Prodrug and Analog Approaches to Improving the Intestinal Absorption of a Cyclic Peptide, GPIIb/IIIa Receptor Antagonist

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Received February 14, 1997; accepted May 12, 1997

Purpose. The purpose of this study was to test whether structural modifications improve the intestinal absorption of DMP 728 (cyclo(D-Abu-NMeArg-Gly-Asp-Amb)), a GPIIb/IIIa receptor antagonist.

Methods. In vitro permeabilities of prodrugs and analogs of DMP 728 across excised rat intestinal segments were determined.

Results. n-Butyl and n-octyl esters of DMP 728 were relatively stable during in vitro permeation of rat intestine. Intestinal permeation rates of these compounds were no greater than that of DMP 728, even though the octyl ester was much more lipophilic. A pivaloyloxymethyl ester, which was hydrolyzed to DMP 728 during intestinal permeation, also did not improve permeability. In another approach, analogs with an additional methyl substituent on various amide nitrogens were evaluated. Cyclo(D-Val-NMeArg-Gly-Asp-NMeAmb), cyclo(D-Abu-diN-MeLys-Gly-Asp-Amb), and cyclo(NMeGly-NMeArg-Gly-Asp-Amb) each had about 2-fold greater permeability than DMP 728. Two other analogs with improved permeability were linear Ac-D-Abu-NMeArg-Gly-Asp-Amb and a DMP 728 derivative in which the Asp was rearranged. An analog in which the charged amino acids were replaced by neutral amino acids had permeability similar to DMP 728.

Conclusions. Within this series of peptides, hydrogen bonding tendency and structural constraint influenced intestinal permeation, but not always in ways consistent with the literature, whereas charge and lipophilicity were not shown to influence intestinal permeability. The failure of these approaches to improve permeation more significantly could be due to the influence of secretory transport.

KEY WORDS: absorption; peptide; prodrug; analog; intestinal permeability; GPIIb/IIIa antagonist.

INTRODUCTION

DMP 728 (cyclo(D-Abu-NMeArg-Gly-Asp-Amb)) has been shown to be a potent GP IIb/IIIa receptor antagonist and an effective inhibitor of platelet aggregation (1). This cyclic peptide is zwitterionic, due to the strongly basic N-methyl arginine and acidic aspartate residues, and is very hydrophilic. The molecular weight of DMP 728 zwitterion is 561 daltons. The hydrophilicity, charge, and molecular weight of DMP 728 are features unfavorable for good intestinal absorption. Indeed, it was reported that the oral bioavailability of DMP 728 was 2–3% in rats and 8–12% in dogs (2,3). Oral administration is preferred for chronic drug therapy of cardiovascular diseases, to obtain high patient compliance. Therefore, various approaches to improving the oral bioavailability of DMP 728 were investigated. Structural modifications (prodrugs and analogs) were considered as possible chemical means to overcome low oral bioavailability. Structural approaches typically aim at increasing the lipophilicity of hydrophilic parent drugs to improve membrane permeability, and prodrugs were prepared for this purpose. Another approach we examined was to reduce the hydrogen bonding tendency of the parent molecule, based on the previous work of others showing that hydrogen bonding of peptides has a greater influence on intestinal permeability than lipophilicity (4). Methylating amide nitrogens was shown to be effective in reducing hydrogen bonding tendency and increasing the permeability of model peptides through Caco-2 membranes (5).

Recently we reported that DMP 728 is subject to secretory intestinal transport, which, in addition to poor lipophilicity, restricts its net absorption (6). DMP 728 and verapamil, a prototypical P-glycoprotein inhibitor which is also secreted by the rat intestine (7), mutually inhibited the secretory transport of each other (6). This suggested that the secretory transport of DMP 728 is P-glycoprotein-mediated. The secretory transport of DMP 728 by intestinal P-glycoprotein is somewhat unusual because intestinal P-glycoprotein substrates are typically hydrophobic drugs or peptides (7–11). There have not been studies reporting how structural features can be manipulated to alter affinity for intestinal secretory transport.

