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Current Industrial Practices of Assessing Permeability and P-Glycoprotein Interaction

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ABSTRACT

Combination of the in vitro models that are high throughput but less predictive and the in vivo models that are low throughput but more predictive is used effectively to evaluate the intestinal permeability and transport characteristics of a large number of drug candidates during lead selection and lead optimization processes. Parallel artificial membrane permeability assay and Caco-2 cells are the most frequently used in vitro models to assess intestinal permeability. The popularity of these models stems from their potential for high throughput, cost effectiveness, and adequate predictability of absorption potential in humans. However, several caveats associated with these models (eg, poor predictability for transporter-mediated and paracellularly absorbed compounds, significant nonspecific binding to cells/devices leading to poor recovery, variability associated with experimental factors) need to be considered carefully to realize their full potential. P-glycoprotein, among other pharmaceutically relevant transporters, has been well demonstrated to be the major determinant of drug disposition. The review article presents an objective analysis of the permeability and transporter models currently being used in the pharmaceutical industry and could help guide the discovery scientists in implementing these models in an optimal fashion.

KEYWORDS: permeability, high throughput, Caco-2 cells, transporters, drug discovery, PAMPA, P-gp

INTRODUCTION

New drug discovery and development is becoming an extremely risky and costly endeavor. A recent report has tagged the final price of bringing a drug to the market at more than a billion dollars, with estimated research time running into multiple years.[1] Despite the considerable investment in terms of finance and resources, the number of drug approvals per year has held steady for the last few years. The advent of combinatorial chemistry, automation, and high throughput screening (HTS) have afforded the opportunity to test thousands of compounds, but the success rate of progressing from initial clinical testing to final approval has remained disappointingly low. Greater than 90% of the compounds entering phase-I clinical testing fail to reach the patients, and as high as 50% entering phase III do not make the cut.[2]

Historically, drug discovery adopted a linear design; the new chemical entities were first selected on the basis of their pharmacological activity/potency followed by their sequential profiling to assess the absorption, distribution, metabolism, elimination, and toxicity (ADMET) characteristics. Such a strategy was generally more time and resource intensive and left little room for any error during the discovery process. Today, the new drug design effort integrates a parallel matrix approach, where the pharmacological efficacy is screened in parallel with ADMET profiling, providing information in a timely manner to maximize the chance for selecting superior drug candidates with better quality for further development. Therefore, the availability of highly accurate, low-cost, and HTS techniques that can provide fast and reliable data on the developability characteristics of drug candidates is crucial. Screening a large number of drug candidates for biopharmaceutical properties (eg, solubility, intestinal permeability, cytochrome P45 (CYP) inhibition, metabolic stability, and more recently drug-drug interaction potential involving drug transporters) has become a major challenge. Determination of the permeability property and the drug-transporter interaction of drug candidates is fast becoming key in characterization studies performed during the lead selection and lead optimization.

Drug absorption across the intestinal membrane is a complex multi-pathway process as shown in Figure 1. Passive absorption occurs most commonly through the cell membrane of enterocytes (transcellular route) or via the tight junctions between the enterocytes (paracellular route). Carrier-mediated absorption occurs via an active (or secondary active process) or by facilitated diffusion. Various efflux transporters, such as P-glycoprotein (P-gp), breast cancer

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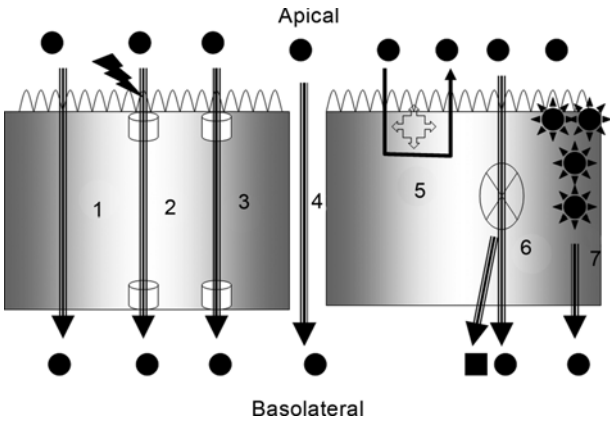

Figure 1. Multiple pathways for intestinal absorption of a compound: (1) passive, transcellular; (2) active or secondary active; (3) facilitated diffusion; (4) passive, paracellular; (5) absorption limited by P-gp and/or other efflux transporters; (6) intestinal first-pass metabolism followed by absorption of parent and metabolite; and (7) receptor-mediated transport.

resistance protein (BCRP), and multidrug resistance protein 2 (MRP-2), are also functional, which could limit the absorption. Intestinal enzymes could be involved in metabolizing drugs to alternate moieties, which might be absorbed. Finally, receptor-mediated endocytosis could also play a role. Because of the multivariate processes involved in intestinal absorption of drugs, it is often difficult to use a single model to accurately predict the in vivo permeability characteristics.

Currently, a variety of experimental models is available when evaluating intestinal permeability of drug candidates.[3-7] A few commonly used models include artificial lipid membrane such as parallel artificial membrane permeability assay (PAMPA); cell-based systems such as Caco-2 cells and Mardin-Darby canine kidney (MDCK) cells; tissue-based Ussing chamber; in situ methods; intestinal single-pass perfusion; and in vivo methods, whole animal absorption studies. Typically, a combination of these models is used routinely in assessing intestinal permeability. A tiered approach is often used, which involves high throughput (but less predictive) models for primary screening followed by low throughput (but more predictive) models for secondary screening and mechanistic studies. PAMPA and cell culture-based models offer the right balance between predictability and throughput and currently enjoy wide popularity throughout the pharmaceutical industry.

Adequate permeability is required not only for oral absorption but also for sufficient drug distribution to pharmacological target organs (eg, tumor, liver). In addition to simple passive diffusion across lipid bilayers, numerous transporters appear to play a critical role in selective accumulation and distribution of drugs into target organs. P-gp is one of the most extensively studied transporters that has been unequivocally known to impact the ADMET characteristics of drug molecules.[8-11] It is a ubiquitous transporter, which is present on the apical surface of the enterocytes, the canalicular membrane of hepatocytes, and on the apical surface of kidney, placenta, and endothelial cells of brain membrane. Because of its strategic location, it is widely recognized that P-gp is a major determinant in disposition of a wide array of drugs in humans. The oral bioavailability of fexofenadine increased significantly when erythromycin or ketoconazole (well known inhibitor of P-gp) was co-administered in humans, suggesting P-gp as a permeability barrier at the absorption site.[12] Similarly, P-gp at the blood-brain barrier limits the entry of drugs into the brain. The biliary elimination of vincristine decreased significantly in the presence of verapamil (a known P-gp substrate/inhibitor).[13] Therefore, the early screening of drug candidates for their potential to interact with P-gp (either as a substrate or inhibitor) is becoming necessary and critical. There are various in vitro and in vivo models used for assessing P-gp interaction.[14-17] In vitro assays such as ATPase activity, rhodamine-123 uptake, calcein AM uptake, cell-based bidirectional transport, radio-ligand binding along with in vivo models such as transgenic (knockout mice) animals are often used to assess the involvement of P-gp. The cell based bidirectional permeability assay is the most popular method for identification of P-gp substrate in drug discovery labs.[16] This cell model provides the right balance of adequate throughput and functional utility. However, there are certain caveats that the user must be aware of to maximize the utility of the assay. In addition to P-gp, other pharmaceutically important drug transporters (eg, MRP2, BCRP, OATP, OAT, OCT) can be examined using a plethora of models (eg, transfected cells, vesicles, recombinant vaccinia, *Xenopus* oocytes). A detailed discussion of these models is beyond the scope of this article.

