Improvement of the Intestinal Absorption of a Peptidomimetic, Boronic Acid Thrombin Inhibitor Possibly Utilizing the Oligopeptide Transporter

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INTRODUCTION

Thrombin is a serine protease which, when activated by a cascade of protease-mediated events, hydrolyzes fibrinogen to insoluble fibrin, resulting in a blood clot. Low molecular weight, potent thrombin inhibitors are thought to have potential utility for preventing arterial and venous thrombosis, and are the target of a number of drug discovery and development programs (1,2). Some tripeptides with arginine in the C-terminus position were found to inhibit thrombin by mimicking a reactive intermediate (1). An extension of this design approach was to prepare peptides having the -COOH group of the C-terminus amino acid replaced by $-B(OH)_2$, boronic acid (3). Ac-(D)Phe-Pro-boroArg-OH (DuP 714) was shown to be a very potent thrombin inhibitor in vitro, and in rabbits dosed intravenously or subcutaneously (3).

For chronic therapy of cardiovascular diseases, oral administration is certainly preferred. However, this route of administration is not always feasible for peptidomimetic drugs like DuP 714, due to poor membrane permeation or presystemic degradation. It was shown that oral DuP 714 produced antithrombotic effects in rats, but a much higher oral dose was required than when dosed intravenously (4). The intestinal absorption characteristics of boronic acid thrombin inhibitors have not yet been described. It was recently shown that m-cyano-substituted borophenylalanine could replace the strongly basic boroarginine, while retaining potency and selectivity as thrombin inhibitors (5). This reduced net charge could benefit intestinal absorption. Such was the case for analogs of the thrombin inhibitor argatroban, where replacement of the highly basic guanidino group with less basic substituents led to improved Caco-2 permeability (6).

In this study we evaluated the in vitro rat jejunal permeability of DuP 714 and several m-cyano-substituted borophenylalanine analogs. We investigated the effects of replacing the strongly basic boroarginine group and of N-terminal α-amino modifications on intestinal permeation. The result presented here demonstrate that one particular structural approach led to very good intestinal permeation, possibly involving the oligopeptide transport system.

MATERIALS AND METHODS

Materials

DuP 714 and four m-cyanoborophenylalanine analogs were prepared at The DuPont Pharmaceuticals Co. (Wilmington, DE). The chemistry has been described previously (3,5). Structures are shown in Fig. 1. Cefazolin, cephradine, cefaclor, L-carnosine, gly-pro, ouabain, and L-glycine were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents used were of the highest grade available.

Permeation Experiments

Intestinal permeation experiments were performed in vitro using excised rat intestinal segments mounted in diffusion cells (Corning Costar, Acton, MA). A portion of intestine was removed from fasted, anesthetized, male Sprague Dawley rats (Crl: CD(SD)BR, Charles River, Kingston, NY) weighing 300–350 g. An intestinal segment of approximately 3 cm length was mounted onto the pins of the diffusion cell, and the half cells were clamped together. The buffer used was Tyrode's buffer, containing 137 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 12 mM NaH_2PO_4, and 6 mM D-glucose. To the donor compartment was added 7 ml of drug solution prepared with Tyrode's buffer, and to the receiving compartment was added 7 ml of drug-free buffer, both of which had been pre-warmed to 37°C. The temperature of the diffusion cells was maintained at 37°C using heating blocks. The donor and receiving fluids were circulated by gas lift with O_2:CO_2. Receiving chamber samples (0.5 ml) were taken at various times and replaced with drug-free buffer. Permeation experiments lasted 2 hr. The surface area available for diffusion was 1.78 cm^2. All permeation experiments were done with 0.2 mM or 1 mM drug solutions at pH 7.4 or pH 6.0. Most studies were done using jejunal segments, but site-dependence was also studied using duodenum, ileum, and colon. This research was done in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Partition Coefficients

Octanol/buffer partition coefficients were determined using the shake-flask method. The buffer was 0.1 M phosphate at pH 7.4. Drug concentrations in each phase were determined by HPLC.

