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

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INTRODUCTION

In many drug discovery programs, poor intestinal permeability of pharmacologically active drug candidates is one of the most common reasons for poor oral efficacy. As such, simultaneous assessment of the structure-permeability relationship as well as the structure-activity relationship can expedite the selection of a drug candidate with good oral absorption. In this regard, the caco-2 cell system as an *in vitro* model appears promising. Caco-2 cells undergo spontaneous enterocytic differentiation in culture and resemble small intestinal epithelial cells (1). When they grow to confluency on a semi-permeable membrane, the cell polarity and tight junctions are established (2). The morphology and cellular properties of the caco-2 cells as an *in vitro* absorption model have been described previously (2). In addition, the presence of di- and tri-peptide transporters (3,4) has also been documented.

However, preliminary studies in our laboratory suggested that compounds that are absorbed by the dipeptide transporter system had relatively low permeability values compared to compounds that were equally well absorbed *in vivo* by passive diffusion. The objectives of the present study were to evaluate the permeability of a heterologous series of fourteen drugs and to investigate whether there is a quantitative difference between drugs that are absorbed primarily *via* the dipeptide transporter system and passive diffusion.

MATERIALS AND METHODS

Materials

[^{14}C]PEG-4000 (specific activity of 0.5 mCi/g) and [^{14}C]mannitol (specific activity of 55 mCi/mmole) were obtained from NEN Research Products (Boston, Massachusetts). Amoxicillin, atenolol, caffeine, cephalexin, guanabenz, propranolol, and salicylic acid were purchased from Sigma Chemical Co. (St. Louis, Missouri). BVaraU, lisinopril, nadolol, pravastatin, and SQ-29852 were obtained from Bristol-Myers Squibb PRI (Princeton, New Jersey). Caco-2 cells (passage #17) were obtained from American Type Culture Collection (Rockville, Maryland). Dulbecco's modified Eagle's medium, nonessential amino acids, L-glutamine, penicillin-G, and streptomycin were purchased from JHR Biosciences (Lenexa, Kansas). Fetal bovine serum was obtained from Hyclone Lab. Inc. (Logan, Utah). Rat tail collagen-type I was purchased from Collaborative Research Inc. (Bedford, Massachusetts). Transwell® inserts (surface area: 4.71 cm^2) with a polycarbonate membrane (3 μm pore size) were purchased from Costar (Cambridge, Massachusetts). Hank's balanced salt solution, 2-N-morpholinoethanesulfonic acid (MES) and N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, Missouri). All solvents were analytical grade.

Cell Culture. Caco-2 cells were seeded onto a collagen coated polycarbonate filter membrane at a density of 80,000 cells/cm^2. The cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin. The culture medium was replaced every two days and the cells were maintained at 37°C, 90% relative humidity, and 5% CO_2. Permeability studies were conducted with the monolayers between 21 and 25 days in culture, and the cell passage numbers were between 40 and 50.

Permeability Study. The transport medium was modified Hank's balanced salt solution (MHBS) containing either 10 mM HEPES, pH 7.4 or 25 mM MES, pH 6.0. Each monolayer was washed twice with MHBS (pH 7.4) and 2.6 mL of MHBS (pH 7.4) was placed on the basolateral side of the monolayer (receiver well). The permeability studies were initiated by adding 1.5 ml of MHBS containing one of the drugs listed in Table I (200 to 800 μM) and [^{14}C]mannitol (0.2 μCi) to the apical side of the monolayer. [^{14}C]mannitol was co-incubated as a hydrophilic marker to check the integrity of the cell monolayer (i.e., to see if the tight junctions are intact) during the incubation. No increase in the mannitol flux was observed with any of the tested drugs. MHBS (pH 7.4) was used for passively absorbed drugs, and MHBS (pH 6.0) was used for carrier-mediated drugs. The monolayers were placed on an orbital shaker (50 cycles/min) and incubated up to 4 hours at 37°C. At hourly intervals, the Transwell® insert was moved to a new receiver well containing fresh MHBS to maintain sink conditions. For salicylic acid, the Transwell® insert was moved every 30 min. Samples were taken from each receiver well and the apical compartment was sampled at the end of the 4-hour period.

