Comparison of the Permeability Characteristics of a Human Colonic Epithelial (Caco-2) Cell Line to Colon of Rabbit, Monkey, and Dog Intestine and Human Drug Absorption

Werner Rubas,[1,2] Nancy Jezyk,[1,3] and George M. Grass[1,4]

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The *in vitro* permeabilities of Caco-2 monolayers and permeabilities in tissue sections from colon of monkey, rabbit, and dog were compared using a series of compounds. The selected compounds differed in their physicochemical properties, such as octanol/water partition coefficient, water solubility, and molecular weight. Their structure included steroids, carboxylic acids, xanthins, alcohols, and polyethylene glycols. A linear permeability relationship was established between Caco-2 and colon tissue from both rabbit and monkey. The results suggest that Caco-2 is twice as permeable as rabbit and five times as permeable as monkey colon. However, no clear relationship could be established between Caco-2 monolayers and dog colon permeability. A relationship between permeability in Caco-2 monolayers and human absorption was found. The results suggest that within certain limits, permeability of Caco-2 monolayers may be used as a predictive tool to estimate human drug absorption.

KEY WORDS: permeability coefficient; Caco-2; dog, monkey, and rabbit intestinal segments; human absorption.

INTRODUCTION

Successful prediction of oral absorption could be used as an integral part of drug development. The *in vitro* determination of the permeability of various intestinal segments and cell monolayers (Caco-2) is a currently recognized method to estimate the barrier function of the gastrointestinal tract (1–8). The procedure involves the mounting of either a specific intestinal segment or cell monolayers grown on a support in an *in vitro* site-by-site diffusion cell. Permeability values are calculated from the change in concentration of the serosal (receiver) solutions over time. To date, little is known about the relationship, if any, of permeability measurements in tissue segments versus Caco-2 cell monolayers.

Permeability may be characterized as the relative magnitude of the interaction of the compound with the aqueous environment and the hydrophobic interior of the membranes. Homologous series of compounds are structurally related and therefore interact similarly with the environment. Thus we investigated a selection of compounds that differed structurally and in physicochemical properties, such as octanol/water partition coefficient, water solubility, and molecular weight (Table I).

The purpose of these studies was to measure permeability values in Caco-2 monolayers and to compare them with previously reported values generated in different intestinal segments of rabbit, dog, and cynomolgus monkey (7). In addition, attempts were made to specify a relationship between permeability in Caco-2 monolayers and percentage absorption in humans.

MATERIALS AND METHODS

Materials

Radiolabeled ^{3}H-hydrocortisone, ^{14}C-polyethylene glycol (PEG) 4000, ^{3}H-PEG 900, and ^{14}C-methanol were from New England Nuclear (Boston, MA 02118). ^{14}C-D-Glucose, ^{14}C-mannitol, and ^{3}H-progesterone were from Amersham (Arlington Heights, IL 60005). ^{3}H-ganciclovir and ^{3}H-naproxen were from Syntex Research (Palo Alto, CA 94304). All other reagents were analytical grade and used as received. Liquid scintillation cocktail (Ready Safe) was from Beckman Instruments, Inc. (Fullerton, CA 93634). Dulbecco's modified Eagle's medium (DMEM/high) with 25 m*M* Hepes and fetal bovine serum (Select) were purchased from JRH Biosciences (Lenexa, KS 66215). MEM nonessential amino acids (100×) solution, L-glutamine (200 m*M*), penicillin–streptomycin solution, and trypsin-EDTA (0.05% trypsin, 0.53 m*M* EDTA) were from GIBCO Laboratories, Life Technologies Inc. (Grand Island, NY 14072). Rat tail collagen was bought from Collaborative Research Inc. (Bedford, MA 01730). Cluster dishes with 12-mm Snapwells (0.4-μm pore size) and T-flasks were obtained from Costar (Cambridge, MA 02140). The Caco-2 cell line was a kind gift from Dr. I. Hidalgo at Smith Kline Beecham Pharmaceuticals (King of Prussia, PA 19406).

