Comparison of Intestinal Permeabilities Determined in Multiple *in Vitro* and *in Situ* Models: Relationship to Absorption in Humans

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In vitro and *in situ* experimental models that are descriptive of drug absorption *in vivo* are valuable tools in the discovery of new chemical entities that are bioavailable after oral administration. The specific objective of the study was to compare the intestinal permeabilities obtained in the three absorption models for consistency, and to assess the utility of the models in predicting the fraction of dose absorbed in human studies. The intestinal absorption models that were compared are widely used: the rat *in situ* single-pass intestinal perfusion system, the rat everted intestinal ring method, and monolayers of human colon adenocarcinoma cell line (CACO-2). The models were compared using small molecular reference compounds, as well as a series of peptidomimetic (PM) analogs. Each model had strong potential for estimating the fraction absorbed. For small organic molecules, excellent correlation was observed when permeabilities from CACO-2 cells and perfusions, or everted rings and perfusions, were compared. Weaker correlation was observed between everted rings and CACO-2 cells. Permeabilities for the set of reference compounds and PMs were positively correlated between any two of the three systems. Variance between correlations for reference compounds and PMs are likely due to structural features and physicochemical properties that are unique to the latter class of compounds. The results support caution in extrapolating correlations based on findings with small organic molecules to the behavior of complex peptidomimetics. Corroboration of permeabilities with two methods of determination is a useful cross-validation of experimental systems, as well as producing a reliable permeability assessment. CACO-2 cell monolayers and rat single-pass intestinal perfusion combine the highest correlation between systems, most defined relationship with fraction absorbed in humans, and experimental logistics in-line with discovery candidates.

KEY WORDS: absorption of peptidomimetics; CACO-2 cell model; intestinal absorption models; fraction absorbed in humans; rat everted intestinal ring model; rat intestinal perfusion model.

INTRODUCTION

In vitro and *in situ* experimental models that are descriptive of drug absorption *in vivo* are valuable tools in estimating the biological transport properties of new chemical entities after oral administration. These experimental models are designed to isolate the barrier or process of interest so as to permit relatively rapid and mechanistic evaluation of drug candidates. *In vivo* studies permit determination of absolute or relative bioavailability, but are also more complex in terms of plasma assay development and assessing where rate-limiting processes occur. Meaningful feedback to the drug discovery effort generally requires a balance between the higher throughput of *in vitro* or *in situ* studies, and the more clinically relevant picture obtained in selected *in vivo* studies. *In vitro* or *in situ* models are of particular utility if they project when 1) absorption is rate-limiting to systemic availability, and 2) permeability is rate-limiting to absorption.

The specific objectives of this study were to compare the intestinal permeabilities obtained in three absorption models for consistency, as well as to use the fraction absorbed data in humans, available in the literature, to assess whether the models were predictive. The models were compared using reference compounds, as well as a series of peptidomimetic (PM) analogs (see Figure 1). The reference compounds were defined as small (<400 Da) organic molecules that were relatively well characterized in terms of transport and metabolism, either in our hands or in the literature. These compounds ranged from those poorly absorbed in humans, such as D-mannitol, to those well absorbed in humans, such as phenytoin, and included compounds that were absorbed by carrier-mediated and passive processes. The PMs originated in a renin inhibitor discovery program (1). Oral delivery of renin inhibitors and other PM-based therapeutic agents (2) has been a formidable obstacle that has resulted in close examination of each aspect of the delivery process. These compounds were selected to represent a wide range of physicochemical properties including lipophilicity, molecular weight and hydrogen-bonding capacity.

Three widely-used intestinal absorption models possessing individual strengths and weaknesses were compared. Each absorption model relied on a different means of determining membrane transport. The rat *in situ* single-pass intestinal perfusion system based permeability calculations on steady-state disappearance of the compound from the intestinal lumen. The rat everted intestinal ring method used tissue accumulation of compound *in vitro* to determine drug uptake rate. The human colon adenocarcinoma cell line, CACO-2, was grown on membrane filters, mounted in diffusion chambers, and the rate of compound appearing in the receiver compartment was the basis of the permeability measurement. This work extends that of other researchers (3-7) by examining how well permeabilities from multiple *in vitro* or *in situ* systems are correlated for a diverse set of molecules, as well as the relationship of intestinal permeability to fraction absorbed *in vivo*.

