# Cell culture-based models for intestinal permeability: a critique

**Praveen V. Balimane and Saeho Chong**

**The model systems that are currently used to determine the intestinal permeability characteristics of discovery compounds often represent a combination of high-throughput, but less predictive, *in silico* and *in vitro* models and low-throughput, but more predictive, *in vivo* models. Cell-based permeability models have been integrated into the discovery paradigm for some time and represent the 'method of choice' across the industry. Here, in addition to an objective analysis of the utility of cell culture models for permeability screening, anticipated future trends in the field of cell culture models are discussed.**

Recent reports have put the final price of bringing a drug to the market at approximately US$1 billion dollars, with an estimated research time running into multiple years [1]. Considering the tremendous amount of time, effort and money that goes into discovering and developing medicines, it is imperative that the pharmaceutical industry constantly reinvents itself to stay afloat and grow in the competitive marketplace. Combinatorial and *in silico* chemistry, proteomics, genomics, robotics and miniaturization have all been steps in the right direction to reducing costs and expediting the drug discovery cycle. In parallel with these technological advances, a pragmatic conceptual awakening is also helping the industry to perform drug discovery with better economic sense. Compared with the old paradigm of drug discovery that was linearly oriented, the smarter drug discovery practiced today is matrixed and parallel in design. In the earlier linear design, new chemical entities were initially selected on the basis of their pharmacological activity, followed by sequential profiling to assess their ADMET characteristics. Such a strategy left only a small margin for error, and was generally more rigid, as well as more time- and resource-intensive. Newer drug design efforts incorporate a parallel matrixed approach to drug discovery, where the pharmacological efficacy is screened parallel to the initial ADMET profiling of compounds, providing more information for selecting superior quality drugs for further development. However, one of the cornerstones of such an approach is the availability of highly accurate, low-cost and high-throughput techniques that can provide fast and reliable read-outs on the developability characteristics of discovery compounds. Such screening techniques facilitate the selection of compounds with a greater probability of succeeding in the clinic, and also provide guidance to chemists on the design of better compounds. Thus, the task of screening discovery compounds for biopharmaceutical properties (e.g. solubility, intestinal permeability and metabolic stability) is now a major challenge facing the industry. Assessing permeability properties is a crucial step in determining the fate of an administered drug. This has provided a great impetus within the pharmaceutical industry to implement appropriate screening models that are high-capacity, cost-effective and highly predictive of *in vivo* permeability and absorption.

For a compound to be a successful medicine, it should have pharmacological activity coupled with adequate structural properties that enable it to reach

**Praveen V. Balimane***
**Saeho Chong**
Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb, Princeton, NJ 08543, USA
*e-mail: praveen.balimane@bms.com

1359-6446/04/$ – see front matter  PII: S1359-6446(04)03354-9

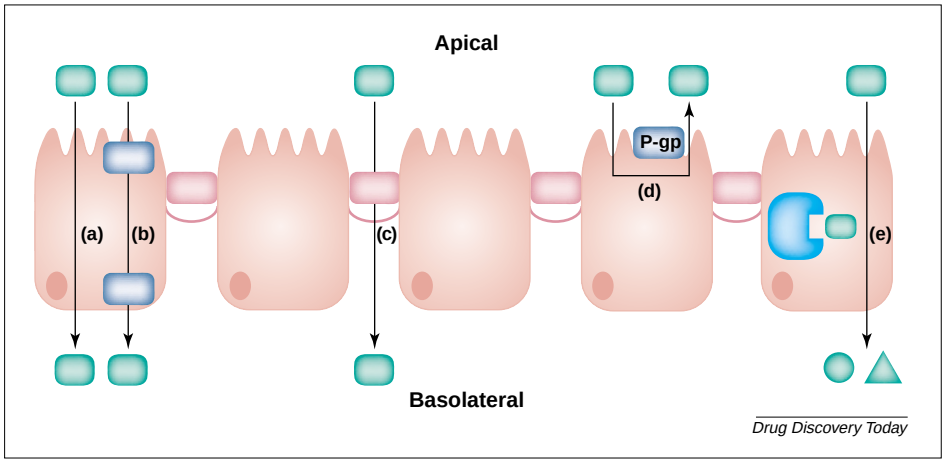


**FIGURE 1**

**Different pathways for intestinal absorption of a compound.** The intestinal absorption of a compound can occur via several pathways: **(a)** transcellular passive permeability; **(b)** carrier-mediated transport; and **(c)** paracellular passive permeability. However, there are also mechanisms that can prevent absorption: **(d)** intestinal absorption can be limited by P-gp, which is an ATP-dependent efflux transporter; and **(e)** metabolic enzymes in the cells might metabolize the compound.

the site of action intact. Consequently, it is also required to have reasonable permeability characteristics (i.e. it can freely travel through the multiple lipid bilayers in the system). Transport of drug substances across the intestinal membrane is a complex and dynamic process that includes the passage of compounds across several functional pathways in parallel. Passive transport occurs through the cell membrane of enterocytes (transcellular) or via the tight junctions between the enterocytes (paracellular). There are various functional influx and efflux mechanisms (via carriers and transporters) that dictate the permeability of compounds. Moreover, several different pathways are available via which molecules can travel from the lumen in to the systemic circulation (Figure 1).

Drug discovery scientists use many techniques when evaluating the intestinal permeability of drug candidates during the drug selection process [2–9]. The most pervasive preclinical methodologies currently used throughout the industry are: *in vitro* methods, for example, animal tissue-based Ussing chamber or membrane vesicles; cell-based assay systems such as Caco-2 cells and Mardin-Darby canine kidney (MDCK); artificial lipid-based systems such as parallel artificial membrane permeability assay (PAMPA) or immobilized artificial membranes (IAM); *in vivo* methods (whole animal pharmacokinetic studies); *in situ* methods (single-pass perfusion); and *in silico* (computer-aided drug design) methods. One, or a combination of these models, is routinely used in permeability assessment in drug discovery. A tiered approach is frequently 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. Cell culture models strike the right balance between predictability and throughput and thus are the method of choice for permeability assessment across the pharmaceutical industry.

