Development and *in Vitro* Evaluation of Systems to Protect Peptide Drugs from Aminopeptidase N

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Purpose. Develop and evaluate systems to prevent aminopeptidase N caused enzymatic degradation of perorally administrated peptide drugs.
Methods. Bacitracin was covalently bound to the unabsorbable carrier matrix poly(acrylic acid) (paa) in order to avoid any dilution effects of the inhibitor in the intestine as well as systemic toxic side effects. The inhibitory effect of this conjugate, of neutralized paa and N-acetylcysteine was evaluated using a brush border membrane model.
Results. Whereas within 6 h of incubation 65.3 ± 3.7 μmol/l of the substrate (L-leucine p-nitroanilide) was hydrolyzed under our assay conditions, this metabolism was reduced to 44.5 ± 6.3 μmol/l and 49.0 ± 8.8 μmol/l (n = 3–5; ± S.D.) using 1.5% bacitracin-polymer conjugate and 0.5% N-acetylcysteine, respectively. The same amount of bacitracin as immobilized to the polymer exhibited a comparably weaker inhibitory effect. Neutralized paa did not inhibit membrane bound aminopeptidase N. Covering the membrane with a thin mucus layer led to a significantly lowered inhibitory effect of all tested agents.
Conclusions. The immobilization of enzyme inhibitors to a carrier matrix and the use of N-acetylcysteine as a novel inhibitor are promising strategies in order to overcome the enzymatic barrier caused by membrane bound peptidases. However the use of effective mucolytic agents seems to be a prerequisite.

KEY WORDS: inhibition of aminopeptidase N; poly(acrylic acid); bacitracin; N-acetylcysteine.

INTRODUCTION

Recently, the oral delivery of therapeutic peptides as an alternative route to parenteral administration has been vigorously investigated. It offers the greatest ease of application, and the number of peptide drugs such as cyclosporine, oxytocin, or gonadorelin as well as pharmaceutical peptide analogues such as desmopressin, sandostatin, or buserelin will increase in the years to come. However, so far, there has been only a few peroral (poly)peptide delivery systems reported which are all considerably ineffective. The main reason for this situation has been in overcoming the enzymatic barrier of the gastrointestinal (GI) tract (1). Although many attempts have been made to develop drug delivery systems shielding the enzymatic attack of luminal proteinases, e.g., trypsin (2), α-chymotrypsin (3,4), and pepsin (5), there is almost nothing reported about systems capable of inhibiting brush border membrane (BBM) bound enzymes. Therefore, it was the aim of the present study to develop and evaluate systems reducing the activity of BBM-bound peptidases in order to overcome this barrier.

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Aminopeptidase N is generally regarded as the most abundant peptidase on the BBM and as the most active exopeptidase (1). Hence, we focused this study on ways to inhibit the proteolytic activity of this enzyme. In order to reach this goal, we followed two promising strategies and evaluated their practicability *in vitro* on a BBM-model.

(I) As the mucus layer covering GI-epithelia prevents a direct contact of enzyme inhibiting agents with a molecular mass higher than 6.5 kDa (6), we investigated inhibitors acting on the uptake of bivalent metal ions (7) which are essential for the proteolytic activity of many endo- as well as exopeptidases. With regard to this mechanism, direct contact with BBM-bound peptidases does not seem to be essential for this type of inhibitor. In the present study we evaluated the inhibitory effect of poly(acrylic acid) (paa) and tried to enhance its depleting capability by the addition of bacitracin, which is well known for its high binding affinity to bivalent cations. Moreover, in order to avoid systemic side effects of bacitracin (8) and a disturbed digestion of nutritive proteins, it was covalently attached to this polymer to develop a locally acting system.

(II) We investigated a non-toxic agent with a molecular mass of 163.2 Da, which should easily pass through the mucus layer and inhibit membrane bound peptidases by direct interaction. As amino acids and slightly modified amino acids are well known to inhibit aminopeptidases (9,10), we focused our interest on N-acetylcysteine exhibiting additionally mucolytic properties (11).

MATERIALS AND METHODS

Materials

Microsomal aminopeptidase (aminopeptidase N; EC 3.4.11.2.), L-leucine-p-nitroanilide, bacitracin, N-acetylcysteine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), and mucin (type II: crude) were obtained by Sigma (St. Louis, MO). Poly(acrylic acid) (Carbopol 940) was purchased from BF Goodrich (Cleveland, OH) and nitrocellulose membrane (Immobiline NC Pure) from Millipore S.A. (Molsheim, France). Sulfo-N-hydroxysuccinimide (SNHS) was obtained by Pierce Europe B.V. (Oud Beijerland, NL). L-cysteine and L-glycine were purchased by Merck (Darmstadt, Germany) whereas all other amino acids were from L. Light Co. Ltd. (Colnbrook, UK).

