In Vitro Permeability Across Caco-2 Cells (Colonic) Can Predict *In Vivo* (Small Intestinal) Absorption in Man—Fact or Myth

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Purpose. To evaluate and optimize the use of Caco-2 cell monolayers to predict the *in vivo* absorption of a broad range of compounds in man.
Methods. Caco-2 cells are derived from human adenocarcinoma colon cells and spontaneously differentiate when grown on porous polyethylene terephthalate membranes (PETP) in a 12 well format to form monolayers of polarized cells possessing function similar to intestinal enterocytes. Transport experiments were conducted using 21 day cultured cells in a shaking water bath at 37°C. Radiolabeled mannitol was used to determine monolayer integrity. Apparent permeability coefficient (P_{app}) was calculated from the appearance of drug in the receiver side.
Results. A strong correlation was observed between *in vivo* human absorption and *in vitro* P_{app} for a variety of compounds ($R = 0.95$, $N = 35$). For compounds that are substrates of p-glycoprotein (Pgp), use of a Pgp inhibitor resulted in a better estimate of absorption in humans. The results of this study suggest that the overall ranking of compounds with $P_{app} < 1 \times 10^{-6}$ cm/sec, between $1–10 \times 10^{-6}$ cm/sec, and $> 10 \times 10^{-6}$ cm/sec can be classified as poorly (0–20%), moderately (20–70%) and well (70–100%) absorbed compounds, respectively.
Conclusions. These data suggest that Caco-2 cells developed under the culturing and transport conditions defined herein can be used to predict *in vivo* human absorption of compounds regardless of transport mechanism, viz., transcellular, paracellular and carrier-mediated.

KEY WORDS: Caco-2; absorption; prediction; human; P-glycoprotein; transport.

INTRODUCTION

In recent years the use of Caco-2 cell monolayers has gained in popularity and momentum as an *in vitro* human absorption surrogate; moreover, the monolayers are generally accepted as a primary absorption screening tool in several pharmaceutical companies. There are several examples of successful application of Caco-2 cell monolayer for prediction of or correlation with human absorption (1,2).

In juxtaposition to the above, some have reported that paracellular and active transport across Caco-2 cell monolayer is less efficient than *in vivo* (3–5). Furthermore, since Caco-2 cells are known to overexpress Pgp, the presence of this efflux pump may cause the absorption of some compounds across the Caco-2 cells to be underestimated.

Thus, with a full appreciation of the perceived limitations of this model as reported in the literature, the objective was to assess the use of the Caco-2 cell monolayers as an *in vitro* tool to predict absorption in man. For application as a general screen for all types of compounds in the discovery setting, the model needs to be capable of encompassing all the major routes of absorption, including the efflux mechanism. The selection of compounds was based on the availability of compounds that were radiolabeled with information on human absorption, ranging from 0 to 100%.

MATERIALS AND METHODS

Materials

Radioactive mannitol, acetyl salicylic acid, acyclovir, diltiazem, fluorouracil, inulin, PEG900, PEG 4000, and propranolol were purchased from NEN-Dupont (Wilmington, DE). Radiolabeled doxorubicin, benzyl penicillin, cimetidine, clonidine, dexamethasone, diazepam, glycine, imipramine, methotrexate, naloxone, D-Phe-L-Pro, prazosin, scopolamine and vinblastine were purchased from Amersham (Arlington Heights, IL). Radioactive caffeine, chloramphenicol, erythromycin, ibuprofen, quinidine, testosterone, tetracycline and valproic acid were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). The remainder of the labeled materials were from Pfizer (Groton, CT). The unlabeled compounds were from Sigma Chemical Co. (St. Louis, MO) and Pfizer Inc. Azithromycin, tenidap, trovoflaxicin, Ziprasidone, CP-W, CP-X, CP-Y and CP-Z were synthesized by medicinal chemists at Pfizer.