**TABLE 1**

**Biological and physical characteristics of the human intestinal tract**

| Gastrointestinal segment | Surface area ($m^2$) | Segment length (cm) | pH of segment |
|---|---|---|---|
| Stomach | 3.5 | 0.25 | 1.0–2.0 |
| Duodenum | 1.9 | ~35 | 4.0–5.5 |
| Jejunum | 184.0 | ~280 | 5.5–7.0 |
| Ileum | 276.0 | ~420 | 7.0–7.5 |
| Colon and rectum | 1.3 | ~150 | 7.0–7.5 |

## Anatomy and physiology of the small intestine

The human small intestine is ~2–6 m in length and is loosely divided into three sections – duodenum, jejunum and ileum, which comprise 5%, 50% and 45% of the length, respectively: the biological and physical parameters of human intestinal tract are listed in Table 1 [10–12]. Approximately 90% of all absorption in the gastrointestinal tract occurs in the small intestinal region, the surface of which has various unique projections that significantly increase the potential surface area available for digestion and absorption. Macroscopic valve-like folds, called circular folds, that encircle the inside of the intestinal lumen are estimated to increase the surface area of the small intestine threefold. In addition, the presence of villi and microvilli increase the surface area by 30-fold and 600-fold, respectively.

The key function of the small intestine is the selective absorption of major nutrients. In addition, it serves as a barrier to digestive enzymes and ingested foreign substances. The epithelial cells in the intestinal region are a heterogeneous population of cells, which include enterocytes or absorptive cells, goblet cells (secrete mucin), endocrine cells, paneth cells, M cells and tuft and cup cells. Enterocytes are the most common epithelial cells and are thus responsible for the majority of the absorption of nutrients and drugs in the small intestine. Because enterocytes are polarized, having distinct apical and basolateral membranes that are separated by tight junctions, molecules are predominantly absorbed via mechanisms such as passive diffusion (paracellular and transcellular) and carrier-mediated processes (facilitated and active).

## Cell culture-based permeability-screening models

Varieties of cell monolayer models that mimic *in vivo* intestinal epithelium in humans have been developed and currently enjoy widespread popularity. Unlike enterocytes, human immortalized (tumor) cells grow rapidly into confluent monolayers that exhibit several characteristics

**TABLE 2**

**Cell culture models commonly used for permeability assessment**

| Cell line | Species or origin | Special characteristics |
|---|---|---|
| Caco-2 | Human colon adenocarcinoma | Most well-established cell model<br>Differentiates and expresses some relevant efflux transporters<br>Expression of influx transporters is variable (differs laboratory-to-laboratory) |
| MDCK | MDCK epithelial cells | Polarized cells with low intrinsic expression of ABC transporters<br>Ideal for transfections |
| LLC-PK1 | Pig kidney epithelial cells | Polarized cells with low intrinsic transporter expression<br>Ideal for transfections |
| 2/4/A1 | Rat fetal intestinal epithelial cells | Temperature-sensitive<br>Ideal for paracellularly absorbed compounds (leakier pores) |
| TC-7 | Caco-2 subclone | Similar to Caco-2 |
| HT-29 | Human colon | Contains mucus-producing goblet cells |
| IEC-18 | Rat small intestine cell line | Provides a size-selective barrier for paracellularly transported compounds |

Abbreviation: ABC, ATP-binding cassette.

of differentiated epithelial cells. Therefore, the cell culture model provides an ideal system for the rapid assessment of the intestinal permeability of drug candidates: examples of cell models that are used routinely for permeability screening are described in Table 2.

### *Caco-2 cells*

The Caco-2 cell model has been the most extensively characterized and useful cell model in the field of drug permeability studies [13–15]. Caco-2 cells, a human colon adenocarcinoma, undergo spontaneous enterocytic differentiation in culture and are polarized with well-established tight junctions. Because the permeation characteristics of drugs across Caco-2 cell monolayers correlate with their human intestinal mucosa permeation characteristics, it has been suggested that Caco-2 cells can be used to predict the oral absorption of drugs in humans. In the past 10–15 years, there has been a tremendous growth in the use of Caco-2 cells for mechanistic studies, and as a rapid *in vitro* screening tool in support of drug discovery within the pharmaceutical industry.

### *MDCK and LLC-PK1 cells*

Apart from Caco-2 cells, the two other cell models that are most frequently used for permeability assessment are MDCK and Lewis lung carcinoma-porcine kidney 1 (LLC-PK1) cells. MDCK differentiate into columnar epithelial cells and form tight junctions (similar to Caco-2 cells) when cultured on semi-permeable membranes. The use of MDCK cell line as a model to evaluate the intestinal permeation characteristics of compounds was first discussed in 1989 [16]. More recently, several other researchers [17–21] investigated the use of MDCK cells as a tool for assessing the membrane permeability properties of early drug discovery compounds. A good correlation between permeation of passively absorbed drugs in MDCK cells and Caco-2 cells was reported. Whereas Caco-2 cells are derived from human colon carcinoma cells, MDCK cells are derived from dog kidney cells, and thus there is a high probability that the expression levels of some transporters would be different in these two cell lines. Hence, more extensive studies are required in MDCK cell line to confirm that the correlation of permeability to human absorption values would be conserved for transporter-mediated uptake and/or efflux compounds. Species difference should also be considered before using MDCK cells as a primary screening tool for permeability in early drug discovery. One of the major advantages of MDCK cells over Caco-2 cells is the shorter cultivation period (three days versus three weeks). A shorter cell culture time becomes a significant advantage considering reduced labor and downtime in case of cell contamination. Similarly, cells derived from LLC-PK1 cells have also been explored as an alternative to Caco-2 cells for assessing the permeability of test compounds. Several investigators [22–24] have reported the utility of porcine cell line for characterizing the passive (transcellular and paracellular) absorption of discovery compounds.

### *Modified cell models: 2/4/A1 line, transfected cells and Caco-2 cell clones*

Despite the widespread use and acceptability of the Caco-2 cell model for permeability assessment, this model suffers from significant short-comings that render it less than optimal as a one-stop cell-based permeability-screening tool. Several modifications and improvements of Caco-2 cells have been investigated to generate a more predictable cell model.