There are other factors that weigh in for each of these models in selecting *in vitro/in situ* methods for evaluation of discovery candidates. These factors are summarized below for each of the experimental systems used in this report. Considerations include ease and cost of preparation and maintenance, control of experimental conditions, reproducibility, uptake versus transcellular measurements, and ease of drug analysis.

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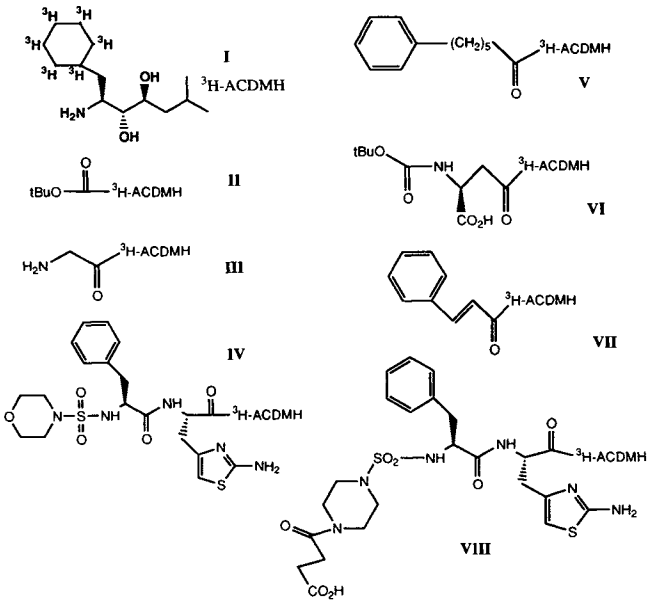

Figure 1. Structures of Tritiated Peptidomimetic Analogs.

Everted rat intestinal rings offer a relatively quick and inexpensive technique for measuring uptake of drug into tissue. These considerations are important in comparing the methods, as the everted ring technique does not entail the considerable time and expense of start-up and maintenance of the CACO-2 cells, nor does it require the greater number of animals, with associated husbandry costs, needed for statistical power with the perfusion method. There is a statistical advantage in that several conditions can be studied in replicate with controls obtained from the same animal. Rings can be prepared from virtually any segment of intestine, permitting study of axial differences in uptake. This technique has been widely used in mechanistic studies of amino acid and peptide transport and has a wide database in the literature (8). Critics of this method take issue with viability of the tissue over the time course of the experiment, although ring incubations require a small fraction of the time used for everted intestinal sac or excised tissue experiments. The technique is limited to uptake of drug by the tissue, unlike excised tissue preparations where transmucosal flux can be monitored (9). One of the most important considerations in whether to use everted rings is if the compound of interest is available in radiolabelled form; analysis of nonradiolabelled drug in intestinal tissue is relatively labor-intensive, although necessary when one is interested in obtaining metabolic information (10).

Rat single-pass intestinal perfusion is an *in situ* technique wherein the blood supply, innervation and clearance capabilities of the animal remain intact. Input of drug can be closely controlled in terms of concentration, pH, osmolality, composition, intestinal region and flow rate (11). The absorption model, *per se*, is physiologically and pharmacologically responsive which may account, in part, for the higher variability observed in some of these experiments. The drug is measured in buffer or perfusate, thus facilitating assay of drug by specific chromatographic means. This technique has been used extensively in establishing a database of permeabilities with correlation to human absorption data, as well as to elucidate absorption mechanism (12,13).

CACO-2 cell monolayers have evolved to a widely used model for intestinal absorption with a burgeoning database in many laboratories. This system offers the convenience of a continuously cultured cell line to model the small intestinal epithelium, despite the origin of CACO-2 from human colon adenocarcinoma. The human origin of the cells is desirable; however, the transformed nature of the cells may result in unpredictable differentiation markers (14). In addition, several reports have associated transepithelial electrical resistance, enzyme expression and some transport properties of the CACO-2 cells more closely with colon than small intestine (6,15). Transport can be measured by either transcellular flux or uptake directly into the cell monolayers, thus isolating apical and basolateral membrane processes. Measurements of drug are made either in buffer or from lysed and precipitated cells, thus minimizing assay difficulty.