Neutralization of Poly(acrylic acid)

10 g of paa were gradually added to 100 ml of a 4% (m/m) methanolic sodiumhydroxyd solution under permanent stirring. The precipitate was filtered, washed with methanol till the pH of the filtrate became neutral and brought to dryness in an exsiccator.

Synthesis of the Bacitracin-Poly(acrylic acid) Conjugate

Bacitracin was coupled to paa by a condensation reaction using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) catalyzed by sulfo-N-hydroxysuccinimide (SNHS) following the procedure described by Staros *et al.* (12). The available NH_2-groups of the peptide were covalently bound

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to the carboxyl groups of the polymer by constituting amide bindings. 100 mg of the neutralized poly(acrylate), 400 mg of EDAC, 22 mg of SNHS, and 200 mg of bacitracin were dissolved in 20 ml of demineralized water and stirred for 6 h at room temperature. The remaining unbound inhibitor and the coupling reagents were removed by dialyzing the reaction mixture for 24 h against demineralized water. The isolated bacitracin-paa conjugate was lyophilized and stored at −20°C. Aliquots were analyzed spectrophtometrically (Perkin-Elmer, Lambda 16 UV/VIS) as well as gravimetrically and enzymatically.

Enzyme Assay with Unbound Aminopeptidase N

Degradation studies with aminopeptidase N were performed using L-leucine-p-nitroanilide as enzyme substrate. Tested polymers or inhibitors were dissolved in TBS (50 mM Tris-HCl buffered saline, pH 7.5) in concentrations as reported in Results and 100 μl aliquots transferred into wells of a microtitration plate. 50 μl of aminopeptidase N (10 μg/ml TBS) were added and the mixtures incubated for one hour at room temperature. After this, 50 μl of the substrate solution (0.5 mg of L-leucine-p-nitroanilide/ml TBS) were added and the increase in absorbance ($\Delta A_{405\ nm}$) caused by the enzymatic reaction at 20 ± 0.5°C was immediately recorded at one minute intervals with a microtitration plate reader (Anthos reader 2001). For controls the polymer or inhibitor was omitted. Concentrations of the hydrolyzed substrate were calculated accordingly by interpolation from a standard curve.

Enzyme Assay with Membrane Bound Aminopeptidase N

A nitrocellulose membrane was clamped between the two halves of a Franz diffusion cell with 10 mm-diameter-holes on both sides. The donor chamber was filled with 250 μl of a solution of aminopeptidase N (50 μg/ml TBS) and incubated for one hour at room temperature. The supernatant was discarded and the enzyme coated side of the membrane washed three times with TBS. The membrane was then incubated with 500 μl of 3% bovine serum albumin (BSA) in TBS for 12 hours at 4°C. The supernatant was removed and the membrane washed again five times with demineralized water. It was directly used for studies with membrane bound aminopeptidase N or additionally coated with a 100–120 μm thin mucus layer. For that purpose 10 μl of 20% mucin in TBS was transferred on the horizontally placed membrane and incubated at room temperature until the mucus layer covering the whole membrane exhibited a plane surface. The addition of polymer preparations had no influence on this mucus layer. The receptor chamber was filled with 3.4 ml of TBS and magnetically stirred (200 rpm). The donor chamber was filled with 1.0 ml of samples as shown in Table 1. Indicated concentrations of polymers were chosen in order to obtain an equal rate of substrate diffusion by all tested samples and also to make the results comparable with studies concerning the unbound enzyme. During all experiments (which were performed at least in triplicate), temperature was maintained at 37 ±1°C by an external water-jacket. For control, neutralized paa was substituted by hydroxyethyl cellulose, as it has no carboxylic acid groups which are essential for the binding of bivalent cations. 200 μl samples were withdrawn from the receptor chamber at one hour intervals and replaced with an equal volume of TBS preequilibrated to temperature. 100 μl of these samples were transferred to a microtitration plate and the concentration of the hydrolyzed substrate was determined by measuring the absorbance at 405 nm. Thereafter, 50 μl of aminopeptidase N (20 μg/ml TBS) were added and, after one hour incubation at room temperature, the absorbance was measured again at 405 nm in order to determine the total amount of substrate diffused to the receptor chamber. In any case, cumulative corrections were made for the previously removed samples in determining the total amount diffused to the receptor phase. Concentrations of hydrolyzed substrate were calculated accordingly by interpolation from a standard curve.

