Transport Characteristics of Peptidomimetics. Effect of the Pyrrolinone Bioisostere on Transport Across Caco-2 Cell Monolayers

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Purpose. To compare the permeation characteristics of amide bond-containing HIV-1 protease inhibitors and their pyrrolinone-containing counterparts across Caco-2 cell monolayers, a model of the intestinal mucosa.

Methods. Transepithelial transport and cellular uptake of three pairs of amide bond-containing and pyrrolinone-based peptidomimetics were assessed in the presence and absence of cyclosporin A using the Caco-2 cell culture model. The potential of the peptidomimetics to interact with biological membranes was estimated by IAM chromatography.

Results. In the absence of cyclosporin A, apical (AP) to basolateral (BL) flux of all compounds studied was less than the flux determined in the opposite direction (i.e., BL-to-AP). The ratio of the apparent permeability coefficients (P_{app}) calculated for the BL-to-AP and AP-to-BL transport ($P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$) varied between 1.7 and 36.2. When individual pairs were ompared, $P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$ ratios of the pyrrolinone-containing compounds were 1.5 to 11.5 times greater than those determined for the amide bond-containing analogs. Addition of 25 μM cyclosporin A to the transport buffer reduced the $P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$ ratios for all protease inhibitors to a value close to unity. Under these conditions, the amide bond-containing peptidomimetics were at least 1.6 to 2.8 times more able to permeate Caco-2 cell monolayers than were the pyrrolinone-containing compounds. The intrinsic uptake characteristics into Caco-2 cells determined in the presence of 25 μM cyclosporin A were slightly greater for the amide bond-containing protease inhibitors than for the pyrrolinone-containing analogs. These uptake results are consistent with the transepithelial transport results determined across this *in vitro* model of the intestinal mucosa.

Conclusions. The amide bond-containing and pyrrolinone-based peptidomimetics are substrates for apically polarized efflux systems present in Caco-2 cell monolayers. The intrinsic permeabilities of the amide bond-containing protease inhibitors are slightly greater than the intrinsic permeabilities of the pyrrolinone-based analogs through Caco-2 cell monolayers.

KEY WORDS: peptidomimetics; pyrrolinone bioisostere; Caco-2 cells; membrane permeability; polarized efflux systems.

INTRODUCTION

In the past twenty years, endocrinologists, pharmacologists and neurochemists have identified many peptides with novel therapeutic potentials (1). However, because of their low permeation characteristics across biological barriers as well as their susceptibility to metabolism and to rapid elimination after parenteral administration, peptides have been difficult to develop as therapeutic entities (2,3).

The metabolic lability of peptides has been resolved in part by modifying the peptide bond using a variety of bioisosteres. The resulting peptidomimetics containing these bioisosteres are resistant to peptidase hydrolysis while retaining their biological activity [e.g., HIV-protease inhibitors (4), antithrombotic agents (5), angiotensin-converting enzyme inhibitors (6), and renin inhibitors (7,8)]. Nevertheless, medicinal chemists have not yet achieved the same degree of success in designing peptidomimetics that exhibit enhanced cellular permeation characteristics. Consequently, peptidomimetics, like peptides, show extremely low oral bioavailability, which can often be attributed to low permeation across the intestinal mucosa (9).

To limit peptide bond hydrolysis, chemists have developed a diverse array of bioisosteres that protect the molecules from peptidase-mediated metabolism (1,10–12). Of particular interest in this study is the pyrrolinone bioisostere mimicking the peptide bond (13). Smith and co-workers have demonstrated that crystalline 3,5-linked pyrrolin-4-ones can adopt backbone conformations strikingly similar to peptoidal β-strands (13). Moreover, the pyrrolinone N-H protons, although displaced from the backbone, were found to serve as interstrand hydrogen-bond donors, permitting these peptidomimetics to form parallel and antiparallel β-plated sheets in the solid state (13). The formation of hydrogen bonds involving their respective amide backbones plays a critical role in the binding of peptidic inhibitors to various proteolytic enzymes [e.g., HIV-1 protease (14)]. From the fact that pyrrolinone-based peptidomimetics can potently inhibit HIV-1 protease, it was concluded that pyrrolinone-based peptidomimetics can generate hydrogen bonds with the amide backbone of proteases. Therefore, pyrrolinone-based protease inhibitors may have significant impact in the treatment of important diseases where it is desired for a small molecule to bind in a β-strand-mimicking conformation to a protein.

