Effects of Structural Modifications on the Intestinal Permeability of Angiotensin II Receptor Antagonists and the Correlation of *In Vitro, In Situ,* and *In Vivo* Absorption

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Purpose. The effects of structural modifications on the membrane permeability of angiotensin II (Ang II) receptor antagonists and the usefulness of *in vitro* and *in situ* intestinal absorption models in predicting *in vivo* absorption or bioavailability were investigated.

Methods. Intestinal permeability was determined *in vitro* using Caco-2 cell monolayers and *in situ* using a perfused rat intestine method. Several physicochemical parameters were either measured or computed, and correlated with intestinal permeation.

Results. Permeation coefficients (Pa) across Caco-2 cell monolayers correlated well with both *in situ* absorption rate constants (ka) and *in vivo* bioavailability or % absorption. For these Ang II antagonists, Pa values larger than 3×10^{-6} cm sec^{-1} and *in situ* ka values of 2×10^{-4} min^{-1} cm^{-1} or above were associated with good *in vivo* absorption. Structural modifications at the R_5 position, where a COOH group was substituted with either a CHO or CH_2OH group, enhanced the permeability of the Ang II receptor antagonists up to 100-fold. There were good correlations between permeability and log P(octanol/buffer), log P_{HPLC}, charge, solvation/desolvation energy and assigned hydrogen bonding potential.

Conclusions. The correlations obtained in this study indicate that both the Caco-2 cell model and the *in situ* perfused rat intestine could be used to predict intestinal absorption *in vivo.* Structural modifications of the Ang II antagonists had a significant impact on the intestinal permeability. Charge, solvation energy, and hydrogen bonding are predominant determinants of intestinal permeability and oral bioavailability of these compounds.

KEY WORDS: Caco-2 cells; intestinal absorption; lipophilicity; solvation energy; hydrogen bonding; bioavailability.

INTRODUCTION

The causes of poor oral bioavailability, a formidable obstacle in the development of new drug therapies, may include low water solubility, poor intestinal permeation, and presystemic metabolism or elimination. Many peptides and structurally related nonpeptides have poor intestinal permeation. An understanding of the factors that influence intestinal absorption can be gained from absorption models, such as the *in situ* perfused rat intestine or Caco-2 cell monolayers, an *in vitro* cell culture model derived from human colon carcinoma. These models are useful in drug discovery programs to provide information on how the structure or physicochemical properties of compounds (e.g. partitioning, charge) affect absorption. The usefulness of these models in drug design relies on the establishment of correlations between *in vitro* or *in situ* permeation and *in vivo* absorption, and between permeation and some physicochemical property. Permeation in an *in situ* perfused rat intestine model has been correlated with percentage absorption in humans (1), as has permeability across Caco-2 cell monolayers (2). Correlations of permeabilities in Caco-2 cell and *in situ* perfused rat intestine models have also been demonstrated (3,4). However, comparisons of these two models as predictors of *in vivo* absorption are only sparingly available in the literature.

This study involves a new category of antihypertensive drugs known as angiotensin II (Ang II) receptor antagonists. Losartan (DuP 753) is the first Ang II antagonist introduced to the market. The Ang II antagonists are nonpeptides, but they mimic a portion of the structure of Ang II, a peptide. Some of the Ang II antagonists have essentially complete oral bioavailability, whereas others have low oral bioavailability (5–12). This study was undertaken to assess the effects of structural variations on the membrane permeability of a series of Ang II receptor antagonists using the *in situ* perfused rat intestine and the Caco-2 cell models. Because *in vivo* bioavailability data exist in several species for these compounds and for those serving as positive and negative controls (5–15), we present correlations between both *in vitro* and *in situ* models and *in vivo* bioavailability in rats, dogs and, where possible, humans.