Table 1. Amounts of Polymers and/or Inhibitors Dissolved in 1.0 ml of Substrate Solution (0.5 mg L-leucine-p-nitroanilide/ml TBS) Used for Experiments with Membrane Bound Aminopeptidase N

Hydroxyethyl cellulose	Neutralized poly(acrylic acid)	Bacitracin-paa conjugate	Bacitracin	N-acetyl-cysteine
32.5 mg	—	—	—	—
32.5 mg	—	—	—	5 mg
32.5 mg	—	—	—	25 mg
—	15 mg	—	—	—
—	15 mg	—	28.5 mg	—
—	—	50 mg	—	—
—	10 mg	15 mg	—	—

RESULTS

Bacitracin-poly(acrylic acid) Conjugate

The amount of bacitracin coupled to neutralized paa was gravimetrically determined. For this reason, the purified sample was prepared in the same way, but omitting EDAC during the coupling reaction served as a negative control.

Because of the different weights of the purified and lyophilized polymer with and without bound inhibitor, it was possible to determine the amount of coupled bacitracin. 58% ± 5.5% (means of three experiments; ±S.D.) of the inhibitor was bound to the polymer, representing 60.6% (m/m) of the coupled product. As the pH-value of the (un)modified polymer did not change using the dialysis procedure, a possible loss of Na^+ ions could be excluded. The UV-VIS spectrum of the conjugate showed absorption maxima below 235 nm and an absorption shoulder at 255 nm. As neutralized paa has no absorption maximum at 255 nm, the successful covalent attachment of the inhibitor, exhibiting a maximum at this wave length to the polymer could be verified. The negative control as described above did not show an absorption shoulder at 255 nm, demonstrating also the efficiency of the purification method.

Inhibition of Unbound Aminopeptidase N

Fig. 1 shows the inhibition of unbound aminopeptidase N activity by neutralized paa. For a positive control, the poly(acrylate) was omitted during the enzymatic reaction. The substitution of neutralized paa by hydroxyethyl cellulose in concentrations between 0.004–1.5% (m/m) demonstrated the

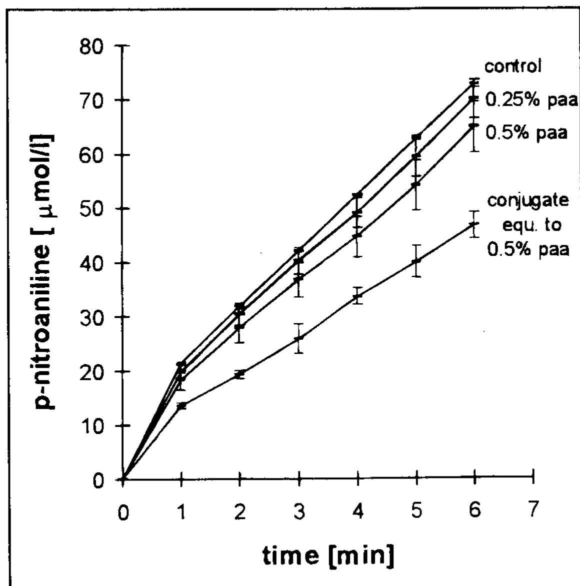

Fig. 1. Inhibition of unbound aminopeptidase N (2.5 μg/ml TBS) by indicated concentrations (%; m/m) of neutralized paa and a concentration of bacitracin-poly(acrylate) conjugate equivalent to 0.5% unmodified polymer; pre-incubation time 1h; substrate: 0.125 mg of L-leucine-p-nitroanilide/ml TBS; Each point represents the mean ± S.D. of at least three experiments.