MATERIALS AND METHODS

MES buffer was prepared from MES, NaCl and KCl (Sigma Chemical Co., St. Louis MO). Gabapentin (1-(aminomethyl)cyclohexaneacetic acid, CI-945, lot XH370889) and [^{14}C]-gabapentin (lot NO286-2701, radiochemical purity ≥98%) were used for studies. Reference compounds and PEG-4000 were also purchased from Sigma Chemical Co. Radiochemical reference compounds were purchased from either New England Nuclear/Dupont (Boston MA: [^{3}H]-D-mannitol, [^{3}H]-acetaminophen, [^{3}H]-L-phenylalanine, [^{3}H]-hydrocortisone, [^{14}C]-phenytoin, [^{3}H]- and [^{14}C]-PEG-4000) or Amersham (Arlington Heights IL; [^{3}H]-prednisolone). Tritiated PMs were prepared using a common tritiated cyclohexyl precursor (Figure 1; 16,17). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma (St. Louis MO). Soluene and Hionic-Fluor were purchased from Packard Instrument Co. (Meriden CT). Neutralizing solution (PGM) was prepared from a saturated solution of sodium pyruvate in methanol, glacial acetic acid and methanol in the ratio of 4:3:1 by volume.

Single-Pass Intestinal Perfusion in Rats. Male white Wistar rats (250 to 470 g) were fasted overnight with water *ad libitum*. Rats were anesthetized with a cocktail of ketamine, xylazine and pentobarbital; animals were sacrificed at the end of the experiment before recovering from the anesthesia. Laparotomy was performed after onset of deep anesthesia and the upper jejunum was identified. Proximal and distal ends of a 3 to 15 cm segment of intestine were cannulated with glass tubing. Perfusion solutions of drug and PEG-4000 were prepared with radiolabelled tracer plus cold material if necessary to achieve desired concentrations in iso-osmotic buffer (10 mM MES, 135 mM NaCl, 5 mM KCl; pH 6.5). Specific activity of the tritium label was 1 to 3 × 10^5 dpm/mL, while the carbon-14 label was 1 to 3 × 10^4 dpm/mL. Drug solution containing the nonabsorbable water marker, PEG-4000 (0.01% w/v), was perfused into the proximal intestine at a constant concentration, C_{in}, and constant flow rate, Q (0.125, 0.20 or 0.25 mL/min), using a Harvard Apparatus Infusion Pump (Model 4200, South Natick MA). Exiting perfusate was collected from the distal cannula at concentration, C_{out}, over 10-min intervals for 90 min. Drug and

PEG-4000 concentrations were determined by dual-label, quench-corrected liquid scintillation spectrometry (Packard TriCarb 4000 Series, Downers Grove IL). The steady-state ratio of outlet to inlet drug concentration, C_{out}/C_{in}, was normalized for Q, intestinal length, L, and calculated drug diffusivity, D, and corrected for water flux using the nonabsorbable marker, then used to calculate dimensionless, steady-state effective permeabilities (12):

$$^{o}P^{*}_{eff} = [\ln (C_{out}/C_{in})]/(-4Gz) \quad (1)$$

where the Graetz Number, $Gz = \pi DL/2Q$. For comparison to everted ring and CACO-2 cell permeabilities, $^{o}P^{*}_{eff}$ was redimensionalized using the ratio of (D/r) where r = radius of rat intestine (0.2 cm).

Uptake of Drug by Everted Rings of Rat Intestine. Male white Wistar rats were fasted, anesthetized and laparotomized as described above. Animals were sacrificed after identification and removal of 15 to 20 cm proximal jejunum. The excised intestine was floated in a tray of ice-cold, oxygenated MES buffer containing 11 mM D-glucose, everted on a slender glass rod and cut into rings (10 to 35 mg) with a razor. Test tubes were equilibrated in a shaking water bath at 37°C. Individual rings were incubated in test tubes containing 1.0 mL radiolabelled drug solution. Specific activities of the tritium label were 1×10^4 to 5×10^5 dpm/nmol and of the carbon-14 label, 1 to 5×10^4 dpm/nmol. Incubations were quenched by emptying the test tube contents onto a cheesecloth-covered beaker and rinsing with ice-cold saline. Tissue was gently blotted dry, placed in a tared vial and weighed. Soluene (1.0 mL) was added to solubilize the tissue overnight, then PGM (0.1 mL) to neutralize the Soluene prior to addition of 15.0 mL Hionic-Fluor, followed by analysis with liquid scintillation spectrometry.