Methods

Cell Culture

Caco-2 cells (passage #52) were a gift from Dr. C. Chandler (Pfizer Inc.). Stocks of Caco-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Grand Island, NY) containing high glucose (4.5 g/l) plus 20% fetal bovine serum (JRH Scientific, Lenexa, KS), 0.1 mM nonessential amino acids, 2 mM L-glutamine, and 40 μg/ml gentamycin (GIBCO). Cells utilized were between passages 52–80. Caco-2 cells were cultured in 75 cm^2 T-flasks (Costar, Cambridge, MA) at 37°C in a 10% CO^2, constant humidity environment. The medium was replaced 2 or 3 times / week. Monolayers were subcultured when they reached ~60–80% confluence ($7–10 \times 10^6$ cells / flask) at a split ratio between 1:15 and 1:20 using 0.05% trypsin-0.02% EDTA (GIBCO).

Single cell suspensions of Caco-2 cells (~100,000) were plated onto 12 mm-diameter high density PETP (Falcon, 0.45 μm pore size, 0.83 cm^2) in a 12 well plate. Upper (apical, A) and lower (basolateral, B) compartments received 0.5 ml and 1.5 ml of medium, respectively.

Monolayer Integrity

To assess the integrity of the monolayers, the flux of radiolabeled mannitol was determined for each insert. Inserts with a flux greater than 0.5%/hr were excluded from the study.

Transport Studies

Caco-2 cells grown on filter supports for 3 weeks were used for all transport studies. Cells were fed 24 hours before

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the transport studies. Complete culture medium was removed from both the apical and basolateral compartments and the monolayers were preincubated with pre-warmed 0.5 ml apical buffer or 1.5 ml basolateral buffer for 0.5 hour at 37°C in a shaker water bath (Precision Scientific) at 70 cycles/min. The apical buffer consisted of Hanks Balanced Salt Solution, 25 mM D-Glucose monohydrate, 20 mM MES Biological Buffer, 1.25 mM $CaCl_2$, 0.5 mM Mg Cl_2 (pH 6.5). The basolateral buffer consisted of Hanks Balanced Salt Solution, 25 mM D-Glucose monohydrate, 20 mM HEPES Biological Buffer, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$ (pH 7.4). At the end of the preincubation, the media was removed and the appropriate drug solution (1 μM) with [^{14}C]- or [^{3}H]-mannitol or blank buffer was added. Samples were collected at regular time intervals (≤3 hr), such that drug disappearance was <10% on the donor side. The pH of the transport buffer did not change significantly during the transport experiment. For transport in the A → B direction, the inserts were moved to wells containing fresh buffer. For transport in the B → A direction, the buffer from the apical side was replaced with fresh buffer. Radioactivity was counted using a liquid scintillation counter.

Flux rate (F, mass/time) was calculated from the slope of cumulative appearance of substrate of interest on the receiver side and the apparent permeability coefficient (P_{app}) was calculated from the following equation.

$$P_{app}\ (cm/sec) = (F * V_D)/(SA * M_D)$$

where SA is the surface area for transport (0.83 cm^2), V_D is the donor volume, M_D is donor amount at t = 0. All data represent the mean of 3 inserts. For Pgp inhibition studies the inhibitor, CP-Y, was added to both compartments before and during transport studies.

RESULTS AND DISCUSSION

Table 1 shows the diversity in structural and physical properties of the 36 compounds investigated, the permeability in the Caco-2 cell monolayers and % dose absorbed *in vivo* in human. A few compounds were identified as substrates for the Pgp efflux pump, such as azithromycin, prazosin, erythromycin and quinidine (Figure 1). For all these compounds, comparison of bidirectional transport indicated that the permeability in the B → A direction was much greater than in the A → B direction. The transport in the A → B direction for these compounds was corrected for the efflux mechanism by coincubating with 10 μM CP-Y (Pgp inhibitor). The transport of paracellularly absorbed compounds were similar in both direction, however, actively absorbed compounds showed much faster transport in the A → B direction. Compounds using transporters for absorption included, glycine, D-Phe-L-Pro, acetyl salicylic acid, taurocholic acid, CP-X and valproic acid. Compounds transported by solvent drag through the tight junctions (paracellular) were sumatriptan, PEG900 and 4000, acyclovir, CP-Z, doxorubicin, glycine, cimetidine, and CP-W. The paracellular transport mechanism was confirmed by perturbing the tight junctions in calcium free buffer that significantly increased flux of paracellular but not transcellular substrates (data not shown).