Cell Culture

Cells were grown as described previously (8,9). Briefly, Caco-2 cells were maintained at 37°C in DMEM/high with 25 m*M* Hepes, supplemented with 10% fetal bovine serum, 1% MEM nonessential amino acids, 1% L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 and 90% relative humidity. Cells grown in 75-cm^2 flasks were passaged every week at a split ratio of 1:3. Cells were trypsinized (10 ml, 5–10 min at 37°C) and seeded at a density of 63,000 cells/cm^2 on prewetted (PBS; 15 min outside and then inside) collagen-coated Transwells polycarbonate filters, which were coated as follows: 1 part rat tail collagen was mixed with 3 parts 60% ethanol. A 100 μl aliquot of this mixture was added to each insert and then dried for 4 hr in the hood with the lid slightly open. The medium was changed every other day. The monolayers were used

[1] Institute of Pharmaceutical Sciences, Syntex Research, Palo Alto, California 94303.

[2] Current address: Pharmaceutical Research and Development Genentech, Inc., 460 Point San Bruno Boulevard, Mailstop 6, South San Francisco, California 94080.

[3] Current address: College of Pharmacy, The University of Michigan, Ann Arbor, Michigan 48109-1065.

[4] To whom correspondence should be addressed at Precision Instrument Design, 205 Alpine Meadows Road No. 4, P.O. Box 1242, Tahoe City, California 96145.

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Table I. Physicochemical Properties, Permeability Coefficients in Caco-2 Monolayers, and Percentage Absorbed in Humans for Investigated Compounds

Compound	MW	log PC (octanol/water)	pH 7.4 solubility (mg/ml)	Permeability $\times 10^{-6}$ (cm/sec)[a]	% absorbed[b]
Hydrophilic					
Ganciclovir	255	−1.65	3.64	2.67 ± 0.72 (5)	8.0
Mannitol	182	−3.10	182	3.23 ± 0.95 (11)	17.0
Methanol	32	−0.74	Miscible	131.83 ± 7.15 (5)	—
PEG 4000	4000	−5.10	2100	0.97 ± 0.26 (5)	0.0
PEG 900	900	—[c]	—	12.10 ± 5.83 (6)	10.0
Lipophilic					
Hydrocortisone	362	1.20	0.78	35.40 ± 1.01 (5)	80.0
Naproxen	250	0.42	197	74.17 ± 8.88 (6)	100.0
Progesterone	315	3.89	0.75	78.93 ± 5.67 (6)	100.0
Transport mediated					
D-Glucose	180	3.89	180	91.35 ± 3.55 (5)	—

[a] Number of experiments in parentheses.
[b] From Refs. 6 and 26–33.
[c] Not available.

between passage 30 and passage 35, after days 20 and 26 of growth.

Permeability Measurements

Radiolabeled compounds were vacuum-dried prior to adding to freshly prepared oxygenated (95% O_2/5% CO_2) Kreb's Ringer bicarbonate buffer (pH 7.4). The following amounts were used: 4.4×10^{-3} m*M* hydrocortisone, 3.0×10^{-3} m*M* PEG 4000, 4.8×10^{-3} m*M* PEG 900, 1.4×10^{-2} m*M* methanol, 9.7×10^{-6} m*M* D-glucose, 3.4×10^{-3} m*M* mannitol, 3.0×10^{-6} m*M* progesterone, 2.2×10^{-5} m*M* ganciclovir, and 8.4×10^{-6} m*M* naproxen. Unlabeled D-glucose (40 m*M*) was added to Kreb's Ringer bicarbonate buffer (serosal solution). To equalize osmotic pressure between the mucosal and the serosal chamber, 40 m*M* unlabeled mannitol was added to the mucosal solution in all cases with the exception of D-glucose transport studies, where 10 m*M* D-glucose and 30 m*M* mannitol were used instead.