This review will focus on 2 experimental models (ie, PAMPA and Caco-2 cells) that are most commonly used in pharmaceutical industry for evaluating permeability/absorption and P-gp interaction potential of drug candidates. PAMPA is used as the primary screening tool and is capable of providing the structure-permeability relationship that enables a successful lead optimization. Caco-2 cell model is often used as the secondary tool to perform in-depth mechanistic studies, to delineate the various pathways of absorption, and to assess the P-gp interaction potential of drug candidates.

Parallel Artificial Membrane Permeability Assay

PAMPA model was first introduced in 1998,[18] and since then numerous reports have been published illustrating the general applicability of this model as a high throughput permeability screening tool.[7,19-21] The model consists of a hydrophobic filter material coated with a mixture of lecithin/phospholipids dissolved in an inert organic solvent such as dodecane creating an artificial lipid membrane

barrier that mimics the intestinal epithelium. The rate of permeation across the membrane barrier was shown to correlate well with the extent of drug absorption in humans. The use of 96-well microtiter plates coupled with rapid analysis using a spectrophotometric plate reader makes this system a very attractive model for screening a large number of compounds and libraries. PAMPA is much less labor intensive than cell culture methods, but it appears to show similar predictability. One of the main limitations of this model is that PAMPA underestimates the absorption of compounds that are actively absorbed via drug transporters. Despite the limitation, PAMPA may serve as an invaluable primary permeability screen during early drug discovery process because of its high throughput capability.

Parallel Artificial Membrane Permeability Assay Study Protocol

A 96-well microtiter plate and a 96-well filter plate (Millipore, Bedford, MA) were assembled into a "sandwich" such that each composite well was separated by a 125-μm microfilter disc (0.45-μm pores). The hydrophobic filter material of the 96-well filter plate was coated with 5 μL of the pION lipid solution and gently shaken to ensure uniform coating. Subsequently, the filter plate was placed on the microtiter plate containing 200 μL of 100 μM test compounds (dissolved in 1% dimethyl sulfoxide[DMSO]/buffer), which constituted the donor well. The donor solution containing the test compound was prepared by dilution (100-fold) from a 10-mM stock solution in DMSO using the pION buffer solution at pH 7.4 followed by filtration through a 0.20-μm polyvinylidene fluoride (PVDF) 96-well filter plate (Corning Costar, Corning, NY). The receiver wells (ie, the top of the wells) of the sandwich were hydrated with 200 μL of the specialized ionic buffer solution. The system was then incubated at room temperature for 4 hours. At the end of the incubation time, samples were removed from the receiver and donor wells and analyzed by a UV-plate reader. The permeability studies were performed in triplicate (ie, 3 wells per compound). The apparent permeability coefficient was estimated using the software provided by pION.

Caco-2 Cells

Caco-2 cell model has been the most popular and the most extensively characterized cell-based model in examining the permeability of drugs in both the pharmaceutical industries and academia.[22-24] Caco-2 cells, a human colon adenocarcinoma, undergo spontaneous enterocytic differentiation in culture and become polarized cells with well-established tight junctions, resembling intestinal epithelium in humans. It has also been demonstrated that the permeability of drugs across Caco-2 cell monolayers correlated very well with the extent of oral absorption in humans. In the last 10 to 15 years, the Caco-2 cells have been widely used as an in vitro tool for evaluating the permeability property of discovery compounds and for conducting in depth mechanistic studies.[25-30]

Caco-2 Cell Culture

Caco-2 cells (passage No. 17) were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described below. The cells were seeded onto 24-well polycarbonate filter membrane (HTS-Transwell inserts, surface area: 0.33 cm^2, Corning) at a cell density of 80 000 cells/cm^2. The cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/mL penicillin-G, and 100 μg/mL streptomycin. The culture medium was replaced every 2 days, and the cells were maintained at 37°C, 95% relative humidity, and 5% CO_2. Permeability studies were conducted with the monolayers cultured for ~21 days with the cell passage numbers between 50 and 80. Caco-2 cell monolayers with trans epithelial electrical resistance (TEER) values greater than 400 ohm.cm^2 were used.

Caco-2 Cells Study Protocol

The transport medium used for the permeability studies was Hank's balanced salt solution (HBSS) buffer containing 10 mM HEPES. Prior to all experiments, each monolayer was washed twice with buffer and TEER was measured to ensure the integrity of the monolayers. The concentration of test compounds ranged from 100 to 200 μM in this assay. The permeability studies were initiated by adding an appropriate volume of buffer containing test compound to either the apical (for apical to basolateral transport; A to B) or basolateral (for basolateral to apical transport; B to A) side of the monolayer. The monolayers were then placed in an incubator at 37°C. Samples were taken from both the apical and basolateral compartment at the end of the incubation period (typically at 2 hours), and the concentrations of test compound were analyzed by a high-performance liquid chromatography (HPLC) method as described earlier.[31] Permeability coefficient (Pc) was calculated according to the following equation: $Pc = dA/(dt \cdot S \cdot C_o)$, where dA/dt is the flux of the test compound across the monolayer (nmol/s); S is the surface area of the cell monolayer (cm^2); and C_o is the initial concentration (μM) in the donor compartment. The Pc values were expressed as nm/s. All permeability data reported in this review were generated at the Bristol-Myers Squibb Pharmaceutical Research Institute (PRI). A minor variation in the experimental procedure often resulted in a significant difference in the permeability measurement making an interlaboratory data comparison very difficult.

Therefore, the study protocol of PAMPA and Caco-2 cell permeability assay is also included as a point of reference.