To investigate what effect the replacement of the amide backbone by a pyrrolinone scaffold has on the cellular transport characteristics, we selected pairs of amide bond-containing and pyrrolinone-based peptidomimetics that exhibit substantial biological activity [i.e., HIV protease inhibitors (15)]. Two of these pairs were exact matching structures, whereas the third pair showed a slightly different side chain (i.e., leucine vs. valine, respectively) between the amide bond-containing peptidomimetic and its pyrrolinone-based counterpart. In this study, we

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ABBREVIATIONS: HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle medium; AP, apical; BL, basolateral.

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compare the transport properties and cellular uptake of amide bond-containing peptidomimetics and pyrrolinone-based analogs using Caco-2 cell monolayers, an *in vitro* cell culture model of the human intestinal mucosa (16–18).

MATERIALS

The amide bond-containing peptidomimetics (Table I, compounds I, III, V) and the pyrrolinone-based analogs (Table I, compounds II, IV, VI) were synthesized as described elsewhere (19–21). All compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and by appropriate parent ion identification by high-resolution mass spectroscopy. D-1[^{14}C]mannitol (spec. act. = 2.07 GBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Cyclosporin A, Dulbecco's phosphate buffer solution (D-PBS; powder form), Hanks' balanced salts (modified), and *N*-(2-hydoxyethyl)- piperazine-*N'*-2-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Co. (St. Louis, MO). L-Glutamine 200 mM (100×), penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and non-essential amino acids 10 mM (100×) in 0.85% saline were purchased from Gibco BRL, Life Technologies (Grand Island, NY). Dulbecco's modified Eagle medium (DMEM) was obtained from JRH Biosciences (Lenexa, KS). Rat tail collagen (type I) was purchased from Collaborative Biomedical Products (Bedford, MA), and fetal bovine serum from Atlanta Biologicals (Norcross, GA). All other chemicals and solvents were of high purity or analytical grade and used as received.

METHODS

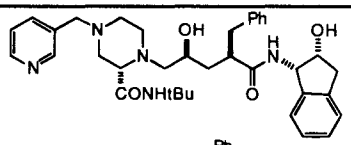
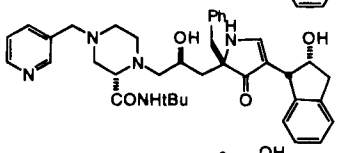
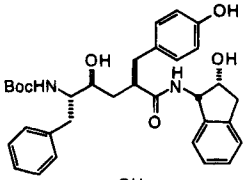
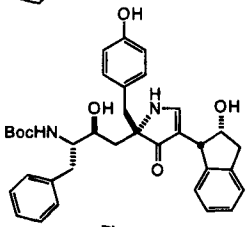
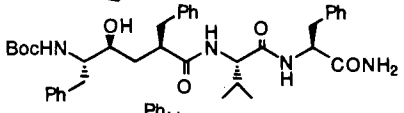
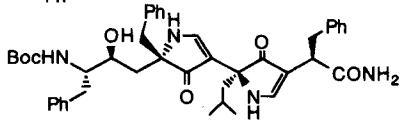
Caco-2 Cell Culture

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 18 and cultured as described previously (22). The cells used in this study were between passage 34 and 70. The integrity of each batch of cells was tested by measuring the flux of [^{14}C]mannitol in representative cell monolayers (n = 3). AP-to-BL flux for this paracellular marker never exceeded 1%/hr (Papp $\leq 3.2 \times 10^{-7}$ cm/s).