In baseline experiments, uptake was determined as a function of time and water bath shaking rate. The unbiased membrane flux, J_o, was obtained from the y-intercept of a plot of uptake versus reciprocal shaking rate. The permeability coefficient, P_o, was calculated from the unbiased flux (J_o), concentration of the incubation medium (C) and conversion factor ($<cf> = 24.0$ cm^2/g) for tissue mass:surface area (18) from the relation (19):

$$J_o = P_o * C * <cf> \quad (2)$$

CACO-2 Cell Transport Experiments. CACO-2 cells from continuous culture (passage 35 to 64) were seeded at high density onto Snapwell polycarbonate filters. Cells were maintained with DMEM/10% fetal calf serum + 0.1 mM nonessential amino acids + 2 mM L-GLN until day of experiment. Experiments were conducted during days 21 to 29 after seeding. Cells on filters were removed from Snapwells, transepithelial electrical resistance (TEER) was measured and cells were washed with buffer. TEER ranged widely, from 200 to 1300 Ω-cm^2, therefore a leakage marker, radiolabelled PEG 4000, was typically used to ensure consistency from experiment to experiment. Cells were placed in side-by-side diffusion apparatus (Precision Instrument, Costar, Cambridge MA) with gas-lift mixing at 37°C. The Lucite chambers were pretreated with 1 mg human serum albumin/mL and/or cold compound when adsorption was problematic. The apical or donor side was filled with drug in buffer (MES, pH 6.5), the basolateral or receiver side with buffer. Buffer contained 25 mM D-glucose. Generally, samples were removed from the donor compartment and assayed as a control against adsorption or secretion of compound from the receiver compartment. Samples were removed from the receiver compartment and the amount of radioactivity transferred as a function of time was used to calculate the permeability.

$$P_{app} = (V/(A*C))(dC/dt) \quad (3)$$

where V is the volume of buffer in the receiver chamber, A is the exposed surface area of the cell monolayer, C is the donor drug concentration and dC/dt is the change in receiver drug concentration over time.

RESULTS AND DISCUSSION

Permeability of Reference Compounds. Permeabilities obtained for reference compounds in the three experimental systems are tabulated in Table I and depicted in Figure 2. The values for fraction absorbed in humans were obtained from the literature (collated in Ref 19) and ranged from 5% for D-mannitol to 100% for L-phenylalanine (at low concentration) and phenytoin. Permeabilities ranged from 0.47 to 234×10^{-4} cm/min. Values for fraction absorbed *in vivo* can be highly variable due to dissolution rate-limits or metabolism, hence compounds were selected that were relatively well-characterized with respect to clinical absorption characteristics. All permeability measurements were conducted with compounds in aqueous solution. Due to its saturable absorption profile, gabapentin provided two data points in a critical region of the curve (at 36% and 74% of dose absorbed in humans).

The results in Figure 2 demonstrated close agreement in the correlation between fraction absorbed and permeability, whether the latter was determined by rat everted intestinal ring uptake, single-pass rat intestinal perfusion or transport across human CACO-2 cell monolayers. The theoretical relationship between fraction absorbed (F_{abs}) and permeability ($^{o}P^{*}_{eff}$) was described by Amidon et al (12):

$$F_{abs} = 1 - \exp(-2\,^{o}P^{*}_{eff}) \quad (4)$$

This correlation indicated that a dimensionless permeability ($^{o}P^{*}_{eff}$) of approximately 1.0, or 25×10^{-4} cm/min (assuming an average aqueous drug diffusivity of 5×10^{-4} cm^2/min) corresponded to a well-absorbed drug. The theoretical curves in Figure 2 were calculated by redimensionalizing $^{o}P^{*}_{eff}$ using the ratio (D/r), with an average drug diffusivity, $D = 5 \times 10^{-4}$ cm^2/min and radius, r = 0.2 cm for rat intestine. The theoretical curve generated was descriptive of the permeability data from rat gut perfusion experiments. Redimensionalizing $^{o}P^{*}_{eff}$ required a 10-fold lower (D/r) factor to fit the CACO-2 data. A different hydrodynamic pattern is generated by stirring in the *in vitro* CACO-2 chambers, as opposed to the laminar flow generated in the single-pass *in situ* perfusion; hence, the steeper slope in the F versus $^{o}P^{*}_{eff}$ in vitro data could be accounted for by a difference in the effective distance for solute transport. Other factors may include the manner in which $^{o}P^{*}_{eff}$ is calculated (loss from lumen versus appearance in receiver compartment *in vitro*); tighter barrier properties for CACO-2 cell monolayers; transport into whole

Table I. Permeability of Reference Compounds in Everted Ring, CACO-2 and Intestinal Perfusions[a]