Tavelin *et al.* [25–27] recently reported a particularly interesting new cell model, 2/4/A1, that originates from fetal rat intestine and might better mimic the permeability of the human small intestine, particularly with regards to passive paracellular permeability. This immortalized cell line forms viable differentiated monolayers with tight junctions, brush-border membrane enzymes, as well as transporter proteins. The paracellular pore radius of 2/4/A1

was determined to be 9.0 ± 0.2 Å, which is similar to the pores in the human small intestine (pore size in Caco-2 is estimated to be ~3.7 ± 0.1 Å). Because the tight junctions in the Caco-2 cell line appear unrealistically tighter than the tight junctions in the epithelial cells in human intestine, the 2/4/A1 cells were proposed as a better model to study compounds passively absorbed via the paracellular route. The trans epithelial electrical resistance (TEER) value in the 2/4/A1 cells reached a plateau of 50 Ω $cm^2$ compared with a plateau of 234 Ω $cm^2$ in the Caco-2 cells (the typical small intestinal TEER is estimated to be in the range of 25–40 Ω $cm^2$). The transport rate of poorly permeable compounds (e.g. mannitol and creatinine) in 2/4/A1 monolayers was comparable with that in the human jejunum, and was up to 300-fold higher than that in the Caco-2 cell monolayers, suggesting that a cell line such as 2/4/A1 will be more predictive for compounds that are absorbed via the paracellular route.

Caco-2 cells are known to express intrinsically the physiologically relevant transporters but the gene expression profiles of the cells and the human intestine can be significantly different [28–30]. A 2–595-fold difference was observed in levels of transporters and metabolizing enzymes expressed in human duodenum and Caco-2 cells. This difference could explain the lack of correlation of *in vitro* Caco-2 cell permeability with *in vivo* absorption for several well-absorbed compounds [e.g. β-lactam antibiotics, glucose and angiotensin-converting enzyme (ACE) inhibitors] that are known to be absorbed via carrier-mediated processes. Several investigators [19,31–34] have suggested the use of cell lines that are transfected with influx transporters, for example, human peptide transporter (hPepT1) and organic anion transporter (OAT), and efflux transporters such as multidrug-resistant proteins (e.g. MDR1 and MRP2). These modified cell lines present efficient *in vitro* permeability models to improve the predictability of carrier-mediated processes.

TC-7, a subclone of Caco-2 cells, is also used for permeability screening. Recently, a comprehensive comparison of the permeability characteristics of TC-7 with parental Caco-2 cells was reported [35,36]. The TC-7 clone exhibited similar cell morphology to Caco-2 cells, displaying the presence of brush-border membrane and microvilli, and the formation of tight junctions. The correlation of permeability values of passively transcellularly absorbed drugs obtained in TC-7 clone corresponded to that generated in parental Caco-2 cells with respect to the extent of absorption in humans. Thus, on the basis of morphological parameters, biochemical attributes and drug permeability characteristics, the TC-7 subclone appears to be similar to Caco-2 cells and presents a suitable alternative to parental cells for intestinal drug permeability studies.

## Issues and short-comings of cell culture models

### *Lack of correlation for carrier-mediated compounds*

The Caco-2 cell model primarily measures passive transcellular and paracellular permeability characteristics of test compounds. Although pharmaceutically important transporters (e.g. peptide transporters, organic cation transporter and OAT) are expressed in Caco-2 cells [30,37,38], they are quantitatively underexpressed when compared with levels of production *in vivo*. For example, β-lactam antibiotics (e.g. cephalexin and amoxicillin) and ACE inhibitors, which are known substrates of dipeptide transporters, have poor permeability across the Caco-2 cell monolayer despite being completely absorbed *in vivo* [39]. This model is likely to generate false negatives with drug candidates that are known to be absorbed by a carrier-mediated process facilitated by transporter proteins [30]. In addition to the lack of expression of various transporter proteins, another obvious issue associated with the use of Caco-2 cell models is the variable levels of expression of those transporter proteins that are produced: mRNA levels of transporters in Caco-2 cells have been shown to be substantially different from human small and large intestine [38]. Expression of several genes that are highly transcribed in the small or large intestine differed fourfold or more in Caco-2 cells.

### *Lack of correlation for paracellularly transported compounds*

The predictability and utility of the Caco-2 cell model to rank a large number of compounds for absorption potential has been demonstrated by several investigators [13,40,41]. However, comparison of the absolute permeability value of individual compounds that permeate primarily via the paracellular route has proven extremely difficult. Walter and Kissel [42] indicated that the permeability of mannitol (paracellular hydrophilic marker) could vary as much as 100-fold (e.g. 2–221 nm $sec^{-1}$) depending on the source of Caco-2 cells. Similarly, it was shown that the TEER value of Caco-2 cells derived from different laboratories could vary as much as 20-fold (e.g. 80–1420 Ω $cm^2$) [7]. The variability observed could be attributed to differences in culture conditions and composition of cell subpopulation. The low molecular weight hydrophilic compounds (e.g. metformin, ranitidine, atenolol, furosemide and hydrochlorothiazide) also showed poor permeability (i.e. equal to or less than mannitol) in this cell model, despite adequate absorption (greater than 50% of dose) in humans. In other words, the Caco-2 cell model can only serve as a one-way screen such that compounds with high permeability in this model are typically well absorbed *in vivo*; however, compounds with low permeability cannot be ruled out as poorly absorbed compounds *in vivo*. Such a limitation poses a problem in providing accurate structure–permeability relationship (SPR) feedback to medicinal chemists. Significant advances in understanding the structure and cellular regulation of tight junctions over the past decade [43,44] have led to the design of agents that can effectively modulate the paracellular permeability to predict *in vivo* absorption more accurately.

