drug absorption was enhanced when drug transit to the colon was retarded. Food increased saquinavir retention in the stomach, in part by allowing more time for the drug to dissolve, and hence be absorbed. It should be noted that fasting data also indicated that drug absorption was enhanced when drug transit to the colon was retarded, since drug absorption was essentially zero after the drug reached the colon.

In addition to scintigraphy, another technique that can be noted as an *in vivo* method to study intestinal drug absorption is the human intestinal perfusion technique. Through the efforts of a few research groups, various human perfusion approaches to measure drug intestinal permeability have been developed, including the double-balloon method (2). In the double-balloon method, human subjects are intubated with a multilumen tube (i.e., an outer tube containing several parallel tubes inside the outer tube). After a subject is intubated with the multilumen tube, pressurized air is introduced into two of the tube lumens, in order to inflate two balloons. The two balloons, which are positioned about 10 cm apart, create an isolated intestinal segment, which can subsequently be perfused with drug solution by pumping drug solution through a third lumen of the tube. The perfused drug solution can be collected from yet another tube lumen(s). Drug permeability across the intestinal mucosa can be calculated, by considering initial and final drug concentrations. The intestinal segment most frequently studied is the jejunum; the colon has also been studied. In the last decade, a range of drugs has been subjected to the human jejunal perfusion technique, providing *in vivo* drug-permeabil-

ity values. In Subheading 5., permeability values from the Caco-2 method will be compared to values from human perfusion. Fistulated animals are also an *in vivo* method noted in Table 1. For example, duodenally fistulated dogs (i.e., dogs with an external access port to the duodenum) can be used to study absorption and/or stability within the duodenum.

## 2.2. *In Vitro Methods*

At the other end of the hierarchical spectrum of techniques, *in vitro* systems are preferentially employed to address mechanistic questions, as well as perform high-throughput screening, as described in Chapter 17. *In vivo* systems represent highly integrated systems and thus are inherently handicapped to address mechanistic questions, although they offer an excellent approach to obtain practical pharmacokinetic data. By virtue of their design, the strength of *in vitro* systems is their simplicity, which is not to suggest that such experiments are readily performed. Perhaps the most complex *in vitro* methods are excised tissues and cell cultures. *In vitro* platforms with greater simplicity are artificial lipid membranes and membrane vesicles.

Numerous cell-culture models have been applied to study and estimate drug permeability. In Chapter 7, several cell-culture models of carrier-mediated transport are described. Two common cell-culture systems to study drug permeability are Caco-2 cell monolayers and Madin-Darby canine kidney (MDCK) cell monolayers. Each system, particularly Caco-2 cell monolayers, is described in greater detail in Subheading 3. It should be noted that a main reason for the utility of Caco-2 and MDCK cells is that each is an adherent cell type that forms a “tight” monolayer. By virtue of being adherent cells (i.e., cell that preferentially grow on surfaces), each can be grown on porous supports. Because of the near-daily attention required to culture cells, excised tissue remains a perhaps more convenient alternative; tissue flap, everted sacs, and intestinal rings are three means to employ intestinal tissue. Artificial lipid membranes include the parallel artificial membrane method (*see* Chapter 17), where a series of lipid bilayers are formed within a filter support, which is subjected to drug flux. A black lipid membrane (BLM) is composed of a single lipid bilayer, and perhaps represents the most simplified system to study membrane permeability, although BLM studies are technically challenging to construct.

## 2.3. *In Situ Methods*

Latin for “in place,” *in situ* lies between *in vivo* and *in vitro*, and is exemplified by methods where drug solution is perfused through the rat intestinal lumen. Several experimental variations of the rat intestinal perfusion method are practiced, such as the single-pass perfusion, the single-pass perfusion with venous collection, and the multiple-pass perfusion. By virtue of being an *in situ* method, the animal is alive during the course of the study, but must be sacrificed after the experiment.

Denoted here as an *in situ* method, the chronically isolated intestinal loop is similar to the perfusion methods mentioned previously because the intestine is suitably prepared and perfused to determine intestinal drug permeability. However, this method is unique because the animal is conscious and is not sacrificed at the end of the experiment. In the chronically isolated intestinal loop method, a segment of the intestine is isolated and externalized to the abdominal wall. The isolated segment is then connected to two cannulas, inserted through the skin and attached to the abdominal wall. The remaining intestine is rejoined by end-to-end anastomosis.

### 3. The Enterocyte and the Caco-2 Cell Model

#### 3.1. Morphology

The GI tract is composed of the mouth, pharynx, esophagus, stomach, small intestine, large intestine, and rectum, and functions to absorb food, nutrients, and water. Arguably, the most important component of the GI tract is the small intestine, because the majority of food digestion and nutrient absorption (and drug absorption) occurs there. The small intestine is a tubular structure with an approximate radius of 1.5 cm. Drug is absorbed from the tube lumen, which can be considered external to the body. As a result, the small intestine is protected by an epithelial barrier, to guard the underlying serosal tissue and avoid systemic exposure of toxic xenobiotics. Epithelial cells of the small intestine are predominately composed of absorptive cells called enterocytes.

