Inhibition of Binding of an Enzymatically Stable Thrombin Inhibitor to Lumenal Proteases as an Additional Mechanism of Intestinal Absorption Enhancement

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Received May 11, 1998; accepted October 14, 1998

Purpose. The objective of the study was to investigate the mechanisms behind increased bioavailability of an enzymatically stable thrombin inhibitor, inogatran, after coadministration with a trypsin inhibitor, aprotinin.

Methods. Rat jejunum, ileum and colon segments were stripped and mounted in modified Ussing chambers, and the permeability to inogatran was determined both in the presence and absence of aprotinin. Inogatran and aprotinin were also coadministered intraduodenally to conscious rats. Competitive binding of inogatran to trypsin was studied using kinetic dialysis and was compared to aprotinin. The fraction of free (unbound) trypsin probe, in the absence of trypsin inhibitors was determined by performing experiments without pancreatine and without inhibitors, respectively.

Results. A 3-fold increased permeability to inogatran in the presence of aprotinin was seen *in vitro*, in some cases correlated with changed barrier properties of the intestinal segments. The *in vitro* results were well correlated with the *in vivo* results. There was a 5-fold increase in the bioavailability of inogatran in the presence of aprotinin. The binding of a trypsin probe was inhibited by both the presence of inogatran and aprotinin. Aprotinin showed a several fold higher displacement than inogatran. The results indicate both an effect of aprotinin on the epithelial membrane and an inhibition of binding of the thrombin inhibitor to trypsin or other serine proteases in the gut.

Conclusions. The coadministration of aprotinin with enzymatically stable peptides, like thrombin inhibitors, may improve their absorption after oral administration. This suggests a new additional mechanism for intestinal absorption enhancement of peptide drugs.

KEY WORDS: thrombin inhibitor; absorption; aprotinin; permeability enhancement; peptide absorption.

INTRODUCTION

Synthesis of potent analogues of hydrophilic drugs, especially to peptides with low oral bioavailability, has often been the main goal in the synthesis strategy and less has been focused on increasing the intrinsic absorption potential (e.g. the permeability coefficient) of the drug itself. Due to this, absorption of hydrophilic drugs and peptidomimetics after oral administration seems generally to be low, mainly due to proteolytic degradation in the gut lumen and brush border membrane or low membrane transport permeability. Apart from synthesizing new stable analogues with higher intrinsic permeabilities, improvement of absorption has been achieved by coadministration with enzyme inhibitors, e.g. protease inhibitors or enhancer systems like medium chain glycerides, bile acids, fatty acids etc. (1–3). The use of protease inhibitors, e.g. trypsin inhibitors to increase the absorption of peptides like insulin and calcitonin is well known (1–2, 4–6). The mechanism behind the increased absorption is the decreased proteolytic activity of trypsin and chymotrypsin, two enzymes which are abundant in the gastrointestinal tract. It is also known that inhibition of the enzymatic activity in the gastrointestinal tract by polymers like polycarbophil will increase the bioavailability of protease sensitive compounds *in vitro* (8) and also *in vivo* (9). Although the mechanism behind the effect of polycarbophil is unknown, a chelating effect on Ca^{2+} in the active site of the protease enzyme can be one mechanism involved. Lundin *et al.* 1995, found an increased absorption of enzymatically unstable as well as stable oxytocin analogues by removal of the pancreatic juice using a pancreatic duct cannulated pig model (10), e.g. in the absence of trypsin and chymotrypsin. This means that apart from inhibiting proteolytic degradation, protease inhibitors might have other mechanisms by which they increase absorption/bioavailability of drugs from the GI tract.

The drug inogatran (pINN) is a low molecular weight thrombin inhibitor with a dipeptide like structure. It is a reversible inhibitor with a Ki of 15 nM and has a 45-fold and >500-fold selectivity over trypsin and over other serine proteases respectively (11). The drug is relatively hydrophilic in nature with a log D oct/water (pH 7.4) of −0.1, three pKa-values 1.3; 7.6; >12 and a molecular weight of 439 g/mole (11–12) and has recently been reported to have a low bioavailability (5%) in the rat (13), probably due to low membrane permeability which has been found both in rats and humans (12). The drug has been reported to be proteolytically stable, since 90% of the drug is found intact in the feces and urine after 72 hrs (11).

The objective of the present study was to investigate the mechanism of the increased bioavailability of a proteolytic stable peptide, inogatran, during coadministration with the trypsin inhibitor aprotinin.

