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Note

Validation of the 96 well Caco-2 cell culture model for high throughput permeability assessment of discovery compounds

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Abstract

The use of Caco-2 cells for permeability screening of discovery compounds is quite well established and serves as the "method-of-choice" across the pharmaceutical industries worldwide. The typical permeability-screening model involves growing cells on a 12 well or 24 well transwell format. In this manuscript, we report the use of Caco-2 cells grown on 96 well transwell plates for screening of discovery compounds to assess their permeability characteristics. A set of standard compounds (marketed compounds) belonging to the various class of Biopharmaceutics Classification System (BCS) were used to assess the utility of the 96 well Caco-2 cells. Extensive validations were also performed with ~160 Bristol-Myers Squibb (BMS) discovery compounds by comparing the permeability values in the 96 well Caco-2 cells with the in-house 24 well Caco-2 cells. Functional Caco-2 cells with intact monolayers could be established in the 96 well format using optimized seeding and culturing conditions. The permeability of BCS compounds in the 96 well format was found to be comparable to the permeability in 24 well format. Similarly, there was very good correlation ($R^2 = 0.93$) between the two formats for the extensive validation performed with in-house discovery compounds. The validated 96 well Caco-2 cell system presents a very attractive permeability screening tool that can perform much more efficiently than the conventional 12 well or 24 well systems while providing the same high quality permeability screening data.
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Keywords: Permeability; Caco-2 cells; High throughput screening; Absorption; Transport; Drug discovery

Abbreviations: ADME, absorption, distribution, metabolism, elimination; BCS, biopharmaceutics classification system; BMS, Bristol-Myers Squibb Company; HBSS, Hank's balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IAM, immobilized artificial membrane; LSC, liquid scintillation counter; NCE, new chemical entity; PAMPA, parallel artificial membrane permeability assay; P_c, permeability coefficient; P-gp, P-glycoprotein; TEER, trans epithelial electrical resistance

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In spite of many innovations in drug delivery methods, the oral route still remains the most desirable route of administration for new chemical entities (NCE). For a NCE to become a successful drug, there are a multitude of desirable characteristics it should possess: potency to a biological target, selectivity, good stability and physico-chemical properties, minimal toxicity and adequate ADME profile. Good permeability through intestinal membranes can lead to adequate systemic absorption that is a critically desirable property for NCE's. Different models are used in early discovery to screen the permeability properties of compounds (Artursson and Borchardt, 1997; Avdeef, 2001; Balimane et al., 2000; Hidalgo, 2001; Hillgren et al., 1995; Kerns, 2001). Commonly used models include cell based assays such as Caco-2, MDCK, LLC-PK1 cells; tissue based models such as ussing chamber, single pass perfusion; whole animal models such as in vivo screens; and even non-empirical in silico models (Artursson, 1991; Cho et al., 1989; Irvine et al., 1999; Tukker, 2000; Wessel et al., 1998). Some key characteristics desirable in a permeability screen in early drug discovery stage are: high efficiency, high accuracy, time, cost and space effectiveness and capability for high throughput.

Combinatorial chemistry and other advances in synthetic chemistry have led to a tremendous inflow of discovery compounds being fed into the screens for permeability assessment. Permeability-screening models have constantly been modified and improved to handle the deluge of compounds from chemistry laboratory. However, Caco-2 cells grown on 12 well or 24 well transwell plates have been the staple of the Pharmaceutical Industry for HT permeability screening of discovery compounds. Increasingly, a lot of companies have incorporated sophisticated levels of automation into these assays to make it amenable to higher throughput (Lenz et al., 1999; Russell et al., 1999). However, there is enough rationale for further miniaturization (i.e., 96 well Caco-2) that could help in streamlining these permeability assays (Alsenz and Haenel, 2003; Balimane et al., 2004). Apart from the obvious increase in throughput, miniaturization would lead to a tremendous cost reduction (decreased cost of media, plates, buffer, etc.). One additional key advantage that 96 well cells have over 12 well or 24 well set-up is the requirement of much less discovery compound to perform the same assay. This can be a significant advantage keeping in mind the hectic pace at which diverse chemotypes are synthesized in early stages of discovery. The use of 96 well Caco-2 cell system that can perform as well as the 12 well or 24 well Caco-2 cells can significantly increase the productivity of the cell based permeability assays. With adequate automation, the 96 well Caco-2 cell system can become an attractive and a valuable tool in early discovery. The article presents the validation data for the 96 well Caco-2 cells using marketed as well as discovery program compounds from active research projects within Bristol-Myers Squibb (BMS).