Transepithelial Transport Studies

The transepithelial transport of the peptidomimetics across Caco-2 cell monolayers was determined in triplicate at 37°C.

Table I. Chemical Structures and Physicochemical Properties of Amide Bond-Containing and Pyrrolinone-Based Peptidomimetics

Compound	Chemical Structure	MW	Membrane Interaction Potential[a] [log k'_{IAM}]
I		614	0.177
II		668	0.137
III		560	0.877
IV		584	0.826
V		658	0.708
VI		706	0.710

[a] Capacity factor determined from partitioning of the solute between 0.02 M phosphate buffer, pH 7.4/acetonitrile (78:22, v/v) and an immobilized artificial membrane of phosphatidylcholine analogs (IAM.PC.DD).

On the day of the experiment, cells were rinsed twice with prewarmed Hanks' balanced salt solution (HBSS, pH 7.4) and preincubated for 30 min at 37°C with HBSS or HBSS containing 25 μM cyclosporin A. The compound of interest dissolved in HBSS in the presence or absence of 25 μM cyclosporin A was applied to the donor compartment, and HBSS with or without 25 μM cyclosporin A was placed in the receiver compartment. Transport experiments were performed measuring the AP-to-BL and BL-to-AP flux of the peptidomimetics in the presence and absence of 25 μM cyclosporin A. The volumes added were 1.0 ml (AP) and 2.6 ml (BL), respectively. Final concentrations of the individual pairs of peptidomimetics were selected based on the aqueous solubility of the compounds, i.e., 200 μM (compounds I and II), 5 μM (compounds III and IV), and 10 μM (compounds V and VI). From the receiver compartment, aliquots (200 μl) were removed up to 120 min as described previously (22).

Cellular Uptake Studies

The uptake characteristics of the peptidomimetics into Caco-2 cells were measured in the presence of 25 μM cyclosporin A. Cell monolayers were preincubated as described for transepithelial transport studies. Uptake was initiated by adding 1.0 ml of the peptidomimetic stock solution in HBSS to the AP compartment, while 2.6 ml of HBSS was added to the BL compartment. Experiments were performed in triplicate with 25 μM cyclosporin A in both compartments. Final concentrations of the individual pairs of peptidomimetics were the same as used for the transepithelial transport studies. After an incubation period of 120 min, cell monolayers were washed 3 times with ice-cold HBSS to stop further uptake and to remove unbound compounds. Cells were scraped from the membrane and transferred in 500 μl ice-cold HBSS to a microcentrifuge tube. After adding 500 μl ethanol, cells were vortexed, sonicated for approximately 10 min and centrifuged for 5 min at 1000 × g. The supernatant was then subjected to HPLC analysis.

HPLC Analysis

The HPLC system (Shimadzu, Inc., Tokyo, Japan) consisted of a LC-6A pump, a SCL-6A controller, and a SPD-6A UV detector connected to a C-R6A Chromatopac integrator. The peptidomimetics were eluted under isocratic conditions using a Vydac C18 column (25 cm × 4.6 mm I.D., Hesperia, CA). The mobile phase (flow-rate = 1.0 ml/min) consisted of 30–45% (v/v) acetonitrile in water with 0.1% (v/v) trifluoroacetic acid as the ion-pairing agent. Detection was performed with the UV detector at λ = 210 nm. Under these conditions, the retention times for the peptidomimetics were between 5 and 10 min.

Membrane Interaction Potential

The ability of the peptidomimetics to interact with biological membranes was estimated by determining their partitioning between a modified aqueous phase (0.02 M phosphate buffer, pH 7.4/acetonitrile 78:22, v/v) and an immobilized artificial membrane (IAM.PC.DD column, 10 cm × 4.6 mm I.D., Regis Technologies, Inc., Morton Grove, IL) as described elsewhere (23). Aliquots (5–10 μl) of the peptidomimetics solutions (~40 μM, in running buffer) were injected on the column (flow rate 1.0 ml/min), and solutes were detected with a UV detector (λ = 210 nm). The capacity factor (k'_{IAM}) of each solute was calculated using Equation 1,

$$k'_{IAM} = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R is the retention time (in minutes) of the compound of interest, and t_0 corresponds to the void volume of the column determined with citric acid.

