In Vitro/in Vivo Models for Peptide Oral Absorption: Comparison of Caco-2 Cell Permeability with Rat Intestinal Absorption of Renin Inhibitory Peptides

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INTRODUCTION

The routine design and development of peptides as orally bioavailable therapeutic agents remain a significant challenge to the pharmaceutical scientist. Among the problems contributing to the generally low bioavailability of such molecules are poor transport across the intestinal mucosa, metabolism in both the intestinal lumen and the systemic circulation, and rapid first-pass clearance (1). In order to understand better the individual contribution and interplay among these various processes, it would be desirable to have appropriate model systems which allow for the convenient examination of one process independent of the others. It is further desirable that these models realistically predict the same events *in vivo*.

Consistent with this goal, we have recently reported the use of the Caco-2 cell monolayer system as an *in vitro* model for the study of peptide transport across the intestinal mucosa (2,3). Caco-2 cells are human colon adenocarcinoma cells which, when grown in culture, differentiate to form monolayers which look and behave much like human small intestinal epithelium (4,5). We have further reported the relationship between peptide permeability results obtained in the Caco-2 model and the oral absorption in the rat of a homologous series of model peptides (6).

While these preliminary results suggest that the *in vitro* model is indeed a reasonable paradigm for the *in vivo* rat, the peptides studied were structurally very similar. In order to establish the more general feasibility of the model, the present study was undertaken. Here we report the relationship of Caco-2 cell permeability with rat intestinal absorption for a heterogeneous series of synthetic peptides which were designed and synthesized as potential inhibitors of the enzyme renin, a key component in the regulation of blood pressure in humans (7).

MATERIALS AND METHODS

Peptides. The structures of the renin inhibitory peptides, synthesized at the Upjohn Company, are shown in Fig. 1. U-71038 (II), U-85087E (VII), and U-84656 (IV) were provided by S. Thaisrivongs (Cardiovascular Diseases Research) and U-86786 (VI) was from J. B. Hester (Cardiovascular Diseases Research). U-82159E (III) and U-85704E (V) were from T. K. Sawyer (Biochemistry) and U-77436 (I) was provided by G. L. Bundy (Medicinal Chemistry). Structural analyses were provided by the Physical and Analytical Chemistry Division.

Permeability Coefficient Determinations in Caco-2 Cell Monolayers. The preparation of confluent Caco-2 cell monolayers on Transwell polycarbonate filters has been described in detail previously (8). Passage numbers of the cells used in these studies were between 26 and 40. The monolayers were 14–21 days postseeding before use. Permeability coefficients (P_{mono}) were calculated from the steady-state rate of transfer of the peptide across the monolayers as described previously (8). Peptide concentrations were determined by HPLC (III, IV, VI) or bioassay (I, II, V, VII) by a previously described method (9). Experiments were performed in, at least, triplicate.

Peptide Oral Absorption in Rats.[3] The procedure for preparation and dosing of bile duct cannulated rats has been described in detail previously (6). Peptides were dissolved in 0.1 *M* citric acid for id administration or 0.003 *M* citric acid for iv dosing, except for the less soluble II and VII, which were prepared in 0.006 *M* citric acid for the iv dose. I, VI, and VII were administered to the rats in 4 mL/kg volumes of 0.25 or 1.25 m*M* solutions for the iv and id doses, respectively. I was used as a standard in each experiment. III, V, and IV, due to poor assay sensitivity, were given 4 mL/kg doses of 2 or 7.5 m*M* peptide iv or id, respectively. Finally, because of its poor solubility, the iv formulation of VII was administered as a more dilute solution (1 m*M* in 0.006 *M* citric acid, 8 mL/kg iv). Generally, four animals were dosed iv and six animals id for each peptide.

Bile was collected for 3 hr after peptide administration. A single terminal urine sample was obtained by aspiration of the bladder contents at sacrifice. Peptide concentrations in the bile and urine were determined by a renin activity assay as previously reported (9). IV, which was relatively inactive in the bioassay, was analyzed by HPLC. Absorption was calculated from the total peptide recovered from bile plus urine after oral administrated compared to that recovered after iv administration (10).

HPLC Methods. A Waters WISP 712 Autosampler was employed along with a Waters 600 pump, a Waters 490 multiwavelength detector, and a DEC-based Waters 840 Data and System Control Station. Columns used were either (A) a Shandon Hypersil BDS C-18 column, 5-μm particle size, 15-cm length, or (B) a Brownlee MPLC Speri-5 RP-18 column, 5-μm particle size, 13-cm length including the guard column. Injection volumes ranged from 50 to 1000 μL depending upon the concentrations being measured.

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[3] All procedures in this study are in compliance with the Animal Welfare Act Regulations, 9CFR Parts 1, 2, and 3, and with the *Guide for the Care and Use of Laboratory Animals* [DHEW Publication (NIH) 85-23, 1985].