PAMPA AND CACO-2 CELL MODEL: SYNERGIES

PAMPA and Caco-2 cell models are often used in combination to evaluate the permeability properties of a large number of compounds at the early drug discovery stage. PAMPA model has been demonstrated in recent years to be an efficient, economical, and high-throughput model. Caco-2 cell model, on the other hand, has been the gold standard model for over a decade for permeability screening in drug discovery phase. PAMPA captures the transcellular passive permeability across lipoidal membrane barrier without the contribution from pores or drug transporters. Caco-2 cell model is capable of incorporating not only the transcellular passive permeability but also the transporter-mediated (efflux and influx) and the paracellular components of transport. Figure 2A and B demonstrates that a reasonably good prediction of the extent of absorption in humans can be obtained for ~22 marketed drugs by both Caco-2 cell and PAMPA models. Table 1 lists the permeability values in these 2 models and the fraction absorbed in humans for the validation set. These drugs were selected from different therapeutic areas, and they represent diverse structures and physicochemical properties. Also, they are well known to be passively absorbed with no known major transporter involvement. It is evident that for passively absorbed drugs, both permeability models showed similar correlation. The 2 models also shared several other characteristics that are quite common. The dynamic range of permeability values (ie, the fold increase in permeability value for highly absorbed drugs compared with poorly absorbed drugs) was close to 2-orders of magnitude in both models with similar slope. The steepness of the slopes may reflect the predictability of the model for drugs that are moderately absorbed, and it appears that the 2 models have similar predictability for drugs with moderate permeability. The figure also demonstrates that it is very difficult to differentiate drugs in the mid range because of a large variability in the mid range of the curve. However, both models may work very well in binning the drug candidates into broad categories (high, medium, low) based on their permeability values but might

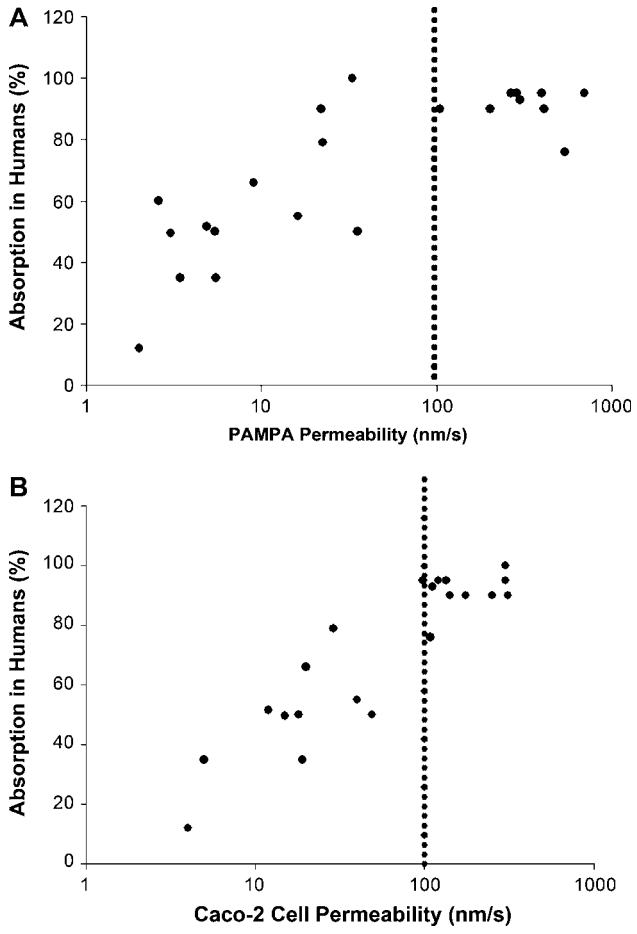

Figure 2. Correlation of (A) PAMPA permeability and (B) Caco-2 cell permeability with the extent of absorption in humans for marketed drugs. These drugs are known to be primarily absorbed via passive diffusive pathway. Each point is the mean of 3 or more repeats. The dotted line represents a cut-off of 100 nm/s.

Table 1. PAMPA and Caco-2 Cell Permeability Values of Marketed Drugs*

Drug	PAMPA Permeability (nm/s)	Caco-2 Cell Permeability (nm/s)	% Fraction Absorbed in Humans
Acebutalol	16 ± 11	40 ± 4	55
Alprenolol	299 ± 68	111 ± 30	93
Amiloride	5 ± 3	49 ± 8	50
Cimetidine	22 ± 3	29 ± 12	79
Desipramine	700 ± 170	300 ± 21	95
Dexamethasone	287 ± 11	134 ± 13	95
Etoposide	35 ± 4	18 ± 10	50
Fenaterol	3 ± 1	NA	60
Furosemide	9 ± 1	20 ± 4	66
Hydralazine	105 ± 4	141 ± 16	90
Ketoconazole	542 ± 37	108 ± 18	76
Ketoprofen	22 ± 6	250 ± 26	90
Metformin	5 ± 2	12 ± 3	52
Metoprolol	266 ± 16	120 ± 10	95
Naproxen	33 ± 3	300 ± 41	100
Norfloxacin	5 ± 1	19 ± 3	35
Phenytoin	204 ± 9	310 ± 15	90
Propranolol	411 ± 110	175 ± 26	90
Sulfasalazine	2 ± 1	4 ± 1	12
Sulpiride	3 ± 1	5 ± 2	35
Terbutaline	3 ± 1	15 ± 6	50
Verapamil	399 ± 112	98 ± 19	95

*NA indicates not available.

lack the sensitivity to accurately predict small differences in permeability values. The 2 models also demonstrate similar variability of data (20%-30% coefficient of variation) and mass balance/recovery issues (many compounds had incomplete recovery).

Figure 3 represents the correlation observed between the 2 models for ~100 research compounds from various drug discovery programs in Bristol-Myers Squibb PRI. The compounds were carefully selected from a dozen research programs, and they reflected a wide diversity of chemical space and physicochemical properties. The permeability values for these compounds were determined in both models using standard experimental conditions described above. Next, the compounds were classified into "low" and "high" permeability bins based on internal calibration data. Permeability value of ~100 nm/s was selected as a cut-off for both models. A cursory glance at the correlation figure might suggest a lack of linear correlation between the 2 models in terms of their permeability values. However, incorporation of the binning strategy (using 100 nm/s as the cut-off value) suggests that the 2 models demonstrate an acceptable agreement. Around 80% of the compounds tested were assigned to the same bin (ie, the results were in agreement) by both the models. These compounds are represented in quadrants 2 and 3 in the figure. Almost half of those compounds were classified as "low" permeability by both methods (quadrant 3) and the other half were "high" permeability (quadrant 2). The compounds that fell in quadrants 1 and 4 were the disconnects between the 2 permeability models. The disagreement observed for ~20% of the compounds highlights the fundamental difference between the 2 models. PAMPA is an absorption model that captures uncontaminated transcellular diffusion across the lipid bilayers. It is completely devoid of any influx/efflux transporters or paracellular pores. On the other hand, cell tight junctions and various drug transporters (influx: PepT1 and efflux: P-gp, MRP2, BCRP, etc) are expressed in the Caco-2 cell monolayer. The compounds shown in quadrant 4 (high PAMPA and low Caco-2 cell permeability) are likely to be substrates of efflux transporters that would limit their permeability in Caco-2 cell model. Because the PAMPA is simple lipid bilayers, the permeability of compounds in quadrant 4 remains high. A handful of compounds from the quadrant 4 were evaluated for their P-gp substrate potential by performing bidirectional transport study in Caco-2 cells. As expected, they were all shown to be substrates with efflux ratio (ratio of B to A/A to B) higher than 3. Quadrant 1 represents compounds that have high Caco-2 cell permeability and low PAMPA permeability. Compounds in quadrant 1 are likely to permeate Caco-2 cell monolayer via paracellular pores and/or influx transporters. In general, a good agreement between the 2 permeability models for passively absorbed compounds is expected. If there is disagreement between these 2 models, it may provide a hint that specific influx or efflux transporters are involved. And the follow-up studies using more mechanistic models (eg, cell line with specific transporter expressed, *Xenopus* oocytes, etc) might be warranted.