The correlation of *in vivo* absorption in humans and log of P_{app} value in the Caco-2 cell monolayers, uncorrected for efflux transport gave a correlation coefficient R of 0.90 (not shown). The correlation improved over the uncorrected correlation when the efflux mechanism for azithromycin, prazosin, quinidine and erythromycin was suppressed by coincubating with the Pgp inhibitor, CP-Y (Figure 2, R = 0.95). This suggests that a correction may be necessary for compounds that are substrates of Pgp; since the cells originate from cancerous cells where Pgp is usually overexpressed. The linear regression line used to fit this curve was as follows:

$$\%\ absorbed = 1.95 * \ln(P_{app}) + 24.4$$

On the basis of this correlation, compounds may be ranked into 3 different categories. Compounds with a P_{app} of $<1 \times 10^{-6}$ cm/sec will be poorly absorbed (0–20%). Compounds with a P_{app} between $1–10 \times 10^{-6}$ and $>10 \times 10^{-6}$ cm/sec will be moderately (20–70%) and well absorbed (70–100%), respectively. The slope of approximately 2 measured in this system indicates that there is a small (2-fold) difference between permeability across Caco-2 and *in vivo* absorption in man.

Table 1. Apparent Permeability in Caco-2 cells, *In Vivo* Human Absorption (F) and mLogP of Various Compounds

Compounds	$P_{app} \times 10^6$ (cm/sec)	$F^{1,15}$ (%)	mLogP
Sumatriptan	3	55	0.51
PEG900	0.83	10	−4.91
PEG4000	0.78	0	ND
Acyclovir	2	30	−0.09
CP-Z	0	0.003	−0.09
Erythromycin	3.73[a]	35	−0.14
Doxorubicin	0.16	5	−1.33
Mannitol	0.65	16	−2.50
Glycine	80	100	−3.44
Azithromycin	1.04[a]	35	0.14
Caffeine	50.5	100	0.20
Cimetidine	3.06	62	0.82
CP-W	3.12	50	1.10
Chloramphenicol	20.6	90	1.23
Noloxone	28.2	91	1.53
Methotrexate	1.2	20	1.60
Acetyl Salicylic acid	30.67	68	1.7
Benzyl Penicillins	1.96	30	1.82
Dexamethasone	23.4	92	1.85
Tenidap	51.2	90	1.95
Prazosin	43.6[a]	100	2.05
Valproic acid	48	100	2.06
Quinidine	20.4[a]	80	2.19
Propranolol	27.5	90	2.53
Trovoflaxicin	30.23	88	2.81
Ibuprofen	52.5	100	3.23
Diazepam	70.97	100	3.36
Clonidine	30.1	95	3.47
Fluconazole	29.8	100	3.49
Desipramine	21.6	100	3.64
Testosterone	72.27	100	3.70
Ziprasidone	12.3	60	3.71
Imipramine	14.1	100	3.88
D-Phe-L-Pro	44.3	100	ND[b]
Taurocholic acid	34.5	ND	ND
CP-X	34.15	> 50	4.41

[a] In the presence of Pgp inhibitor (10 μM CP-Y). mLogP; calculated LogP as defined by Moriguchi.
[b] ND: not determined.