For this paper, Caco-2 cells were seeded onto filter membranes at a density of ~80,000 cells/cm^2 for both 24 well and 96 well Caco-2 cell plates. The cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential 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 approximately 21 days with the cell passage numbers between 22 and 40. Physiologically and morphologically well developed Caco-2 cell monolayers with TEER values greater than 400 Ω cm^2 were used for the studies reported in this manuscript. The formation of confluent Caco-2 cells with functional tight junctions in the 96 well transwells was confirmed by microscopy, TEER value measurements and permeability studies performed with mannitol (paracellular probe with low permeability). Direct visual inspection of Caco-2 cells under the microscope demonstrated uniform cell growth with no structural damage. Measurement of TEER values presented a quick confirmation of tight junction formation. Mannitol flux was monitored in the 96 well Caco-2 cells that had been cultured for 7, 14, 21, 28 and 38 days, respectively, post-seeding. Results from the growth study (Fig. 1) provided evidence of formation of intact tight junction as early as 14 days post-seeding with cell monolayers remaining intact for up to 28 days post-seeding. Low permeability values of mannitol ($P_c < 30$ nm/s) demonstrated that 96 well Caco-2 cells were optimum from 21 days to at least up to 28 days post-seeding.

For investigating the utility of 96 well Caco-2 cells, permeability studies were performed in parallel in both 96 well and the more conventional 24 well Caco-2

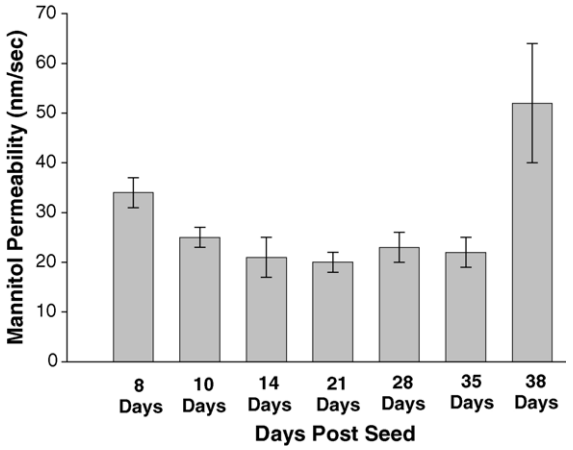

Fig. 1. Permeability values for ^{14}C-mannitol plotted against days post-seeding to assess the integrity of Caco-2 cell monolayers in 96 well format. Permeability studies were conducted at 5 μM concentration at 37 °C for 4 h. Each data point represents mean ± S.D. of three to six repeats.

cells. The transport medium used for the permeability studies was modified Hank's balanced salt solution (HBSS) buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid). The pH of the apical compartment was 6.5 and the basolateral compartment was pH 7.4. Prior to all experiments, each monolayer was washed twice with the transport buffer followed by TEER measurement to ensure the integrity of the monolayers. The concentration of test compound (added to the apical compartment) was typically 200 μM in this assay. The permeability studies were initiated by adding an appropriate volume of buffer containing test compound to the apical side of the monolayer. Apical and basolateral volumes were 0.2 and 1.1 mL, respectively, in the 24 well plates and 0.15 and 0.3 mL, respectively, in the 96 well plates. The monolayers were then placed in an incubator for 4 h at 37 °C. Samples were taken from both the apical and basolateral compartment at the end of the 4-h period and the concentrations of the test compound were analyzed by either a modified version of an earlier published high performance liquid chromatography method (Chong et al., 1996) or by scintillation counting (for mannitol and digoxin). Permeability coefficient (P_c) was calculated according to the following equation:

$$P_c = \frac{dA}{dt}\frac{1}{SC_0},$$

where dA/dt is the flux of the test compound across the monolayer (nmol/s), S the surface area of the cell monolayer and C_0 is the initial concentration in the apical compartment. The P_c values were expressed as nm/s.