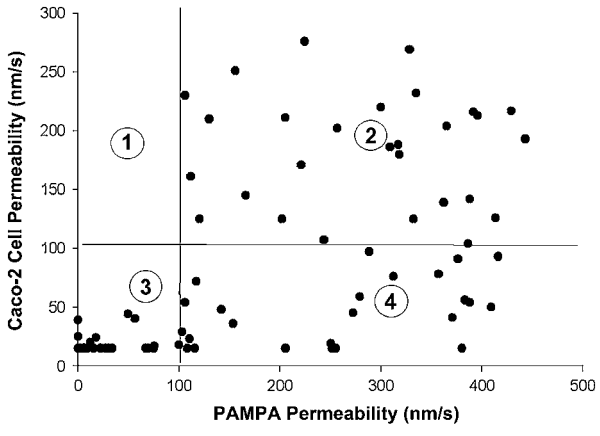

Figure 3. Correlation of PAMPA permeability with Caco-2 cell permeability for ~100 internal research compounds from the Bristol-Myers Squibb labs. Each point is the mean of 3 or more repeats.

A combination of PAMPA and Caco-2 cells is often used to assess the permeability of test compounds in the most cost-effective manner.[32,33] PAMPA model is preferred by virtue of its easy set-up and rapid operation. It is a low-cost assay (cost per test is a fraction of Caco-2 cells' cost) and is less resource and time intensive. Automated robotic driven PAMPA model is capable of screening thousands of compounds per week. Caco-2 cells, on the other hand, is a cost- and resource-intensive assay and not amenable to ultra high throughput screening. To obtain the maximum permeability/absorption information in the least amount of time with minimal use of resources, a combination of PAMPA along with unidirectional Caco-2 cells (A to B permeability) is increasingly becoming popular. Figure 4A shows the Caco-2 cell permeability values obtained for a series of compounds within a chemotype from a typical research program. The majority of the compounds had "low" permeability values below 100 nm/s in the Caco-2 cell model. These compounds with low Caco-2 cell permeability would generally be assumed to be poorly absorbed in humans and may not make the initial cut to move forward (ie, low priority). However, a combination assay (PAMPA and unidirectional Caco-2 cell) provided a better understanding of the absorption characteristics of these compounds. A randomly selected set of compounds with Caco-2 cell permeability values lower than 100 nm/s was evaluated for their PAMPA permeability as well as their bidirectional permeability in Caco-2 cell model. The results from these 2 permeability models are shown in Figure 4B. The combination assay demonstrated that the low A to B Caco-2 cell permeability of many compounds was because they are substrates of P-gp (ie, B to A

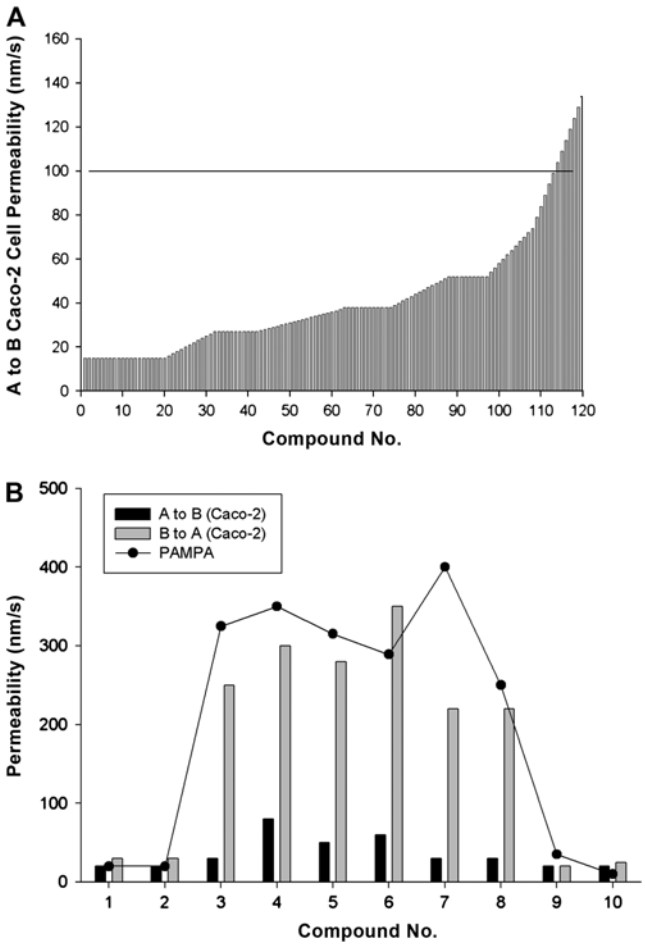

Figure 4. (A) Caco-2 cell permeability (A to B) for a list of compounds within a research program. Permeability values were obtained using Caco-2 cells cultured for ~21 days followed by transport studies at 200 μM. (B) Bidirectional Caco-2 cell permeability and PAMPA permeability values for some randomly selected compounds within the program. PAMPA study was performed at 100 μM.

permeability is much higher than A to B permeability) rather than because of low intrinsic permeability of the compound. The high PAMPA permeability coupled with low Caco-2 cell permeability would indicate an active efflux process (in Caco-2 cells) and not necessarily low intrinsic permeability of the test compound. It can be expected that these compounds may have more than adequate intestinal permeability if intestinal P-gp is saturated due to high drug concentration in the gut. In fact, many of these compounds were shown to exhibit good oral bioavailability in preclinical animal models. Despite being a substrate of P-gp, these compounds were well absorbed in vivo, and the high PAMPA permeability value was reflective of in vivo absorption. A combination of PAMPA and unidirectional (A to B) Caco-2 cell permeability models can synergistically provide invaluable permeability/absorption assessment of test compounds. If both PAMPA and A to B Caco-2 cell permeability are low, then the compound can be deprioritized to have low intrinsic permeability and thus likely to have poor absorption in humans. However, if A to B Caco-2 cell permeability is low but PAMPA is high, then the compound is likely to be a substrate of P-gp and not necessarily a poorly permeable compound in vivo. At relevant oral dose level, P-gp can be saturated to allow adequate absorption to occur in humans. A parallel combination assessment of PAMPA and A to B Caco-2 cell permeability followed by more detailed bidirectional Caco-2 cell assay might be a prudent path for research programs.