Figure 1 illustrates the small intestine at the organ level, the enterocyte level, and three levels in between (3). In panel 1, circular folds and longitudinal and circular muscles facilitate absorption by enhancing surface area and mixing action, respectively. In panel 2, the surface area of the circular folds is enhanced by villi. In panels 3 and 4, the villus is covered by a layer of enterocytes; other specialized cells, which function in mucous secretion, endocrine secretion, and immunity, are also present. In the small intestine, cells at the base of the villi undergo mitosis. These cells subsequently differentiate, as they migrate to the top of the villi, replacing older cells, and release into the lumen. The center of each villus is occupied by lymphatic vessels, which combine with other lymphatic vessels from other villi to form a lymph duct. The lymphatic vessel within a villus is called a lacteal. Also within a villus is a microcirculation system (i.e., arterioles, capillaries, and venules), which is situated around the lacteal. From panel 4, enterocytes present the main resistive barrier between the gut lumen and the blood and lymph, and present a physical and biochemical barrier to permeation. The physical barrier is constituted by the cell membrane and the presence of tight junctions, which restrict transcellular and paracellular permeability of solutes, respectively. The presence of various metabolic enzymes (e.g., Cytochrome P450) and efflux proteins (e.g., P-glycoprotein [P-gp]) contribute toward the biochemical barrier.

The absorbed nutrients and drugs enter the blood capillaries, while absorbed fat enters the lacteals. The enterocyte is illustrated in panel 5. Cell structures and their functions are described in Chapter 1. Enterocytes are columnar and highly polarized, with apical and basolateral membranes that are separated by tight junctions. Microvilli on the apical membrane (or mucosal side) allow for enterocytes to function as absorptive cells. These finger-like projections enhance the absorptive surface area in a multiplicative fashion, as microvilli further amplify the effects of villi and circular folds.

Since the early 1990s, the Caco-2 cell monolayer model has been and continues to be a frequently employed model system to answer questions and test hypotheses concerning intestinal drug absorption. Caco-2 cells were derived from a primary colon tumor from a 72-yr-old man (4). Like most tumor cell lines, Caco-2 cells possess a transformed phenotype, which has given rise to an immortalized cell line. Daughter cultures of a continuous cell line have similar growth characteristics and genetic properties as the parent culture. The Caco-2 cell monolayer model initially became a model of interest because Caco-2 monolayers mimic enterocytes, in terms of cell morphology and biochemistry. Most notably, these cells can be cultured with ease *in vitro* and undergo spontaneous, enterocyte-like differentiation after reaching confluence (5). In fact, Caco-2 cells are frequently used as a model to study cell differentiation. Efforts to

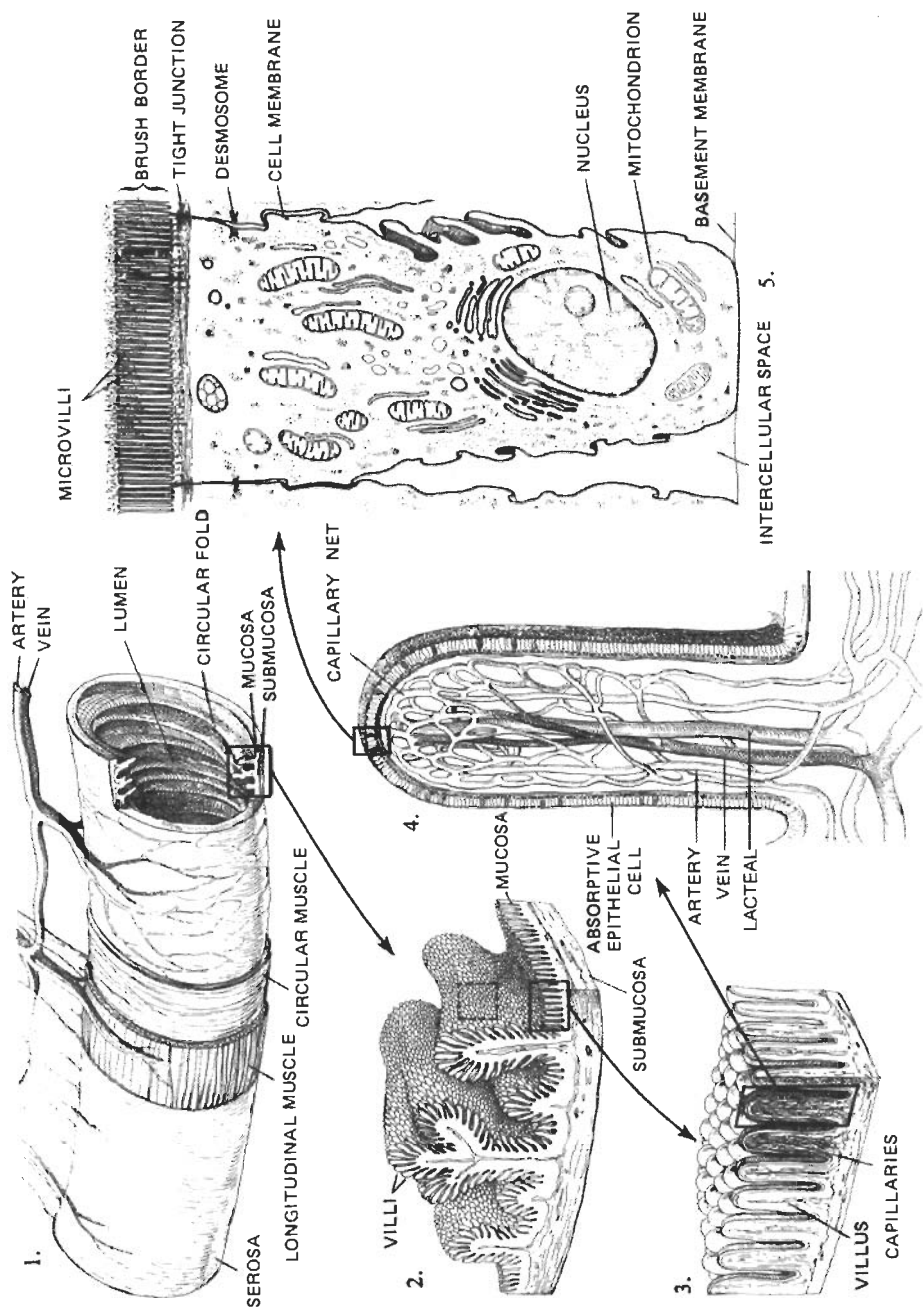


Fig. 1. Increasingly magnified views of the small intestine. Adapted with permission from ref. 3.