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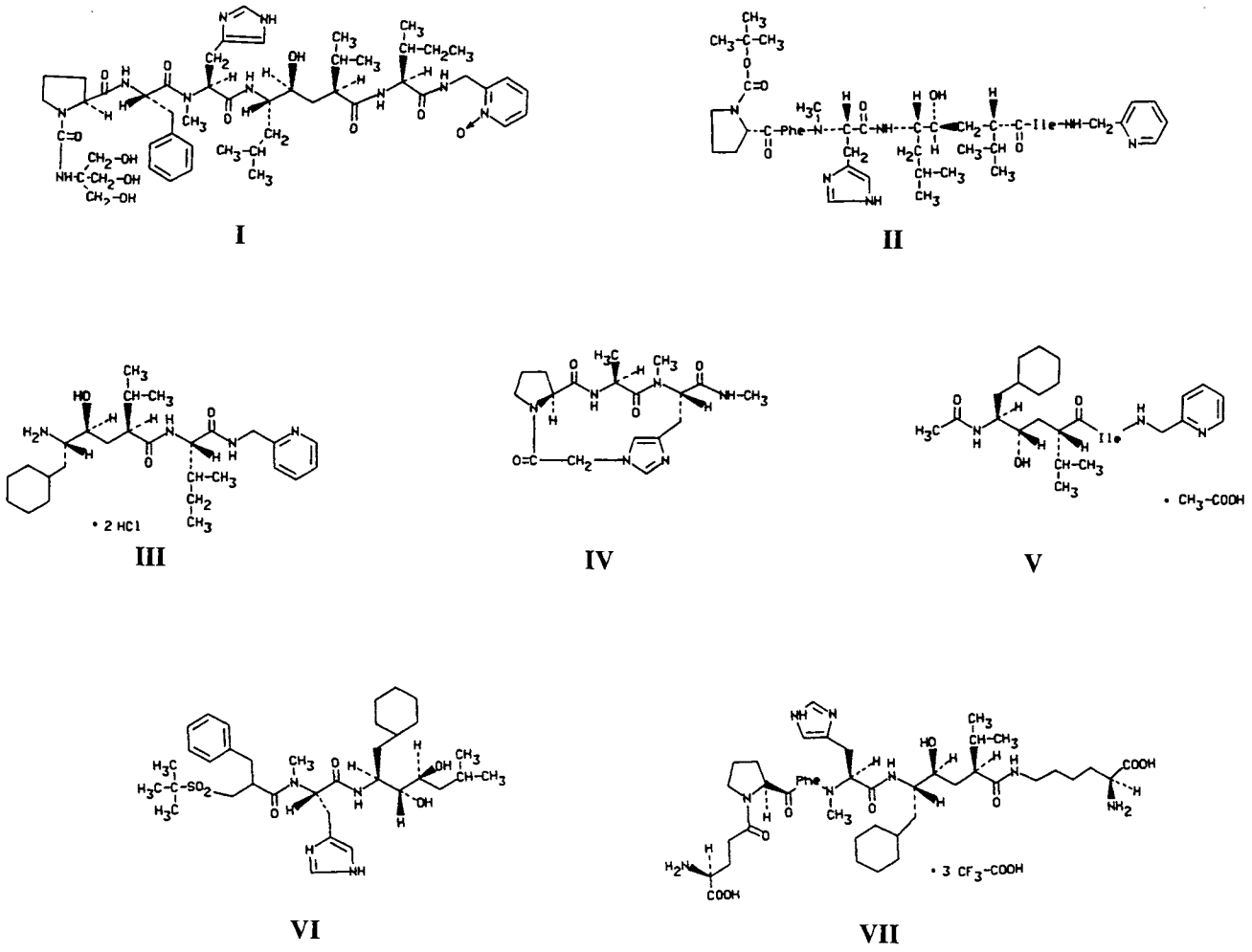

Fig. 1. Structures of the renin inhibitory peptides.

For III, column B was used with a mobile phase consisting of 80% water, 18% acetonitrile, 2% methanol, 0.08%H_3PO_4, and 0.025% *N,N*-dimethyloctylamine (DMOA). The flow rate was 1.5 mL/min, giving a retention time of 2.2 min with detection at 262 nm.

For IV, column A was used with a mobile phase consisting of 25% methanol, 75% 5 m*M* phosphate buffer, pH 7.3. The flow rate was 1.4 mL/min, giving a retention time of 2 min with peak detection at 206 nm.

For VII, column A was used with a mobile phase consisting of 40% water, 40% methanol, 20% acetonitrile, 0.06% H_3PO_4, and 0.02% DMOA. Detection was at 206 nm and the flow rate was 1.4 mL/min, giving a 4.4-min retention time.

For VI, column A was employed with a mobile phase of 82% methanol, 18% 2 m*M* phosphate buffer, pH 8. Detection was at 206 nm with a flow rate of 1.4 mL/min, to give a 3-min retention time.