Caco-2 monolayers growing on inserts (1.13 cm^2, 0.4-μm pore size) were washed free of medium with Kreb's Ringer bicarbonate buffer prior to placement into an acrylic half-cell (Precision Instrument Design). The matching half-cell was joined to seal the diffusion apparatus and the chambers were then immediately placed in an aluminum block heater. Mucosal (donor) and serosal (receiver) solutions, 6.0 ml each, were placed into their respective chambers. Solutions were circulated by gas lift (95% O_2/5% CO_2). Samples (0.1 ml) of the donor phase (mucosal side) were taken prior to the experiment as well as upon its conclusion. Serosal samples (1.0 ml) were taken at appropriate time points with replacement of the sampled volume. Samples were placed in scintillation vials, scintillation cocktail was added, and samples were counted in a scintillation counter using an external standardization method. All experiments were conducted at 37°C and continued for 2 hr. The permeability coefficient (P) was calculated according to the following equation:

$$P = \frac{V \cdot dC}{A \cdot C_0 \cdot dt} \quad (1)$$

where $V \cdot (dC/dt)$ is the change in mass as micrograms per unit time in the receiver chamber; A is the surface area of the serosal site of the intestinal tissue or the monolayers, respectively; and C_0 is the starting concentration of the donor chamber.

Statistics

Results are presented as means ± SEM. Unless otherwise noted, statistical comparisons were made with analysis of variance (ANOVA) at a 95% confidence level, using Fisher's protected least significant difference (PLSD) as the test statistic.

RESULTS

Permeability values of the series of compounds examined in this study using Caco-2 monolayers ranged between 9.7×10^{-7} cm/sec for PEG 4000 and 1.32×10^{-4} cm/sec for methanol (Table I). With the exception of methanol, which is highly soluble in both water and lipid, lipophilic compounds (log PC > 0) demonstrated the highest permeabilities. Among the hydrophilic compounds (log PC < 0) permeability appeared dependent upon molecular weight, except for PEG 900, which, despite a significantly higher molecular weight, was more permeable than both ganciclovir and mannitol. The measured permeability for the transport-mediated compound D-glucose (10 m*M*) was much greater than mannitol, a passively transported compound of similar physicochemical characteristics.

The passively transported compounds examined in this study constitute a range of more than eight orders of magnitude in octanol/water partitioning and approximately two orders of magnitude in permeability (Table I). Correlations between measured permeabilities in Caco-2 cells and measured permeabilities of the same compounds in different segments of rabbits, dogs, and cynomolgus monkey were determined using the values measured for these tissues in an earlier study (7). Figure 1 illustrates the correlation of the apparent permeability of these compounds in Caco-2 cells vs

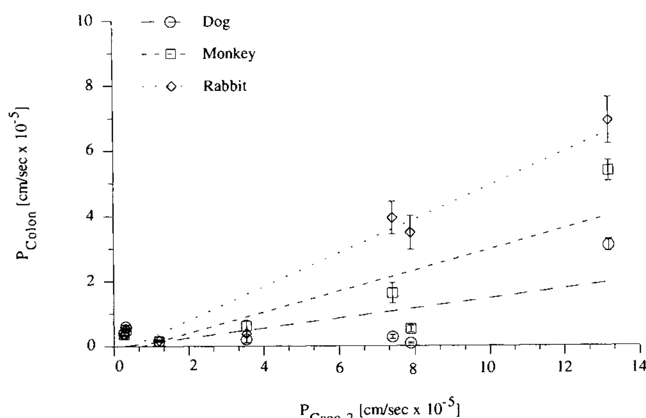

Fig. 1. Relationship between permeabilities of rabbit, cynomolgus monkey, and dog colon and permeability in human colonic (Caco-2) monolayers. Rabbit: $y = 0.52x - 2.32 \cdot 10^{-6}$; $r^2 = 0.969$. Monkey: $y = 0.32x - 2.35 \cdot 10^{-6}$; $r^2 = 0.840$. Dog: $y = 0.15x - 4.00 \cdot 10^{-7}$; $r^2 = 0.684$.