culture noncancerous enterocytes is technically challenging, because cells dedifferentiate (i.e., lose their specialized properties *in vitro*). Although these cells are colorectal adenocarcinoma cells, Caco-2 cells are broadly representative of enterocytes (e.g., form a tight monolayer of polarized, columnar cells with microvilli, containing brush-border enzymes such as peptidases, disaccharidases, and alkaline phosphatase) (6). These cells are an enterocyte model in many research areas, from cellular biochemistry and signaling to cellular physiology. These cells have also been used to study the transport of various nutrients and endogenous compounds, including amino acids, bile acids, and neurotransmitters (7). These studies indicate the expression of a variety of active transport systems by these cells similar to those expressed in the small intestine, which help in the translocation of various nutrients. In addition, these cells also express various receptors involved in the endocytotic transport pathways, such as vitamin B<sub>12</sub>.

However in spite of these similarities, there are significant differences between the *in vivo* intestinal epithelium and the Caco-2 system, because a simple tumor cell line is inherently unable to duplicate all attributes of a normal cell, much less a collection of cells that form a complex epithelium. Another inherent challenge is the maintenance of phenotypic stability of cultured cells, which varies with each passage (8). Phenotypic stability is also affected by differences in culture conditions.

As described later, recent research efforts have focused on: (1) differences in physical barrier function between the Caco-2 system and the small intestine, (2) Caco-2 expression properties relevant to drug transport and metabolism, (3) methods to induce Caco-2 biochemical barrier function, and (4) cell-culture methods to facilitate phenotypic stability and rapid assay.

### **3.2. Differences in Physical Barrier Function Between the Caco-2 System and the Small Intestine**

#### **3.2.1. Relative Homogenous Nature of the Caco-2 Model and Absence of Mucus**

The intestinal epithelium comprises of a heterogeneous population of cells, including enterocytes as well as goblet and vacuolated cells, tuft cells, microfold cells, theliolymphocytes, Paneth cells, and basally granulated cells. The enterocytes and goblet cells represent the two major phenotypes in the intestinal epithelium (9). Mucus-producing goblet cells are of particular potential interest with regard to drug absorption, but are absent in the Caco-2 system. Absence of mucin is just one of the many imperfections of the Caco-2 system, in terms of its suitability as a comprehensive model for the *in vivo* epithelium. The role of mucus as a diffusion barrier to drug absorption is not clearly understood, owing to the limited number of studies and conflicting results observed so far (10). HT-29 cells are a mucus-producing goblet-cell line that potentially may address this limitation. These cells form monolayers with a large proportion of mature goblet cells, which secrete mucin to form a layer of mucus at the apical-cell surface. A limited number of studies have also grown co-cultures of Caco-2 cells with HT-29 goblet-cell lines and apparently have shown limited success (9).

#### **3.2.2. Paracellular Permeability**

Two major routes for passive drug transport across an epithelial monolayer are the paracellular and transcellular pathways. The transcellular pathway refers to the movement of solutes through the cell via the apical and basolateral plasma membranes. The paracellular transport refers to the passage of solute molecules through the intercellular spaces or pores between cells. Paracellular permeability is a function of the intercellu-

lar spaces, which are gated by tight junctions. This barrier function of the tight junctions is a significant factor in the transport of hydrophilic drugs across epithelial barriers. Caco-2 monolayers exhibit relatively low paracellular permeability (10–100 times lower) compared to human intestinal permeability (11). This difference can result in underestimation of permeabilities for compounds with low transcellular permeability. Nevertheless, Caco-2 cells have been used to study qualitative and quantitative aspects of paracellular transport. There have also been efforts to develop alternate cell lines that exhibit paracellular permeabilities comparable to human intestine. A majority of these efforts focus on goblet-cell lines (e.g., HT29) or co-cultures (9). Recent efforts use normal intestinal cells expressing a temperature-sensitive mutant of Simian Virus 40 (SV40) T oncogene (2/4/A1). These cells are based on a temperature-switch mechanism, where a change in culture temperature promotes cell differentiation, such that the cells exhibit high paracellular permeability.

### **3.3. Caco-2 Expression Properties Relevant to Drug Transport and Metabolism**

Historically, the first models of drug permeability assume passive permeability, with little attention focused on active transport and the contribution of intestinal metabolism, largely because models for active transport and intestinal metabolism were lacking. Also lacking was an understanding about the interplay between the various components of the biochemical barrier to the absorption of xenobiotics. Cell-culture models now provide critical tools to uncover and understand mechanisms controlling this drug transport. Mechanisms include influx transporters, efflux transporters, and intestinal metabolism.