Apical to basolateral permeability coefficients were calculated according to the following equation: Permeability Coefficient (Pc) = $dA/(dt \cdot S \cdot C_o)$, where dA/dt is the flux of drug across the monolayer (nmole/sec), S is the surface area of the cell monolayer (4.71 cm^2), and C_o is the initial concentration (μM) in the apical compartment. The permeability coefficient values are expressed as nm/sec.

Analytical Methods

The concentrations of drugs were analyzed by a specific HPLC-UV assay. A C_{18} μ-Bondapak reverse phase column

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Table I. List of Compounds Tested and Their Physicochemical Properties

Drug	M.W.	Acid/Base Property	cLogP[a]	% Abs. in Human	Perm. Coef.[b] (nm/sec)
Passively absorbed drugs					
Caffeine	194	neutral	−0.1	100	214 ± 5
Mannitol	182	neutral	−4.7	16	5 ± 1
PEG-4000	3000–3700	neutral	ND	<1	0.8 ± 0.2
BVaraU	349	weak acid	−1.6	82	40 ± 4
Pravastatin	425	weak acid	0.5	34	23 ± 2
Salicylic acid	138	weak acid	2.2	100	419 ± 55
Atenolol	266	weak base	−0.1	50	40 ± 3
Guanabenz	231	weak base	3.0	79	209 ± 11
Nadolol	309	weak base	0.2	35	45 ± 2
Propranolol	259	weak base	2.8	90	148 ± 10
Carrier-mediated drugs					
Amoxicillin	365	monoacid/monobase	−1.9	100	8 ± 2
Cephalexin	347	monoacid/monobase	−1.9	100	5 ± 1
Lisinopril	442	diacid/monobase	−1.7	25	0.5 ± 0.1
SQ-29852	440	diacid/monobase	ND	60	0.2 ± 0.1

[a] cLogP, calculated intrinsic Log octanol/water partition coefficient, was obtained with the Daylight program (Daylight C.I.S., Inc. Irvine, CA). ND, not determined.
[b] Mean ± SD (n = 3).

(3.9 mm x 30 cm; Waters Chromatography Division, Millipore Corp., Milford, Massachusetts) was used. The mobile phase, consisting of solvent A (water:acetonitrile:trifluoroacetic acid, 95:5:0.1 v/v) and solvent B (water:acetonitrile: trifluoroacetic acid, 20:80:0.1 v/v), was programmed as a linear gradient. The flow rate was 1.2 ml/min and the absorbance was monitored at either 210 or 220 nm. The concentrations of [^{14}C]mannitol were determined by liquid scintillation counting (Model 2500, Packard Instr. Co., Downers Grove, Illinois)

RESULTS AND DISCUSSION

Despite the fact that this heterologous series of ten passively absorbed compounds cover a wide range of physicochemical properties (*e.g.*, size, charge, and lipophilicity; see Table I), the permeability coefficient across the caco-2 cell monolayer was fairly well correlated to the extent of *in vivo* absorption (figure 1A, open squares). These results are consistent with the observations reported by other investigators (5). Figure 1B (open circle) indicates that a reasonably good prediction of the extent of absorption in humans can be obtained for passively absorbed drugs when the Amax algorithm (described in the figure legend) is used. However, the lipophilicity estimates (represented as cLogP values) were not correlated with the extent of absorption for this heterologous series of passively absorbed drugs (figure 2), indicating that the lipophilicity is not the exclusive determinant for oral absorption. As an initial rapid screening tool in support of the drug discovery process, the observed variability and the predictive power of this model appear acceptable, provided that adequate controls (e.g., compounds with known *in vivo* absorption in humans) are included in each experiment.