To ensure the consistency and quality control of Caco-2 cells grown on the 96 well transwells, a standard set of compounds were tested multiple times. Mannitol (a probe to confirm the integrity of the monolayer), metoprolol and dexamethasone (transcellular highly permeable compounds), sulfasalazine (transcellular low permeable compound), cimetidine and atenolol (paracellular low permeable compounds) and digoxin (P-gp substrate with efflux ratio >8) were the control compounds used. TEER values were also monitored to ensure monolayer consistency during the studies. These compounds were studied multiple times in the 24 well Caco-2 cells as well. Quality control permeability studies demonstrated that both 24 well and 96 well Caco-2 cells produced similar permeability values for the standard compounds selected. High permeability probe compounds (metoprolol and dexamethasone) had permeabilities greater than 100 nm/s in both systems. Low permeability probe compounds (sulfasalzine, cimetidine and atenolol) had permeabilites below 30 nm/s. Digoxin (a P-gp substrate) had efflux ratio (ratio of permeabilities: (B to A)/(A to B)) greater than 10 in both 24 well and 96 well Caco-2 cells demonstrating adequate expression of efflux transporter P-gp.

The quality control studies were followed by transport studies (performed on both 24 well and 96 well Caco-2 cells) with a set of marketed compounds with known human absorption values. A comprehensive set of 30-marketed drugs (Table 1) having diverse physicochemical properties and human absorption values were studied in both the 24 well and 96 well Caco-2 cells. Compounds included in this set had human absorption values ranging from a low of 5% to a high of 100% with a very broad range of physico-chemical properties. Great care was taken to ensure human absorption values of the compounds selected and the primary reference for the absorption values are listed in an alternate publication (Wessel et al., 1998). Permeability values were assessed in triplicate and the mean values were used for correlating the data. Mean permeability values were plotted against the known human absorption values to determine the predictability of 96 well Caco-2 cells. As is evident from Fig. 2, both 24 well and 96 well

Table 1
Permeability values for 30-marketed compounds in 24 well and 96 well Caco-2 cells

Compound	Human absorption (%)	P_c (nm/s) in 24 well, mean ± S.D.	P_c (nm/s) in 96 well, mean ± S.D.
Acebutalol	40	48 ± 4	15 ± 2
Acetaminophen	95	199 ± 14	135 ± 12
Antipyrine	100	288 ± 35	427 ± 36
Atenolol	50	25 ± 4	40 ± 6
BMS 189664	10	20 ± 4	5 ± 2
BMS-180291	100	195 ± 30	215 ± 22
Caffeine	100	331 ± 27	410 ± 33
Cimetidine	95	49 ± 12	29 ± 4
Desipramine	95	215 ± 21	134 ± 12
Dexamethasone	100	120 ± 13	114 ± 11
Guanabenz	79	111 ± 14	62 ± 8
Hydralazine	90	141 ± 16	152 ± 18
Ibuprofen	100	395 ± 40	523 ± 41
Inulin	5	10 ± 2	10 ± 1
Ketoconazole	76	95 ± 8	208 ± 21
Mannitol	15	32 ± 5	23 ± 2
Metoprolol	95	137 ± 10	127 ± 10
Naproxen free A	100	424 ± 41	452 ± 37
Naproxen sodium	100	401 ± 38	486 ± 40
Propranolol	90	111 ± 14	277 ± 24
Ranitidine	50	24 ± 5	58 ± 4
Salicylic acid	100	440 ± 37	166 ± 12
Nadalol	35	17 ± 4	17 ± 3
Pravastatin	34	33 ± 5	29 ± 4
BvAraU	82	41 ± 6	68 ± 6
Sulfamethoxazole	100	263 ± 44	377 ± 33
Sulfasalazine	13	22 ± 5	32 ± 4
Sulfisoxazole	100	245 ± 35	147 ± 16
Terbutaline	73	29 ± 6	28 ± 4
Timolol	72	47 ± 6	69 ± 8

Caco-2 data showed the typical sigmoid correlation. Both the cell architectures lead to highly predictable data at the extremities (i.e., for very highly permeable compounds and very poorly permeable compounds, the predictions were very accurate). However, the steepness of the slope for compounds having human absorption between 30 and 70% limits the predictability in the middle. This was consistent for both cell architectures and quality of the data was similar for both 24 well and 96 well Caco-2 cells. The recoveries were also calculated in both cell systems and were found to be comparable and acceptable (>80% for all compounds; data not shown). Most of the compounds selected in this set are absorbed via passive transcellular transport. However, some additional compounds transported by more complicated transport mechanisms (active transporter mediated, parcellular mediated, efflux mediated) were also purposely chosen. Caco-2 cells are known to express P-gp and dipeptide transporters, important mechanisms in intestinal influx of nutrients and efflux of xenobiotics. The functional differences between the two cell architectures could be better characterized when such compounds were included in the data set. Our set included the following actively transported, efflux mediated and paracellular compounds: salicylic acid, sulfasalazine, cimetidine and ranitidine (Collett et al., 1999; Lee et al., 2002; Liang et al., 2000). There were no significant differences between the two systems with regards to the permeability values for these compounds.