For a molecule to passively permeate the intestinal epithelium it must partition into the cellular membrane. The energetics of this transfer process is dependent on the strength of the molecule's interactions with water and lipid. Experimentally, the energetics of this transfer has been approximated by octanol/water partition coefficients ($P_{octanol/water}$ or log P). Caco-2 cell permeation rates for separate groups of structurally diverse drugs and model compounds correlated with their log P values (2). However, for a series of model peptides there was no correlation between log P values and *in situ* permeation rates (4). The *in situ* permeation of these compounds did correlate with the number of hydrogen bonds the peptides could make with water (4), or with partitioning between heptane and ethylene glycol (17). Computed molecular descriptors like computed log P (cLog P) and the number of hydrogen bond donor and acceptor groups in a molecule have been correlated with or used to predict membrane permeability (17–21). Although these simple computational approaches have been predictive of permeability for several groups of compounds, assigning these parameters, in particular hydrogen bond number, can be arbitrary for some functional groups and fragments. An alternative computational approach is to determine the solvation/desolvation energy of a molecule. Computed solvation energy represents the energy necessary to transfer a molecule from the gas phase to an aqueous environment. While not directly related to the energetics of partitioning into the membrane, solvation energy does reflect the process of "stripping" a molecule from

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its waters of hydration and it is therefore indicative of hydrogen bonding potential.

In this study we have examined the relationships between membrane permeability of nonpeptide Ang II antagonists and typically determined physicochemical parameters (log P, log P_{HPLC}, charge, hydrogen bonding). In addition, we present correlations of computed solvation energies and molecular surface areas with membrane permeability.

MATERIALS AND METHODS

Materials

The Ang II antagonists, DMP 728, and DuP 996 were synthesized at The DuPont Merck Pharmaceutical Company. Propranolol was obtained from Sigma Chemical Co. ^{14}C-PEG 4000 and ^{14}C-mannitol were obtained from the DuPont Company, NEN Research Products (Boston, MA). Caco-2 cells were grown on Costar Transwell® inserts (4.7 cm^2 surface area and 0.4 μm pore size) using standard tissue culture conditions (2–4).

Caco-2 Transport Studies

The integrity of the Caco-2 monolayers was monitored by determining transepithelial electrical resistance (TEER) before each permeation experiment and ^{14}C-PEG 4000 or ^{14}C-mannitol permeation after each study (excluding monolayers with >1% permeation per hour per well). Normal TEER values were 270–300-Ω cm^2. The flux across empty filter inserts was also measured for prototypical compounds (losartan, DMP 811, and XM970) and in each case the cell monolayer was demonstrated to be the rate-limiting permeability barrier. The transport of each Ang II antagonist across confluent Caco-2 cell monolayers was tested in triplicate at three different concentrations (typically 10^{-3}, 10^{-4} and 10^{-5} M) at pH 7.4. The positive control compounds, propranolol and DuP 996, were tested at lower concentrations because of their high membrane permeability. Drug concentrations were determined using HPLC. The amount transported across the monolayer per unit time (flux) was determined. Flux was corrected for the total monolayer surface area and initial drug concentration to give the permeation coefficient (Pa).

In Situ Perfusion Methods

Fasted male Sprague-Dawley rats weighing 250–325 g were anesthetized with i.p. sodium pentobarbital and a section of the small intestine, 10 cm from the pylorus and approximately 20 cm long, was isolated without disrupting the mesentery. Silicone tubing was inserted through incisions at each end of the segment and tied in place. A 9 ml perfusion solution (0.1 mM drug in pH 7.4 buffer) was held in a reservoir maintained at 37°. The perfusion solution was circulated through the intestinal segment and back into the reservoir. The flow rate was 2.8 ml/min. Samples (0.075 ml) were removed from the reservoir over a 120 min period. There was no replacement of the sample volume. At the end of the experiment the intestine and tubing were drained and the volume of perfusate remaining was determined to provide an estimate of solvent absorption. The actual length of intestine perfused was measured. The amount (nmoles or %) remaining in the perfusate was plotted versus time on semi-logarithmic coordinates. The first-order decay rate constants were divided by the intestine length to give the apparent absorption rate constant per unit length, ka. This research was done in compliance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Octanol/Buffer Log P and Log P_{HPLC} Measurements

Each Ang II antagonist was prepared as a 0.1 mM solution in 0.1 M phosphate buffer at pH 7.4. This was equilibrated with an equal volume of n-octanol. Phases were separated and assayed by HPLC. Log P is the logarithm of the ratio of the concentration in octanol to the concentration in buffer. Another measured index of lipophilicity was based on HPLC retention time, using methods similar to those described previously (22). The HPLC system consisted of a 25 cm × 4.6 mm octadecylsilane column (Zorbax SB C18, Macmod), UV detection, and mobile phases containing varying proportions of methanol and 0.02 M, pH 7.4, phosphate buffer. The capacity factor was determined as $(t_x-t_0)/t_0$, where t_x is the compound's retention time and t_0 is the retention time of the void volume. Capacity factors were plotted versus mobile phase methanol concentrations on semi-logarithmic coordinates. Extrapolation to 0% methanol gives log P_{HPLC}, which is representative of the compound's partitioning between the stationary phase of the column and pH 7.4 buffer.