Many β-lactam aminocephalosporin antibiotics (3,4) and several angiotensin II converting enzyme inhibitors (6) have been reported to be absorbed primarily *via* the intestinal DTS. To evaluate the predictive capability of caco-2 cell model for drugs that are absorbed *via* the DTS, amoxicillin, cephalexin, lisinopril, and SQ-29852 were evaluated. Because the DTS demonstrated a maximal efficiency with an inward proton gradient (4), the permeability was measured with an apical pH of 6.0 and a basolateral pH of 7.4 for this class of compounds. The initial concentration used was 200 μM which is substantially smaller than the reported Km value (> 2mM) for DTS in the Caco-2 cells to avoid possible saturation. Surprisingly, these compounds showed relatively poor permeability across the caco-2 cell monolayer compared to drugs that are equally well absorbed *in vivo* by passive diffusion (figure 1A, closed circles) and the caco-2 cell model significantly under-predicted the extent of oral absorption (figure 1B, closed circles). The poor permeability is not due to the difference in pH (6.0 vs 7.4), because the permeability of SQ-29852 was also studied at an apical pH of 7.4 and the Pc value was similar to that at an apical pH of 6.0. The poor permeability of this class of compounds was also not due to physical losses (*e.g.*, adsorption to plastic devices) or cellular uptake because in all cases, there was quantitative recovery in the donor and receiver samples. The total recoveries were 99.2 ± 0.8% (n = 12) and 99.6 ± 1.0% (n = 30, mean ± sem) for the actively transported and passively diffused drugs, respectively. Although comparison of Pc values among different laboratories must be done carefully, the reported caco-2 cell permeabilities of lisinopril (7) and cephalexin (4) were also low (about 3 and 8 nm/sec, respectively), as was the case in the present study. Based on the current permeability-absorption relationship defined for passively absorbed drugs, the predicted *in vivo* absorption for the four carrier-mediated drugs tested (see figure 1B, ≤ 15%) was as much as ten-fold lower than the actual *in vivo* absorption value (25 to 100%).

The presence of the DTS in caco-2 cells has been convincingly demonstrated by several investigators (3,4,8).