### *Lack of metabolic enzymes in cell models*

Cytochrome P450 (CYP) 3A4 is the most prominent oxidative CYP enzyme present in the intestine and plays a significant role in first-pass metabolism [45]. An ideal cell-based intestinal permeability tool would be one that simulates the human gastrointestinal enterocytes not only in lipid bilayer characteristics but also in metabolic enzyme activity. Such a model would facilitate the simultaneous study of drug transport and intestinal metabolism of test compounds. Although Caco-2 cell-based models are known to express adequate amounts of hydrolase, esterase and brush-border enzymes, they fail to simulate the complete *in vivo* intestinal environment because they do not express appreciable quantities of CYP3A4, the principle CYP present in human epithelial cells. Efforts have been made to develop Caco-2 cells expressing high levels of cDNA-derived CYP3A4 either by modification of the growth media or using the transfection technique [46,47]. CYP3A4 catalytic activity was demonstrated to be appreciably higher in these specialized Caco-2 cells compared with control cells. However, a shortcoming of such transfected models is the lack of stringent control of expression levels of the enzymes and their overall relevance to the *in vivo* situation. These CYP-expressing cell lines can serve as a valuable tool if the objective is to incorporate permeability and intestinal metabolism in a single assay. If only 'intrinsic permeability' is desired, the overexpression of the CYP enzymes is undesirable.

### *Non-specific binding: cacophilicity*

One universal difficulty encountered with most cell-based models is the potential for non-specific drug-binding that can confound data interpretation. A significant physical loss that is the result of non-specific drug-binding to plastic devices and/or cells could lead to underestimation of permeability (i.e. false negatives). Cacophilicity has often been used as a term to define the potential of a drug to reversibly bind to Caco-2 cells, thus leading to incomplete recovery and resulting in an underestimation of permeability values. There are several experimental strategies that can be used to minimize the problem of non-specific binding. One approach involves the analysis of drug concentration in the donor compartment, as well as the receiver compartment at the end of the study, which will provide the recovery data to the analyst and suggest a probable underestimation of permeability caused by non-specific binding. In cases where compounds are extensively bound, the ratio of the final concentration of receiver to donor compartment might be more relevant than permeability values. A second methodology involves addition of serum proteins (e.g. bovine serum albumin) to the receiver compartment in the Caco-2 cell setup [48–50]. The presence of these proteins at a concentration of 1–4% improves the recovery of highly bound lipophilic compounds, thus providing more realistic permeability values. However, the addition of proteins only affects those compounds that are extremely lipophilic (log P >3) and highly protein bound (i.e. >95% of compound bound). Miniaturization (involving the use of 24- and 96-well cell models with higher surface area to volume ratio) and lower concentration studies (with an extremely high surface area to drug-amount ratio) generally exacerbates the non-specific binding issues. Therefore, a balance of size and concentration with reasonable throughput can provide the best protocol for cell-based permeability studies in a discovery setting.

### *Solubility complications: imperfect model for compounds with low and/or pH-dependent solubility*

Transport studies in cell-based models are often performed at concentrations of 10 μM to 200 μM in a Hanks' balanced salt solution (HBSS) or other buffer systems. Frequently, discovery compounds are synthesized with a view to optimizing potency and pharmacological activity, leading to generally lipophilic and poorly soluble compounds (solubility in aqueous buffer <0.1 mg $ml^{-1}$). Despite the judicious use of co-solvent and solubilizer systems, a significant percentage of new drug candidates cannot be evaluated in these permeability models because of their poor aqueous solubility. In addition to the solubility obstacle, another important variable for absorption in the human gut is the variability in pH from the upper small intestine to the distal large intestine. The bulk of absorption occurs in the small intestine, where the pH varies from acidic (~pH 5) to neutral or even slightly basic pH [51]. Thus, in the upper small intestine, a weak acid will be primarily unionized, leading to the passive transcellular pathway being the dominant permeation route (at a slightly basic pH, a weak base will be unionized and also absorbed via permeation). However, the Caco-2 cell studies are typically performed under a fixed pH condition (apical pH of 6.5 and basolateral pH of 7.4) and thus are incapable of simulating the dynamic pH environment that the compound is exposed to *in vivo*. Permeability studies can be performed at a range of pH conditions, but this affects assay throughput and cost. Moreover, it is not uncommon for the change in apical pH to have pronounced effects on the permeability of passively and actively transported compounds, which makes interpretation of data difficult [52,53].

### *Analytical limitation: phosphate-based transport buffers make analysis challenging*

One of the prerequisites for the success of cell-based permeability models is the availability of a high-throughput, sensitive and accurate method to analyze the samples generated, with the majority of laboratories using LC–UV HPLC or LC–MS tools for sample analysis. To meet the requirement of the analysis of the significant volume of samples produced in discovery, these methods often involve reduced run times, shorter LC columns and other such features to improve throughput. Although such analytical modifications might be satisfactory for the majority of compounds, in particular scenarios, for example, cassette studies [where more than one compound is co-incubated

(i.e. N in 1)] and the use of special solubilizers and/or additives in the transport buffer, traditional methods do not work optimally. In addition, the high salt content of transport buffers (e.g. HBSS) interferes with ionization, thus complicating the development of sturdy LC–MS methods. An analytical method that combines the speed of a UV-plate reader with the sensitivity of LC–MS would be ideal for preventing the bottleneck at the analytical end of permeability studies. Modifications to an analytical method that simultaneously quantitate marker [e.g. atenolol for paracellular, propranolol for transcellular and talinolol for p-glycoprotein (P-gp)] and discovery compounds can improve the efficiency of the analysis, as well as the quality of cell-based data [54].