RESULTS

Physicochemical Characteristics

The replacement of the amide bond in the peptidomimetics by a 3,5-linked pyrrolin-4-one surrogate did not dramatically change their molecular weights (Table I). On average, the molecular mass of the pyrrolinone-based analog was ~5% greater than that of the amide bond-containing peptidomimetic. The ability of the peptidomimetics to interact with biological membranes was assessed by IAM chromatography. The results presented in Table I show for the first two pairs (i.e., compounds I, II and compounds III, IV) slightly stronger interactions between the amide bond-containing compounds and the immobilized phosphatidylcholine analogs than were found for the pyrrolinone-containing analogs. The log k'_{IAM} values of compounds V and VI were not substantially different. It should be noted that these results suggest that all the peptidomimetics studied have a significant propensity to interact with biological membranes (III > IV > VI≥ V >> I ≥ II).

Transport Across Caco-2 Cell Monolayers

When the peptidomimetics were applied to the donor compartment, linear fluxes were observed to the receiver compartment, independent of whether the peptidomimetic was initially applied to the AP or BL compartment. However, for all peptidomimetics, the flux in the BL-to-AP direction was substantially greater than the flux in the AP-to-BL direction. For example, Figure 1 shows the time courses of bidirectionally performed experiments for the amide bond-containing compound I (Panel A) and its pyrrolinone-based counterpart, compound II (Panel B). The ratio of the apparent permeability coefficients (P_{app}) calculated from the BL-to-AP and AP-to-BL transport of the peptidomimetics ($P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$) varied between 1.7 and 36.2 (Table II).

When comparing the amide bond-containing peptidomimetic to its corresponding pyrrolinone-based analog, the pyrrolinone-based compound appeared to be substantially less able to permeate Caco-2 cell monolayers from the AP-to-BL direction than was its amide bond-containing counterpart (Table II). Similarly, the $P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$ ratios calculated for the pyrrolinone-based analogs were consistently greater than those determined for the corresponding amide bond-containing compounds. Inclusion of 25 μM cyclosporin A in the transport buffer resulted in an equal flux of the compounds from the AP-to-BL and the BL-to-AP side (Figure 1). In the presence of cyclosporin A, P_{app} values calculated for AP- to-BL transport (Table III) were significantly greater than those determined in the absence of cyclosporin A (Table II). In contrast, BL-to-AP transport of the peptidomimetics was reduced in the presence