#### **3.3.1. Influx Transporters**

In concert with broad developments in gene-based advances, a significant amount of research has been performed in the last decade to identify and characterize previously unknown transporters that serve to enhance absorption (i.e., influx transporters). There are transport systems for amino acids, peptides, monosaccharides, monocarboxylic acids, organic cations, organic anions, phosphates, nucleosides, and vitamins (12,13). Table 2 lists several influx transporters in Caco-2 cells. Several transporters have been cloned and studied in detail and play a major role in determining the intestinal absorption of xenobiotics. With the commercial success of the anti-viral prodrug valacyclovir, researchers have made significant progress to understand the role of transport proteins in drugs absorption. For example, a large body of work has been reported on the peptide transporter and its various isoforms. Peptide transporters are known to play an important role in the oral absorption of various classes of drugs, including  $\beta$ -lactam antibiotics and ACE inhibitors. The peptide transporters have also been exploited in a prodrug fashion to improve the intestinal uptake of the otherwise poorly absorbed nucleoside analogs acyclovir and zidovudine (14). In contrast to nutrient transporters, which often exhibit narrow substrate specificity, other transporters exhibit broad substrate specificity, such as monocarboxylic acid transporters (MCTs) (12).

Although the Caco-2 system expresses a significant proportion of the influx transporters, this system does not reflect the degree of expression of active transporters in vivo. Many studies indicate that the level of transporter expression in Caco-2 monolayers differs by more than fivefold (15,16) relative to their expression in vivo levels. The level of expression of influx transporters is typically low in vitro, resulting in an under-

**Table 2**  
**Representative Influx Active Transporters in Caco-2 Cells**

Transporter	Natural substrate	Drug substrates
Large neutral amino acid system	Valine	Gabapentin, L-dopa, $\alpha$ -methyldopa
Pept1	Small peptides	$\beta$ -lactam antibiotics, valacyclovir, ACE inhibitors
Glucose transport systems	Glucose	Dehydroascorbic acid
Monocarboxylic acid transport system	Lactic acid	Atorvastatin,
Inorganic phosphate transport system	Short-chain fatty acids	Salicylic acid
	Inorganic phosphates	Foscarnet
Bile-acid transport system	Bile acids	Chlorambucil-bile acid conjugates (S2521)
Organic cation transport system	Carnitine	Cimetidine
Multivitamin transport system	Biotin, pantothenic acid	Tat9-K-biotin (bioactive peptide conjugated with biotin)

estimation of drug permeability. It should be noted that *in vivo* expression varies. For example, a significant correlation was observed between Pept1 expression and cephalixin permeability, indicating that variability in the intestinal expression of Pept1 is a major determinant of variable *in vivo* cephalixin absorption (16). Moreover, *in vitro* transporter expression level can be dependent on passages and age of the culture (8). Not surprisingly, considerable variability is observed between different labs. The transport of bile acids across Caco-2 cells varies almost 20-fold with regard to the contribution of the transporter, with one report showing a 60-fold enhance of bile-acid permeability owing to the transporter, whereas another report indicates only a 3.6-fold effect (7,17,18). Similarly, the activity of the amino acid transporter differs by as much as fivefold between labs when comparing bi-directional transport ratios.

### 3.3.2. Efflux Transporters

The intestinal epithelium expresses a variety of efflux transporters and metabolic enzymes, which constitute a biochemical barrier to drug absorption. Efflux serves as a detoxification system in the small intestine and limits the absorption of toxic xenobiotics from the small intestine. Several efflux transporters are members of the adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transporter family and are detailed in Chapter 8. Efflux proteins generally exhibit broad substrate specificity and high capacity. The low and variable bioavailability of drugs such as digoxin and HIV protease inhibitors is partly owing to efflux transporters. Owing to overlapping substrate specificity, various efflux proteins may operate in parallel to limit intestinal drug absorption in a complex manner. Caco-2 cells express several efflux transporters such as P-gp and multiple drug resistance proteins (MRPs). Expression levels vary significantly and can depend on age in culture, culture conditions, and previous drug exposure (19).

A few studies correlate level of expression in the Caco-2 system with in vivo human tissue (20,21). Taipalensuu et al. (20) compared the expression of 10 members of the ABC transporter family in Caco-2 cells to expression levels in human jejunum. Results indicate that the Caco-2 system expresses most of the genes responsible for efflux proteins at levels similar to those observed in human jejunum. In most cases, the Caco-2 values were within a factor of 2.5 of human jejunum values. The exception was BCRP, which was found to be expressed at very high levels in the jejunum compared to Caco-2. The in vivo level of expression of BCRP was even higher than the transcript for MDR1. This finding indicates that the role of BCRP may have a greater role in limiting drug absorption than previously thought. Indeed, studies with mice have indicated that BCRP has a major role in limiting the bioavailability of topotecan. Similar findings were reported by Anderle et al. (15), who examined mRNA expression of various transporter and ion-channel genes using a microarray chip and compared the transcript levels between Caco-2 and human intestine. Their study indicated lack of significant differences in the expression levels of ABC transporters in Caco-2 cells and human intestine. Minor differences were seen in expression levels of Pept1, with a higher level in small intestine than Caco-2 cells. A more comprehensive study has been carried out by Sun et al. (21), who compared the gene expression profiles of Caco-2 cells and human duodenum for various transporters, channels, and metabolic enzymes. They further related these results to drug permeability differences between human duodenum and the Caco-2 system. Their study indicates at least a 10-fold difference in influx transporter-expression levels between Caco-2 and small intestine. P-gp was expressed at a much higher level in the duodenum than Caco-2 cells; MRPs were expressed at comparable levels between the Caco-2 and small intestine.

### 3.3.3. Intestinal Metabolism

Metabolic enzymes comprise another biochemical barrier to drug absorption and can be broadly classified into two main groups: the phase I oxidative cytochrome P-450s (CYPs) and the phase II conjugative enzymes (e.g., UDP-glycosyltransferases [UGTs] and sulfotransferases [SULTs]). These enzymes can present a major barrier, particularly in coordination with efflux transporters.