MATERIALS AND METHODS

Solutions and Substances

Inogatran, labeled (^3H; 71.4 kBq/nmol) and unlabeled, was obtained from Astra Hässle AB Mölndal, Sweden. Albumin (Sigma A-1887), Aprotinin (Sigma A-1153), Trypsin (Sigma T-8003), Human thrombin (Sigma T-6759), pancreatine (Sigma P-7545), BPTI (Bovine Pancreatic Trypsin Inhibitor, Sigma T-0256), and SOY-3 (Biozyme), and S-2222 (Bz-Ile-Glu(g-OR)-Gly-Arg-pNA; where R = H (50%) and R = CH_3 (50%); Chromogenix, trypsin substrate).

Animals

Male Sprague-Dawley rats of about 100 days old were obtained at least one week prior to use. The rats had free

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0724-8741/99/0100-0074$16.00/0

access to food and water prior to sacrifice for the permeability experiments and were fasted 16 hours before *in vivo* experiments.

Performance of Ussing Chamber Experiments

Preparation of the Intestinal Segments

During anesthesia with Isofluran, Forene® (Abbott), the intestine was removed and washed twice with cold Krebs-Bicarbonate Ringer's solution (KBR), bubbled with a O_2/CO_2 (95/5%) gas mixture to a constant pH of 7.4.

The proximal jejunum 15 cm distally to pylorus, distal part of the ileum, 5 cm proximal to the ileocecal junction, and the descending colon were used and prepared for the Ussing chamber submerged in KBR (12°C) as described earlier (14–15).

Transport Experiments

Transport experiments were performed at 37°C in modified Ussing chambers with effective stirring conditions, as described previously (14–15). The potential difference (PD), tissue resistance (Rt) and the short-circuit current (SCC) were monitored simultaneously before and during the experiments for evaluation of the viability and integrity of the tissues. After 30–40 minutes of equilibration, the experiments were started by exchange of the solutions on both sides of the intestinal membrane for fresh KBR (37°C). The transport media was maintained at pH 7.2–7.4 during the transport experiments by continuous bubbling with an O_2/CO_2 (95/5%) gas mixture. Aprotinin was added to the mucosal side 1:1 and 3:1 on weight basis (mg) in relation to inogatran. As the permeability coefficients for unlabeled and ^{3}H-labeled inogatran were found to be almost identical, 3.1 ± 0.2 and $3.51 \pm 1.04 \times 10^{-6}$ cm/s respectively in ileum segments from the rat, ^{3}H-labeled inogatran was used for analysis of inogatran permeability in all the *in vitro* experiment. ^{3}H inogatran and unlabeled inogatran were added to the mucosal KBR solution to a final concentration of 1.1 mg/10 ml. Samples of 50 μL were withdrawn from the serosal side at regular time intervals and were replaced with fresh KBR solution. The transport experiments were carried out for 150 minutes. The analysis of content of inogatran in the samples was made by measuring of the radioactivity using a Wallac Win Spectral (1414 Liquid Scintillation Counter). Corrections in the samples were made for changed specific activity due to the addition of unlabeled compound.

Performance of *In Vivo* Experiments

The *in vivo* experiments were performed using conscious animals which have had at least one day of rest after the surgical insertion of the arterial and duodenal catheters before the experiments.

Surgical Insertion of Intestinal Catheter

The rat was anesthetized with a mixture of Zylazine (5 ml/kg) and Ketamine (100 mg/kg) which was given ip. The skin on the abdomen and the peritoneum were opened with a 2 cm midline incision, 2 cm distally of the breastbone bow. A duodenal catheter (PE 50; polyethylene) was inserted about 2–2.5 cm distally to pylorus and was introduced 20 mm in the direction of the intestinal transport into the segment. The catheter was secured in the intestine by a purse-string suture and was brought through a hole in the abdominal wall up to the neck of the rat by the use of a trocare and fixed. For protection and fixation of the position of the catheter where it leaves the abdominal wall a bubble on the catheter was needed. The peritoneum and skin of the abdomen were then stitched separately. For more detailed information see also Griffiths *et al.*, 1996 (16).

Surgical Insertion of the Artery Catheter

During anesthesia (see above) an approximately 2 cm median ventral neck skin incision was made. The left common carotid artery was carefully freed from surrounding tissue and nerves for a length of approximately 2 cm using blunt dissection. By using an open forceps to stop the blood flow, a ligature (Silk®, 4-0) was tied around the artery, a small incision was made and finally a prefilled (0.9% NaCl) catheter (PE-50 jointed with PE-90) was inserted. The ligature was then tied tightly around the artery and the inserted catheter, and strengthened by a second adjoining ligature. The catheter was flushed with NaCl (0.9%) and congested with a stopper at the free end. A tunnel to the neck was made by a trocar and the catheter was led through the tunnel and cut to an appropriate length. For more detailed information see also Griffiths *et al.*, 1996 (16).