Computational Methods

For each molecule, a single three-dimensional conformation was built and the geometry was optimized using a molecular mechanics method (SYBYL Molecular Modeling System, Version 6.03, TRIPOS Associates, St Louis, MO). For DMP 728, the NMR solution confirmation was used. Solvation energies were then calculated using two methods (available from Biosym Software Insight II, Biosym Technologies, Inc. San Diego, CA); Cramer and Truhlar's SM2 method (23,24) as implemented in the AMSOL computer program (method A) and the Eisenberg and Wesson method implemented with Sharp et al.'s parameterization (25,26) (method B). Molecular charges were assigned based on the molecule's pKa(s) and assuming a physiological pH of 7–7.4. Partial atomic charges were computed using the AM1 method as implemented in the J. J. P. Stewart MOPAC 5.0 semi-empirical software (Quantum Chemistry Program Exchange, Indiana University, Bloomington, IN, QCPE #455). Molecular surface areas were calculated using the Eisenberg and Wesson method (26). For DuP 996, clog P was calculated using Medchem software Version 3.54. (Pomona College). The number of hydrogen bonds (N) were estimated according to Stein (19) and by assigning N = 4 for the tetrazole ring, N = 0.5 for carbonyl ester, and N = 1 for CF_2CF_3 and all other substituents.

Data Analysis

There were single determinations of log P (octanol/buffer), log P_{HPLC}, and of the computed parameters, hydrogen bond number, solvation energy, and molecular surface area. The values of Caco-2 Pa and *in situ* ka represent the mean ± SD. Correlation coefficients (r) were determined using linear regression.

RESULTS

Caco-2 Permeation

The structures of DuP 996, DMP 728 and the Ang II antagonists examined are shown in Figure 1. DuP 996 and propranolol were evaluated in the Caco-2 cell model as representative well-absorbed drugs. DMP 728 is a representative poorly-absorbed drug. For each Ang II antagonist the amount permeating versus time was proportional to the donor concentration, with the exception of DuP 532, for which most samples were below the limit of detection. Since permeation coefficients (Pa) were concentration-independent, the Pa values at the various concentrations were averaged. Table I shows Pa values for all Ang II antagonists, and for the model compounds used for comparison. The Ang II antagonists had a broad range of permeabilities through Caco-2 cell monolayers with Pa values of the most permeable compounds being 100 to 1000-fold greater than those of the least permeable analogs. DuP 532, DMP 811, and E3174 had permeabilities similar to PEG 4000.

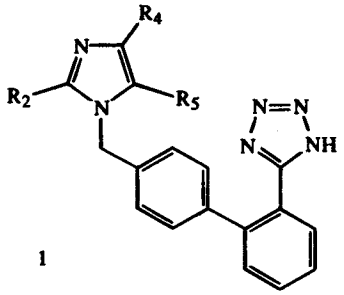
Absorption in Rat Intestinal Perfusion Model

The profiles of drug disappearance from rat intestinal perfusates *in situ*, when plotted using semi-logarithmic coordinates, represent apparent first-order absorption rate constants. The apparent absorption rate constants (ka) are shown in Table I. As with the Caco-2 epithelial permeation model, there was a broad range of *in situ* absorption rates within this series of Ang II antagonists, although the difference between the least and most permeable compounds was less than that for the Caco-2 model. There was a good correlation (r = 0.92) between *in situ* ka and *in vitro* Caco-2 Pa values, as shown in Figure 2.