CYP3A4 is the principal component of the oxidative enzymes in the small intestine. Intestinal CYP3A4 contributes significantly to the poor bioavailability of certain drugs. As both CYP3A4 and P-gp functions to protect the body from toxic xenobiotics, there is substantial overlap in substrate specificity between these two systems (e.g., cyclosporin, verapamil, etoposide). Likewise, CYP3A4 inhibitors, such as ketoconazole and erythromycin, significantly inhibit the activity of P-gp.

It has been proposed that P-gp not only functions as a transport barrier but also promotes intestinal metabolism by CYP3A4, resulting in a synergistic effect. Two mechanisms have been suggested to explain the enhancement of metabolism by P-gp. One entails the repeated cycling of the compound in the intestine, such that there is increased exposure of drug to intestinal CYP3A4. An alternate mechanism points to P-gp effluxing primary metabolites from the cell, thus avoiding product inhibition of the enzymes.

Glucouronidation and sulphate conjugation are two major phase II metabolic pathways in enterocytes and are mediated by UGTs and SULTs, respectively. Phase II conjugation enzymes usually work in conjunction with organic anion efflux mechanisms in restricting the absorption of drugs. Flavanoids are one class of compounds

whose absorption is adversely affected by the activity of these enzymes. Moreover, these compounds are reported to promote their own metabolism by the inducing expression of these enzymes. Once flavanoids are conjugated inside the enterocytes, their metabolites are usually extruded from the enterocytes via organic anion transporters.

### **3.4. Methods to Induce Caco-2 Biochemical Barrier Function**

In vitro cell culture has potential to predict drug interactions. Specific efforts have been directed towards understanding drug interactions involving drug absorption, with particular interest in the role of metabolic enzymes and efflux transporters. However, systems such as Caco-2 cells do not express significant amounts of CYP3A4 and phase II metabolic enzymes. Several approaches have been taken to circumvent this limitation. One method is to stably transfect Caco-2 cells or other cells with cDNA encoding for CYP3A4. Another approach is enzyme induction by exposing cells to inducing agents. Treatment of Caco-2 cells with 1- $\alpha$ ,25-dihydroxy vitamin D<sub>3</sub> is used to induce CYP3A4 expression, as well as P-gp, in a dose dependent fashion (22). UGT has been induced by the aryl hydrocarbon receptor agonist t-butylhydroquinone (TBHQ) and the anti-oxidant type inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This induction is isoform-specific, with UGT1A6 and UGT1A9 being inducible by TBHQ, and UGT1A6, 1A9, and 2B7 responding to TCDD.

Recent studies indicate the potential of drugs to induce or repress metabolic enzymes expression, which can result in modulated exposure of other co-administered drugs. For example, P-gp and CYP3A4 induction has been proposed as a mechanism to explain decreased verapamil absorption after repeated oral rifampin administration. Similarly, reduced HIV protease-inhibitor exposure after repeated administration has been ascribed to self-induction of intestinal P-gp and CYP3A4. Dietary components like grapefruit juice and other citrus fruits have been observed to increase oral drug bioavailability by downregulation of CYP3A4 and P-gp inhibition.

To study such drug interactions, subclones of Caco-2 (e.g., TC-7) and other cell lines (e.g., LS-180) have been used. These alternatives may prove useful to predict induction of the biochemical barrier by drugs and the resultant potential for drug interactions. In recent years, orphan nuclear receptors such as the pregnane X receptor (PXR), constitutive androgen receptor (CAR), arylhydrocarbon receptor (AhR), retinoic acid receptor (RAR), and farnesoid X receptor (FXR) have been established as xeno-sensors that regulate the expression of phase I and II enzymes and efflux transporters. This transcriptional regulation is achieved by the binding of these xenobiotic receptors to the nuclear-response elements found within the promoter regions of target genes. There is significant evidence indicating common regulatory mechanisms at the molecular level controlling the expression of metabolic enzymes and efflux proteins. A study of these controlling elements will be of significant importance in delineating the expression variability of efflux proteins and metabolic enzymes in different individuals. For example, the CYP3A4 and P-gp share a common regulatory-expression control through PXR, which partly explains why inducers of CYP3A4 expression increase P-gp (23).

### **3.5. Cell-Culture Methods to Facilitate Phenotypic Stability and Rapid Assay**

Traditional Caco-2 cell culture for permeability studies employs a rather generic cell-culture approach, resulting in a 3-wk period to attain functional, differentiated monolayers. Medium changes during this culture period are cumbersome and expensive. Moreover, the use of 10% fetal bovine serum (FBS), which varies in composition from lot to lot, contributes to phenotypic instability. Owing to these reasons, the traditional Caco-2 system is typically a low-through-put system that is susceptible to phenotypic drift. We developed a rapid culture system for growing Caco-2 cell monolayers that reduces the need for serum (24). In this system, confluent and polarized Caco-2 cell monolayers are formed in 4 d. One salient feature of this system is the use of reduced serum (2% calf serum compared to 10% FBS), which results in reduced variability (usually associated with serum composition) and significantly lowers costs. Another advantage of this system is the reduction in transporter expression, which better allows the estimation of passive permeability. Liang et al. found this system to provide permeability values comparable to the 21-d system, with a low level of P-gp expression (25). This model has been proposed as a rapid, accurate, and economical mean of classifying drugs candidates within the Biopharmaceutics Classification System (BCS).