Finally, a random assortment of ∼160 BMS compounds (from ∼10 active research programs from different therapeutic areas) was studied in both the Caco-2 cell models (24 well and 96 well) to assess the agreement of permeability values obtained form the two formats. These compounds were selected randomly from on-going discovery projects within the company with

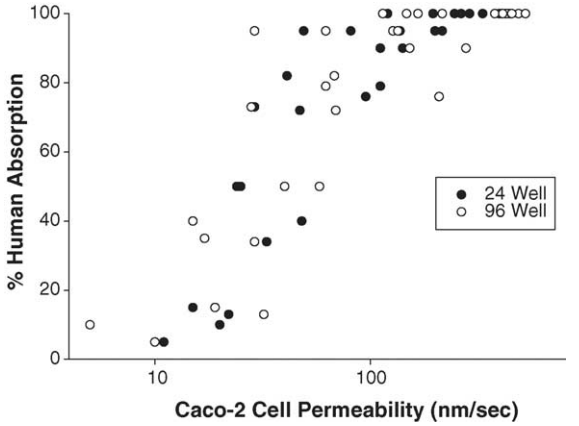

Fig. 2. Correlation of 24 well and 96 well Caco-2 cell permeabilities for marketed compounds with known human fraction absorbed values. Permeability studies were conducted at 100–200 μM concentration at 37 °C for 4 h. Each data point represents mean of three to six repeats.

no bias towards structure, physico-chemical properties or potencies. Permeability values were determined both in the 24 well and the 96 well Caco-2 cells. As shown in Fig. 3, there was a very good agreement between the permeability values obtained by the two methods. The correlation lead to a regression value of 0.93 suggesting a high degree of correlation between the two cell formats. Similarly, a comparison of the recovery data (mass balance) also suggested that recoveries were similar by both methods. A majority of the compounds

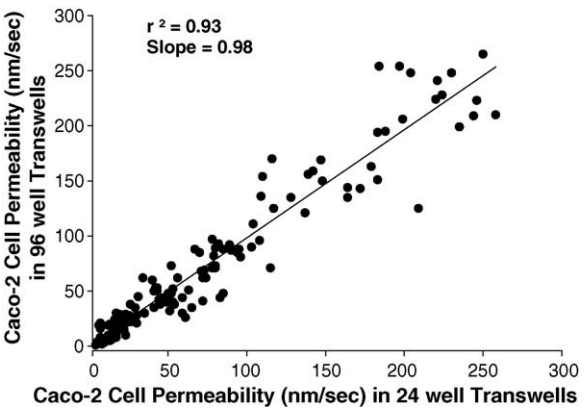

Fig. 3. Correlation of 24 well Caco-2 cell permeability data with 96 well Caco-2 data for 160+ BMS compounds. Caco-2 studies were conducted at 37 °C for 4 h. Each data point represents mean of at least three repeats.

demonstrated adequate recovery values (>60% recovered) in both 24 well and 96 well Caco-2 cells (data not reported in this publication).

In contrast to the successful use of miniaturization and automation in biological activity screening, intestinal permeability and absorption screening have not made similar advancements. Cell culture model such as the Caco-2 cell monolayer model is the pharmaceutical industry's "method of choice" for permeability assessment but little has been done to drive on its miniaturization potential. Currently, permeability studies using cell monolayer are conducted in an automation-friendly 12 well or 24 well transwell plates (Artursson and Karlsson, 1991; Fung et al., 2003; Polli et al., 2001; Rubas et al., 1996; Yamashita et al., 2000). This paper presents one of the first systematic validation of the 96 well Caco-2 cells for use in screening compounds for their permeability characteristics (Alsenz and Haenel, 2003). Conventionally, culturing the cells on 96 well transwell plates has been a technical challenge because of manufacturing issues, smaller surface area and generation of multi-layer cells along the edges of wells. This process of clumping of cells would lead to non-reproducible permeability results. Seeding density was optimized both with regards to number of cells/cm^2 as well as the volume of the media (containing the cells). Collagen treated plates were tested, however, untreated plates worked fine. Contrary to 24 well Caco-2 cells, the cells growing in 96 wells were much more sensitive to feeding regimen. The culture media were replaced every 2 days without fail. Significant modifications in the design of the 96 well plates that included optimization of the angles of the transwell walls, redesign of the basolateral access port and the specialized bottom feeder tray afforded minimum disruption to the growing cells. Thus, an extensive optimization of culturing conditions with the modification of the plate design lead to successful culturing of the Caco-2 cell monolayers on 96 well plates.