Correlations Among Permeation, Bioavailability, and Physicochemical Properties

In vivo percentages of oral absorption and bioavailability for these Ang II antagonists are also given in Table I. In general, these Ang II antagonists are not subject to first-pass metabolism, so bioavailability is similar to the percentage absorption. Several of these compounds had similar bioavailabilities when dosed in solution or as a solid powder, so dissolution was not limiting bioavailability. Bioavailability (F) was similar in rats and dogs, and for losartan the bioavailability in humans was similar to the rat and dog values. Both Caco-2 Pa values (on a logarithmic scale) and *in situ* rat intestinal perfusion ka values correlated well (r = 0.97 and 0.92, respectively) with F in rats (Figure 3).

One of the goals of this study was to identify relationships between structural features or physicochemical properties of these compounds and their membrane permeabilities. The substituent at the R_5 position (Figure 1) greatly influenced intestinal



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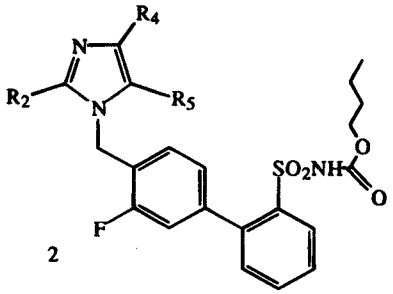

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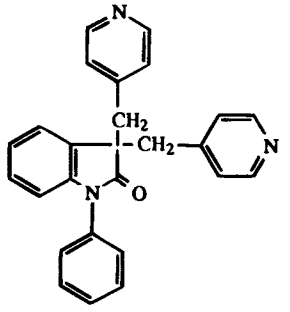
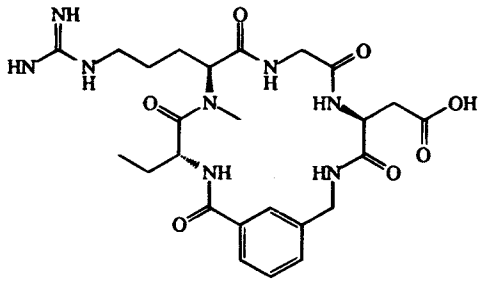
Compound	Structure	R_2	R_4	R_5
Losartan	1	nC_4H_9	Cl	CH_2OH
E-3174	1	nC_4H_9	Cl	COOH
DMP 581	1	nC_3H_7	C_2H_5	CHO
DuP 532	1	nC_3H_7	CF_2CF_3	COOH
DuP 167	1	nC_4H_9	Cl	CHO
DMP 811	1	nC_3H_7	C_2H_5	COOH
XM970	2	nC_3H_7	C_2H_5	CH_3CO



DuP 996



DMP 728

Fig. 1. Structures of the Ang II antagonists tested (templates 1 and 2) and two reference compounds used, DuP 996 (well absorbed) and DMP 728 (poorly absorbed).

Table I. *In vitro* Caco-2 cell permeation coefficients (Pa), *in situ* rat intestinal perfusion absorption rate constants (ka), and *in vivo* absorption characteristics for Ang II antagonists and reference compounds

Compound	*In Vitro* Caco-2 Pa (cm/sec) × 10^6 Mean ± SD	*In Situ* Perfusion ka ($min^{-1}cm^{-1}$) × 10^4 Mean ± SD	% Absorption in Rats (Radiolabel Study)[a,b]	% F in Rats[b]	% F in Dogs[b]	% F in Humans[b]
DMP 728	0.35 ± 0.19	ND[c]	2	2–4	5–12	ND
^{14}C-PEG	0.02[e]	ND	ND	ND	ND	ND
DuP 532	0.009[e]	0.56 ± 0.04	6[d]	8	13	ND
DMP 811	0.021 ± 0.006	1.1 ± 0.1	ND	8	13	ND
E3174	0.069[e]	0.4 ± 0.1	ND	12	ND	ND
Losartan	1.15 ± 0.33	2.2 ± 0.03	41[d]	33	27	35
DMP 581	4.20 ± 2.38	4.4 ± 0.2	ND	60	28	ND
XM970	6.35 ± 1.95	6.6 ± 0.6	ND	52[f]	ND	ND
DuP 167	9.72 ± 0.19	5.6 ± 1.1	ND	60	28	ND
DuP 996	29.0 ± 0.62	ND	80	60[g]	93[g]	38[h]
Propranolol	26.7 ± 0.48	ND	100	ND	27[i]	26[i]