Compound	Fraction Absorbed	10^4 * Diffusivity (cm^2/min)[b]	10^4 * Permeability (cm/min)		
			Rings	CACO-2	Perfusion
D-Mannitol	5%	6.22	14.0 ± 0.6	0.5 ± 0.1	1.5 ± 0.9
Gabapentin[c]	36%	5.95	-	0.9 ± 0.4	3.4 ± 1.1
Gabapentin[d]	74%	5.95	52.5 ± 2.7	2.6 ± 1.1	12.9 ± 1.4
Acetaminophen[e]	80%	6.86	-	-	51.6 ± 3.2
Hydrocortisone	95%	4.33	-	26.8 ± 0.4	-
Prednisolone	95%	4.13	34.2 ± 1.6	-	18.1 ± 1.0
Phenytoin	100%	4.92	-	53.9 ± 3.2	-
L-Phenylalanine[f]	100%	6.36	127 ± 23.0	17.7 ± 0.5	107 ± 6.3

[a] Values for perfusion experiments represent the mean of 4 to 8 animals; for CACO-2 experiments, the mean of 3 to 6 monolayers; for ring experiments, the extrapolated value from 3 time courses where each time course consisted of 21 to 27 rings from one animal. Standard errors are tabulated with the mean values.

[b] Calculated by the Hayduk-Laudie correlation, using the Le Bas method for calculation of molal volumes by additive-volume increments (30).

[c] 50 mM.

[d] 0.01 or 0.04 mM.

[e] 0.50 mM.

[f] 0.1 mM.

tissue with mucus-coated villi in perfusions and rings versus mucus-free epithelial cell monolayers with microvilli for CACO-2.

From Figure 2, experimentally-derived permeabilities greater than approximately 20 × 10^{-4} cm/min were descriptive of well-absorbed compounds, regardless of the method of permeability determination. The data reach the plateau region at 100% absorbed at comparable permeabilities; however, analysis of the individual profiles revealed differences at less than 100% absorbed. CACO-2 permeabilities have the steepest slope between 0 to 100% absorbed. This observation was consistent with results published by Artursson and Karlsson (3), in which CACO-2 permeabilities were determined for a set of 20 compounds in which lipophilicities and

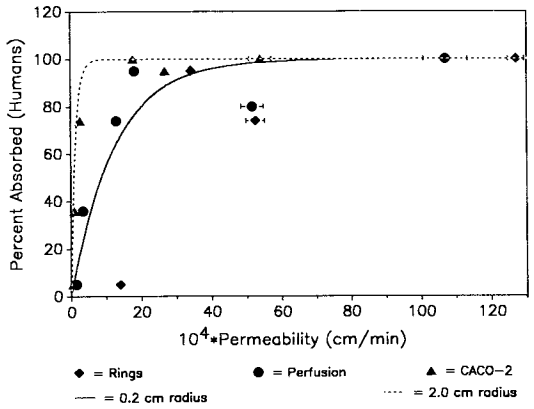

Figure 2. Fraction Absorbed in Humans Versus Permeabilities Determined by Rat Everted Intestinal Rings, Rat Intestinal Perfusion or CACO-2 Cell Monolayers. The theoretical curve is described by $F_{abs} = 1 - \exp(-2°P^*_{eff})$, EQN 4. Values for perfusion experiments represent the mean of 4 to 8 animals; for CACO-2 experiments, the mean of 3 to 6 cell monolayers; for ring experiments, the extrapolated value from 3 time courses where each time course consisted of 21 to 27 rings from one animal. Standard errors are tabulated with the mean values.

absorption in humans covered a wide range. CACO-2 permeabilities for compounds with intermediate absorption varied approximately one order-of-magnitude in both the previous and present studies. Permeabilities from perfusion studies were shifted to the right of the CACO-2 results. The relationship of these results with fraction absorbed agree qualitatively with those of Amidon et al (12), although prednisolone was the only compound studied in common. The more moderate slope of the theoretical curve correlating perfusion results with fraction absorbed facilitates the predictive use of these data. Everted ring results were the least conclusive because of the limited number of data points. The data suggested that permeabilities from ring experiments may be used to group compounds as well absorbed or poorly absorbed, but that compounds with intermediate absorption may not be predictively ranked using this method. With regard to predictive ability, more encouraging results were obtained by Porter et al (20) when the ring method was compared to uptake by isolated intestinal cells. The ring technique was also deemed more suitable than intestinal brush border membrane vesicles for uptake studies based on lower variability (21).