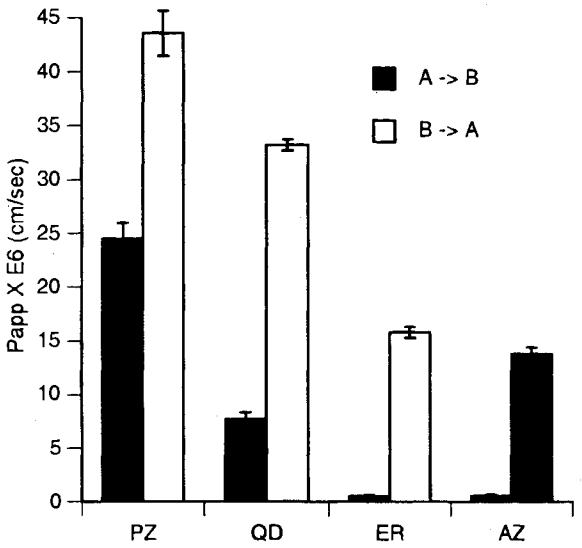

Fig. 1. Bidirectional permeability of prazosin (PZ), quinidine (QD), erythromycin (ER) and azithromycin (AZ), (1 μM each) in the Caco-2 cell monolayers.

The permeability of transcellularly absorbed compounds, dexamethasone, testosterone, and propranolol across these Caco-2 cells were similar to those reported in literature (1,2). Permeability reported by Artursson and Karlsson (1) for the paracellularly absorbed compounds were about 4-fold lower than that measured in the present system, indicating that these monolayers were leakier. Overall, the P_{app} of compounds transported by the paracellular route in our system were reasonable, ranging from 0 to 3.12 × 10^{-6} cm/sec.

Chong *et al.* (6) and Lenneras *et al.* (5) reported that the effective permeabilities of solutes with carrier-mediated absorption were much slower in Caco-2 cell monolayers than *in vivo*. In contrast, P_{app} determined in these Caco-2 cells for carrier-mediated compounds were very high (30.67 to 80 × 10^{-6} cm/sec). Thus, under the culturing and experimental conditions defined herein, the Caco-2 cell monolayer does seem to give a reasonable estimate of carrier-mediated transport. In total, the present data indicate that permeability across Caco-2 cell monolayers can be used to give a fair assessment of *in vivo* absorption independent of the mechanism of transport. The higher apparent permeability measured from rat and human jejunal perfusions (5) than that across Caco-2 cells may result from the manner in which the values are determined (appearance versus disappearance of compound).

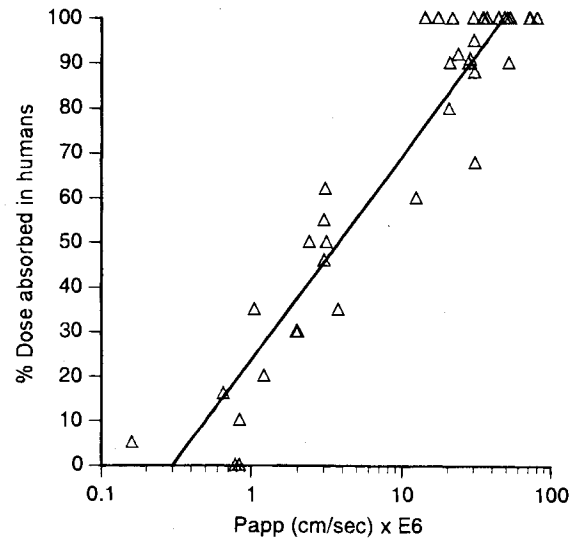

Fig. 2. Correlation between % dose absorbed *in vivo* in humans versus P_{app} across Caco-2 cell monolayers treated with 10 μM CP-Y (R = 0.95, n = 35).

Few authors have reported that Caco-2 cells can only be used to predict passive drug transport, mainly transcellular, and do not predict carrier-mediated and paracellular transport. The reason was explained by low expression of carriers in the Caco-2 cells and smaller pore size (similar to colonic epithelia). Several factors may explain the discrepancies between the data generated in this lab versus data from others, including cell culturing and transport conditions and the source of Caco-2 cells.