[a] Based on amount of radioactivity excreted in urine and bile.
[b] After solution administration (see references 7–22).
[c] Data not available.
[d] Personal communication, R. M. Williams, DuPont Merck Pharmaceutical Co.
[e] Results from one donor concentration.
[f] Personal communication, T. A. Emm, DuPont Merck Pharmaceutical Co.
[g] Personal communication, D. C. Rakestraw, DuPont Merck Pharmaceutical Co.
[h] Personal communication, W. D. Fiske, DuPont Merck Pharmaceutical Co.
[i] Subject to dose-dependent first-pass elimination.

permeability, with DuP 167 > losartan > E3174, which had respectively, CHO, CH_2OH, and COOH at R_5, but were otherwise identical. DMP 581 and DMP 811 were also structurally identical except at R_5, and permeabilities again had the relationship CHO >> COOH. XM970 was structurally dissimilar, with a sulfonamide replacing the tetrazole, and a methyl ketone at R_5. These changes did not adversely affect permeability. The three compounds with poorest absorption, DMP 811, DuP 532, and E3174, all have a COOH group at position R_5, in addition to the anionic tetrazole group.

The physicochemical properties determined for this series of Ang II antagonists were the octanol/pH 7.4 buffer partition coefficient (log P), a lipophilicity index based on HPLC retention time (log P_{HPLC}), solvation energy, hydrogen bond number, and charge. Values for these determinations are reported in Table II. The correlations between each physicochemical determinant and *in vitro* or *in situ* permeabilities were assessed. Generally, correlations were better when the log of the Pa or ka values were used. The correlation coefficients (r) given in Table II used log Pa or log ka. Intestinal permeability (Pa or ka) was well correlated with the indices of lipophilicity (log P or log P_{HPLC}). Figure 4 illustrates the correlation of Caco-2 Pa and log P. Log P (octanol/buffer) provided better correlations than log P_{HPLC}. Hydrogen bond number strongly correlated with intestinal permeabilities as well (r > 0.9). Calculated solvation energies were also highly correlated with intestinal permeability (r = 0.90 and 0.93 for Pa vs. solvation energy by methods A and B, respectively; r = 0.88 and 0.93 for ka vs. solvation energy by methods A and B, respectively). In general, the Ang II antagonists with two charged groups had poor permeability and those with a formal charge of one or zero had good permeability. Log P and solvation energy are related to the formal charge, indicating that the partition coefficients and solvation energies of these compounds are strongly dependent on electrostatics. Solvation energies were also correlated with the measured log P (octanol/buffer) values (r = 0.89 using method A; r = 0.98 using method B). Hydrogen bonding, partitioning, and solvation energies are interrelated, since each is reflective of the strength of drug/solvent interactions.

The surface areas of these molecules are generally within 10–20% of each other and are therefore not expected to be a determining factor in the permeability of this specific set of molecules. The molecular weight of the compounds in this

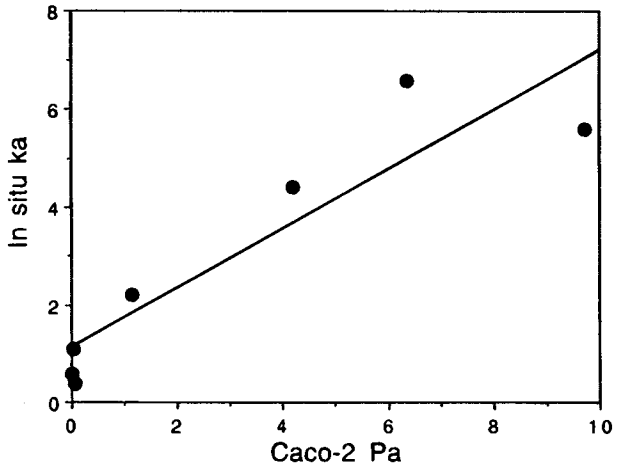


Fig. 2. Correlation between the *in vitro* Caco-2 permeation coefficients (Pa) and *in situ* intestinal absorption rate constants (ka) of Ang II antagonists.