Administration In Vivo

The test solution was administered as a bolus through the duodenal catheter. After administration NaCl (0.9%) was flushed through the catheter to eliminate loss of substance due to the inner volume of the catheter (0.15 mL). Inogatran was given in a dose of 20 μmol/kg (or 8.8 mg/kg) in all experiments with or without different amount of aprotinin (1:1 or 1:3 w/w inogatran:aprotinin), dissolved in a phosphate buffer solution (pH 8, 0.1 M). The dose volume was 5 mL/kg. Blood samples of 0.3 mL were taken prior to administration and at regular time intervals until 240 minutes after administration and 0.3 mL NaCl (0.9%) was given to substitute the loss of blood volume after every withdrawn. The blood samples were put into Eppendorf® tubes containing citrate buffer (0.13 mol/L) and immediately mixed to prevent coagulation. The Eppendorf® tubes were put on ice before centrifuged for 10 minutes (10,000*G, + 4°C). Thereafter the plasma was withdrawn and transferred to new Eppendorf® tubes and was kept in the refrigerator until further analysis the same day or frozen at −20°C pending analysis.

Thrombin Time Elongation Measurement

The concentration of inogatran in the plasma samples was determined as thrombin time elongation (TTe) using a coagulometer KC 10A (Amelung, Germany). The citrate rat plasma, 100 μl, was diluted with 100 μl of NaCl solution (0.9%), and the plasma analysis was started by addition of human thrombin (see above) in a Tris-HCl buffer solution with 100 μl albumin pH 7.4.

Calculations

Permeability Coefficients

All rate constants were obtained under "sink" conditions (i.e. the concentration of the drug on the receiver side never exceeded 5% of the drug on the donor side). The apparent permeability coefficient (P_{app}, cm/s) was determined for excised segments according to the following equation:

$$P_{app} = dQ/dt \times 1/AC_0 \quad (1)$$

In which dQ/dt is the transport rate (steady-state flux, mol/s), C_0 is the initial concentration in the donor chamber (mol/mL), and A is the exposed surface area of the membrane (cm^2).

Bioavailability

The thrombin time elongation (TTe) was plotted against time from zero to 240 minutes and the area under the curve (AUC) was obtained by integration. The AUC was thereafter related to the AUC of an iv dose of inogatran and the bioavailability was calculated according to Equation 2. Inogatran is not metabolized either in the gut or in the systemic circulation. Additionally, there is a linearity between TTe and concentration of inogatran in plasma, within certain limits (11). The REA (relative effect area) values obtained can therefore be assumed to reflect the bioavailability which in turn reflects the fraction absorbed and is given in % of the total dose administered (see Equation 2). No further recalculation of concentration from thrombin elongation time or analysis of concentration of inogatran was performed. AUC, TTe_{max}, t_{max} and F% for the three different formulations of inogatran were calculated from the thrombin elongation time versus time plasma profiles. All other pharmcokinetic parameters of inogatran in rats can be obtained from the work of Eriksson *et al.*, 1998 (11).

$$(AUC_{exp}/AUC_{iv}) \times (dose_{iv}/dose_{exp}) \times 100 = REA\ (\%) = F\ (\%) = fa\ (\%) \quad (2)$$

Trypsin Binding Studies

Trypsin Activity

Trypsin activity in solutions of pancreatine was determined using the substrate S-2222 according to methods described earlier (15). Briefly, a 2.0 µl sample was added to a mixture of Tris buffer (pH 8.3) with 20 mM $CaCl_2$ and S-2222 solution. The absorbance at 405 nm was recorded vs time and the trypsin activity was calculated from the slope of such a curve. Pure trypsin solutions was analyzed in the same way to correlate trypsin concentration to activity. The intrinsic rate of degradation of trypsin in the solution during the course of the experiment was determined. During the 1 h duration of the kinetic dialysis experiments, see below, the trypsin activity was reduced to 50%.