Firstly, Hu and Borchardt have shown that the apparent maximum carrier flux of L-α-methyldopa was significantly increased when post-feeding time was prolonged from 8.5 to 56 hr (7,8). Transport experiments in the present system were conducted using cells that were fed 24 hr before the study. Secondly, Cogburn *et al.* have noted that filters, especially those coated with collagen (without cells) may act as barriers to transport of a drug molecule and should be tested prior to use (9). The filters used in the present system were uncoated high density PETP. This material is preferrable to cellulose filters because of larger population of pores, such that it does not act as a transport barrier, with minimal non-specific binding (data not shown). Thirdly, it has been shown that compounds absorbed by specialized transporters and transcellular diffusion are greatly affected by temperature, pH gradient and hydrodynamics in the system (7,8,10), therefore, one has to tightly control all external variables when conducting these types of studies. Fourthly, the source of Caco-2 cell is an important parameter in the use of Caco-2 cells as an absoprtion predictive model. Hu *et al.* have shown that the two Caco-2 cell clones had significantly different levels of peptide transporter function and prolidase activity (8). The S-K cells seem to transport loracarbef at a faster rate via the transporter with higher maximal flux but had lower total prolidase activities, when compared to ATCC cells. Additionally, Caro *et al.* (11) have shown that the late passages of Caco-2 cells (TC-7) express bile acid transporter and phase I and II enzymes better than the low passages of Caco-2 cells. The Caco-2 cells used in this study were donated to us by Dr. Chandler (Pfizer Inc.). Using these cells, Chandler *et al.* estimated the K_m and V_{max} (maximal flux) of cholyltaurine to be similar to those obtained in isolated human ilieal brush border vesicles (12). These data and other data generated in this laboratory (unpublished data) suggests that the expression level of peptide, amino acid, bile acid and monocarboxylic acid transporters are similar to their expression *in vivo*.

Finally, confusion in the literature regarding the leakiness of the monolayers may arise from the measured transepithelial electrical resistance (TEER) which is used to assess the integrity of monolayers (3,13). TEER is determined primarily by the ion flux through paracellular space. However, there can be a large change in TEER with little or no change in solute flux because

solute flux is dependent on the size and charge of the permeant (14). A range of values between 150–400 ohms.cm^2 have been reported indicating that in different laboratories, under different culture conditions, Caco-2 monolayers can display the electrical properties of either small intestinal or colonic enterocytes (13). This suggests that the flux of an impermeable marker like mannitol is a more sensitive indicator of the size of the tight junction than the resistance assay. As stated above, lack of standardization in cell culturing and experimental procedures makes it impossible to compare permeability data across laboratories. To use this model successfully, it is necessary to validate the use of Caco-2 cells using model compounds under a defined culturing and experimental conditions.

In conclusion, the overall advantage of this model in discovery is to rank order compounds in a high throughput format. The data generated support the application of this model for all major mechanisms of GI absorption, therefore this is applicable in discovery setting where little is known about the compound. Selection of candidates for clinical studies should be made in conjunction with solubility and metabolism (gut and hepatic) data to assure that pharmacokinetics in man will be optimal. Implementation of human surrogates (Caco-2 cells, human microsomes, liver slices and intestinal homogenate) as primary screens earlier in the discovery mode will reduce the chance of attrition in the clinic.

SUMMARY

Excellent correlation was observed between *in vivo* absorption and *in vitro* P_{app} for a variety of compounds encompassing transcellular, paracellular and carrier-mediated mechanisms. Therefore, the Caco-2 cells can be used as a predictive as well as a screening tool, provided dissolution and GI metabolism are not limiting the portal availability. For compounds that are substrates of Pgp, use of inhibitors gives a better estimate of absorption in humans. In general, compounds with $P_{app} < 1 \times 10^{-6}$ cm/sec, 1 to 10×10^{-6} cm/sec, and $> 10 \times 10^{-6}$ cm/sec will be poorly (0–20%), moderately (20–70%) and well (70–100%) absorbed.

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