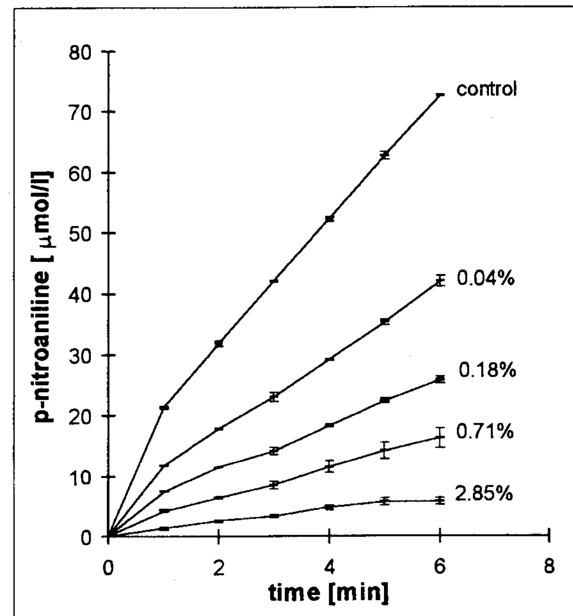

Fig. 2. Inhibition of unbound aminopeptidase N (2.5 μg/ml TBS) by indicated concentrations (%; m/m) of bacitracin; pre-incubation time 1h; substrate: 0.125 mg of L-leucine-p-nitroanilide/ml TBS; Each point represents the mean ± S.D. of at least three experiments.

same increase of hydrolyzed substrate as determined for the positive control. According to this result, hydroxyethyl cellulose seems to have no influence on the enzymatic activity of aminopeptidase N. It was therefore used as a carrier matrix for the substrate for studies with the membrane bound enzyme. Furthermore, the inhibitory effect of neutralized paa could be significantly enhanced by the covalent attachment of bacitracin (Fig. 1). Since the concentration of the conjugate which is equivalent to 0.5% neutralized paa corresponds to 0.77% bacitracin, it can be concluded that the same concentration of unbound inhibitor has a comparably stronger inhibitory effect. Fig. 2 shows this inhibitory effect of increasing bacitracin concentrations.

On the other hand, studies with increasing concentrations of N-acetylcysteine demonstrated a very effective inhibition of aminopeptidase N as shown in Fig. 3. N-acetylcysteine concentrations higher than 0.25% allowed a complete inhibition of L-leucine-p-nitroanilide degradation. Agents exhibiting a free thiol group such as β-mercaptoethanol and L-cysteine demonstrated, in the same molarity, a weaker but marked inhibitory effect. Whereas certain amino acids such as L-methionine, L-alanine, L-leucine, and L-valine also had a weak inhibitory effect compared to N-acetylcysteine, the influence of L-serine and L-glycine on the enzyme activity was negligible (data not shown).

Inhibition of Membrane Bound Aminopeptidase N

In order to investigate also the influence of the mucus layer on the inhibition of aminopeptidase N, all studies were carried out with and without this mucus layer. A schematic diagram of the BBM-model developed by us is illustrated in Fig. 4. In contrast to experiments with unbound aminopeptidase N, no inhibitory effect of neutralized paa used in comparable concentrations could be observed towards the membrane linked enzyme. However, the bacitracin-polymer conjugate still exhibited an inhibition of enzymatic activity as shown in Fig. 5. Furthermore, an inhibition of membrane bound aminopeptidase N could also be achieved by unbound bacitracin (2.85%; m/m) and N-acetylcysteine (0.5%; m/m). Comparing the inhibitory effect of 1.5% conjugate—corresponding to approximately 0.9% unbound bacitracin—with the inhibitory effect of 2.85%

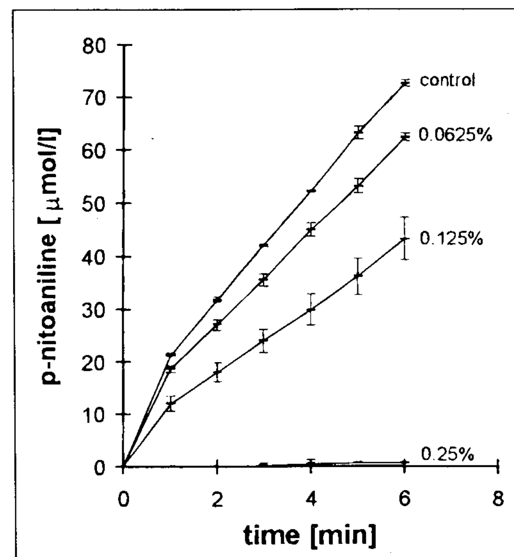

Fig. 3. Inhibition of unbound aminopeptidase N (2.5 μg/ml TBS) by indicated concentrations (%; m/m) of N-acetylcysteine; pre-incubation time 1h; substrate: 0.125 mg of L-leucine-p-nitroanilide/ml TBS; Each point represents the mean ± S.D. of at least three experiments.