RESULTS

Permeability coefficients, calculated from the steady-state cumulative flux of the peptides across the Caco-2 cell monolayers, ranged from 1.2×10^{-7} cm/sec (II) to 12.6×10^{-7} cm/sec (IV). Although not possible to ascertain without a specific assay, metabolism was not expected to be a complication during transport since these peptides were prepared to be inert protease inhibitors. In support of this, the total calculated mass of peptide in the donor and receiver compartments was generally equal to the initial mass at the beginning of the experiment.

For the *in vivo* studies, the absorption after duodenal administration ranged from low, about 3% for II, to significantly higher, 39% for III. In Fig. 2, the relationship between oral absorption and Caco-2 cell permeability is shown. It can be seen that, in general, the percentage absorption for the renin inhibitory peptides is paralleled by the permeability across the Caco-2 cell monolayers. This relationship is similar to that reported previously for a model series of D-phenylalanine oligomers (6). These earlier results are also included in Fig. 2.

DISCUSSION

For the initial estimation of the intestinal absorption potential of a solute, the *in vitro* Caco-2 cell model system has several advantages over *in vivo* measurements. In general, very small quantities of the solute are required. Further, since the transport experiments are performed in well-

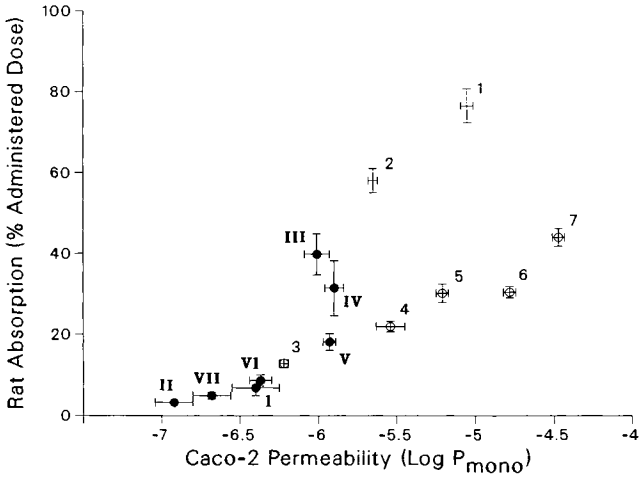

Fig. 2. Relationship of Caco-2 cell permeability with rat intestinal absorption for the renin inhibitors. Included are earlier results (6) for a homologous series of D-phenylalanine oligomers: $AcPheNH_2$ (1), $AcPhe_2NH_2$ (2), $AcPhe_3NH_2$ (3), $AcPhe_2(NMePhe)NH_2$ (4), $AcPhe(NMePhe)_2NH_2$ (5), $Ac(NMePhe)_3NH_2$ (6), and $Ac(NMePhe)_3NHMe$ (7).

defined buffer medium, HPLC analysis is uncomplicated by interference found in most biological fluids obtained from *in vivo* studies. These properties of the model make it attractive for comparing absorption potential within groups of several investigational compounds. However, in order to be generally useful, it is necessary to establish the correlation between the permeability results obtained from the *in vitro* Caco-2 experiments and the *in vivo* intestinal absorption gained in the whole-animal work.

Several reports have recently appeared attempting to establish such relationships. In one study, Caco-2 permeability coefficients were shown to compare favorably with rat intestinal permeability for a series of model β-blockers (11). The same author reported a significant correlation between *in vitro* Caco-2 cell permeability and oral absorption in humans (12).

In our earlier work with model peptides, we reported a good correlation between Caco-2 cell permeability and absorption in the rat. However, the model peptides used in that study were a homologous, structurally related series (6). The results presented here further substantiate our earlier correlation with a group of structurally dissimilar peptides.

In making comparisons between Caco-2 cells and the normal intestinal mucosa, it should be pointed out that the Caco-2 model is derived from a single population of cells and is, in effect, a cloned cell system. Thus it is quite different from the *in vivo* situation, where a very heterogeneous cell distribution is found, along with a mucus layer and other lumenal components (13), all of which may play a role in the transport of solutes. The good correlations obtained in this, and the earlier studies, suggest that the principal determinants of intestinal absorption are present in the much simpler Caco-2 cell monolayer system.

The two most significant outliers in Fig. 2 (1 and 2) are also the smallest peptides among the series. As pointed out earlier, the absorption in the rat for these small solutes was greater than that predicted from the Caco-2 cell permeability coefficients, which may suggest the presence of a larger population of effective "pores," resulting in a larger contribution from paracellular transport *in vivo* than in the *in vitro* model (6). This is supported by the higher electrical resistance found in the cell monolayer system than that present in the normal intestinal epithelium (4). If these two peptides are ignored, the remaining 12 yield a correlation coefficient (r^2) of 0.85 for the comparison between *in vivo* and *in vitro* absorption.

In summary, the good correlation between Caco-2 cell permeability and rat intestinal absorption for peptides of greater than two amino acids in length suggests the utility of the *in vitro* model for predicting the oral absorption potential of this class of molecules. As such, it should prove useful in the discovery and design of novel peptide therapeutic agents.

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