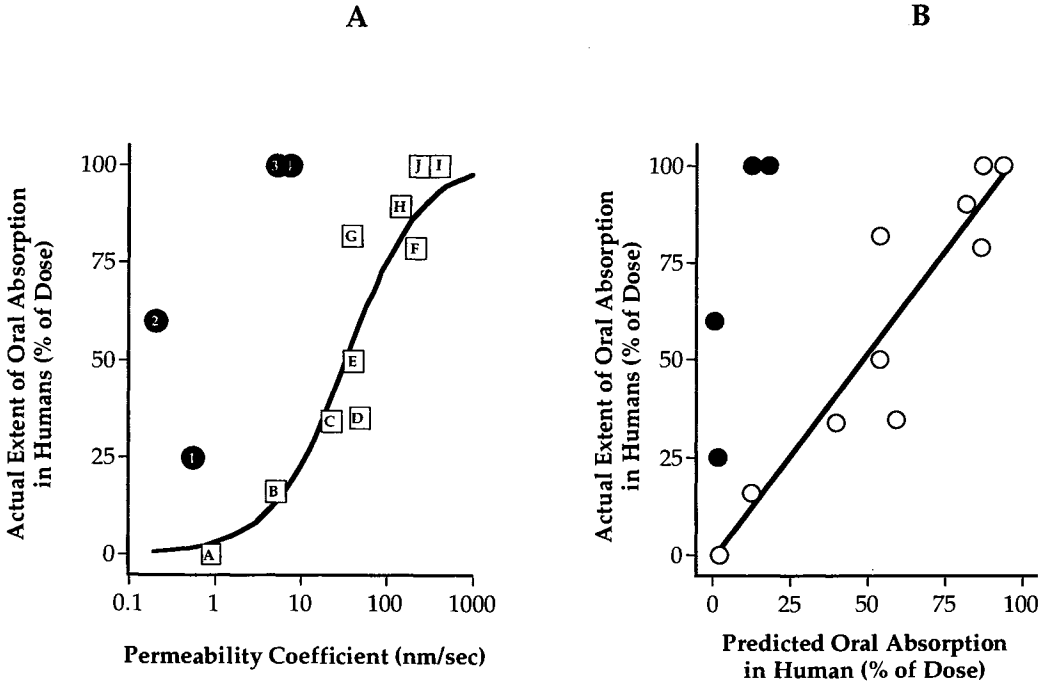

Fig. 1A. Correlation between *in vivo* oral absorption in humans and *in vitro* permeability coefficient across the Caco-2 cell monolayer. The squared capitalized letters represent compounds known to be absorbed by passive diffusion in humans: A, PEG-4000 (5); B, mannitol (5); C, pravastatin (9); D, nadolol (10); E, atenolol (5); F, guanabenz (11); G, BVaraU (12); H, propranolol (5); I, salicylic acid (5); J, caffeine (13). The circled numbers represent those compounds reported to be absorbed by the DTS (1, lisinopril (14); 2, SQ-29852 (15); 3, cephalexin (16); 4, amoxicillin (17)). The line was drawn by fitting the data with a sigmoidal Amax model described below:

$$\% \text{ Absorption in humans} = (\text{Amax} * Pc^{\gamma})/(Pc_{50}{}^{\gamma} + Pc^{\gamma})$$

where Amax is set at 100% and the Pc_{50} represents the permeability coefficient value which is required to achieve 50% absorption in humans. Pc_{50} and γ were 35 ± 19 and 1.1 ± 0.7 (mean ± sem), respectively.

Fig. 1B. Correlation between predicted (based on relationship defined for passively absorbed drug)*vs.* actual extent of oral absorption in humans; passively absorbed (○), carrier-mediated (●). The straight line represents the linear regression equation $Y = 1.0\ X - 0.1$ ($r = 0.92$, $p < 0.01$) for ten passively absorbed compounds.

Most of the previous studies, however, examined the uptake/accumulation of β-lactam aminocephalosporins by caco-2 cells rather than the trans-monolayer permeation. The results from the present study suggest that the dipeptide transporter system in the caco-2 cell monolayer is, perhaps, quantitatively under-expressed when compared to that *in vivo*. This could involve the carrier on the apical membrane (brush border) or the basolateral membrane, since separate transporter systems have been suggested for the polarized enterocyte (4).

In support of the drug discovery process, the ideal screening tool can tolerate false positive results, which will be further screened with more rigorous studies, but should not provide a false negative result, as is the present case for the caco-2 cell model and carrier-mediated drugs. If there is a false negative, potentially useful compounds might be abandoned. For example, if the caco-2 model had been used during the discovery of amoxicillin and cephalexin, very low absorption in humans would have been predicted based on their relatively low Pc values (7.5 and 5 nm/sec, respectively); however, the actual extent of absorption of these drugs is substantial (>90%).

In conclusion, compounds with Pc values greater than

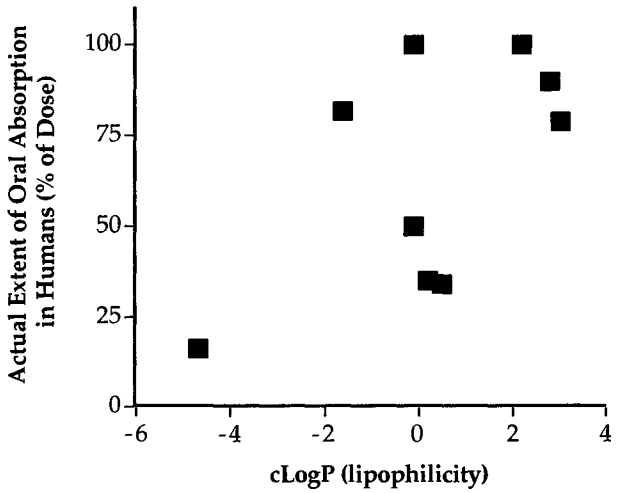

Fig. 2. Lack of correlation between *in vivo* absorption in humans and cLogP values for nine passively absorbed drugs. PEG-4000 was not included in this correlation because of unreliable cLogP value ($p > 0.05$, NS).

10 nm/sec were predicted to have acceptable absorption in humans (>20%). To date, there are no examples within our laboratory or in the literature when poorly absorbed compounds show high permeability through the caco-2 cells. However, when a compound has a Pc value less than 10 nm/sec, the results obtained with the caco-2 cells must be interpreted cautiously because absorption might be acceptable *in vivo* if it is absorbed by a specialized transport process, such as the dipeptide transporter system.

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