This study examined how structural modifications influence intestinal permeability of DMP 728, a compound for which poor lipophilicity, hydrogen bonding, and intestinal secretory transport are barriers to its intestinal absorption. Various prodrugs and analogs of DMP 728 were obtained and their in vitro intestinal permeabilities were compared with that of DMP 728, using rat jejunum in diffusion cells.

MATERIALS AND METHODS

DMP 728 and its prodrugs and analogs were supplied by The DuPont Merck Pharmaceutical Co. (Wilmington, DE). Their purities were rechecked chromatographically. Bacampicillin and ampicillin were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade available.

The buffer for all permeation experiments was Tyrode's buffer, which contained 137 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4 and 6 mM D-glucose. The pH was 7.4 in most experiments, unless stated otherwise. Permeation experiments were done in vitro as described previously (7), using side-by-side diffusion cells (Precision Instrument Design, Los Altos, CA). We have previously shown that intestinal permeation rates using this experimental system were well correlated with in vivo absorption properties (6). Jejunal segments were excised from ether-anesthetized, fasted, male, Sprague-Dawley rats (Crl:CD(SD)BR, Charles River, Kingston, NY). After rinsing in ice-cold saline, a segment was cut open longitudinally to form a sheet. A sheet was mounted onto the pins of a diffusion cell, and the other half-cell was clamped into place. The surface area for diffusion was 1.78 cm^2. The mucosal chamber contained 7 ml of drug solution, and the serosal chamber contained 7 ml of drug-free

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NOTATIONS: Amb, m-(aminomethyl) benzoic acid; D-Abu, D-2-aminobutyric acid.

0724-8741/97/0800-1026$12.50/0

buffer. All experiments were performed using 0.2 mM drug concentrations. The mucosal and serosal solutions were bubbled with 95% O_2:5% CO_2, which also provided efficient mixing, and the temperature was maintained at 37°C. Serosal samples (0.5 ml) were taken at various times and replaced with drug-free buffer. Samples were quenched with 0.5 ml of 0.1% trifluoroacetic acid (TFA). Drug concentrations were determined by HPLC as follows: column, Nova-Pak C-18 (4μm, 3.9 × 150 mm, Waters, Milford, MA); mobile phase, 12–15% acetonitrile and 0.1% TFA in water; flow rate, 0.6–1 ml/min; wavelength, 220 nm; and column temperature, 35°C.

The amounts of drug permeating the intestine were calculated for each sample interval and were plotted vs. time. The slope of the linear portion of this plot was corrected for membrane surface area and donor drug concentration to obtain the permeability coefficient (P). Results are expressed as mean ± SE. Statistical comparisons between control (DMP 728) and test groups were made using t-tests.

HPLC capacity factors were used to measure relative lipophilicities. Capacity factors are retention times of the compounds of interest relative to the void volume. These were determined using mobile phases containing varying proportions of pH 7.4, 0.1 M phosphate buffer and methanol, using an octadecylsilane column. The logarithms of the capacity factors were plotted against mobile phase methanol concentrations, and the extrapolated logarithm of the capacity factor at 0% methanol was determined. This is termed log k_{hplc}. Usually four methanol concentrations were used to construct these plots.

This research was done in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

RESULTS

Intestinal Permeability of DMP 728 Prodrugs

As representative simple ester prodrugs, n-butyl and n-octyl esters on the carboxylate of the aspartic acid residue were obtained (Fig. 1). These prodrugs exist as organic cations in the experimental buffer (pH 7.4). The lipophilicity of the octyl ester was much greater than that of DMP 728, with a log k_{hplc} value of 4.3, vs. 1.9 for DMP 728. The log k_{hplc} of the butyl ester (1.9) was the same as that of DMP 728. These ester prodrugs of DMP 728 were relatively stable against enzymatic attack during intestinal permeation, since only a very small amount of DMP 728 appeared as a hydrolysis product in the serosal fluid during the 120 minute permeation study. Permeation of the intact prodrugs are therefore reported. As shown in Table I, jejunal permeabilities of these prodrugs were no greater than that of DMP 728. Therefore, the addition of a lipophilic moiety to this position of the DMP 728 structure, and an increase in apparent lipophilicity with the octyl ester, did not lead to an improvement of intestinal absorption.