There were three apparent outlier permeabilities in Figure 2. The everted ring uptake of D-mannitol was greater than expected, thus tending to shift the results from the origin. This high apparent permeability for D-mannitol may have been due to not correcting the uptake for extracellular water. An underestimate in the fraction of D-mannitol absorbed is also possible, which may have compounded the problem. The permeability of acetaminophen in the perfusion system was higher than anticipated. Experiments were conducted with a relatively high concentration of acetaminophen in an effort to saturate metabolizing enzymes; however, it appears that metabolism to a more permeable species may have occurred, thus resulting in a higher apparent permeability. The permeability of gabapentin in everted rings was also high, and may have been the result of a paracellular contribution to transport (see below, Figure 3).

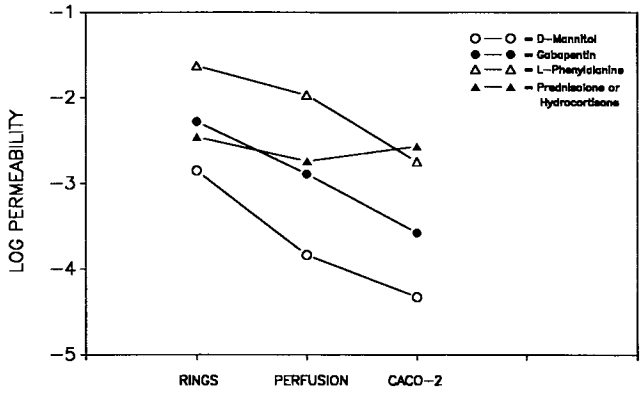

Figure 3. Rank Order Comparison Between Absorption Models for Reference Compounds. Four curves representing the rank order of log permeability determined in the three absorption models are shown. Prednisolone and hydrocortisone have been treated as same compound for purposes of model correlations.

Rank order comparison of permeabilities obtained for several reference compounds using the three absorption models is shown in Figure 3. Transport of three of the four compounds fit the trend of rings > perfusion > CACO-2 cells. Two of these three compounds, gabapentin and L-phenylalanine, are substrates for the System L transporter in the intestine (19,22); thus, the permeability of these compounds will be dependent on affinity and activity of System L in the models. System L activity may have been highest in the everted rings and lowest in CACO-2. Gabapentin and L-phenylalanine are also zwitterionic at physiological pH and have molecular weights less than 200; hence, there may be a paracellular contribution to transport of these molecules, particularly gabapentin, that may be more pronounced in the ring system. Parallels between the absorption models for the transport of gabapentin, L-phenylalanine, and D-mannitol were evident in the results. D-Mannitol is absorbed exclusively through the paracellular pathway. Given the lack of correction for extracellular water in the ring method and the tight barrier presented by the CACO-2 monolayer, the intermediate permeability of D-mannitol from *in situ* perfusion was readily explicable. Prednisolone and hydrocortisone are closely related physicochemically, and are depicted as a single data set in Figure 3. Both compounds are absorbed by a diffusive, transcellular mechanism. Permeabilities obtained from the three models were indistinguishable for prednisolone and hydrocortisone, suggesting that drug partitioning and diffusion through membrane was the dominant transport mechanism and similar in each system (23).

Permeability of Peptidomimetic Series. The structures of the PM compounds are given in Figure 1. Compound I (ACDMH) was the common structural moiety comprising the right-hand side in all of the analogs. Three of the eight analogs were nonionizable (II, V, VII), the other five had pKa's ranging from 4.7 to 9.3 and were in solution with some element of negative (VI, VIII) or positive (I, III, IV, VIII) charge. Selected physicochemical properties are listed in Table II. Partition coefficients were determined at pH 6.5 using an HPLC correlation method (24). The compounds were very lipophilic, with log partition coefficients of 1.5 or greater. Molecular weights varied from 243 to 808. The availability of hydrogen-bonding functionalities on each molecule

Table II. Selected Physical Properties of Peptidomimetics

Compound	log P[a]	MW[b]	H-bond #[c]
I	1.9	243.4	6
II	4.0	343.5	6.5
III	1.5	300.4	8
IV	3.0	708.9	13.5
V	4.7	417.6	6
VI	2.1	458.6	11
VII	3.6	373.5	6
VIII	2.0	808.8	16.5

[a] Log partition coefficient, determined at pH 6.5 by HPLC correlation method.
[b] Molecular weight (Da).
[c] Number of sites capable of forming hydrogen bonds, adapted from Stein (1967).

was calculated using an adaptation of the method of Stein (25). Hydrogen-bonding capability was extensive, with H-bond numbers ranging from 6.0 to 16.5.