Drug absorption primarily occurs in the small intestine, where the pH may vary from acidic to neutral and slightly basic.[34,35] In the upper small intestine where pH is likely to be more acidic, weakly acidic drugs exist primarily as unionized form, and the passive transcellular pathway becomes the dominant permeation route. On the contrary, weakly basic drugs will be mostly in the form of ionized species, and consequently the passive transcellular route plays a minor role. Therefore, the apical pH used in the permeability assay becomes critical. Both PAMPA and Caco-2 cell permeability assays are typically performed at a single donor pH 6.5 to maintain adequate throughput (ie, maximum number of compounds evaluated in minimum time frame). Single donor pH is perfectly appropriate for neutral and zwitterionic compounds, where a change in pH is not expected to affect the ionization status. However, most new drug candidates are either weak bases or weak acids. Therefore, the permeability of drug candidates with ionizable groups depends significantly on the experimental pH used. Table 2 lists a handful of drugs (acids and bases) along with their permeability values in the PAMPA model under 2 different experimental pH conditions. As expected, acidic drugs (eg, ibuprofen, ketoprofen, piroxicam) showed significantly higher permeability when studied at pH 5.0 than 7.4, and the basic drugs (eg, metoprolol, timolol) had much

Table 2. Effect of pH on PAMPA Permeability*

	PAMPA (nm/s)		
	pH 5.0	**pH 7.4**	**% FA**
ACIDS			
Glipzide	562 ± 27	14† ± 1	95
Ibuprofen	756 ± 69	5† ± 1	100
Ketoprofen	567 ± 49	9† ± 1	90
Naproxen	500 ± 85	17† ± 2	100
Piroxicam	621 ± 42	21† ± 3	100
BASES			
Timolol	34† ± 2	595 ± 17	90
Hydralazine	2† ± 2	177 ± 44	90
Metoprolol	40† ± 8	512 ± 124	95

*% FA indicates percentage fraction absorbed in humans.
†Permeability value is inconsistently low despite complete absorption in humans.

higher permeability at pH 7.4 than 5.0. All drugs listed are almost completely absorbed in humans (>90%). But the permeability of these drugs when inappropriate pH was used is comparable to drugs that are not absorbed at all. Thus, it appears that an appropriate donor pH should be used depending on the physicochemical properties of test compounds. The degree of pH dependency is often very difficult to estimate because the pK_a of a large number of drug candidates cannot be measured experimentally due to resources limitation. To minimize the occurrence of false negatives (ie, classified as poorly permeable when it is not), it becomes necessary to run the assay at 2 different pH condition (low of ~pH 5.0 and high ~pH 7.4) simulating the dynamic pH environment in the intestine to capture true intrinsic permeability. Because of a higher throughput potential, the PAMPA model can be used in the first tier pH-dependent permeability study for similar chemotypes. Once an optimum pH is identified, all subsequent studies (including cell-based, tissue-based studies) can be performed at that pH. However, it should be recognized that the permeability studies performed at multiple pH would negatively impact the throughput and cost involved.

Caveats in PAMPA and Caco-2 Cell Models

Transporter- and Paracellular-mediated Absorption

PAMPA is a high throughput, non-cell-based permeability model that provides estimates of the passive transcellular permeability property. The lack of any functional drug transporters and paracellular pores in PAMPA makes it an inappropriate model for compounds that are absorbed via transporter- and pore-mediated processes. However, the lack of transporter- and pore-mediated permeability might be an advantage of the PAMPA model. Because the PAMPA provides uncontaminated transcellular passive permeability data, it could be more useful in constructing the structure-permeability relationship at the chemistry bench. Lipophilicity (most commonly expressed as Log P or Log D values) plays a major role in passive diffusion. An adequate lipophilicity is required for a permeant to travel across the phospholipid membrane. However, as shown in the Figure 5, the PAMPA permeability of 22 marketed drugs (listed in Table 1) did not correlate well with lipophilicity alone because other factors (eg, polar surface area, molecular volume/flexibility, hydrogen bonding) are also involved in passive diffusion. Although pharmaceutically important drug transporters (eg, PEPT1, OCT, OAT) are functionally expressed in Caco-2 cells,[36-38] they are quantitatively underexpressed when compared with in vivo situation. For example, beta-lactam antibiotics (eg, cephalexin, amoxicillin) and ACE inhibitors, which are known substrates of dipeptide transporters, are poorly permeable across the Caco-2 cell monolayer despite the fact that they are completely absorbed in vivo.[31] This model is likely to generate false

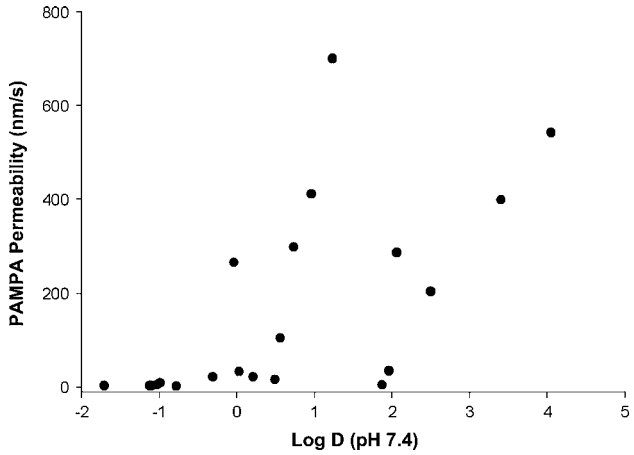

Figure 5. Correlation of PAMPA permeability with lipophilicity. Log D (pH 7.4) value was calculated using ACD/Log D module.

negatives with drug candidates that are transported by carrier-mediated process. Caco-2 cells have tight junctions that are significantly tighter compared with human intestine, and thus Caco-2 cells normally under-predict the permeability value of drugs that are absorbed primarily via paracellular pathway. The low molecular weight hydrophilic compounds (eg, metformin, ranitidine, atenolol, furosemide, hydrochlorothiazide) showed poor permeability (ie, equal or less than mannitol) in Caco-2 cells despite adequate absorption (greater than 50% of dose) in humans. Therefore, models such as PAMPA and Caco-2 cells can only serve as a one-way screen such that compounds with high permeability in these models are typically well absorbed; however, compounds with low permeability cannot be ruled out as poorly absorbed compounds in humans.