The MDCK model is a nonintestinal epithelial cell line that also serves as a rapid approach to measuring passive permeability. A main advantage of MDCK cells is their adaptability to high-throughput screening. Native fast growers, MDCK cells may be used for permeability studies in as little as 3 d. MDCK monolayers are a model for studying tight-junction functioning and provide low paracellular permeability. Passive permeability values from 3-d MDCK cells are comparable to Caco-2 cells (18,26). Additionally, MDCK monolayers express a subset of several major transporters, such as peptide transporter, P-gp and MCTs (18).

MDCK monolayers have been used to stably overexpress specific transporters to develop simplistic models for the specific transporter in question. Relative to a system such as Caco-2 that expresses a variety of transporters, the advantage of a transfection approach is the resulting high expression of the single transporter, essentially without confounding effects of other transporters. MDCK cells transfected with the MDR1 gene (i.e., MDRI-MDCK cells) are often used to study the effect of P-gp on drug permeability. Although only one transporter is typically overexpressed, doubly transfected systems have been generated to study nucleoside transport.

## **4. Cell Plasma Membrane and Drug Permeability**

### **4.1. The Lipid Bilayer**

The apical plasma membrane is generally thought to be the main barrier for passive drug absorption, as the apical membrane defines the interface between self and the external environment. The plasma membrane is a bilayer, composed primarily of amphipathic phospholipids, as well as other components (e.g., proteins, sugars, cholesterol). Phospholipids contain a hydrophilic phosphate head and two hydrophobic fatty-

**Table 3**  
**Phospholipid Composition of Apical Membranes of the Small Intestine**

Phospholipid	Percent of total phospholipid			
	Pig	Rat	Rabbit	Mouse
Phosphatidylethanolamine	34.0	38.3	30.3	49.1
Phosphatidylcholine	34.0	32.6	35.0	25.1
Phosphatidylserine	12.0	13.0	7.5	6.0
Phosphatidylinositol	10.0	8.7	8.3	11.6
Sphingomyelin	10.0	6.4	10.5	8.3
Lysophosphatidylethanolamine	—	—	3.	—
Lysophosphatidylcholine	—	1.0	3.1	—
Phosphatidic acid	—	—	1.2	—
Cardiolipin	—	—	—	—
Phosphatidylglycerol	—	—	—	—

**Table 4**  
**Fatty Acid Tail Composition of Apical Membranes in Rat Colon**

Fatty acid	Composition (%w/w)
16:0	28.0
16:1	2.5
18:0	19.2
18:1	24.0
18:2	15.2
20:4	11.2

acid tails. The phosphate head group is polar, with the phosphate functional group being negatively charged. Table 3 lists several types of phospholipid-head groups found in the apical membranes of various animals (27). Phosphatidylethanolamine and phosphatidylcholine are the two most common phospholipids, with each phospholipid possessing a net neutral charge. Phosphatidylserine carries a net negative charge. Phospholipids also differ in fatty-acid tail composition. Because there are two chains present per phospholipid, the tails can exhibit symmetry or nonsymmetry (i.e., identity or nonidentity in fatty-acid tail composition). Each fatty-acid tail is characterized by its carbon-chain length, as well as its degree of unsaturation. The vast majority of fatty-acid tails are 16, 18, or 20 carbons in length. Unsaturation generally can occur between carbon 9 and 10, and 12 and 13. In Table 4, two common phospholipid fatty-acid tails are 16:0 and 18:1 (28). These designations denote a fatty-acid tail of length 16 carbons and no double bond, and fatty-acid tail of length 18 carbons and one double bond, respectively. Hence, a range of phospholipids, that differ in phosphate-head group and fatty-acid tails, exist.

The phosphate-head group and fatty-acid tails vary in chemical composition, such that phospholipid properties change, resulting in modulated membrane properties. Different head groups and tails alter lipid packing, membrane-surface charge, and fluidity or anisotropy. For example, the head group's bulkiness influences both phospholipid

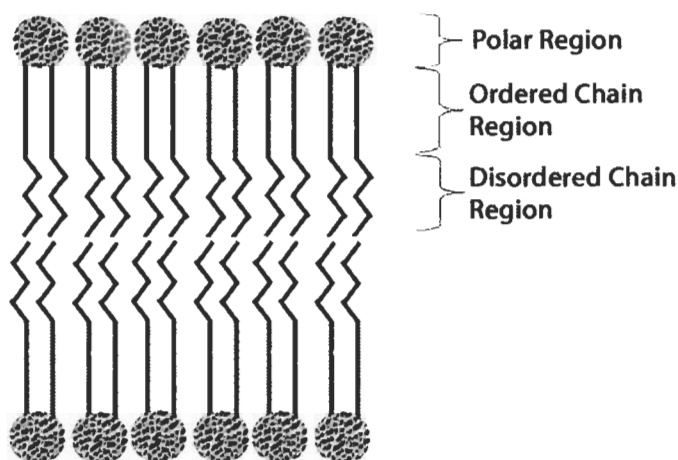


Fig. 2. Schematic of phospholipid bilayer and three regions in a bilayer leaflet.

packing density and nominal membrane charge. Also, fatty-acid tails composition impacts the lipophilic region of the lipid bilayer. Because fatty-acid double bonds are generally *cis* in conformation, unsaturation introduces sharp bends in the carbon chain, resulting in less membrane order and greater membrane fluidity.