The successful integration of 96 well Caco-2 cell system in early discovery has some very significant advantages: increased throughput, reduced cost (media and plate costs) and much less compound requirement for permeability assessment. These advantages can have a major impact on drug discovery and play a key role in boosting the productivity of R&D laboratory. A higher throughput 96 well Caco-2 cell system presents a very attractive permeability screening tool

Table 2
Comparison of 24 well with 96 well Caco-2 cell system[a]

Property	24 well Caco-2 cells	96 well Caco-2 cells
Cost to perform permeability study for 1 compound (1 monolayer, A to B only)	US$ 19	US$ 8
Maximum throughput achievable (1 operator, 1 incubator), if no analytical bottle-necks (compounds/weeks)	∼200	∼800
Total labor time[b] (seeding, feeding, culturing, transport, study, etc.) for 200 test compounds (h/week)	10–15	10–15
Amount of compound required for study (mg)	∼2	<1
Total space requirements to support typical discovery work (i.e., 500 compounds/week)	1 incubator, 1/2 laboratory bench	1/4 incubator space, 1/2 laboratory bench
Compatibility with analytical methods	Sample volumes enough to perform HPLC, LC/MS	Sample volumes enough to perform LC/MS but a challenge for HPLC

[a] Table reflects the internal Bristol-Myers Squibb experience.
[b] Labor times calculated using manual mode of operation. Automation will lead to significant timesavings and also take 1/4 time for 96 well compared to 24 well.

that can perform much more efficiently than the conventional 12 well or 24 well systems while providing the same high quality screening data. Table 2 presents a head-to-head comparison of the 24 well versus 96 well Caco-2 cell model and presents the functional advantages of the miniaturized cell model for a discovery organization.

The future success of pharmaceutical companies hinges on the successful integration of efficient high throughput permeabillity screening models for early selection of developable compounds. Fig. 4 demonstrates the significant increase in productivity that has been achieved in the 24 well Caco-2 cell permeability model over the last few years. Apart from automation of these permeability studies, simultaneous advancements made in the field of analytical methods has contributed to the surge in productivity. Continuous optimizations in all aspects of permeability study (experimental front-end, sample analysis mid-end and data manipulation at the back-end) have all played a key role in increasing throughput. Automation led the first wave of productivity increase in this model and now the implementation of miniaturized cell models (i.e., 96 well) presents another opportunity for increasing throughput of these permeability models. In the near future, further optimization (robotic automation, streamlining of the analytical technique and cassette incubations studies, etc.) in this system can be explored to establish the ideal permeability-screening model for drug discovery.

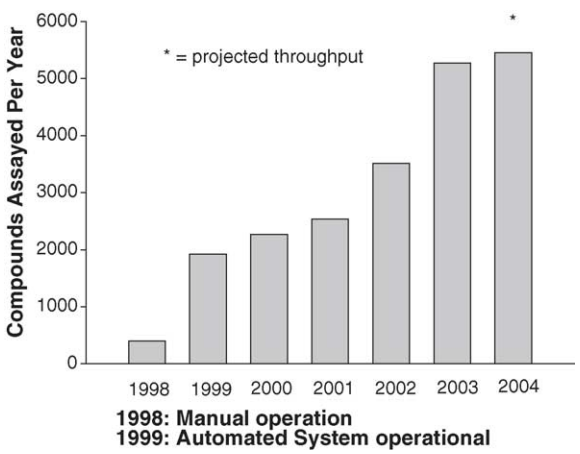

Fig. 4. Productivity increase in 24 well Caco-2 cell permeability-screening model over the years. Automation leads to an initial surge in throughput in 1998–1999. Miniaturization coupled with automation can lead to the next surge in productivity in the near future.

In conclusion, we have demonstrated that 96 well Caco-2 cells perform as effectively as the 24 well Caco-2 cells in predicting the intestinal permeability. A higher throughput 96 well Caco-2 cell system presents a very attractive permeability screening tool that can perform much more efficiently than the conventional 12 well or 24 well systems while providing the same high quality permeability screening data. With adequate automation, the 96 well Caco-2 cell systems will have

enough capacity to handle the permeability screening demands in a typical drug discovery organization.

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