rabbit, monkey, and dog colon. A good correlation coefficient ($r^2 = 0.972$) was achieved only between rabbit colon and Caco-2. The slope value of 0.51 suggests that the permeability of Caco-2 monolayer is approximately twice as high as that of rabbit colon. The correlation between monkey colon and the cell monolayer was moderate ($r^2 = 0.840$), and no correlation was found between dog colon and Caco-2. Monkey colon was one order of magnitude less permeable than the cell monolayers. No correlation was found between Caco-2 monolayers and all other segments examined.

A relationship between percentage drug absorbed in human and permeability in Caco-2 monolayers was established (Fig. 2). Compounds that showed 100% absorption in human had a permeability value of approximately $>7 \times 10^{-5}$ cm/sec. Incompletely absorbed drugs such as ganciclovir demonstrated a permeability that was approximately one order of magnitude lower.

DISCUSSION

The objective of this study was to evaluate the relationships between intestinal segment of rabbit, cynomolgus monkey, and dog and the Caco-2 human colonic cell line.

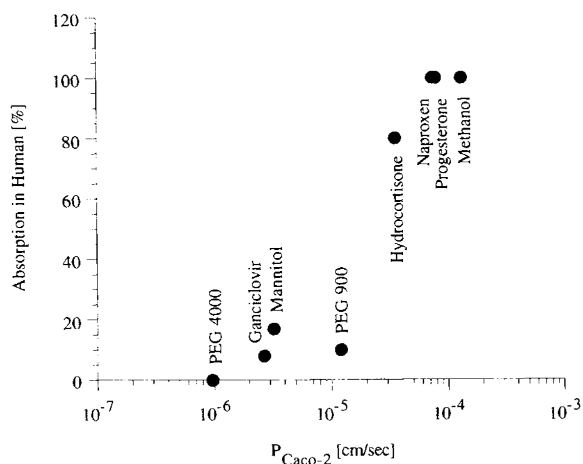

Fig. 2. Oral absorption in humans as a function of permeability values in Caco-2 monolayers. Percentage absorbed from Refs. 6 and 29–36.

Transport of Hydrophilic Compounds

For polar compounds, with log PC < 0, transport across tissue is slow due to poor membrane partitioning. These solutes cross the epithelium primarily via the paracellular shunt and are restricted by the space between tight junctions. Because permeability has been shown to correlate with molecular size rather than molecular weight, it was not anticipated that molecular weight would be a direct predictor of permeability (10). However, as similar studies in humans reported a slightly decreased constant urinary recovery of individual fractions of low molecular weight PEGs (MW range, 240–370) after oral ingestion (11–14), a rank order of permeabilities, which corresponded to molecular weight, was anticipated. Despite its high purity (>99%) and a molecular weight distribution which has been determined by the vendor as 350 (3.9%), 470 (40.6%), 800 (8%), 1000 (6.5%), 1250 (4.9%), and 1650 (36.1%), respectively, the permeability of PEG 900 was significantly higher than of the lower molecular weight compounds mannitol and ganciclovir. If all of the observed material in the receiver chamber were of low molecular weight, the permeability would be even higher than we calculated, since we take the total, and not a fraction of the, PEG concentration into account. Our observation is contrary to the rank order observed in *in vitro* studies using the same hydrophilic compounds in various segments of animal intestine (7). A rank-order correlation has also been found for PEGs ranking in MW from 630 to 1070 (15–17).

Because the permeability values obtained for the extracellular space marker PEG 4000 are similar to those previously reported in the literature, it is unlikely that this discrepancy is caused by leaky tight junctions within the monolayer (6,9). At this time, there is no obvious explanation for this rather unusual result.