Figure 2 is a schematic of phospholipid bilayer. Each lipid leaflet consists of three regions: the polar region, the highly ordered chain region, and the disordered chain region. The polar region consists of the hydrated phosphate-head group and the ester/ether linkages between the head groups and acyl chains. The highly ordered chain region is the fatty-acid chain region with no unsaturation. The disordered chain region includes the fatty-acid chain regions with unsaturation. Phospholipid composition changes impact these regions. For example, small phosphate-head group moieties and the addition of cholesterol each increase lipid-packing density, to result in a more rigid membrane. Of course, the charge of the phosphate-head group influences membrane charge. Also, fatty-acid saturation generally will reduce the degree of disorder in the disordered chain region.

#### 4.2. Bilayer Properties and Passive Drug Permeability

A model that relates bilayer properties and solute permeability through the bilayer is the solubility-diffusion model. Both solute solubility in the bilayer and solute diffusivity across the bilayer dictate solute permeability across the bilayer. Solute solubility in the bilayer, as a determinant of permeability, has received greater attention. Typically, increased membrane lipophilicity—for example, by increasing fatty-acid chain length—enhances the permeability of lipophilic drugs. Meanwhile, reduced membrane lipophilicity—for example, by increasing membrane protein content—enhances the permeability of hydrophilic drugs. For various solutes, Overton's Rule suggests that solute permeability coefficients follow oil/water partition coefficients. The hydrocarbon domain of bilayers has been compared with various hydrocarbon solvents (e.g., hexane, dodecane, hexadecane, octanol, heptane). Additionally, the chain ordering in lipid bilayers, particularly in the ordered chain region, serves as a diffusional resistance and entropic barrier to partitioning. For example, after correcting for its hydrophobic

effect, the incorporation of cholesterol into a membrane reduced solute permeability, owing to cholesterol's membrane-ordering effect (29).

### **4.3. Bilayer Properties and Active Drug Transport**

Plasma-membrane properties, such as membrane fluidity, affect not only passive drug permeability, but also the conformation and functioning of membrane-bound transporters. Because most of transport proteins are membrane-bound, the physical state of the cell membrane is generally important in transporter function. Hence, transporter-mediated permeability is modulated by changes in plasma bilayer. Several studies have investigated the influence of cholesterol content and cell-membrane fluidity on P-gp function. Fluidization usually resulted in decreased P-gp activity. Studies of the effect of rigidizing the cell membrane on P-gp activity have yielded contradictory results, with some reports suggesting an increase in P-gp activity, and others suggesting a decrease.

We previously studied the effect of three pharmaceutical surfactants on membrane fluidity and transporter function in Caco-2 cells (30). Tween-80 and Cremophor EL increased fluidity in the hydrophobic region of Caco-2 plasma membranes. In contrast, vitamin E TPGS rigidized the hydrocarbon region. All three surfactants contained poly(ethylene oxide) and were effective in inhibiting P-gp, although Vitamin E TPGS showed a markedly different pattern of P-gp inhibition than Tween-80 and Cremophor EL. Tween-80 and Cremophor EL progressively increased AP-BL permeability of rhodamine 123 over a concentration range of 0–1 mM.

Vitamin E TPGS's effect was equally large, mainly owing to reduced BL-AP permeability of rhodamine 123. Moreover, its effect was already maximal at 0.025 mM. These P-gp inhibition effects would appear to be related to these excipients' ability to modulate membrane fluidity. Tween-80 and Cremophor EL fluidized lipid bilayers, whereas vitamin E TPGS rigidized lipid bilayers. However, among the three surfactants, only Tween-80 inhibited the peptide transporter. Likewise, only Cremophor EL inhibited the monocarboxylic acid transporter. Nevertheless, P-gp, the human intestinal peptide transporter, and the monocarboxylic acid transporter were each inhibited by at least one of these surfactants. A common functional feature of these surfactants was their ability to modulate fluidity, although the present studies indicate that strong membrane-fluidity modulation by itself was not sufficient to reduce transporter activity. The protein kinase C (PKC) inhibitors failed to inhibit transporter activity, suggesting inhibition of PKC was not the mechanism of surfactant-induced transporter inhibition. In total, these results suggest that surfactants can inhibit multiple transporters, but that changes in membrane fluidity itself are not sufficient to reduce transporter activity.

## **5. In Vitro Caco-2 Passive Drug Permeability and Its Relation to In Vivo**

In addition to Caco-2 monolayers mimicking the gut epithelium, a second rationale for the use of Caco-2 monolayers is their ability to be correlated with in vivo oral absorption. Figure 3 exemplifies the relation between the oral percent dose absorbed in humans and Caco-2 permeability. Each data point is derived from measuring the Caco-2 permeability of a drug and plotting its permeability value against its oral percent dose absorbed from human pharmacokinetic studies. Each compound is known to be passively absorbed (i.e., not a substrate for active intestinal transport), not limited by dissolution, and not suspect to luminal degradation. Similar plots appear throughout the literature (24,31).

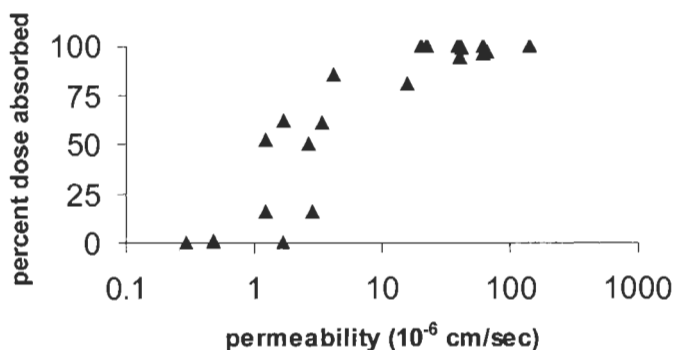


Fig. 3. Relationship between percent oral dose absorbed in humans and Caco-2 permeability.