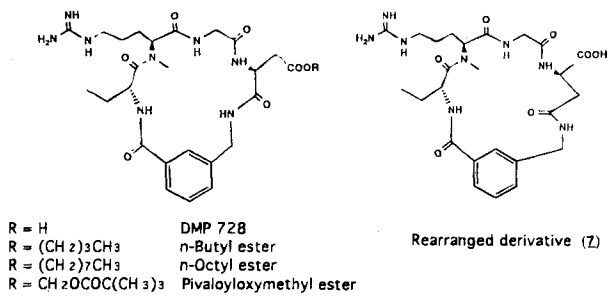


Fig. 1. Chemical structures of DMP 728, the prodrugs evaluated, and a rearranged derivative (7).

As an alternative, we examined the jejunal permeability of a pivaloyloxymethyl ester of DMP 728 (Fig. 1). The pivaloyloxymethyl ester of DMP 728 was unstable in solution at neutral pH, rapidly forming the rearranged structure also shown in Fig. 1 (not the ester hydrolysis product). However, the pivaloyloxymethyl ester was stable at pH 5, so a permeation experiment was performed using pH 5 mucosal and serosal buffers. To confirm the suitability of this experimental condition, we examined intestinal permeability of ampicillin and its prodrug, bacampicillin, using pH 5 buffers. Bacampicillin concentrations in the mucosal solution decayed due to hydrolysis to ampicillin, and conversion was approximately complete by the end of the permeation experiment. Unchanged bacampicillin was not detected in the serosal solution at any sampling time point, so bacampicillin was completely converted to ampicillin as it permeated the intestinal membrane. The permeability coefficient of ampicillin was significantly greater using bacampicillin as the donor ($P = 5.79 \times 10^{-6}$ cm/sec) than using ampicillin as the donor ($P = 3.42 \times 10^{-6}$ cm/sec), indicating that bacampicillin improved ampicillin absorption. These results are consistent with reported in vivo data (12). The hydrolysis and permeation results with bacampicillin indicate that a pH 5 medium should be acceptable for the study of the DMP 728 pivaloyloxymethyl prodrug.

When 0.2 mM prodrug was used in a pH 5 mucosal solution, intact pivaloyloxymethyl ester and rearranged products were not detectable in the serosal fluid after 120 min, indicating that the ester was rapidly converted into DMP 728 during the membrane permeation process. This prodrug was also hydrolyzed in the mucosal donor solution, with 36% remaining as the intact prodrug after 120 minutes exposure to the intestine. The rate of DMP 728 appearance in the serosal solution was almost identical to the case in which DMP 728 was used as the donor at pH 5 (data not shown). These results again indicated

Table I. Rat Jejunal Permeability Coefficients (P) for DMP 728 and Various Prodrugs and Analogs, as Well as for Ampicillin and Atenolol Also Given are log k_{hplc} Indices of Lipophilicity

	P (10^{-6} cm/sec)	log k_{hplc}
DMP 728	1.29±0.08	1.9
DMP 728 butyl ester	1.24±0.16[a]	1.9
DMP 728 octyl ester	1.16±0.10[a]	4.3
1	2.52±0.40[b]	1.2
2	2.37±0.65[b]	1.6
3	2.50±0.47[b]	1.9
4	2.80±0.34[b]	2.6
5	0.95±0.30	3.4
6	2.41±0.55[b]	1.8
7	3.50±0.77[b]	1.7
Ampicillin	3.86±0.24	
Atenolol	12.5±1.3	

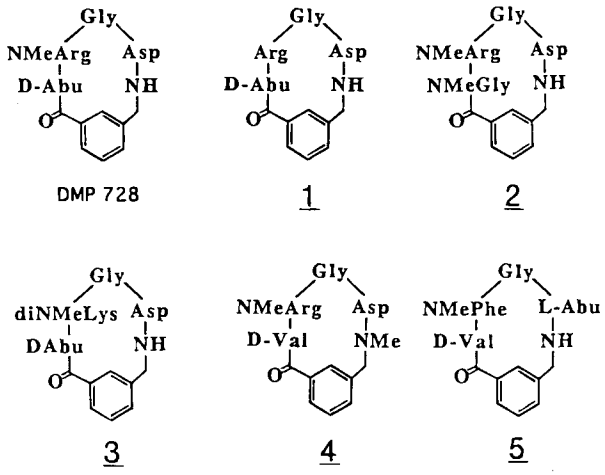
[a] Permeation of the ester.
[b] Significantly different ($p < 0.05$) from DMP 728.

that this prodrug approach to improving low intestinal permeability of DMP 728 was unsuccessful.