Incomplete Mass-balance Due to Nonspecific Binding

Nonspecific drug binding to plastic devices and cells during the permeability study is a common problem, which often makes the data interpretation difficult. Permeability is calculated based on several factors: the amount of drugs that appeared in the receiver compartment, the initial concentration in the donor compartment, and the surface area of the physical barrier (eg, lipid bilayers and cell monolayer). When significant drug loss occurs during the incubation due to a nonspecific binding, 2 things occur: (1) the concentration in the donor compartment (a driving force) is reduced; (2) the concentration in the receiver compartment is artifactually reduced. This will lead to an underestimation of permeability estimates, and potentially lead to false negatives. "Cacophilicity" or "membrane retention" have been used to describe the drug binding to Caco-2 cell monolayer, and the significant binding results in an incomplete recovery. Incomplete recovery is particularly common with lipophilic drug candidates. A few approaches may be able to minimize the nonspecific binding. Rather than using the initial donor

concentration, the final donor concentration at the termination of incubation can be used. Assumption is that the nonspecific binding occurs relatively quickly, therefore, the final donor concentration is a better estimate for the concentration gradient between 2 compartments. For certain drug candidates with poor recovery, the results can be significantly different depending on the calculation method used. Another approach involves the addition of serum proteins (bovine serum albumin in case of Caco-2 cells) or surfactants (in case of PAMPA) to the receiver compartment to minimize nonspecific binding, therefore, improve the assay recovery and overall predictability of the model.[25,39,40]

Inadequate Aqueous Solubility

Drug candidates at the early discovery stage are often optimized in terms of structure-activity relationship (SAR) around potency and pharmacological activity, and the SAR generally leads to rather lipophilic and poorly soluble candidates (solubility in aqueous buffer < 0.01 mg/mL). As a result, significant percentage of new drug candidates cannot be evaluated in the permeability models due to their poor aqueous solubility. This is particularly problematic in the cell-based model because the cells do not tolerate the typical organic cosolvents (eg, DMSO, propylene glycol [PG]) very well. Beyond a small percentage of cosolvents (>1%), the cell tight junction is easily compromised, making the data interpretation difficult or impossible.[41-43] Consequently, for some discovery programs, the permeability data cannot be provided for a majority (>95%) of new compounds because they tend to come from a similar chemotype, which may share a problematic pharmacophore in terms of aqueous solubility. PAMPA model has a significant advantage over Caco-2 cells in this regard. Figure 6 shows the effect of various concentrations of DMSO on the PAMPA permeability of some probe compounds (high and low Pc compounds).

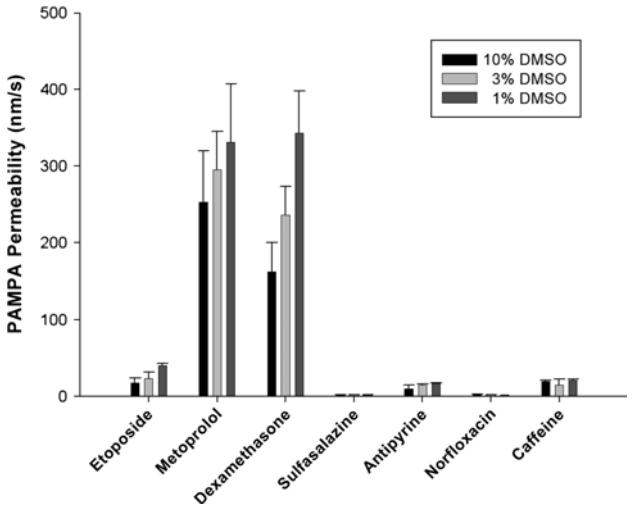

Figure 6. Effect of DMSO on the PAMPA permeability.

Contrary to a cell-based model, where higher than 1% DMSO would compromise the cell monolayer leading to unreliable results, the PAMPA permeability was consistent in the presence of DMSO up to 10%. Having higher cosolvent concentration not only increases the percentage of compounds that can be successfully studied in a permeability assay, but it also improves the mass-balance recovery. A higher cosolvent concentration is likely to minimize the physical loss (eg, nonspecific binding to device and membrane) during the permeability study.

Other Experimental Variability

It is well documented that the Caco-2 cell permeability of the same set of drugs obtained from different laboratories varies significantly.[29,30,44,45] There are a variety of factors that can influence the outcome. Minor differences in cell culture conditions (eg, seeding density, feeding frequency, composition of the cell media), experimental protocol (eg, initial concentration of drugs, composition of the permeability buffer, pH, monolayer washing steps), and age of the cells (eg, passage number, culture duration, tightness of junction) can produce dramatic differences in the permeability values. In addition, the function of drug transporters expressed in the cell-based models can fluctuate significantly with difference in culture conditions.[36,45] PAMPA permeability model, on the other hand, is a relatively rigid model less prone to interlab variability problems. The lipid solution used to create the bilayers membrane is the only component of the model that can incorporate fluctuations in permeability value. Moreover, since PAMPA captures only the passive diffusive permeability and is devoid of any transporter proteins, other experimental factors play a negligible role.

P-gp Substrate Assay: Bidirectional Permeability in Caco-2 Cells

The bidirectional permeability assay, where the basolateral to apical (secretory direction, B to A) permeability is compared with the apical to basolateral (absorptive direction, A to B) permeability, is regarded as the gold standard in identifying P-gp substrates because it is functionally the most direct method of measuring efflux characteristics of drug candidates. Compounds with an efflux ratio (ratio of B to A/A to B) greater than 2 to 3 are typically considered as P-gp substrates. However, it is well known that efflux transporters other than P-gp (eg, MRP2, BCRP) are also functionally expressed in the Caco-2 cells. Therefore, a simple bidirectional difference may not ascertain that the compounds being tested are indeed P-gp substrates. As a confirmatory study, a follow up bidirectional experiment is routinely repeated in the presence of known inhibitors of P-gp, BCRP, and MRP2. GF120918 at 2 to 4 uM is often

used to selectively inhibit the P-gp-based efflux transport. However, some recent publications have reported that GF120918 interacts not only with P-gp but also with the BCRP.[46,47] Similarly, MK-571 and fumitremorgin C (FTC) are used to selectively inhibit the MRP2[48,49] and BCRP activity,[50,51] respectively. Comparison of efflux ratio in the absence and presence of these specific inhibitors can delineate the potential role of the individual efflux transporter. Because it is a cell-based assay with a physical barrier (lipid bilayer), the test compounds must have adequate cell permeability for the bidirectional P-gp assay. One major drawback of the bidirectional permeability assay is that the P-gp substrates with insufficient transcellular permeability cannot be identified. Drugs such as famotidine and ranitidine are substrates for secretory transporter proteins,[52] but often they fail to be detected as P-gp substrates owing to their low passive permeability.