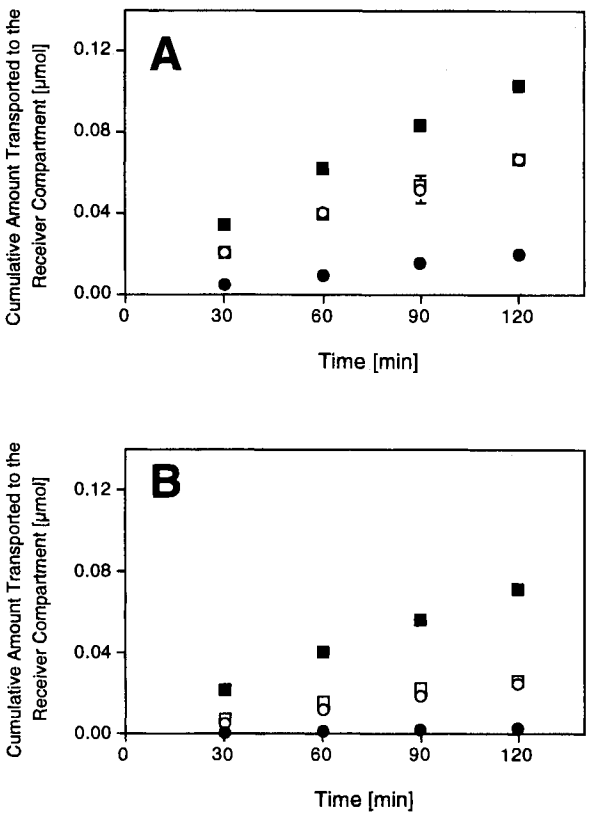


Fig. 1. Transepithelial transport of amide bond-containing and pyrrolinone-based peptidomimetics across Caco-2 cell monolayers. Panel A shows the flux of the amide bond-containing compound I and Panel B the corresponding profile of the pyrrolinone-based analog compound II. Initial donor concentration in these experiments was consistently 200 μM. Samples for the AP-to-BL (●, ○) and BL-to-AP (■, □) transport were determined in the presence (open symbols) and absence (closed symbols) of 25 μM cyclosporin A. Experiments were performed in triplicate (average ± SD).

of cyclosporin A compared to the transport experiment performed in the absence of cyclosporin A (Table II). $P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$ ratios determined in the presence of cyclosporin A were approximately one for the pairs I and II and III and IV, and close to one for the V and VI pair. Therefore, we were able to compare the intrinsic permeability coefficients of these different peptidomimetics. The results suggested that the amide bond-containing peptidomimetics have slightly greater membrane permeation characteristics than the pyrrolinone-based analogs.

Table II. Transport Characteristics of Amide Bond-Containing and Pyrrolinone-Based Peptidomimetics Determined Across Caco-2 Cell Monolayers

	$P_{app} \times 10^6$ [cm/s]		
Compound	AP→BL	BL→AP	BL→AP/AP→BL
I	2.96±0.09	21.47±0.22	7.3
II	0.36±0.02	13.03±0.23	36.2
III	13.01±0.21	23.91±0.45	1.8
IV	4.70±0.06	12.69±0.53	2.7
V	3.12±0.73	5.44±0.74	1.7
VI	0.61±0.05	11.95±0.60	19.5

Note: Transport experiments were performed in triplicate (average ± SD) using initial donor concentrations of 200 μM (compound I, II), 5 μM (compound III, IV), and 10 μM (compound V, VI).

The cellular uptake of these peptidomimetics into Caco-2 cells was measured in the presence of 25 μM cyclosporin A after a 120 min incubation period with initial application of the compounds to the AP compartment (Table III). Similar to the transepithelial transport results, the intrinsic uptake characteristics of the amide bond-containing peptidomimetics were slightly greater than those of the pyrrolinone-based analogs.

DISCUSSION

The amide bond in peptides is the major target for enzyme-catalyzed degradation mediated by endo- and exopeptidases (2,3). Medicinal chemists have developed numerous chemical strategies using peptide bioisosteres to modify the labile amide bond in order to prevent rapid proteolysis (1,10–12). However, little is known about how these peptide bioisosteres affect membrane permeation characteristics. In this study, we have examined how the pyrrolinone bioisostere affects permeation of peptidomimetics across Caco-2 cells.

Because the replacement of an amide bond with a bioisostere can result in greater enzymatic stability and/or modified membrane permeation characteristics, it was important to determine whether or not incorporation of this bioisostere has an impact on the metabolic profile of the peptidomimetic. When applied to the AP side of Caco-2 cell monolayers, the amide bond-containing peptidomimetics and the pyrrolinone-based analogs used in this study were metabolically stable over an incubation period of 120 min (recovery ≥ 97.4%; data not shown). This implies that in these peptidomimetics the amide bond per se is not susceptible to enzymatic hydrolysis. Similar results are reported by Smith and colleagues (21) who determined the metabolic stability of compound I in the presence of chymotrypsin. Consequently, the differences observed in the permeation characteristics of these peptidomimetics are exclusively related to the structural differences between these two classes of peptidomimetics and are not superimposed by increased metabolic stability after replacement of the amide bond with the pyrrolinone scaffold.