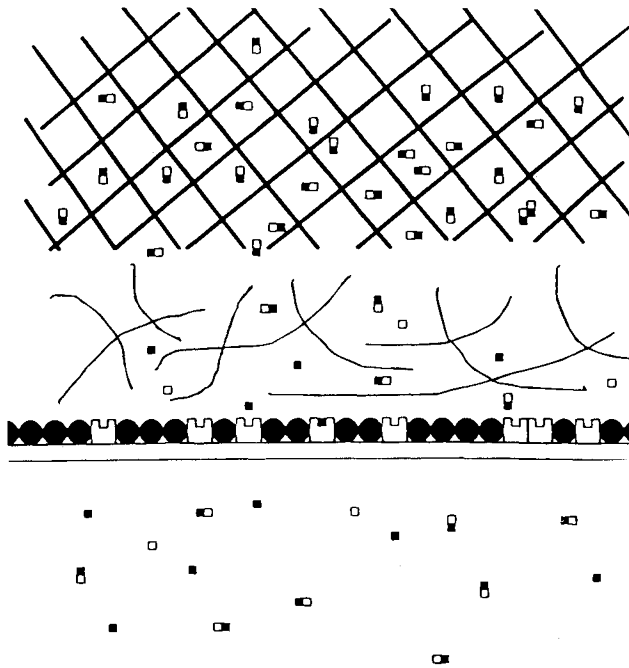

Fig. 4. Schematic diagram of the BBM-model; aminopeptidase N: ◻; bovine serum albumin: ●; L-leucine-p-nitroanilide: ◻■; L-leucine: ◻; p-nitroaniline: ■; carrier matrix: ▬; nitrocellulose: ▭; mucus: ‿.

bacitracin, demonstrated a stronger protective effect of the lower concentrated but immobilized inhibitor. Covering the enzyme coated membrane with a thin mucus layer revealed its significant influence on all inhibitory agents. After 4 h of incubation, the percentage of degradated substrate in the receptor chamber was photometrically determined. Compared to the control (3.25% hydroxyethyl cellulose) leading to a substrate degradation of 65.5 ± 0.7% (means of three experiments; ±S.D.), the bacitracin-polymer conjugate (5%; m/m) reduced this metabolism to 62.3 ± 3.55% (means of three experiments; ±S.D.). Under the use of 2.5% (m/m) of N-acetylcysteine, a degradation of 54.3 ± 3.1% (means of three experiments; ±S.D.) could be determined.

DISCUSSION

Aminopeptidase N is a metalloprotease exhibiting a binding site for the bivalent cation Zn^{2+} which is essential for the proteolytic activity of the enzyme. It has been described that depletion of Zn^{2+} from the enzyme structure by bivalent cation binding agents such as bacitracin can inhibit the activity of aminopeptidase N (13). In this study, we revealed a weak inhibitory effect of neutralized paa which is also able to bind bivalent cations. In order to enhance this inhibitory effect, bacitracin exhibiting a high association constant of zinc bacitracin, estimated to be 2.5×10^3 at pH 6.34 (14), was covalently bound to the polymer. Whereas the same amount of bacitracin as coupled to the polymer, demonstrated a comparable stronger inhibitory effect in the enzyme assay with unbound aminopeptidase N, the situation was completely changed under the use of the membrane bound enzyme. Since unbound bacitracin can pass through the membrane, this additional dilution effect which can also be expected under *in vivo* conditions, might be the explanation for the stronger protective effect of the immobilized inhibitor. Moreover, the high binding affinity of the conjugate to bivalent cations seems to be sufficient for the uptake of Zn^{2+} from membrane bound aminopeptidase N covered with a thin mucus layer. However, comparable high concentrations of the modified polymer (5%) were necessary in order to achieve a significant inhibition of the enzyme. In contrast, neutralized paa did not show any inhibitory effect in the same experiment, demonstrating the essential need of matrix systems which exhibit a higher binding affinity to bivalent cations than the unmodified poly(acrylate).