Bidirectional Permeability Using Engineered Cell Lines

Several efflux (eg, P-gp, MRP2, BCRP) and influx transporters (eg, PEPT1) are expressed in Caco-2 cells under standard cell culture conditions. However, the functional expression of drug transporters in Caco-2 cells may vary significantly depending on the passage number and minor changes in culture conditions. For example, the efflux ratio of digoxin or sulfasalazine (well-known P-gp substrate) varied drastically with the passage number. It is interesting to note that the poor or lack of P-gp functional expression becomes problematic sporadically. There was no identifiable trend with the change in passage number. Figure 7 demonstrates that the levels of P-gp expression in Caco-2 cells can vary significantly depending on the cell passage number. Mannitol (a hydrophilic paracellular marker) and metoprolol (a lipophilic transcellular marker) demonstrated consistent permeability values over the different passage numbers. The reproducibility for these 2 compounds confirms that the cell monolayers are not compromised. However, sulfasalazine, a P-gp substrate that typically has low A to B permeability value (<30 nm/s), showed significantly higher permeability (> 120 nm/sec) for only certain passage numbers. The variability in the levels of expression of P-gp was directly reflected by the variability in the permeability values of sulfasalazine. Similarly, the levels of P-gp expression have also been known to fluctuate widely amongst the wells even in a single-passage study. Digoxin efflux ratio can vary from a low of 8 to a high of 20 in different wells of the same passage number, Caco-2 cells highlighting the variability in the expression of efflux transporter.

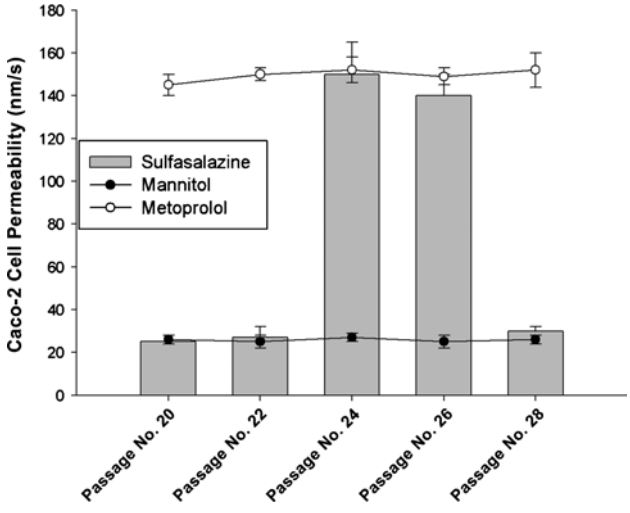

Figure 7. Effect of Caco-2 cell passage number on the permeability of sulfasalazine, mannitol, and metoprolol.

Because of inconsistent P-gp functional expression in Caco-2 cells, the cell line, which overexpresses efflux transporters might be a better in vitro tool to examine the drug-transporter interactions. The engineered cell lines selectively express the transporter of interest and facilitate interaction studies with a specific transporter in isolation. Bidirectional studies performed in these cell lines (overexpressing only one transporter) would specifically demonstrate the involvement of a single transporter without any interplay with other transporters. Follow-up studies can be conducted in the presence of a selective inhibitor to further confirm the involvement of the transporter (viz, transporter phenotyping). MDCK and LLC-PK1 cell lines stably transfected with a specific efflux transporter (eg, MDR1, mdr1, MRP2, BCRP) may be used to tease out transporter interactions at early discovery stage. The functional expression of transporters in these cell lines appears to be more stable compared with Caco-2 cells.

Experimental Factors on Efflux Ratio

Currently there is no universally accepted standard study protocol for conducting the cell-based bidirectional permeability assay. Different laboratories have performed these studies under different experimental conditions that have often showed a large interlaboratory variability. Standardization of experimental conditions (eg, pH, drug concentration, duration of incubation) can provide better consistency of the results and significantly minimize the occurrence of false negatives (ie, unable to identify a true substrate). The use of an optimal substrate concentration is a key parameter for attaining accurate results from this model. Performance of bidirectional studies at high concentrations (50 μM or more) often lead to saturation of the efflux transporter and result in efflux ratio of unity even for well-known P-gp substrates. Figure 8 demonstrates the dramatic effect of using the lower (3 μM) concentration for improving the utility of this model. Classical P-gp substrates such as verapamil, quinidine, and 2 internal research compounds are shown to have efflux ratio (ratio of B to A/A to B) of ~1 when studied

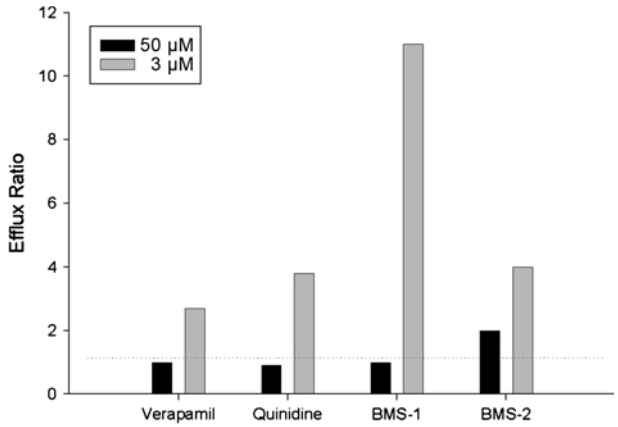

Figure 8. Effect of substrate concentration (3 μM vs 50 μM) on the efflux ratio (ratio of B to A/A to B) in the Caco-2 cell bidirectional study. The efflux ratio was calculated using the mean data. The coefficient of variation in the permeability data (both directions) is typically less than 25% amongst replicates.

at 50 μM. However, when they were studied at lower concentration of 3 μM, they demonstrated unequivocal efflux characteristics with efflux ratio > 2. The P-gp is saturable at high substrate concentrations, and thus at 50 μM, the P-gp is knocked-out functionally leading to false negative data. Using lower substrate concentrations (<5 μM) could potentially minimize the saturation of the efflux transporters and significantly improve the predictability of the assay. It should be recognized that lowering the substrate concentration imposes an analytical challenge, and the analytical technique must be capable of measuring the sample concentrations with adequate accuracy.

The preliminary screen at a low substrate concentration is an efficient approach to evaluate a large number of compounds and maintain sufficient throughput. However, "yes or no" classification may not be very useful because it is difficult to quantify (if possible) the clinical significance of the potential role of P-gp in the drug disposition. As a follow-up study, it is recommended that a range of substrate concentrations be tested, and the estimated Km value be related to in vivo relevant drug concentrations either in the intestine (during absorption) or systemic circulation (during distribution and excretion). One of the common limitations in conducting a concentration-dependency experiment is the lack of adequate aqueous solubility of new drug candidates (ie, poor solubility does not allow a wide range of concentration to be tested).

