Potent and Selective Inhibition of Zinc Aminopeptidase A (EC 3.4.11.7, APA) by Glutamyl Aminophosphinic Peptides: Importance of Glutamyl Aminophosphinic Residue in the P_1 Position†

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ABSTRACT: Through the development of a new chemical strategy, aminophosphinic peptides containing a pseudoglutamyl residue (GluΨ(PO_2-CH_2)Leu-Xaa) in the N-terminal position were synthesized and evaluated as inhibitors of aminopeptidase A (APA). The most potent inhibitor developed in this study, GluΨ(PO_2-CH_2)Leu-Ala, displayed a K_i value of 0.8 nM for APA, but was much less effective in blocking aminopeptidase N (APN) (K_i = 31 μM). The critical role of the glutamyl residue in this phosphinic peptide, both in potency and selectivity, is exemplified by the P_1 position analogue, AlaΨ(PO_2-CH_2)-Leu-Ala, which exhibited a K_i value of 0.9 μM toward APA but behaved as a rather potent inhibitor of APN (K_i = 25 nM). GluΨ(PO_2-CH_2)Leu-Xaa peptides are poor inhibitors of angiotensin converting enzyme (K_i values higher than 1 μM). Depending on the nature of the Xaa residue, the potency of these phosphinic peptides toward neutral endopeptidase 24−11 varied from 50 nM to 3 μM. In view of the in vivo role of APA in the formation of brain angiotensin III, one of the main effector peptides of the renin angiotensin system in the central nervous system, highly potent and selective inhibitors of APA may find important therapeutic applications soon.

Aminopeptidase A (glutamyl-aminopeptidase, EC 3.4.11.7, APA)[1] is a homodimeric type II membrane proteinase (*1*), which specifically cleaves in vitro NH_2-terminal glutamyl or aspartyl residues from peptide substrates (*2*−*4*). APA shares significant sequence homology with another aminopeptidase of broader specificity, aminopeptidase N (APN, EC 3.4.11.2) (*5*−*8*), able to cleave neutral or basic NH_2-terminal residues from peptides (*3, 9*). The sequence of these two enzymes contains the zinc-binding motif, HEXXH, found in most zinc-dependent metalloproteinases (*10, 11*). In APA, the presence of this metal was confirmed by metabolic labeling with radioactive zinc, and amino acid ligands of this metal have been identified by site-directed mutagenesis (*12, 13*).

Recently, brain APA and APN were shown to be involved in vivo in the metabolism of angiotensin II (Ang II) and angiotensin III (Ang III), respectively (*14, 15*). In the central nervous system, several lines of evidence support the notion that the brain renin angiotensin system (RAS) controls cardiovascular functions and body fluid homeostasis (*16*−*19*). More specifically, it was demonstrated that angiotensin III, resulting from the conversion of angiotensin II by APA in the brain, was one of the main effector of the brain RAS in the control of vasopressin release and vasopressinergic neuron activity (*14, 20*). These results lend credence to the hypothesis that angiotensin III could also play an imporant role in the control of blood pressure by central nervous system, justifying the development of highly potent and selective inhibitors.

Several highly potent pseudo-peptide inhibitors of APN have been described (*21, 22*). Among them, several free aminophosphinic peptides were observed to block APN with K_i values in the nanomolar range (*23*) (Figure 1). On the basis of these structures and taking into account the specificity of APA for acidic amino acid residues, free aminophosphinic peptides bearing a pseudoglutamyl residue at the N-terminal position should behave as highly potent APA inhibitors (Figure 1). Despite many attempts, the synthesis of these types of phosphinic peptides by standard procedures is unsuccessful. By developing a completely new chemical strategy, we recently prepared GluΨ(PO_2-CH_2)Xaa phosphinic synthons (Scheme 1) (*24*), making possible for the first time to synthesize phosphinic peptides, containing a pseudoglutamyl residue at the N-terminal.

In the present study, glutamyl aminophosphinic peptides were synthesized and their potencies were evaluated against

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[1] The abbreviations used are: APA, aminopeptidase A (EC 3.4.11.7); APN, aminopeptidase N (EC 3.4.11.2); ACE, angiotensin converting enzyme (EC. 3.4.15.1); NEP, neutral endopeptidase 24−11 (EC. 3.4.24.11); Ψ indicates that the peptide bond has been modified, and the formula of the group that has replaced this peptide bond is in parentheses; tBu, *tert*-butyl; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl; Ad, adamantyl; Boc, *tert*-butyloxycarbonyl.