Intestinal Permeability of DMP 728 Analogs

Structural analogs of DMP 728, which had been prepared primarily for evaluation of structure vs. pharmacologic activity relationships, were examined for their jejunal permeabilities. These compounds are shown in Fig. 2. The permeability coefficients for these analogs across rat jejunum are presented in Table I. One of the factors investigated was the effect of methylating amide and amine nitrogens. Compound *1* is structurally the same as DMP 728 except that it lacks the methyl substituent on the arginine amide. Compounds *2, 3,* and *4* can be considered as having an additional nitrogen methylated, although these also differ in one of the substituents. The permeabilities of compounds *2, 3,* and *4* were greater than DMP 728, suggesting that the introduction of an additional N-methyl group into a peptide bond of this cyclic peptide could be useful in improving its intestinal permeability. However, compound *1,* lacking the N-methyl on the arginine, also had higher permeability than DMP 728. So, in this case, permeability clearly does not always correlate with methylation of hydrogen bonding groups. Compound *5* was selected as an uncharged analog, to test the hypothesis that poor DMP 728 permeation is related to its zwitterionic character. The log k_{hplc} of *5* was 3.4, which is considerably greater than 1.9 obtained for DMP 728. The permeability of *5* was almost identical to DMP 728, however, suggesting that charge (or lipophilicity) was not a major determinant of intestinal permeability in this cyclic peptide series. Compound *6* is a linear analog of DMP 728. This linear analog exhibited increased permeability compared with cyclic DMP 728. Compound *7* (Fig. 1) is an analog of DMP 728 in which there is structural rearrangment at the aspartate group. This compound showed the greatest permeability among the analogs tested (2.7-fold greater than DMP 728). Overall, there was no relationship between rat intestinal permeability and lipophilicity, as indicated by log k_{hplc} values.

Ampicillin and atenolol were used as a reference compounds to gauge in vivo absorbabilities of these analogs based on their in vitro permeabilities. Ampicillin is poorly to moderately absorbed from the gastrointestinal tract in humans, and atenolol is approximately 50% absorbed in humans and rats. Although several analogs improved the permeability of DMP 728 2-fold or more, permeability was still relatively low when compared with these reference compounds (Table I).



Ac-DAbu-NMeArg-Gly-Asp-mAmb

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Fig. 2. Abbreviated structures of DMP 728 and the analogs tested in this study.

DISCUSSION

Previous work (6) has shown that low oral bioavailability of DMP 728 is due to poor intestinal permeation. The structural or physicochemical features contributing to the poor membrane permeability of DMP 728 could include its zwitterionic charge, hydrophilicity, and hydrogen bonding tendency. In addition, active secretory transport by P-glycoprotein also contributes to its low intestinal absorption (6). The relative importance of each of these barriers to intestinal absorption has not been clarified. We investigated how structural changes that influence charge, lipophilicity, and hydrogen bonding, affect intestinal permeability. These would be expected to primarily influence passive transcellular permeation. However, for polar compounds such as these, the paracellular route of membrane permeation may also be important. Since these compounds have a tripeptide core, it may also be possible that some of these compounds interact with the intestinal oligopeptide transport system. Any particular structural change could therefore affect more than one mechanism of absorption or secretion. We have not attempted to characterize the mechanisms of permeation of each of the analogs or prodrugs examined. Our initial effort here was to focus on applying approaches previously shown to be successful in improving intestinal absorption by changing lipophilicity and hydrogen bonding, and to assess the overall effect of these on permeability.