Transport of Lipophilic Compounds

In these Caco-2 monolayers, it was noted that progesterone was more permeable than hydrocortisone, in contrast to previous reports, which suggest that the partition coefficient of hydrocortisone falls in an optimal range (log PC, 1–3) for absorption (18,19).

Progesterone and hydrocortisone differ in their number of hydroxyl groups, zero and three, respectively. The addition of hydroxyl groups in a homologous series of compounds results in a positive change in the Gibbs free energy ($d\Delta G$) value (20), which indicates increased interactions in the hydrophilic phase relative to those that occur in the hydrophobic phase. Such a substituent will decrease permeability. Conversely, a substituent that is characterized by a negative $d\Delta G$ value, such as $-CH_2-$, will facilitate the aqueous-to-membrane transition, thereby increasing permeability. An increased $d\Delta G$ value suggests a greater interaction for hydrocortisone with hydrophilic groups in the cell membrane. Thus, hydrocortisone permeability should be reduced compared to progesterone. Given these relationships, one could speculate that log PC measurements in octanol are not a good predictor of brush border polarity, as has been previously suggested (21).

Unstirred Water Layer Effects

Transport of lipophilic compounds such as progesterone is aqueous boundary layer controlled, in contrast to the membrane-controlled kinetics of less lipophilic molecules, e.g., hydrocortisone. Previously conducted permeability studies using compounds with different lipophilicities were performed at different thicknesses of the unstirred water layer (UWL) generated by varying the gas flow rate in the diffusion chamber (8). The authors found maximum permeability at zero flow rate for compounds with a log PC between 1.90 and 2.90 (18,19). Increasing the flow rate from 0 to 15 ml/min increased the permeability of testosterone (log PC = 3.31) from $4.07 \pm 1.13 \times 10^{-5}$ to $10.90 \pm 1.56 \times 10^{-5}$ cm/sec, while there was no significant increase in the permeability of corticosterone (log PC = 1.89), which changed only slightly, from $5.39 \pm 0.70 \times 10^{-5}$ to $6.16 \pm 0.64 \times 10^{-5}$ cm/sec. Assuming that progesterone transport, like testosterone transport, is diffusion controlled, the thickness (h) of UWL can be calculated using the equation

$$h = D/P \quad (2)$$

where h is the apparent thickness of the UWL (cm), D the diffusion coefficient, and P the measured permeability coefficient. The aqueous diffusion coefficient for progesterone was assumed to be 8×10^{-6} cm^2/sec (22). Thus, the thickness (h) was calculated to be 1014 μm, which is approximately half the value (1966 μm) in unstirred diffusion cells (8). It is noteworthy that this estimation of UWL thickness is in relatively good agreement with data from humans, where a value of approximately 600 μm was reported (23).

In the presence of UWL the concentration of the probe molecule at the aqueous–lipid interface, C_2, is reduced below the concentration of the molecule in the bulk solution, C_1. The magnitude of this reduction is given by the expression

$$C_2 = C_1 - J\,(h/D) \quad (3)$$

where J is the flux rate of the probe molecules across the unstirred layer, h is the thickness of the barrier, and D is the free diffusion coefficient for the molecule. Consequently, the concentration of the molecule at the aqueous–lipid interface, C_2, increases with decreased UWL. Since thinning of the UWL progressively increases the permeability of less polar compounds (8,24), these results confirm the concept that transport of hydrocortisone is membrane controlled, while transport of progesterone is limited by the aqueous boundary layer.