*Effect of co-solvents and transport media on permeability*

Solubilizers are often used to study the Caco-2 cell permeability of poorly soluble compounds, and the effect of commonly used co-solvents on the physiology of Caco-2 cells and their functionality has been the focus of several investigations [55–58]. Organic solvents (e.g. methanol, ethanol, polyethylene glycol and Tween 80) introduced at a low concentration (~1–2% v/v) generally have little effect on the integrity of Caco-2 cells tight junctions [55]. However, the use of a substantial volume of organic co-solvent compromises the integrity of the cells and renders them unusable. This presents a problem for the permeability assessment of poorly soluble compounds that are negligibly soluble in the aqueous transport buffer used in the Caco-2 cell studies. Although desirable for their solubility enhancement properties, some solubilizers (e.g. Tween 80, cremophor and pluronic) are known to inhibit efflux transporters such as P-gp and thus confound the permeability results [56–58]. Although extensive research is directed towards finding compatible solubility enhancers, there is insufficient understanding of the impact of these solubilizers on the influx and efflux transporters and paracellular transport mechanism of drugs [59]. Some attempts have also been made to investigate the use of more physiologically relevant solutions based on human intestinal fluid (e.g. using simulated fasted and fed small intestinal fluids as transport buffers) in Caco-2 cell studies. These too are known to interfere with efflux transporter systems and their clinical effects are not fully validated [54,60,61]. There is a significant diversity in the material and protocols used by different laboratories for cell-based permeability assessment, which precludes the implementation of a universal method for such studies. An improved scenario would be if each laboratory incorporated a set of standard internal reference compounds as controls and provided acceptance criteria for their cell culture models [59].

*Effects of passage number, culturing condition and intra- and inter-laboratory variability*

Despite the use of generally similar transport study protocols, the Caco-2 cell permeability values obtained from different laboratories are widely different [7,42,59]. Small differences in culturing conditions (seeding density, cell feeding routine and composition of the media), experimental conditions (composition and osmolarity of the transport buffer and washing steps) and age of the cells (passage number and culture duration) are all factors that are known to produce dramatic inconsistencies in permeability values among different laboratories. Expression levels of known and unknown transporter proteins in the cell-based models also fluctuate with varying culture conditions [30,37,38,42]. The expression level of P-gp, a significant efflux transporter, is documented to vary significantly with the age of cell cultures. Caco-2 cell subclones expressing high levels of efflux transporters, which are useful for establishing structure–transport relationship for efflux transporters, have been characterized [62]. These disparate approaches to optimize the Caco-2 cells model have added great variability between the clones and there is now an urgent need to standardize cell culture procedures and/or protocols across all laboratories.

*High cost of cell culture: resource intensive model*

The preparation of a fully functional cell monolayer generally requires a three-week cell culture period with 8–9 laborious cell-feeding steps. Despite cell-based models being relatively economical (compared with *in vivo* models), this three-week long process puts strain on the finances and resources of discovery groups (which are already overstretched) within pharmaceutical organizations. Currently, it is estimated that a typical Caco-2 cell permeability study involving a 21-day culturing followed by a uni-directional (apical to basolateral) permeability assessment costs the drug discovery organizations ~US$19 per compound. Research efforts such as modification of the coating material and growth media can substantially reduce the preparation time (less than one-week) and drive cost down for drug discovery [39,63,64]. A shorter cell-culturing period to generate functional monolayers not only increases the overall productivity but also reduces the chance of bacterial and/or fungal contamination, thus minimizing costly downtimes. Despite these limitations, the Caco-2 cell model is still the most widely used intestinal cell culture model, and is providing valuable information for the decision-making process in early drug discovery.

## Future trends

Recent advances in molecular biology and combinatorial chemistry have changed the way in which pharmaceutical companies conduct drug discovery research. The biggest challenge lies in screening a large number of drugs in a short period of time. Smart use of cell culture techniques has exponentially improved the efficiency of permeability screening at the discovery end and has helped push down costs and time taken to progress a drug. There have been substantial advances in the fields of miniaturization and automation, with respect to cell culture techniques, that

have aided in the HTS of discovery hits. Concerted efforts involving the use of novel media and transwell plates have led to a compression of the time-lines required for growing and culturing cell lines. Analytical advances have also enabled the exploration of cassette incubation studies (N in 1) in cell culture models to drive the efficiencies even higher. Trends that we believe will continue to evolve in the coming years in cell culture permeability techniques are discussed below.

### *Miniaturization*

Most biological screening assays are performed in the miniaturized mode (384- or 1536-well plates). In contrast to the successful use of miniaturization in biological activity screening, cell-based intestinal permeability screening has not made similar advances. In the Caco-2 cell monolayer model, the pharmaceutical industries method of choice for permeability assessment, moderate success has been achieved with miniaturization. Currently, permeability studies using cell monolayer are conducted in an automation-friendly 12- or 24-well transwell plates. Unfortunately, further miniaturization (use of 96- and 384-well cell models) has been technically difficult to achieve because of the small surface area of the monolayer. Recently, there have been reports of the successful implementation of Caco-2 cells in 96-well plate format for both permeability and P-gp interaction studies [65,66]. The successful integration of a 96-well Caco-2 cell system in early discovery would have significant advantages: increased throughput; reduced cost (media and plate costs); and a considerable decrease in the amount of compound required for permeability assessment. These advantages could potentially have a major impact on drug discovery and could have a key role in boosting the productivity of R&D laboratories.

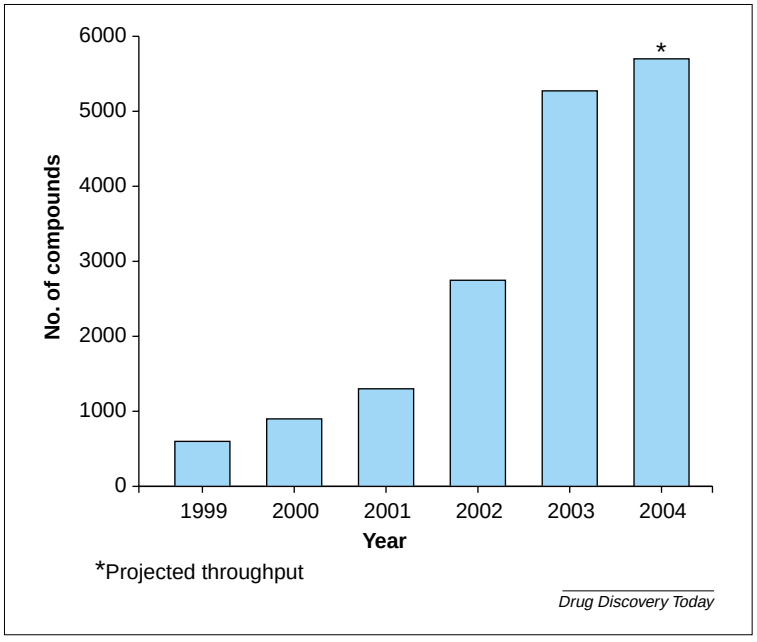


**FIGURE 2**

**Throughput of cell-based permeability models.** A snap-shot of the total number of discovery compounds for which permeability is assessed each year using cell-based models: data are representative of a mid–large pharmaceutical company. Although automation efforts have provided significant increases in capacity in the past few years, analytical processes can be potential bottlenecks, limiting the overall throughput of cell-based models. Data shown represent the experience within Bristol-Myers Squibb.