Kinetic Dialysis

The competitive displacement of inogatran from trypsin in comparison with well known trypsin inhibitors was tested using kinetic dialysis. The experiments were carried out in a thermostated (37°C) well-stirred diffusion chamber (modified Ussing chamber) where the donor and the receiver compartments were separated by a dialysis membrane (Spectrapor 2, cut-off 15000, area 3.14 cm^2, thickness 23 µm). In each compartment 12.5 ml of USP intestinal fluid (pH 7.5) was added. Fifteen minutes before the start of an experiment pancreatine (6.0 g/l) was added to the donor compartment giving a dispersion (soluble and insoluble material) of approximately 8.0 µM trypsin. The experiment was started by adding a trypsin probe (8.0 µM + ^{3}H-labeled) (Astra Hässle AB; binding constant to trypsin 9.5 × 10^{-7} M^{-1}) and the trypsin inhibitors to the donor compartment. The different trypsin inhibitors used were aprotinin, SOY-3 (Soy bean trypsin inhibitor), BPTI (bovine pancreatic trypsin inhibitor) and inogatran and additionally the chelating effect of EDTA was tested. The amount of aprotinin used was equimolar to the trypsin concentration and the ^{3}H-trypsin probe. All other compounds tested were used in equal weight compared to aprotinin. The amount of trypsin probe that crossed the membrane was detected by liquid scintillation counting and normalized to the initial concentration, C/C_0. Only the free unbound trypsin probe can cross the membrane. The fraction of free (unbound) trypsin probe, f, in the absence of trypsin inhibitors was determined by performing experiments without pancreatine (f = 1.0) and without inhibitors (f determined to 0.09).

Statistics

Results are presented as mean values ± SD. Regional permeability (separate analysis for each intestinal region) and bioavailability data were analyzed with one-way analysis of variance. Confidence intervals of the mean difference between control group and each of the two dose groups were calculated, using Dunnet correction, giving an overall confidence level of 95% for the two intervals within each separate analysis. If the interval did not contain zero the difference was concluded to be significant at a 5% significance level.

RESULTS

The concentration dependent increase in the permeability coefficient (P_{app}) of inogatran in the absence and presence of

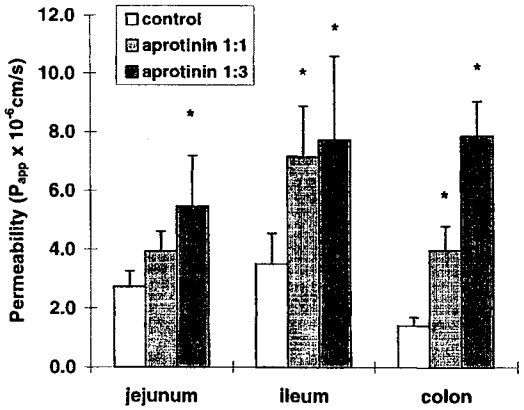

Fig. 1. Apparent permeability coefficients (P_{app}) of inogatran in three different regions of the rat intestinal tract with or without two concentrations of aprotinin, 1:1 or 1:3 (inogatran:aprotinin) w/w basis. Each group consists of segments taken from 4–7 different rats. Values represent mean ± SD. Asterisks indicate significantly different from the control group at a 5% level, analysed with one-way analysis of variance.

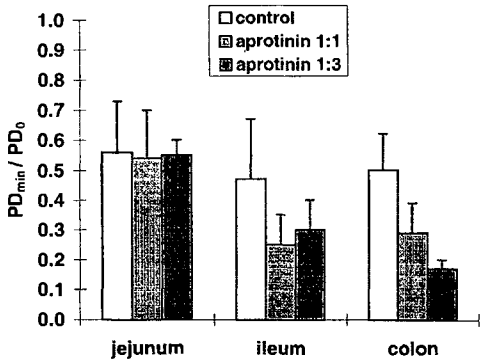

Fig. 2. The ratio of the minimum potential difference (PD_{min}) and the potential difference at the start of the experiment (PD_0) for the three different segments jejunum, ileum and colon of the rat with or without exposure to aprotinin.