Such plots are generally similar in permeability values and sigmoid curvature, with three general areas of the plot. On the left side of the plot with low-permeability values, a plateau persists with percent dose absorbed equal to about 0%; for a range of sufficiently small permeability values, such as permeability less than  $1 \times 10^{-6}$  cm/s, the percent dose absorbed is 0%. Likewise, a second area of the plot with sufficiently high permeability (e.g., permeability greater than  $10 \times 10^{-6}$  cm/s) exhibits a plateau with the percent dose absorbed equal to about 100%. In between these two areas, the percent dose absorbed increases approximately log-linearly with permeability (between  $1 \times 10^{-6}$  cm/s and  $10 \times 10^{-6}$  cm/s). As described in Subheading 3, even passive Caco-2 permeability values are subject to incompletely explained variation with culture conditions, time in culture, and passage number. Between laboratories, and also within a laboratory over the course of time, the greatest variation in plots such as Fig. 3 manifests in low-permeability values. For example, permeability value of the hydrophilic solute mannitol easily vary 10-fold between laboratories from  $0.1 \times 10^{-6}$  cm/s to  $1 \times 10^{-6}$  cm/s. This variability presumably is owing to variation in tight-junction formation and/or imperfection in monolayer integrity.

The two extremes of the sigmoid curve in Fig. 3 indicate that sufficiently low-permeability values result in essentially no drug absorption, and sufficiently high-permeability values result in essentially complete drug absorption. In the middle region of such plots, the general slope is about one, or even greater than one (24,31). This characteristic indicates that Caco-2 monolayers mimic the relative passive permeability barrier function of the human intestine in a reasonable fashion. Absolute comparisons have been made between Caco-2 permeability and human jejunal permeability obtained from human perfusion (2). For low-permeability compounds, Caco-2 values are typically about 50-fold less than human jejunal permeability values. This difference is attributed to a lower paracellular permeability and/or a larger surface area *in vivo* in humans. For high-permeability compounds, Caco-2 values are typically about three-fold less than human jejunal permeability values.

It should be noted that plots such as Fig. 3 have intrinsic difficulty in showing a strong correlation (i.e., a tight degree of association) between Caco-2 permeability and percent dose absorbed. A main reason for a limiting correlation is the intrinsic variability in the percent dose absorbed parameter, which is obtained from human pharmacokinetic studies. For many drugs, particularly those with incomplete absorption, oral bioavailability is suspect to at least modest variability (e.g., %CV = 15%). Addition-

ally, first-pass metabolism adds to the uncertainty of percent dose absorbed estimates. In contrast to the variability in y-axis values of Fig. 3, we have found the intra-day variability in Caco-2 permeability generally to be 10% or less (30).

## 6. Future Directions

Many efforts have been directed to develop an ideal cell line to duplicate drug absorption across the small intestine. Of course, ideal characteristics depend on the application. Ideal characteristics in one circumstance may contrast desirable properties in other applications. For example, a general advantage of Caco-2 cells is the low native expression of metabolic enzymes, in order to measure permeability values without bias from metabolism. However, as described in Subheading 3., Caco-2 cells induced to express metabolic enzymes have been described as a model system for coupled transport and metabolism.

Given the explosion in our understanding of drug absorption across epithelia, particularly transporter-mediated absorption, the need for an even wider array of transport models continues, particularly at the molecular level for specific individual processes. Future approaches should include the development of singly and co-transfected cell lines to facilitate a better understanding of the interaction between the drugs and cell proteins (e.g., transporter proteins or metabolic enzymes), along with any co-factors. Practical applications of such cell lines with high selective expression will be the design and development of new therapeutic agents with acceptable pharmacokinetic properties. Also, reporter-gene assays are needed for drug-interaction studies that involve induction or inhibition of enzymes and transporters by drugs and dietary components. Additionally, the identification of nuclear receptors as xeno-sensors represents a major step forward in understanding the genetic mechanisms controlling the expression of drug-metabolizing enzymes. Such efforts may potentially explain intra-individual and inter-individual variability in drug bioavailability. There is tremendous potential for a cell-based screening system incorporating the role of nuclear receptors to mechanistically screen for potential drug candidates with low drug-interaction potential, thus facilitating the development of safer drugs. Such systems will further lead to the development of therapeutic options based on an individual's genetic profile.

Future progress is also needed in our understanding of passive permeability, including the role of the lipid bilayer. Although the solubility-diffusion model provides a basis for understanding the interacting roles of drug and lipid bilayer in determining drug permeability through a specific lipid bilayer, it is clear that much remains to be understood. Moreover, given the diversity of plasma-membrane composition in different organs, tissues, and even different parts of the same cell, it can be expected that future efforts will explore the effects of these differences on drug permeability and organ disposition, and their basis. Furthermore, a future direction is the elucidation of excipients on passive and active drug transport, with the aim to control and modulate drug bioavailability and disposition.

Finally, the need to better integrate our understanding of drug permeability with other arenas continues, including applied areas of drug design and development. For example, the FDA Biopharmaceutics Classification System (BCS) is widely viewed as conservative, with potential to extend biowaivers to highly soluble drugs with moderate (rather than simply high) permeability. One major limitation in this regulatory consideration is a comprehensive analysis of allowing biowaivers of moderately permeable

drugs. A comprehensive analysis needs to consider the risk of formulation changes to result in bioinequivalence for moderately permeable drugs. Although permeability models have been related to pharmacokinetic measures such as percent dose absorbed, and although some studies have assessed the potential for excipients to modulate drug permeability, permeability is only infrequently considered in assessing the overall robustness of the drug product.

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