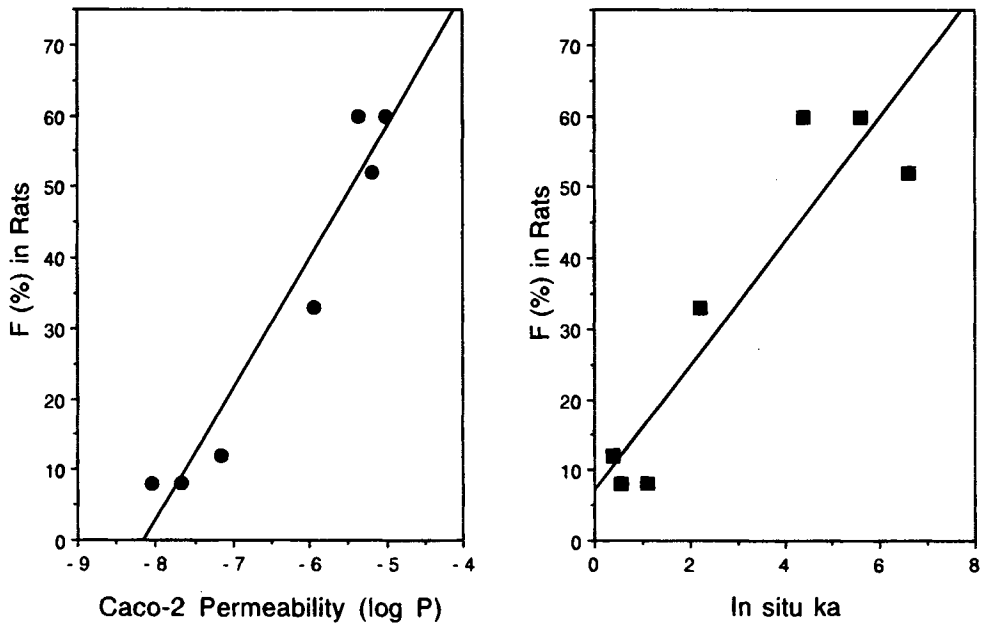


Fig. 3. Correlation between Caco-2 Pa values or *in situ* rat intestinal perfusion ka values and oral bioavailability in rats, for various Ang II antagonists.

series are not significantly different and are all below 550 daltons. Molecular weight was therefore not be expected to be a discriminating factor for membrane permeability.

DISCUSSION

The data summarized in Table I and Figure 3 indicates that the Caco-2 cell model and the *in situ* perfused rat intestine model yield comparably predictive data and either could be used to assess the *in vivo* intestinal absorption of related compounds. Although intestinal permeation is only one of several processes that can influence bioavailability, it is the critical determinant of oral bioavailability for this series of compounds.

The relationship between Caco-2 Pa values or *in situ* intestinal perfusion ka values and bioavailability, as shown in Figure 3, have been sigmoidal in other studies. We did not have any Ang II antagonists with permeability as great as DuP 996 or propranolol, the well absorbed reference compounds. If so, a

Table II. Correlations Among Measured or Computed Physicochemical Properties and Intestinal Permeability

Compound	Molecular Weight	Molecular Charge	Log P Octanol/ pH 7.4	Log P HPLC	H-Bond Number[a]	Solvation Energy		Molecular Surface Area
						Method A	Method B	
DMP 728*	560.6	+/−	−1.9	1.9	8	−136.2	−34.4	772
DuP 532	506.4	−2	−1.31	3.7	9	−162.9	−15.4	632
DMP 811	416.2	−2	−0.63	3.3	8	−174.2	−15.5	631
E3174	436.1	−2	−1.22	3.9	8	−177.9	−16.2	659
Losartan	461.0	−1	1.19	4.2	7	−69.6	−8.8	626
DMP 581	400.0	−1	1.03	4.2	6	−14.7	−7.9	627
XM970	543.7	−1	1.72	4.0	6.5	−66.6	N.D.	701
DuP 167	460.4	−1	1.35	4.2	6	−68.5	−8.5	606
DuP 996*	391.5	0	2.9[b]		3	−10.9	−0.01	620
Propranolol*	295.8	+1	3.1[c]		3	−50.1	−0.02	508
Correlation (r) with log *in vitro* Pa[d]			0.94	0.82	0.97	0.90	0.93	0.07
Correlation (r) with log *in situ* ka[d]			0.96	0.59	0.91	0.88	0.93	0.06

[a] Number of hydrogen bonds (N) was estimated by assigning N = 4 for tetrazole ring, N = 0.5 for carbonyl ester, N = 1 for CF_2CF_3 and all other substituents.