Comparison of Caco-2 Permeability to *in Vitro* Tissue Permeability

In view of the available literature on permeability in gastrointestinal tissues and the colonic cell line Caco-2, it is surprising that there have been only limited attempts to correlate these data (4). In the present study, several compound classes were used to determine, whether or not Caco-2 monolayers behave in a manner similar to that of the excised tissues previously examined in this laboratory (7). Compared to colonic segments, the results obtained show a very good correlation between rabbit colon and Caco-2 monolayers, while the correlation between monkey colon and cell monolayers was moderate, and no correlation was found between dog colon and the cell monolayers (Fig. 1). There was no linear correlation among any of the other segments and the Caco-2 monolayers observed. This is in agreement with previous studies by Artursson (4), who also failed to find a linear correlation between cell monolayers and rat ileum using a homologous series of β-blocking agents. Caco-2 monolayers lack goblet cells and, hence, mucin. In addition, anatomical structures such as villi are also absent. This may explain the poor correlations obtained when comparing Caco-2 monolayers to *in vitro* permeability measurements using small intestine tissues. In addition, transepithelial electrical resistance (TEER) was found to be similar for rabbit colon and Caco-2 monolayers but not for Caco-2 monolayers and small intestinal tissues (4).

Transport studies of glucose in biopsies from human and animal jejunum revealed a maximum active transport per square centimeter of serosa of 5 ± 2 μmol/hr/cm^2 (25). Transport in the ileum, however, is about half that in the jejunum. From the equation

$$J = P \cdot C \quad (4)$$

where J is drug flux, P is the permeability coefficient, and C is the concentration of the drug at the absorbing surface. It is noteworthy that in Caco-2 cells we determined a flux of approximately 1 μmol/hr/cm^2, which is in good agreement with literature values for ileal transport, considering the fact that maximum carrier-mediated transport takes place at concentrations that are two to three times as high as those examined in this study (25). At normal rates of fluid absorption and at the saturation concentration of the carrier, passive transport may be as high as 50% (26,27). In diffusion cells, however, no net water transport was observed in either rabbit jejunum or colon (7). Therefore, primarily a carrier-mediated glucose transport is suggested in Caco-2 cells, which has been demonstrated by several investigators (28). This is also supported by the large difference in permeabilities for mannitol and D-glucose, 3.24×10^{-6} and 91.35×10^{-6} cm/sec, respectively. The similarity in physicochemical properties of these compounds suggests that an alternate mechanism of transport is available for D-glucose. This is consistent with observations for similar studies in tissues (7).

Comparison of Caco-2 Permeability to Human Absorption

Methods to estimate oral drug absorption in humans using physicochemical parameters such as partition coefficient have been developed but can be limited in their use. In general, octanol/water partition data are marginally effective, since the polarities of the brush border membrane and octanol may be different (21). Therefore, a system which uses actual measured permeability as an indication of the interaction of a compound and the tissue may be more effective. For this reason, a correlation was attempted by plotting percentage absorption in humans vs permeability values in Caco-2 monolayers. Compounds that are completely absorbed in humans demonstrated permeability values $>7 \times 10^{-5}$ cm/sec. Compounds with poor absorption, <20%, had permeability values $<1 \times 10^{-5}$ cm/sec. The steep increase in compound absorption for those with permeability values >1

$\times 10^{-5}$ cm/sec reflected the switch from mainly paracellular to transcellular pathway. Although the present studies used a limited number of compounds, there is evidence that a correlation of percentage absorption in humans vs permeability in Caco-2 cells can allow prediction of absorption in human within certain limitations of drug solubility and intestinal reserve length. These limitations are imposed because drug permeability does not account for the relationships between dose size and solubility within the intestine. Since absorption is directly related to drug flux, Eq. (3) describes the interaction of permeability and solubility. A relationship between permeability and percentage absorbed in humans should be valid only in cases where the drug solubility (and hence flux) is great enough to allow complete absorption in a time period before the drug is eliminated from the body via gastrointestinal transit.

A similar relationship involving Caco-2 monolayer permeability and absorption in human has also been examined by Artursson and Karlsson (6). In their work, similar ranges of permeability values for complete and poor absorption were identified, however, their absolute values were up to one order of magnitude less than in the current study. This probably results from the differences encounter between a stirred system, as used in this current study, and the stagnant fluid system utilized by Artursson.

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