Analyses

Drug concentrations in all samples were determined by HPLC using UV absorbance detection. For each permeation study, the cumulative amount of drug permeating the intestine was calculated and plotted vs. time. Permeation rates were determined from the linear portion of amount permeating vs. time plots. These were normalized for membrane surface area and divided by the initial donor drug concentration to give the

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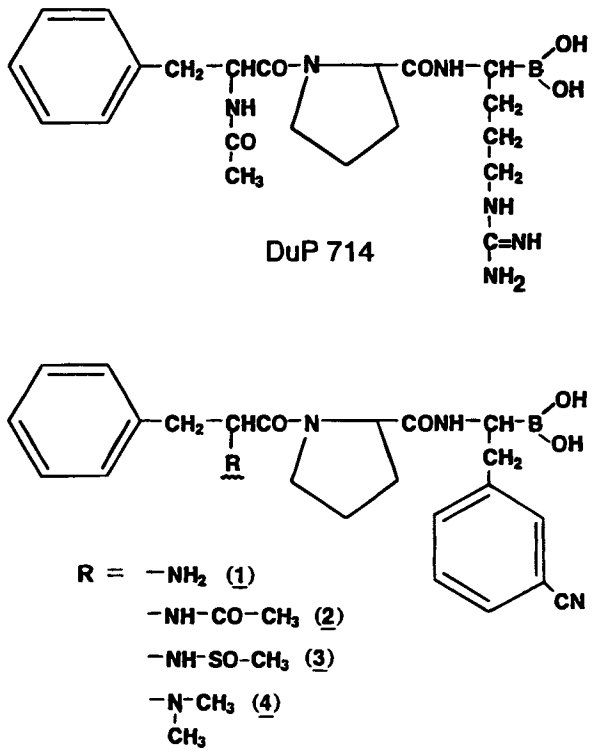

Fig. 1. Structures of DuP 714 and the m-cyanoborophenylalanine analogs tested.

permeability coefficient. There were generally four permeation experiments for each experimental group. The results are expressed as the mean ± SE. Statistical comparisons were made using t-tests, and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Intestinal Permeation Comparisons

The intestinal absorption characteristics of DuP 714 and the four m-cyano-substituted borophenylalanine analogs (Fig. 1) were compared with two reference compounds. The reference compounds were theophylline, which is well absorbed, and cefazolin, which is poorly absorbed. Permeability coefficients for rat jejunum at pH 7.4 are reported in Table I. Donor concentrations in these experiments were 1 mM. DuP 714 had much poorer intestinal permeability than theophylline (P_{app} = 23.3 ± 1.0 × 10^{-6} cm/sec). Since its permeability coefficient was similar to or lower than that of cefazolin (P_{app} = 3.46 ± 0.14 × 10^{-6} cm/sec), low in vivo absorption would be expected.

Table I. Rat Jejunal Permeability and Octanol/pH 7.4 Buffer Partition Coefficients (log P) of Boronic Acid Thrombin Inhibitors

Compound	P_{app} (10^{-6}, cm/sec)	log P
DuP 714	1.49 ± 0.20	−1.54
1	18.26 ± 1.95	0.42
2	1.34 ± 0.23	1.35
3	2.38 ± 0.56	1.13
4	1.06 ± 0.10	0.59

Recently, we and others have shown that low oral bioavailability of some peptidomimetic drugs can be at least partly due to the involvement of an energy-dependent secretory transport system or systems (7–9). In order to assess if secretory transport contributes to the poor absorptive intestinal permeation of DuP 714, mucosal-to-serosal (absorptive) and serosal-to-mucosal (secretory) permeation rates of DuP 714 were compared, at concentrations of 0.2 mM and 1 mM. Greater permeation in the secretory direction versus the absorptive direction would be evidence of transport by a secretory transport system. Consistent with our previous report (8), cefazolin demonstrated about 4-fold greater permeation in the serosal-to-mucosal direction than in the mucosal-to-serosal direction. In contrast, the permeation rate of DuP 714 across rat jejunum was slightly greater in the absorptive direction than in the secretory direction (data not shown). Accordingly, there was no evidence that secretory transport restricts DuP 714 intestinal absorption. Poor passive membrane permeability of DuP 714 seemed to be the major reason for the relatively poor oral absorption of DuP 714.

Of the four DuP 714 analogs tested, compound **1** exhibited much greater permeation than the other three analogs, whose permeability coefficients were similar to DuP 714 (Table I). Compound **1** has a free amino group on the N-terminal phenylalanine (Fig. 1). Partition coefficients in an octanol/pH 7.4 buffer system were determined and are included in Table I. There was a clear increase in lipophilicity accompanying replacement of the boroarginine with m-cyanoborophenylalanine. However, there was no correlation between log P and intestinal permeability. The increase in permeability of **1** was not related to an increase in lipophilicity.