Permeabilities for the set of PMs obtained using the three absorption models are tabulated in Table III. Values ranged from a low of 2.56×10^{-4} cm/min for VIII in the perfusion model to a high of 316×10^{-4} cm/min for II in the ring model. Both of these compounds were lipophilic (log P = 2.0 and 4.0, respectively, but widely different in terms of molecular weight (808 versus 344 Da) and hydrogen-bonding potential (16.5 vs 6.5 sites). The relationships between physical properties and permeabilities by the perfusion and CACO-2 methods have been presented in preliminary form (16,17,26,28). Relationships were examined between permeability and lipophilicity (partition coefficient), molecular weight and hydrogen bonding functionality. In general, permeability of the peptidomimetics increased with increasing lipophilicity, and was inversely related to increasing molecular weight of H-bonding; however, these relationships were not straightforward (27).

The correlations between the permeabilities obtained from each of the three experimental systems are shown for the reference compounds, as well as the PM analogs, in Figure 4. Log-log plots were used to better depict the wide

Table III. Permeability of Peptidomimetics: Everted Ring, CACO-2 and Perfusion Permeabilities[a]

Compound	10^4 * Permeability (cm/min)		
	Rings	CACO-2	Perfusion
I	109 ± 14.0	14.8 ± 0.9	27.1 ± 2.5
II	316 ± 98.3	40.0 ± 6.7	88.5 ± 9.5
III	19.9 ± 6.8	16.1 ± 1.2	12.1 ± 1.6
IV	15.9 ± 6.5	3.4 ± 0.8	21.6 ± 3.2
V	54.2 ± 21.0	4.2 ± 0.1	90.8 ± 10.3
VI	36.7 ± 21.1	5.4 ± 0.5	5.9 ± 0.9
VII	212 ± 109	51.2 ± 8.6	82.0 ± 8.2
VIII	nd	3.9 ± 0.2	2.6 ± 0.7

[a] Values for perfusion experiments represent the mean of 4 to 8 animals; for CACO-2 experiments, the mean of 3 to 6 cell monolayers; for ring experiments, the extrapolated value from 3 time courses where each time course consisted of 21 to 27 rings from one animal. Standard errors are tabulated with the mean values.

range of permeabilities observed. Permeability results are summarized for the reference compounds and PM analogs in Tables I and III, respectively. In each plot, a regression line was drawn for the reference compounds. The reference line can be used as a basis to compare the correlation between the models for small organic molecules versus more complex peptidomimetics. The models can be compared in terms of absolute permeabilities, as well as relative to the regression line for reference compounds.

Results from CACO-2 and perfusion studies are depicted in Figure 4. CACO-2 permeabilities were generally higher for the peptidomimetics than would have been predicted by the reference compound correlation, and perfusions were somewhat lower. For compounds such as these, with very high lipophilicity, lower $^{o}P^{*}_{eff}$ may be the result of higher aqueous resistance and in turn, effective resistance to transport (29). Excellent correlation was observed between systems for the reference compounds (r = 0.99), although this decreased when all compounds were regressed together (r = 0.77). The only PM falling below the reference line is V, which has the highest partition coefficient (log P 4.7) in the set, but intermediate molecular weight and relatively low hydrogen-bonding potential. In terms of absolute numbers, perfusion permeabilities were higher than CACO-2 for the reference compounds, with the exception of prednisolone/hydrocortisone for which values were comparable. Perfusion values were higher than or comparable to CACO-2 values for the PMs. This observation is more clearly illustrated in Figures 3 and 5, where the rank orders of reference compound and PM permeability are compared. This finding differs from that of Kim et al (5) in which the permeabilities for a set of model peptides perfused through rat intestine by recirculation were less than that of the CACO-2 permeabilities. The difference may be explained in that the earlier work obtained permeabilities after nonsteady-state perfusion and measured appearance of compound in the mesenteric circulation, whereas in this report, perfusion provided steady-state drug