For all peptidomimetics studied, BL-to-AP transport was consistently greater than that observed in the opposite direction (Table II). Similar findings have been linked to the presence of apically polarized efflux systems (24). Research related to the phenomenon of multidrug resistance in cancer chemotherapy has led pharmaceutical scientists to focus on a membrane protein termed P-glycoprotein, which is assumed to reduce the intracellular accumulation and/or the transcellular flux of a wide variety of drugs across the gastrointestinal mucosa, including peptides [e.g., gramicidin D and valinomycin (25)]. P-glycoprotein is a 170–180 kDa membrane glycoprotein acting as an ATP-dependent efflux pump. It is highly expressed not only in cancer cells but also in various normal tissues including small intestine, colon, brain, and kidney (26). Although there are many examples in the literature in which cellular efflux can be directly related to P-glycoprotein (27), there is increasing evidence for the presence of other efflux pumps that might be involved in these processes (ATP-binding cassette (ABC) transporter superfamily). The role of polarized efflux systems

Table III. Cellular Uptake and Transcellular Transport of Amide Bond-Containing and Pyrrolinone-Based Peptidomimetics Across Caco-2 Cell Monolayers Determined in the Presence of 25 μM Cyclosporin A

Compound	Cellular Uptake [μg/mg protein]	$P_{app} \times 10^6$ [cm/s]		
		AP→BL	BL→AP	BL→AP/AP→BL
I	1.50±0.08	8.96±0.12	9.19±0.25	1.0
II	0.87±0.18	3.83±0.02	3.75±0.24	1.0
III	0.49±0.03	16.59±1.10	17.83±0.90	1.1
IV	0.25±0.01	5.84±0.24	6.04±0.13	1.0
V	0.81±0.05	3.23±0.76	4.72±0.18	1.5
VI	0.28±0.05	1.50±0.03	2.89±0.20	1.9

Note: Transport and uptake experiments were performed in triplicate (average ± SD) using initial donor concentrations of 200 μM (compound I, II), 5 μM (compound III, IV), and 10 μM (compound V, VI).

as a barrier to the absorption of peptides and peptidomimetics has recently been reviewed by Burton and colleagues (27,28).

Since the $P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$ ratios of the pyrrolinone-based peptidomimetics were consistently greater than those determined for the amide bond-containing analogs, we conclude that the pyrrolinone-based peptidomimetics are better substrates for these apically polarized efflux systems in Caco-2 cell monolayers. However, these results are not sufficient to make a statement about the relative affinities of these peptidomimetics to the efflux pumps. For that purpose, it would be necessary to determine the transport parameters (i.e., K_M, v_{max} values) of the peptidomimetics using a wide concentration range.

A variety of compounds have been reported to efficiently inhibit polarized efflux [e.g., verapamil, cyclosporin A, and chlorpromazine (27)]. In the presence of 25 μM cyclosporin A, $P_{BL\rightarrow AP}/P_{AP\rightarrow BL}$ ratios for all peptidomimetics studied were close to unity (Table III), suggesting significant inhibition of the efflux systems. Verapamil up to a concentration of 300 μM partially inhibited the efflux of these peptidomimetics (data not shown). P_{app} values determined in the presence of 25 μM cyclosporin A, therefore, represent the intrinsic permeability coefficients of these peptidomimetics. Under these conditions, the amide bond-containing peptidomimetics appear to be more able to permeate Caco-2 cell monolayers than are the pyrrolinone-based analogs (Table II). These findings are again consistent with the results obtained from the uptake studies into Caco-2 cells (Table III).