Further Characterization of 1 Absorption

It is known that β-lactam antibiotics having a free α-amino group, such as cephradine, cephalexin, and cefaclor, are generally well absorbed after oral administration and that they are transported by the oligopeptide transport system in the small intestine (10). To examine the reason for relatively high intestinal permeation of compound **1**, we investigated whether its permeation had characteristics similar to substrates of the oligopeptide transporter. One characteristic of transport by the oligopeptide transporter is stimulation by a proton gradient. To evaluate the effects of a proton gradient on **1** permeability, jejunal permeability at 0.2 mM **1** was determined using pH 6.0 and pH 7.4 donors. There was no apparent stimulation of **1** permeation in the presence of a proton gradient, with P_{app} values (10^{-6}, cm/sec) of 12.9 ± 0.7 at pH 6.0 vs. 14.8 ± 1.3 at pH 7.4. The inhibitory effects of various compounds on **1** permeation were investigated. Cephradine was studied similarly, for comparison. As shown in Table II, 0.2 mM cephradine permeation was significantly inhibited by the dipeptides, L-carnosine and gly-pro, at 20 mM concentrations. These two dipeptides also significantly inhibited **1** permeation. However, L-glycine and DuP 714 had no inhibitory effects on **1** permeation. Ouabain, a metabolic inhibitor, also significantly reduced **1** permeability. These results suggest the possible involvement of the oligopeptide transport system in absorption of **1**.

Site dependence of intestinal permeation of **1** was investigated using rat duodenum, jejunum, terminal ileum, and colon.

Table II. Inhibitory Effects of Various Compounds on Rat Jejunal Permeability of **1** and Cephradine

Inhibitor	P_{app} (10^{-6} cm/sec)	
	1	Cephradine
Control	14.8 ± 1.3	9.5 ± 1.2
L-carnosine (20 mM)	10.3 ± 0.8[a]	6.4 ± 0.5[a]
Gly-Pro (20 mM)	10.7 ± 0.8[a]	3.2 ± 0.4[a]
Cefaclor (20 mM)	9.8 ± 0.9[a]	N.D.[b]
DuP 714 (10 mM)	15.4 ± 1.7	N.D.
L-glycine (20 mM)	14.3 ± 1.5	N.D.
Ouabain (0.1 mM)	11.8 ± 0.4	N.D.

[a] Significantly ($p < 0.05$) different from control.
[b] Not determined.

Results are presented in Table III. Compound **1** permeated all regions of the rat intestine relatively well, although permeability of the colon was slightly lower than small intestinal permeability.

DISCUSSION

In the gastrointestinal tract are various carrier-mediated systems for efficiently absorbing various nutrients, such as amino acids, oligopeptides, monosaccharides, bile acids, and water soluble vitamins. One approach to improving the oral bioavailability of poorly absorbed, peptidomimetic drugs might be to modify the structure in a way that allows recognition by the oligopeptide transporter, which seems to exhibit broad substrate specificity relative to other nutrient transporters. A variety of drugs are absorbed by the oligopeptide transporters, including some β-lactam antibiotics and angiotensin converting enzyme inhibitors (10,11). The boronic acid thrombin inhibitor, DuP 714, and the m-cyano-substituted borophenylalanine analogs, structurally mimic a tripeptide.

Our results show that DuP 714 and three of the four m-cyano-substituted borophenylalanine analogs have rat intestinal permeability at least as poor as that of cefazolin. One analog (**1**) having a free α-amino group had relatively good absorptive permeation, and its permeation was inhibited by known substrates of the oligopeptide transporter. These results imply that high intestinal permeation of **1** is at least partly mediated by the oligopeptide transport system. Since **1** permeation was still quite high in the presence of these inhibitors, mechanisms other than the oligopeptide transport system may contribute to its relatively high permeation. Permeation of **1** was not stimulated by a proton gradient. It has been reported that some substrates of the oligopeptide transport system are transported at acidic and neutral pH, whereas others are only transported at acidic pH (12). The key structural feature of **1** would seem to be the free α-amino group. The presence of a free α-amino group has been shown to increase affinity for the oligopeptide transporter for some compounds (13), although other studies have clearly shown that an α-amino group is not required for interaction with the oligopeptide transporter (14). The presence of the boronic acid functional group of **1** did not preclude its apparent interaction with the transporter.

Although the oligopeptide transporter has relatively broad substrate specificity, as mentioned above, not all compounds in a particular structural family may be recognized. For the intestinal oligopeptide transport system to be utilized to improve the oral absorption of peptidomimetic drugs, the key structural features required for transport need to be understood. In order to acheive success in this approach, pharmacologic activity must also be retained. The results presented here raise the possibility of designing peptidomimetic, boronic acid thrombin inhibitors with good intestinal permeability, apparently absorbed by the oligopeptide transporter.

Table III. Site Dependence of In Vitro Rat Intestinal Permeation of Compound **1**, Using 0.2 mM Concentrations at pH 7.4

Site	P_{app} (10^{-6} cm/sec)
Doudenum	13.6 ± 1.2
Jejunum	14.8 ± 1.3
Ileum	12.0 ± 0.5
Colon	9.4 ± 0.5

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