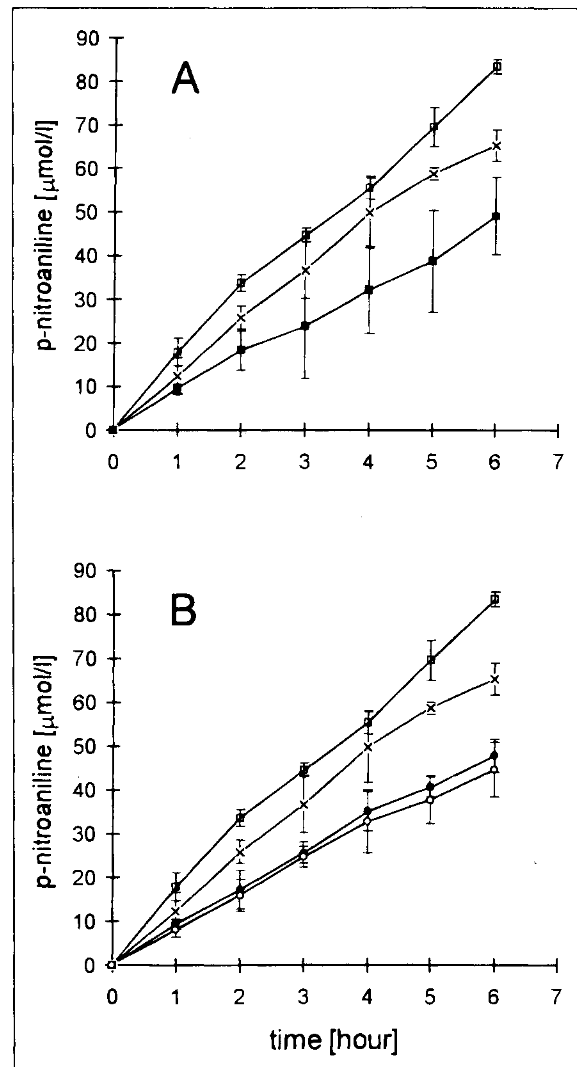

Fig. 5. Inhibition of membrane bound aminopeptidase N; The inhibitory effect of 0.5% N-acetylcysteine (■; A), 1.5% conjugate (O; B) and 2.85% bacitracin (●; B) was compared to 3.25% hydroxyethyl cellulose (x; A, B) used as control by determining the concentration of p-nitroaniline in the receptor chamber at one hour intervals. Concentrations of the total amount of metabolized as well as unmetabolized substrate diffused to the receptor chamber were for all samples in the same range (◻; A, B). Each point represents the mean ± S.D. of at least three experiments.

The present study revealed N-acetylcysteine as a very potent inhibitor of aminopeptidase N. With regard to the peroral administration of peptide drugs, it promises several advantages:

(I) The peroral co-administration of N-acetylcysteine demonstrates no remarkable toxicity risks which were to be expected for enzyme inhibitors used in the past (e.g., amastatin, bestatin, puromycin). (II) Because of its low molecular mass, it can easily pass through the mucus layer covering intestinal epithelia which is a prerequisite for inhibitors directly interacting with the enzyme. (III) Due to its mucolytic properties, N-acetylcysteine is also capable of reducing the mucus-barrier as recently reported by Bernkop-Schnürch and Fragner (6).

The reason for the inhibitory effect of N-acetylcysteine might be seen in the involvement of the free thiol group in overall catalysis or in the binding of metal ions since we could also observe an inhibition of aminopeptidase N by β-mercaptoethanol and L-cysteine. Moreover, the substitution of the thiol group of L-cysteine by a hydroxyl group (=L-serine) led to an almost complete loss of the inhibitory effect. On the other hand, McClellan et al. (15) demonstrated a competitive inhibition of aminopeptidase N especially by L-methionine, L-alanine, L-leucine, and some other nonpolar amino acids which can be confirmed by our studies. As N-acetylcysteine also represents a slightly modified nonpolar amino acid, its inhibitory effect could be explained by such a competitive inhibition.

CONCLUSIONS

From the results of the *in vitro* studies described here, it may be concluded that a direct contact of a drug delivery system exhibiting zinc depleting capabilities, is in principle not a prerequisite for the inhibition of membrane bound aminopeptidase N. However, the separation of the delivery system from the enzyme by the mucus layer leads to a significant decrease in inhibition, demonstrating the substantial need of very effective mucolytic agents for such systems. The immobilization of enzyme inhibitors to a carrier matrix—excluding disturbing dilution effects as well as inhibitor absorption—leads to an enhanced protective effect. It therefore seems to be a promising strategy in overcoming this enzymatic barrier. However, following the strategy of low-sized, non-toxic agents inhibiting the enzyme by direct interaction, N-acetylcysteine might be a novel promising candidate in increasing stability of perorally administrated peptide drugs.

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