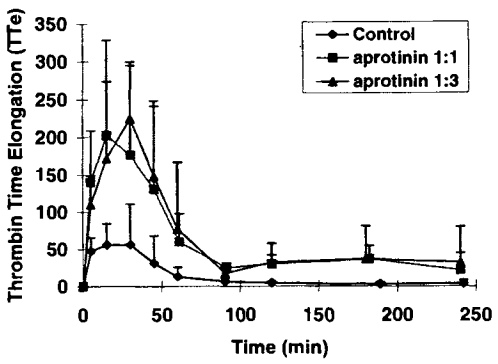

Fig. 3. Plasma thrombin elongation time versus time profiles of inogatran *in vivo* in rats after intraduodenal administration of inogatran in the presence or absence of aprotinin in two doses, 1:1 or 1:3 (inogatran: aprotinin) w/w basis. All values are mean values ± SD (N = 3–5). For more information regarding pharmacokinetic data see Table I.

aprotinin in three different regions of the rat intestinal tract is shown in Fig. 1. There was approximately a 2- to 3-fold increase in the permeability to inogatran in the small intestinal segments in the presence of aprotinin. The largest increase in P_{app} was seen in the colon segments, 3- and 5-fold for 1:1 and 1:3 (inogatran:aprotinin) respectively. Each of the groups showed significant effect of aprotinin. For jejunum the mean P_{app} was significantly higher in the high dose group compared to the control group. For ileum and colon mean P_{app} was significantly higher in both high and low dose groups compared to control. The electrical data from these experiments show no change in PD during aprotinin treatment in the small intestinal segments, however, for the distal colon, a concentration dependent decrease in PD could be seen during treatment with aprotinin (Fig 2).

The results from intraduodenal administration of the mixture of inogatran and aprotinin into conscious rats also showed a dose dependent enhancement of inogatran absorption as can be seen in Fig. 3. The bioavailability (F = fa = REA%; see methods for clarification) increased from 4% in the absence to 17–20% in the presence of aprotinin (Table I). Due to high variability only the mean bioavailability was significantly higher in the high dose group compared to the control group without aprotinin present.

The *in vitro* results from the kinetic analysis showing the inhibition of binding of a specific trypsin probe by different trypsin inhibitors are presented in Fig. 4. Without any inhibitor present and in the presence of pancreatine, the transport rate of the probe was very low. EDTA did not have any effect on the binding of the trypsin probe to trypsin and resulted in low transport rates of the trypsin probe. Both aprotinin and the other trypsin inhibitors inhibited the trypsin probe binding and aprotinin showed a high binding constant as it displaced the trypsin probe from trypsin at equimolar concentration to the trypsin probe. Inogatran also showed displacement of the trypsin probe from the binding site at trypsin, but to a lesser extent as the concentration needed for the same transport rate of the trypsin probe through the membrane was 15 times higher than for aprotinin.

DISCUSSION

Enhancement of the absorption of low permeability drugs with different types of enhancer systems has been shown in the literature (for ref see introduction). These include mechanisms like opening of the paracellular pathway by change in the tight junctional resistance, increased lipid fluidity of the bilayer membrane, inhibition of proteolytic activity and/or inhibition of p-glycoprotein efflux. The bioavailability of inogatran in rats has been shown to be low and dose dependent and an increase in dose from 1 mg/kg rat to 100 mg/kg increased the bioavailability from 5% to 30% (11, 13). This dose dependent increase could not be attributed to saturation of enzymatic degradation since the drug has been found to be enzymatically stable in the rat (11). Instead, the present study indicates a binding of inogatran to proteases in the gut, e.g. trypsin and chymotrypsin. Increased concentration by increased dose will

Table I. Pharmacokinetic Data of Inogatran After Oral Administration of Three Different Formulations[a]

Formulation	TTe_{MAX}[a] (s)	t_{MAX}[b] (min)	AUC[c] (s × min)	F[d] (%)	N
Inogatran	66.5 ± 48.9	18.7 ± 7.5	3334.7 ± 2155.6	3.8 ± 2.5	4
Inogatran:aprotinin (1:1)	206.2 ± 120.3	11.7 ± 5.8	14589.8 ± 5113.4	16.9 ± 5.8	3
Inogatran:aprotinin (1:3)	224.9 ± 75.0	26.3 ± 7.5	15499.5 ± 9014.6	19.4 ± 11.8*[e]	4

[a] All values are expressed as mean ± s.d.
[b] TTe = thrombin time elongation.
[c] t = time to reach a maximum thrombin time elongation in plasma.
[d] AUC = area under the TTe vs time curve.
[e] F = bioavailability, for calculations sz methods.
* Significantly different from the control group (inogatran) at a 5% significance level, analysed with one-way analysis of variance.