### *Robotics and automation*

Taking a cue from the hi-tech and automobile industries, pharmaceutical companies have also invested heavily in automating routine processes within drug discovery and development. Cell-based permeability studies are now regularly conducted by robotic equipment (e.g. liquid handler and articulated arm) with minimum human intervention [67]. In addition, automated cell culturing (i.e. generation of functional cell monolayer) could significantly improve productivity by minimizing or eliminating laborious cell feeding. A fully automated Caco-2 cell system has a throughput in the order of 500 to 2000 compounds studies per month without a proportional increase in resources (Figure 2) [68]. The use of 24-well monolayer (cell surface area ~0.33 $cm^2$) coupled with the use of LC–MS can also significantly reduce the amount of compound (no more than 50 μg) required to perform a permeability experiment using this model. Although automation is a logical direction of future advances in this area, there are currently no commercially available automation systems that are capable of handling permeability studies and cell culturing. Robotic systems coupled with the miniaturization devices can be extremely valuable because of the precision and high-speed handling of repetitive tasks (e.g. pipetting and performing dilutions) and will definitely lead the way to an era of hi-tech laboratories working smoothly with minimal human intervention for 24 hours a day, seven days a week.

### *Shorter is better*

One major application of the cell-based permeability model is the screening of chemical libraries for structures that have favorable permeability characteristics. Conventional Caco-2 cells require 21 days of culturing for appropriate differentiation of the cells and thus utility for screening studies. Some other cell models (MDCK and LLC-PK1) can differentiate much faster and be available for studies in 5–7 days. The 21-day culture requirement for Caco-2 cells is too long for the high-throughput desired by the drug discovery departments. Efforts from our laboratories and other investigators have led to modified cell models that are ready for permeability analysis in a culturing time of 3–7 days [39,64,65,69]. Improvements in culturing media (addition of 10% fetal bovine serum), manipulation of the seeding density and the use of modified transwell plates (BIOCOAT® plates) are some of the technological innovations that are used to reduce the time required for cell growth. These shortened cell models are not only known to perform well for passive transcellular permeability compounds but also for compounds that interact extensively with influx and efflux transporters. Further

reductions in culturing time have the potential to provide cost and time benefits, and this area will certainly be the focus of development efforts in the near future.

### *The more the merrier*

To expedite the discovery of orally bioavailable drugs, several approaches have been applied to cell-based models with the aim of increasing their screening throughput. Experimentally, cell-based assays have two distinct steps, a transport step followed by a quantitative analysis step. Several laboratories have successfully demonstrated that cassette incubations (i.e. N in 1) in permeability studies could provide qualitatively similar results with a much higher throughput [70–72]. Five to ten compounds have been co-incubated and their permeability values assessed singly, as well as in groups. Similarly, with advances in analytical techniques, pooling of multiple analytes and their simultaneous analysis for various compounds can also significantly improve turnaround time [73]. Thus, it appears to be acceptable to perform the N in 1 cocktail permeability study for the HTS of discovery compounds. When performing co-incubation studies, it should be clearly understood that there is the potential for drug–drug interactions to occur, particularly in cases where two or more compounds interact with efflux and/or influx transporters. Such cassette mode studies are applicable when the purpose is to triage a large chemical library and select some favorably absorbed compounds. If used judiciously, this technique has the potential to save valuable time, money and resource in early discovery.

## Conclusions

One of the most important challenges presently facing the pharmaceutical industry is to develop high-throughput, cost-effective and highly predictive screening models of drug permeability and/or absorption that can be used during the decision-making process in the early stages of drug discovery. Automated high-throughput cell culture-based models are currently the method of choice of the pharmaceutical industry for permeability assessment. Despite the widespread use and acceptability of Caco-2 cell model in this analysis, this model has several shortcomings that render it less than optimal as a one-stop cell-based permeability-screening tool. Caution must be exercised in interpreting the results obtained from cell-based permeability models. However, Caco-2 cells can be powerful tool provided that the caveats associated with the model are understood and appreciated. Moreover, various modifications and enhancements of cell-based models, as well as their combination with other *in vitro, in vivo* and *in silico* models, have been suggested to facilitate improvements in the prediction of the levels of absorption of drug candidates in humans.

## References


1 Tufts Center for the Study of Drug Development (2003) Post-approval R&D raises total drug development costs to $897 million. *Impact Report* 5, May–June

2 WaterBeemd, V. and Jones, C. (2003) Predicting oral absorption and bioavailability. *Prog. Med. Chem.* 41, 1–59

3 Penzotti, J. *et al.* (2004) Building predictive ADMET models for early decisions in drug discovery. *Curr. Opin. Drug Discov. Devel.* 7, 49–61

4 Miret, S. *et al.* (2004) Comparison of *in vitro* models for the prediction of compound absorption across the human intestinal mucosa. *J. Biomol. Screen.* 9, 598–606

5 Balimane, P.V. *et al.* (2000) Current methodologies used for evaluation of intestinal permeability and absorption. *J. Pharmacol. Toxicol. Methods* 44, 301–312

6 Avdeef, A. (2001) Physicochemical profiling (solubility, permeability and charge state). *Curr. Top. Med. Chem.* 1, 277–351

7 Hidalgo, I. (2001) Assessing the absorption of new pharmaceuticals. *Curr. Top. Med. Chem.* 1, 385–401

8 Kerns, E. (2001) High throughput physicochemical profiling for drug discovery. *J. Pharm. Sci.* 90, 1838–1858

9 Lipinski, C. *et al.* (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 46, 3–26

10 Daugherty, A. and Mrsny, R. (1999) Transcellular uptake mechanisms of the intestinal epithelial barrier: part 1. *Pharm. Sci. Technol. Today* 4, 144–151