Another potential issue with cell-based models is that the integrity of tight junctions in the Caco-2 cell monolayers can be compromised (ie, becomes leaky) when incubated with test compounds, and the extent of damage is typically concentration dependent. The permeability across the compromised monolayer is often much higher compared with the intact monolayers, and the permeability becomes artifactually high in both directions. In that case, the efflux ratio often becomes unity, and the test compound is classified as a nonsubstrate even if it is a true substrate (again false negative). To detect cell damage during the incubation, the TEER value can be measured before and after the study. But the change in the TEER value is not very sensitive to detect a minor cell damage. A more definitive way to detect cell damage is to co-incubate a paracellular route marker such as radiolabeled mannitol in the test run. It is also possible to see false positives. It appears that the basolateral membrane of Caco-2 cell monolayer is more sensitive than the apical membrane (ie, the cell damage occurs more frequently in the B to A direction than the A to B direction). When this occurs, B to A permeability is significantly greater than A to B permeability, and the test compound is classified as a P-gp substrate even if it is a nonsubstrate (false positives). Figure 9 shows that the cell damage due to BMS research compound (BMS-X) is concentration dependent because the mannitol permeability was significantly higher when incubated with 50 μM compared with 10 μM BMS-X, and it also illustrated that the only basolateral membrane was sensitive to BMS-X. When the substrate concentration was reduced to 10 μM, the B to A permeability was reduced and became similar to A to B permeability. Therefore, lowering the substrate concentration is a good way to minimize the potential cell damage, and consequently the incidence of both false negatives and false positives.

Another experimental factor that needs to be fully optimized is the pH used in the bidirectional permeability studies. Optimization of the apical pH is not as straightforward as the substrate concentration. Based on the pK_a of the test compounds, the change in apical pH can lead to dramatically

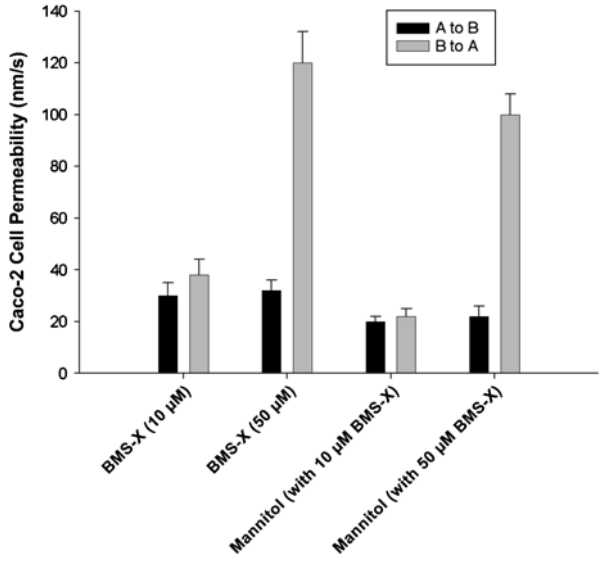

Figure 9. Effect of drug concentrations on the integrity of Caco-2 cell monolayer.

different extent of ionized and un-ionized fractions in the apical compartment and consequently significant changes in the permeability value. Figure 10 demonstrates the effect of apical pH on the efflux ratio observed for well-known P-gp substrates. Digoxin, a neutral P-gp substrate, had no ionization changes with pH and showed a uniform efflux ratio ~10 at all 3 pH. However, saquinavir, a weak base, showed a significant changes in the efflux ratio, where the ratio was greater than 50 at an apical pH of 5.5 as compared with the ratio of ~10 at pH 7.4. On the contrary, sulfasalazine (contains a carboxylic acid) demonstrated a higher efflux ratio at an apical pH of 7.4. The higher efflux ratio observed with saquinavir and sulfasalazine at an apical pH of 5.5 and 7.4, respectively, is primarily due to lower apical to basolateral permeabilities (ie, smaller denominator in the ratio calculation) rather than the changes in the basolateral to apical permeability. Therefore, the apical pH needs to be optimized depending on the type of ionizable function group of the compounds of interest. Prior information on the pK_a and acidic/basic nature of the compound will be very useful in the P-gp substrate assay. Other experimental variables such as incubation duration, composition of transport buffer, presence/absence of serum, cell-culturing conditions, and so forth, need to be calibrated using a set of known P-gp substrates. Factors such as culturing conditions, composition of media, cell-plate architecture (12-well vs 24-well Transwell) all have a significant effect on the expression of P-gp, and the interlaboratory variability is often significantly large. Therefore, a direct comparison of the results obtained from different laboratories should be done cautiously.

CONCLUSIONS

One of the most important challenges facing the pharmaceutical industry today is to develop high-throughput, cost-effective and predictive permeability/absorption screening models that can be used during the lead optimization process early in drug discovery. As discussed, Caco-2 cells and PAMPA are valuable research tools that are currently used in screening compounds for absorption and P-gp interaction potential. Despite the popularity and acceptability of PAMPA and Caco-2 cell models, it is important to recognize the caveats associated with these models to fully realize their potential. Standardization of experimental variables to develop a uniform methodology is an important first step prior to implementation of these models in high throughput mode. Calibration of the models with appropriate reference probes (known marketed drugs as well as internal research compounds) is essential to maintain the validity of the model. These reference compounds should cover a broad structural and physicochemical space and should cover a range of solubility/permeability. Other probes to represent paracellular and efflux (P-gp) transport are also recommended. These reference compounds should be included as quality control set in every test run performed with unknown compounds. Like all other in vitro models, both PAMPA and Caco-2 cells have strengths and weaknesses. A thorough understanding of the rationale underlying the caveats (such as low recovery and solubility) associated with these models could certainly help putting the results in the proper perspective. The results obtained from these assays should not be interpreted in isolation but should be assessed in conjunction with solubility and stability characteristics of the compounds. Finally, it is important to realize that simplistic in vitro models such as PAMPA and Caco-2 cells are inadequate to represent the complicated absorption machinery in the human intestinal tract. Ultimately, it is the judicious use of these models in combination with in vivo studies that enhances our ability to predict the drug disposition in humans.

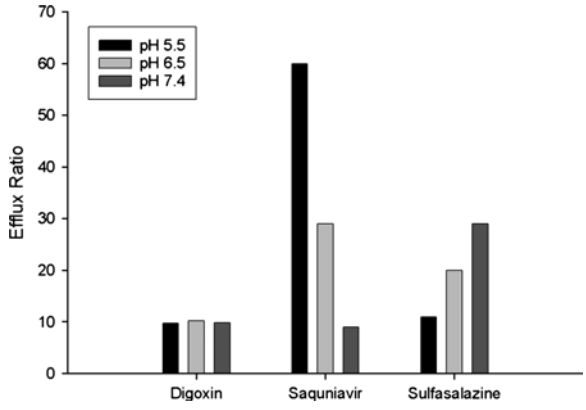

Figure 10. Effect of apical pH (5.5, 6.5, and 7.4) on the efflux ratio of acidic, basic, and neutral P-gp substrates in the Caco-2 cell bidirectional study. The efflux ratio was calculated using the mean data. The coefficient of variation in the permeability data (both directions) is typically less than 25% amongst replicates.

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