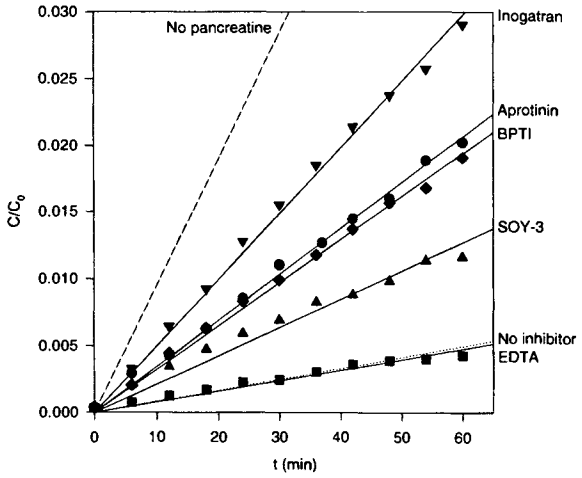

Fig. 4. Result from kinetic dialysis showing *in vitro* inhibition of transport of a trypsin probe by some trypsin inhibitors. The amount of trypsin probe that crossed the membrane is normalized to the initial concentration, C/C_0, of the probe in each experiment. The fraction of free trypsin probe, f, was calculated from the slopes of linear fits to the experimental data points. The values of f in the absence of trypsin inhibitors was determined by performing experiments without pancreatine (f = 1.0) (dashed line) and without inhibitors (f determined to 0.09) (dotted line).

saturate binding sites and contribute to higher bioavailability. However, the effect of aprotinin on the permeability for inogatran has to have some other explanation.

An enhancement effect of bacitracin on small intestinal and colonic epithelium without any concomitant mucosal damage using phenol red and fluorescein isothiocyanate dextran as test drugs has been reported *in vivo* in the rat (18–19). The increase in absorption suggested bacitracin to be a good model adjuvant for poorly absorbable drugs in general. This indicates that apart from inhibition of proteolytic activity serine protease inhibitors can also act as membrane breaking agents increasing the intestinal permeability to drugs. The changed barrier properties (PD) of the rat ileum and colon segments during addition of aprotinin in the present study suggests changed permeability induced by a similar mechanism as suggested for bacitracin (18–19).

Absence of pancreatic juice increased the absorption of both enzymatically stable and unstable oxytocin analogues in the pig (10). Several reasons for this phenomenon were suggested from the study: apart from changed hormone levels in the gut, which could alter peptide transport, some unknown factor seems to be present in the pancreatic juice that could act directly on the membrane. However, the effect could also be due to the absence of binding of the enzymatically stable oxytocin analogue to pancreatic enzymes and thereby the effective concentration at the cell surface increases and subsequently an increased absorption can be seen. In the present study, competitive displacement of inogatran from trypsin, as can be concluded from the kinetic dialysis, greatly enhanced the absorption of the drug *in vivo*. The binding of the trypsin probe to trypsin was inhibited by aprotinin as expected. Due to the large difference in concentration between aprotinin and inogatran in the *in vivo* study, aprotinin clearly demonstrated a higher binding constant to trypsin *in vivo* than does the more abundant available inogatran (1:15 on molar basis). This shows that only a few moles of aprotinin has to be available for trypsin binding to inhibit the binding of inogatran to trypsin *in vivo*. These data suggest a new mechanism of intestinal absorption enhancement of peptide drugs which is not enzymatically cleaved in the GI tract but still has low bioavailability.

The increased P_{app} value of inogatran from the *in vitro* Ussing chamber predicts an *in vivo* absorption in man of between 20–30% according to the correlation curve presented earlier by Lennernäs *et al.* 1997 (12). This enhancement is in the same magnitude as the results from the *in vivo* rat experiments in the present study indicating a good correlation between these models of absorption not only for prediction of absorption in man, but also of the effect of absorption enhancement.

Our results from the present study support the fact that peptide inhibitors synthesised for systemic effects and which are enzymatically stable can act like inhibitors to trypsin or other serine proteases in the gut even if they are not enzymatically cleaved. Indeed thrombin inhibitors are synthesised to be inhibitors for the serine protease thrombin and the selectivity towards trypsin or other serine proteases are not always optimal. As the trypsin-like enzymes are abundant in the GI tract, binding of thrombin inhibitors to these enzymes might severely reduce their bioavailability.

CONCLUSIONS

It can be concluded from the study that a coadministration of aprotinin also with enzymatically stable peptides can improve their absorption after oral administration. A new additional mechanism of peptide absorption enhancement from the gut is suggested.

ACKNOWLEDGMENTS

Dr. Olof Bengtsson, Department of Biostatistics, Astra Hässle AB, for statistical evaluation of data, Mrs Daga Hölmark and Mrs Ann-Sofi Lindh, Animal Resources, Astra Hässle AB, for surgical assistance during the *in vivo* experiments, are all gratefully acknowledged.

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