[b] Log P calculated using Medchem software Version 3.54, Pomona College.

[c] Measured log P from Zaagsma, J. and Nauta, W. J. Med. Chem. 17, pp 507, 1974.

[d] Compounds with* were not included in the correlation and are shown for comparison only.

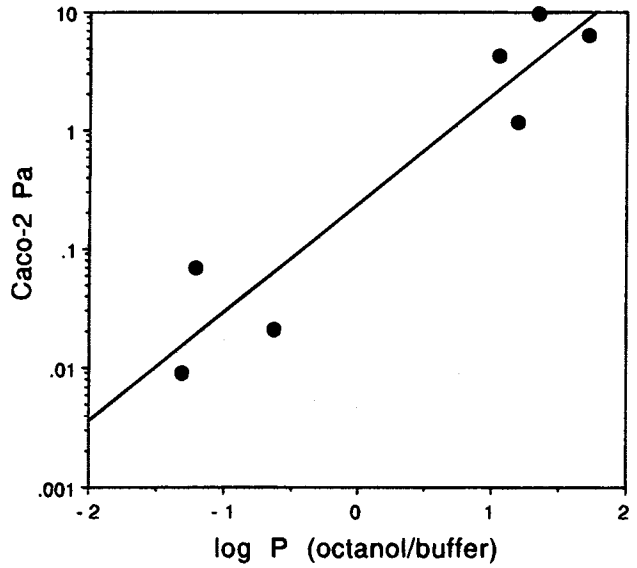


Fig. 4. Correlation of Caco-2 permeability and log P (octanol/buffer) for a series of Ang II antagonists.

plateau in the permeability vs. F plot would probably have been seen.

Our correlations between permeability and lipophilicity are consistent with passive diffusion through the cell membranes as the predominant mechanisms of absorption for the moderately and well absorbed Ang II antagonists. These compounds are monoacidic with pKa values in the 4–5 range. The poorly absorbed compounds were all diacidic. These may be absorbed primarily by a paracellular mechanism. One difference between the Caco-2 and *in situ* rat intestinal perfusion models was in the separation between the least and most permeable compounds. This difference was 100-fold for Caco-2 Pa values, but only about 10-fold for *in situ* ka values (see Table I). It has been suggested that Caco-2 epithelial membranes have more discriminating tight junctions than non-cultured intestinal membranes, which was evident in greater electrical resistance and lower permeability to low molecular weight, hydrophilic drugs such as atenolol (3).

Recent studies using Caco-2 cell monolayers (20, 21, 28) demonstrated that hydrogen bonding is a major determinant of passive transcellular permeability for model peptides. Partitioning systems more discerning of hydrogen bonding potential (e.g. the difference between octanol/water and cyclohexane or isooctane/water partition coefficients, or heptane/ethylene glycol partition coefficients) may be better correlated with membrane permeability than octanol/buffer partition coefficients (17, 18). However, for the Ang II antagonists examined in this study, log P (octanol/buffer) or hydrogen bond numbers correlated with intestinal permeability.

We also tested another potentially useful approach in which the solvation energies of these analogs were calculated and correlated with either their physicochemical properties or their membrane permeabilities. Desolvation energy has been demonstrated to be predictive of intestinal permeabilities of some peptides (28). Good correlations were observed between computed solvation energies and permeability or *in vivo* bioavailability. Very good correlations between solvation energy and hydrogen bond number or log P were also obtained for this set of structurally diverse molecules. Because the solvation energies within this series of compounds are strongly related to charge, we have not addressed whether solvation energies are predictive of permeation for a series of molecules which have no formal charge.

In conclusion, our data affirms that Caco-2 cells and the *in situ* perfused rat intestine are good models for predicting the *in vivo* oral drug absorption of these Ang II antagonists. This study also demonstrates the utility of determining and manipulating the physicochemical properties of drug candidates and analyzing their effects on membrane permeability. A certain degree of lipophilicity is essential for membrane permeation. As with peptides, low solvation energies of these Ang II antagonists, which are reflective of low hydrogen bonding potential, favor absorption.

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