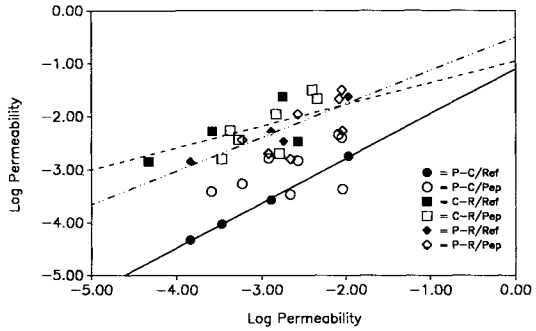

Figure 4. Comparison of Experimental Systems, Double-Log Plot of Permeabilities, where P = Perfusion, C = CACO-2, R = Ring and Ref = Reference, Pep = Peptidomimetic compounds. Prednisolone and hydrocortisone have been treated as same compound for purposes of model correlations. Values for perfusion experiments represent the mean of 4 to 8 animals; for CACO-2 experiments, the mean of 3 to 6 cell monolayers; for ring experiments, the extrapolated value from 3 time courses where each time course consisted of 21 to 27 rings from one animal. Standard errors are tabulated with the mean values. Regression lines are shown for Reference compound permeabilities: ●—● = P-C/ref, r-value: 0.99, ■--■ = C-R/ref, r-value: 0.65, ◆ -◆ = P-R/ref, r-value: 0.95.

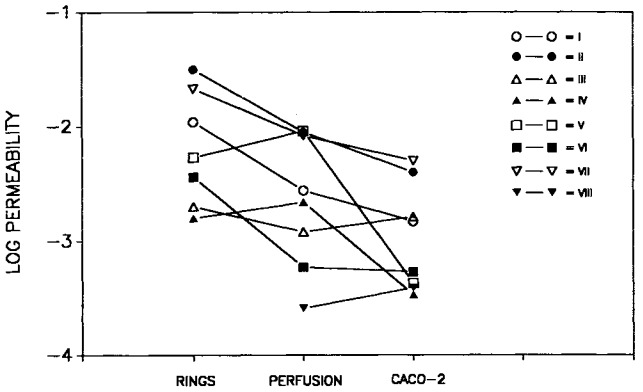

Figure 5. Rank Order Comparison Between Absorption Models for Peptidomimetic Compounds. Eight curves representing the rank order of log permeability determined in the three absorption models are shown.

input and permeability was assessed by disappearance of drug from the intestinal lumen.

Ring and CACO-2 permeabilities are also compared in Figure 4 and provided the lowest correlation between systems for both reference and PM compounds (r = 0.65 to 0.69). Although data points are somewhat evenly distributed around the regression line, the scatter is pronounced and there is no apparent trend to the variance. Absolute permeabilities of the reference compounds were higher in rings than in CACO-2, except for prednisolone and hydrocortisone, which had comparable values (Figure 3). All PM permeabilities were higher from everted ring than from CACO-2 with the exception of Compound III (Figure 5). It is of interest to note that the physicochemical properties of Compound III are very close to those of the passively absorbed reference compounds, prednisolone and hydrocortisone; i.e., log P: 1.5, H-bond: 8, molecular weight 300 vs 360). It is also noteworthy that if prednisolone and hydrocortisone were omitted from this data set, the r-value would rise to 0.99 for the reference compounds (figure not shown). This observation suggests that a significant difference in permeability measurement exists between everted rings and CACO-2 cells for compounds that are absorbed by a transcellular mechanism.

Comparison between ring and perfusion permeabilities also provided good correlation for reference compounds (r = 0.95) which weakened when the PMs were added to the regression (r = 0.78) (Figure 4). Data points were evenly distributed around the regression line for reference compounds, with greater confidence from the higher correlation coefficient suggesting similar behavior for reference and PM compounds. Permeabilities from ring experiments were higher than or comparable to values obtained from perfusions for both reference and PM compounds.

CONCLUSIONS

For small organic molecules, good-to-excellent correlation between systems was observed with permeabilities obtained from everted rat intestinal ring uptake, rat *in situ* intestinal perfusion and CACO-2 cell monolayers. Each model also had strong potential for predicting the fraction absorbed, although the everted ring system was least charac-

terized due to fewest data points. Permeabilities for the sets of reference and PM compounds were positively correlated between the experimental systems. Results suggested good agreement between the three methods in ranking test compounds. Although the reference compounds span a wide range of physicochemical properties, several of the more polar reference compounds are absorbed by carrier-mediated mechanisms which are not available to the PMs. Variance between correlations for reference compounds and PMs are likely due to structural features and physicochemical properties that are unique to the latter class of compounds. The results support caution in extrapolating correlations based on findings with small organic molecules to the behavior of complex peptidomimetics. Corroboration of permeabilities with two methods of determination is a useful cross-validation of experimental systems, as well as producing a reliable permeability assessment. CACO-2 cell monolayers and rat single-pass intestinal perfusion combine the highest correlation between systems, most defined relationship with fraction absorbed in humans, and experimental logistics in-line with discovery candidates.

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