Characterization of the physicochemical properties of the peptidomimetics revealed that all compounds used in this study have a significant propensity to interact with biological membranes (Table I). The fact that the log k'_{IAM} values of compound V and VI, the bisamide and bispyrrolinone, respectively, were not substantially different is not understood. It could be related to the slight difference in the side chain between these two molecules whereas the other two pairs of peptidomimetics studied (i.e., compound I, II and III, IV) were exact matches. Pidgeon and colleagues (29) have correlated membrane interaction with IAM columns and Caco-2 cell permeability for the same compounds. These studies revealed that the membrane interaction potential determined on an IAM column (log k'_{IAM}) correlates quite nicely ($r = 0.85$) with transport properties across Caco-2 cell monolayers (log P_{app}), provided the molecule permeates the cellular barrier by transcellular passive diffusion. However, limitation of IAM chromatography in predicting oral bioavailability was observed for compounds permeating the cell monolayer predominantly via the paracellular pathway and/or mediated by active transport processes. The strong interaction observed for all peptidomimetics with this immobilized artificial membrane implies that these peptidomimetics traverse the cellular barrier via the transcellular pathway. The fact that these peptidomimetics are substrates for apically polarized efflux systems supports this hypothesis since substrate activity for efflux pumps requires that a compound first diffuses to the inner leaflet of the phospholipid membrane (25). Our results show that the pyrrolinone-based peptidomimetics are better substrates for these efflux systems than are the amide bond-containing analogs. In general, lipophilicity is viewed as a crucial factor for substrate activity with apically polarized efflux systems (27). Surprisingly, the amide bond-containing peptidomimetics exhibited stronger interactions with the immobilized artificial membrane of phosphatidylcholine analogs (Table I) and are, therefore, considered to be more lipophilic than the pyrrolinone-based analogs. These results imply that other factors than the lipophilicity which indicates the propensity of the molecule to partition into the membrane must contribute to substrate activity with these efflux pumps (e.g., spatial arrangement of the molecule).

Smith and colleagues (15) have shown that pyrrolinone-based HIV protease inhibitors are less potent against the isolated enzyme than are the amide-containing counterparts. Importantly, however, the relative potencies in the cellular assay were reversed which implied that pyrrolinone-based protease inhibitors are more readily transported across biological membranes than are the amide-containing analogs. To test this hypothesis, the transport properties of amide-containing and pyrrolinone-based peptidomimetics across biological membranes were determined using the Caco-2 cell culture model. The results from this study did not confirm the earlier conclusions from Smith and co-workers (15). However, we realize that the two experimental systems used (i.e., HIV infected lymphocytes and Caco-2 cell monolayers) are quite different in their characteristics. As a consequence, our results could be explained by differences in substrate specificity as well as the individual kinetic parameters between polarized efflux mechanisms present in lymphocytes (30,31) and differentiated enterocytes. Furthermore, the pharmacological activity of these peptidomimetics in the cellular assay using HIV infected lymphocytes was determined in the presence of various macromolecular binding proteins (e.g., albumin and α-glycoproteins) that were not included in the characterization of the transport

properties across Caco-2 cell monolayers. Recent findings reported by Bilello and co-workers (32) suggest that macromolecular binding proteins such as serum α-glycoproteins can reduce cellular uptake, intracellular concentration and antiviral activity of HIV-1 protease inhibitors.

In fact, preliminary transport experiments performed in the presence of 10% (v/v) fetal bovine serum revealed that under these conditions pyrrolinone-based peptidomimetics are more able to permeate this *in vitro* model of the intestinal mucosa than are the amide bond-containing analogs (data not shown). This implies that the amide bond-containing peptidomimetics, which have been shown to interact more strongly with an artificial membrane than their pyrrolinone-based counterparts, appear to interact as well to a greater extent with macromolecular binding proteins. A lower apparent drug concentration due to an increased fraction of protein bound drug would then result in a more effective efflux of the peptidomimetics mediated by the polarized efflux systems during transcellular transport.

Taken together, these studies and those reported earlier (15) reemphasize the complexity of the factors determining oral bioavailability of drugs. At the same time, they visualize the importance to include parameters that reflect the membrane permeation properties of a molecule into the optimization process of drug discovery.

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