If successful, perhaps the most facile approach to improve the intestinal absorption of DMP 728 is through prodrugs. Both n-butyl and n-octyl esters of DMP 728 were expected to be more lipophilic than DMP 728, and were highly resistant to enzymatic hydrolysis during their intestinal permeation. It was, therefore, expected that these prodrugs might exhibit greater intestinal permeability than DMP 728. However, this was not the case. It is possible that these DMP 728 esters, which are cations at pH 7.4, are also recognized as substrates of intestinal P-glycoprotein and that their intestinal permeation is restricted by this efflux system. This has not yet been studied. A pivaloyloxymethyl ester was much more rapidly converted to DMP 728 during jejunal permeation. However, this prodrug also failed to exhibit improved absorption of DMP 728. In contrast, a similar prodrug approach was recently shown to improve the in vitro intestinal permeation and in vivo oral bioavailability of a poorly permeable angiotensin II antagonist (13). Possibly, when the pivaloyloxymethyl prodrug is hydrolyzed to DMP 728 on the brush-border membrane or in the cytosol, the DMP 728 formed is immediately secreted into the lumen side by P-glycoprotein.

The second approach was to evaluate structural analogs of DMP 728, to examine how specific structural changes influence intestinal permeability. Compounds were selected from those available from structure-pharmacologic activity screening. Con-

radi et al. (4,5) previously reported that intestinal permeation within a series of model peptides inversly correlated with the number of hydrogen bonding groups in the structure, and that alkylation of amide bonds reduced the overall hydrogen bonding potential of peptides and increased intestinal permeability. DMP 728 analogs with an additional N-methyl substituent (compounds *2, 3,* and *4*) exhibited about 2-fold greater jejunal permeability than DMP 728. However, the permeation of desmethylated analog *1* was also greater than DMP 728, being inconsistent with the above explanation.

The effects of charge were investigated with the neutral analog *5*. Although this compound was more lipophilic than DMP 728, as measured by log k_{hplc}, *5* did not have greater permeability than DMP 728. Linear analog *6* permeated the intestinal membrane better than cyclic DMP 728. The lipophilicity of *6* was not greater than that of DMP 728. The lack of charge and lipophilicity effects could be due to differences in secretory transport by P-glycoprotein.

The rearranged analog of DMP 728, *7*, exhibited the greatest intestinal permeability among the analogs tested. Its permeation was on average 2.7-fold greater than that of DMP 728, and it would be expected to have improved oral bioavailability as well. However, this compound was not bioactive.

Changes in conformational constraint can influence membrane permeability. Comparisons of Caco-2 membrane permeabilities for several linear and cyclic hexapeptides were recently reported, in which cyclization resulted in a 2- to 3-fold increase in permeability (14,15). Cyclization of those peptides appeared to reduce the hydrogen bonding potential, increased lipophilicity, and reduced the hydrodynamic radius, all possibly due to increased structural constraint. Within the family of cyclic Arg-Gly-Asp peptides examined here, conformational constraint has been evaluated previously using circular dichroism (16) and NMR (17). DMP 728 is more conformationally constrained than *1*, but was less permeable. Also, the unconstrained linear peptide was more permeable than DMP 728, in contrast with the previous literature.

In conclusion, structural approaches to improving the oral absorption of DMP 728 are complicated because of the multiple barriers to acheiving membrane permeation. Increasing lipophilicity and reducing the charge, which would conventionally be expected to increase membrane permeability, had no effect. Permeability increases of 2- to 3-fold were seen with N-methylated analogs, with a linear analog, and with a rearranged analog. These results were not always consistent with the hypotheses that permeability can be increased by reducing hydrogen bonding or increasing conformational constraint. This could be because of the confounding influence of secretory transport. The mechanisms of permeation of these prodrugs and analogs have not been determined, though. Even with a 3-fold increase in permeability, rat jejunal permeability was still lower than permeabilities of ampicillin and atenolol. Thus it is expected that these analogs would still have relatively low oral bioavailability.

ACKNOWLEDGMENTS

This work was supported by the DuPont Merck Postdoctoral Program. The technical assistance of Christine Gerard is greatly appreciated, as are the chemical syntheses of Sharon Jackson, Arlene Rockwell, and Chu-Biao Xue.

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