11 Carr, K. and Toner, O. (1984) Morphology of the small intestine. In *Pharmacology of the Intestine* (Csaky, T. ed.), pp. 1–50, Springer–Verlag

12 Madara, J. and Trier, J. (1987) Functional morphology of the mucosa of the small intestine. In *Physiology of the Gastrointestinal Tract* (Johnson, L. ed.), pp. 1209–1249, Raven Press

13 Artursson, P. (1991) Cell cultures as models for drug absorption across the intestinal mucosa. *Crit. Rev. Ther. Drug Carrier Syst.* 8, 305–330

14 Artursson, P. and Karlsson, J. (1991) Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelia (Caco-2) cells. *Biochem. Biophys. Res. Commun.* 175, 880–890

15 Rubas, W. *et al.* (1996) Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue. *J. Pharm. Sci.* 85, 165–169

16 Cho, M. *et al.* (1989) The MDCK epithelial cell monolayer as a model cellular transport barrier. *Pharm. Res.* 6, 71–77

17 Irvine, J. *et al.* (1999) MDCK cells: a tool for membrane permeability screening. *J. Pharm. Sci.* 88, 28–33

18 Tang, F. and Borchardt, R. (2002) Characterization of efflux transporter(s) responsible for restricting intestinal mucosa permeation of an acycloxyalkoxy-based cyclic prodrug of the opoid peptide DADLE. *Pharmaceutical Research* 19,780–796

19 Tang, F. *et al.* (2002) Are MDCK cells transfected with the human MDR1 gene a good model of the human intestinal mucosa? *Pharm. Res.* 19, 765–772

20 Troutman, M. and Thakker, D. (2003) Efflux ratio cannot assess P-gp-mediated attenuation of absorptive transport: asymmetric effect of P-gp on absorptive and secretory transport across Caco-2 cell monolayers. *Pharm. Res.* 20, 1200–1209

21 Putnam, W. *et al.* (2002) Functional characterization of monocarboxylic acid, large neutral amino acid, bile acid and peptide transporters, and P-gp in MDCK and Caco-2 cells. *J. Pharm. Sci.* 91, 2622–2635

22 Li, H. *et al.* (2002) Characterization of the transport of uracil across Caco-2 and LLC-PK1 cell monolayers. *Pharm. Res.* 19, 1495–1501

23 Adachi, Y. *et al.* (2003) Quantitative evaluation of the function of small intestinal P-gp: comparative studies between *in situ* and *in vitro*. *Pharm. Res.* 20, 1163–1169

24 Thwaites, D. *et al.* (1993) Passive transepithelial absorption of thyrotropin-releasing hormone (TRH) via a paracellular route in cultured intestinal and renal epithelial cell line. *Pharm. Res.* 10, 674–681

25 Tavelin, S. *et al.* (1999) A conditionally immortalized epithelial cell line for studies of intestinal drug transport. *J. Pharmacol. Exp. Ther.* 290, 1212–1221

26 Tavelin, S. *et al.* (2003) An improved cell culture model based on 2/4/A1 cell monolayers for studies of intestinal drug transport: characterization of transport routes. *Pharm. Res.* 20, 373–381

27 Tavelin, S. *et al.* (2003) Prediction of the oral absorption of low permeability drugs using

small intestine like 2/4/A1 cell monolayers. *Pharm. Res.* 20, 397–405
28 Nakamura, T. *et al.* (2003) Gene expression profiles of ABC transporters and cytochrome P450 3A4 in Caco-2 and human colorectal cancer cell lines. *Pharm. Res.* 20, 324–327
29 Taipalensuu, J. *et al.* (2004) Exploring the quantitative relationship between the level of MDR1 transcript, protein and function using digoxin as a marker of MDR1-dependent drug efflux activity. *Eur. J. Pharm. Sci.* 21, 69–75
30 Sun, D. *et al.* (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequence tags and correlation with permeability of 26 drugs. *Pharm. Res.* 19, 1400–1416
31 Han, H. *et al.* (1998) Cellular uptake mechanism of amino acid ester prodrugs in Caco-2/hPepT1 cells overexpressing a human peptide transporter. *Pharm. Res.* 15, 1382–1386
32 Braun, A. *et al.* (2000) Cell cultures as tools in biopharmacy. *Eur. J. Pharm. Sci.* 11 (Suppl. 2), S51–S60
33 Adachi, Y. *et al.* (2001) Comparative studies on *in vitro* methods for evaluating *in vivo* function of MDR1 P-gp. *Pharm. Res.* 18, 1660–1668
34 Tang, F. *et al.* (2002) Are MDCK cells transfected with MRP2 gene a good model of the human intestinal mucosa? *Pharm. Res.* 19, 773–779
35 Gres, M. *et al.* (1998) Correlation between oral drug absorption in humans, apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line: comparison with the parental Caco-2 cell line. *Pharm. Res.* 15, 726–733
36 Pontier, C. *et al.* (2001) HT29-MTX and Caco-2/TC7 monolayers as predictive models for human intestinal absorption: role of the mucus layer. *J. Pharm. Sci.* 90, 1608–1619
37 Behrens, I. *et al.* (2004) Variation of peptide transporter (PepT1 and HPT1) expression in Caco-2 cells as a function of cell origin. *J. Pharm. Sci.* 93, 1743–1754
38 Anderle, P. *et al.* (2004) Intestinal membrane transport of drugs and nutrients: genomic membrane transporters using expression microarray. *Eur. J. Pharm. Sci.* 21, 17–24
39 Chong, S. *et al.* (1996) *In vitro* permeability through Caco-2 cells is not quantitatively predictive of *in vivo* absorption for peptide-like drugs absorbed via the dipeptide transporter system. *Pharm. Res.* 13, 120–123
40 Artursson, P. and Borchardt, R. (1997) Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharm. Res.* 14, 1655–1658
41 Chong, S. *et al.* (1997) Evaluation of biocoat intestinal epithelium differentiation environment (3-day cultured Caco-2 cells) as an absorption screening model with improved productivity. *Pharm. Res.* 14, 1835–1837
42 Walter, E. and Kissel, T. (1995) Heterogeneity in the human intestinal cell line Caco-2 leads to differences in transepithelial transport. *Eur. J. Pharm. Sci.* 3, 215–230
43 Daugherty, A. and Mrsny, R. (1999) Regulation of the intestinal epithelial paracellular barrier. *Pharm. Sci. Technol. Today* 2, 281–287
44 Ward, P. *et al.* (2000) Enhancing paracellular permeability by modulating epithelial tight-junctions. *Pharm. Sci. Technol. Today* 3, 346–358
45 Benet, L. *et al.* (2003) Transporter–enzyme interactions: implications for predicting drug–drug interactions from *in vitro* data. *Curr. Drug Metab.* 4, 393–398
46 Crespi, C. *et al.* (2000) Analysis of drug transport and metabolism in cell monolayer systems that have been modified by cytochrome P4503A4 cDNA-expression. *Eur. J. Pharm. Sci.* 12, 63–68
47 Schmiedlin-Ren, P. *et al.* (1997) Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix coated permeable supports in the presence of 1alfa, 25-dihydroxyvitamin D3. *Mol. Pharmacol.* 51, 741–754
48 Aungst, B. *et al.* (2000) The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV protease inhibitors and other lipophilic compounds. *Pharm. Res.* 17, 1175–1180
49 Krishna, G. *et al.* (2001) Permeability of lipophilic compounds in drug discovery using *in vitro* human absorption model, Caco-2. *Int. J. Pharm.* 222, 77–89
50 Saha, P. and Kou, J. (2002) Effect of bovine serum albumin on drug permeability estimation across Caco-2 monolayers. *Eur. J. Pharm. Biopharm.* 54, 319–324
51 Dressman, J.B. *et al.* (1993) Gastrointestinal parameters that influence oral medications. *J. Pharm. Sci.* 82, 857–872
52 Yamashita, S. *et al.* (2000) Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *Eur. J. Pharm. Sci.* 10, 195–204
53 Neuhoff, S. *et al.* (2003) pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: Implications for drug–drug interactions. *Pharm. Res.* 20, 1141–1148
54 Augustijns, P. and Mols, R. (2004) HPLC with programmed wavelength fluorescence detection for the simultaneous determination of marker compounds of integrity and P-gp functionality in the Caco-2 intestinal absorption model. *J. Pharm. Biomed. Anal.* 34, 971–978
55 Takahashi, Y. *et al.* (2002) Common solubilizers to estimate the Caco-2 transport of poorly water-soluble drugs. *Int. J. Pharm.* 246, 85–94
56 Bogman, K. *et al.* (2003) The role of surfactants in the reversal of active transport mediated by multidrug resistance proteins. *J. Pharm. Sci.* 92, 1250–1261
57 Rege, B. *et al.* (2001) Effect of common excipients on Caco-2 transport of low-permeability drugs. *J. Pharm. Sci.* 90, 1776–1786
58 Rege, B. *et al.* (2002) Effect of non-ionic surfactants on membrane transport in Caco-2 cell monolayers. *Eur. J. Pharm. Sci.* 16, 237–246
59 Ingels, F. and Augustijns, P. (2003) Biological, pharmaceutical, and analytical considerations with respect to the transport media used in the absorption screening system, Caco-2. *J. Pharm. Sci.* 92, 1545–1558
60 Deferme, S. *et al.* (2003) P-gp attenuating effect of human intestinal fluid. *Pharm. Res.* 20, 900–903
61 Ingels, F. *et al.* (2002) Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. *Int. J. Pharm.* 232, 183–192
62 Horie, K. *et al.* (2003) Isolation and characterization of Caco-2 subclones expressing high levels of multidrug resistance efflux transporter. *Pharm. Res.* 20, 161–168
63 Lentz, K. *et al.* (1998) Development of a more rapid culture system for Caco-2 monolayers. *American Association of Pharmaceutical Scientists Eastern Regional Meeting,* October 1998, New Brunswick (NJ, USA)
64 Yamashita, S. *et al.* (2002) New and better protocols for a short term Caco-2 cell culture system. *J. Pharm. Sci.* 91, 669–679
65 Alsenz, J. and Haenel, E. (2003) Development of a 7-day, 96-well Caco-2 permeability assay with high-throughput direct UV compound analysis. *Pharm. Res.* 20, 1961–1969
66 Balimane, P.V. *et al.* (2004) Utility of 96-well Caco-2 cell system for increased throughput of P-gp screening in drug discovery. *Eur. J. Pharm. Biopharm.* 58, 99–105
67 Russell, J. *et al.* (1999) An automated system for the determination of intestinal permeability (Caco-2 cells) in support of drug discovery. *International Symposium on Laboratory Automation and Robotics*, 17–20 October 1999, Boston, MA, USA, pp. 97
68 Fung, E. *et al.* (2003) Higher-throughput screening for Caco-2 permeability utilizing multiple sprayer liquid chromatography/tandem mass spectrometry system. *Rapid Commun. Mass Spectrom.* 17, 2147–2152
69 Liang, E. *et al.* (2000) Evaluation of an accelerated Caco-2 cell permeability model. *J. Pharm. Sci.* 89, 336–345
70 Laitinen, L. *et al.* (2003) N-in-one permeability studies of heterogenous sets of compounds across Caco-2 cell monolayers. *Pharm. Res.* 20, 187–197
71 Palmgren, J. *et al.* (2004) Characterization of Caco-2 cell monolayer drug transport properties by cassette dosing using UV/fluorescence HPLC. *Eur. J. Pharm. Biopharm.* 57, 319–328
72 Taneergren, C. *et al.* (2001) Compound mixtures in Caco-2 cell permeability screens as a means to increase screening capacity. *Pharmazie* 56, 337–342
73 Bu, H. *et al.* (2000) High-throughput Caco-2 cell permeability screening by cassette dosing and sample pooling approaches using direct injection/on-line guard cartridge extraction/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 14, 523–528