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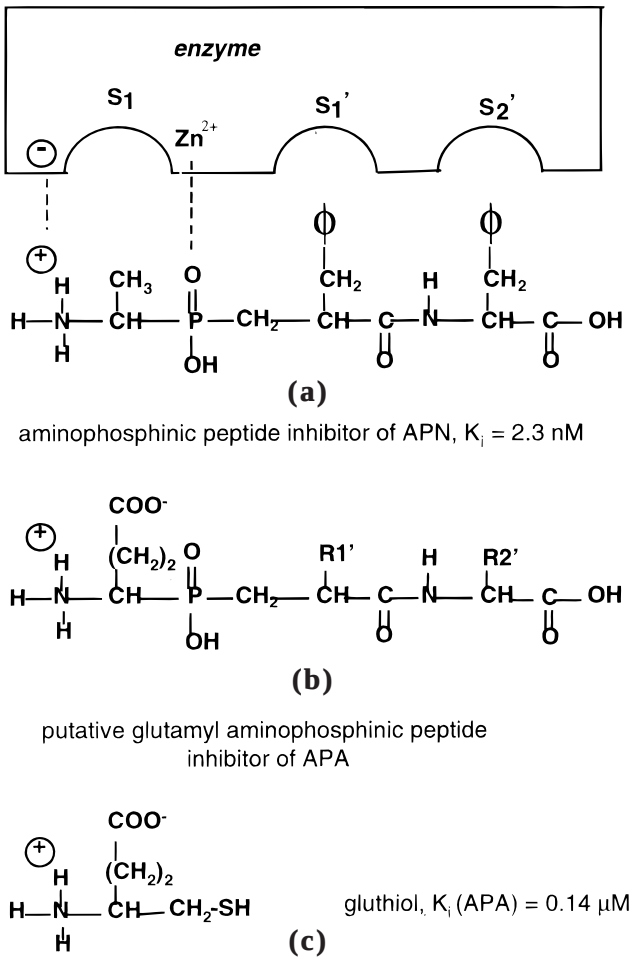
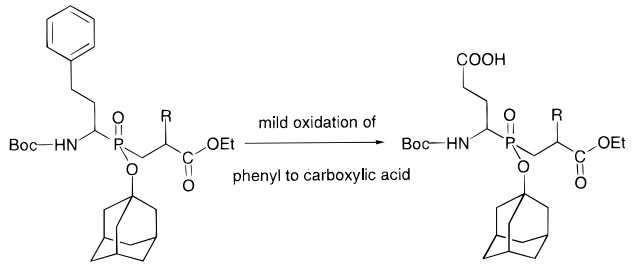


FIGURE 1: Schematic representation of the interaction of aminophosphinic peptide inhibitors with (a) aminopeptidase N and (b) aminopeptidase A. The proposed mode of interaction of gluthiol with APA is depicted in (c).

Scheme 1



APA and APN, but also angiotensin converting enzyme (ACE, EC 3.4.15.1) and neutral endopeptidase 24.11 (NEP, EC 3.4.24.11), two other zinc-metallopeptidases also involved in the metabolism of vasoactive peptides in the central nervous system (*25*, *26*). Both the potency and selectivity of the inhibitor toward APA were observed to depend critically on the presence of a P_1 pseudoglutamyl residue in these phosphinic peptides, pointing out the importance of appropriate chemical strategy to synthesize glutamyl aminophosphinic peptides.

MATERIALS AND METHODS

Purified rabbit kidney neutral endopeptidase 24.11 (NEP) was kindly provided by Drs. P. Crine and G. Boileau (Département de Biochimie, Université de Montréal, Canada) (*27*). Human placental APN was from Sigma. 2-Aminobenzoyl-Ala-Gly-Leu-Ala-*p*-nitrobenzylamide, α-L-glutamyl-β-naphthylamide (GluNA), and α-L-alanyl-β-naphthylamide (AlaNA) were from Bachem. 7-Methoxycoumarin-2-acetic acid (McaOH) was from Aldrich. *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid (DpaOH) was synthesized according to Knight et al. (*28*). Mca-Ala-Ser-Asp-Lys-DpaOH was synthesized by Fmoc solid-phase peptide synthesis, as described previously (*29*).

Enzyme Assays and Inhibition Studies. APA Assays. Mouse recombinant APA was prepared from a stable transfected CHO cell line overexpressing this enzyme, as described previously (*30*). Assays were performed at 25 °C in 50 mM Tris·HCl buffer, pH 7.4, 4 mM $CaCl_2$. APA activity was assayed in microplates by monitoring the rate of hydrolysis of GluNA substrate, following the procedure described elsewhere (*31*). The K_m value determined for this substrate was 100 μM.

APN Assays. Assays were performed at 25 °C in 50 mM Tris·HCl buffer, pH 7.4. APN activity was assayed in microplates by monitoring the rate of hydrolysis of AlaNA, as previously described (*3*). The K_m value determined for this substrate was 100 μM.

ACE Assays. Human wild-type somatic ACE was obtained through stable expression in Chinese hamster ovary cells transfected with appropriate ACE cDNA and purified as previously described (*32*). Assays were performed at 25 °C in 50 mM Hepes buffer, pH 6.8, 200 mM NaCl, and 10 μM $ZnCl_2$. ACE activity was assayed in microplates by monitoring the rate of hydrolysis of Mca-Ala-Ser-Asp-Lys-DpaOH, as previously described (*33*). The K_m value determined for this substrate was 44 μM.

NEP Assays. Assays were performed at 25 °C in 50 mM Tris·HCl buffer, pH 7.4. NEP activity was assayed in microplates by monitoring the rate of hydrolysis of 2-aminobenzoyl-Ala-Gly-Leu-Ala-*p*-nitrobenzylamide, as previously described (*34*). The K_m value determined for this substrate was 138 μM.

Determination of K_i Values. For each inhibitor, percentage inhibition was determined in triplicate experiments at five concentrations. Inhibitor concentrations were selected in order to observe a 20−80% range of inhibition. K_i values were determined using the method proposed by Horovitz and Levitski (*35*). This approach explicitly takes into account the effect of the substrate, enzyme, and inhibitor concentrations and applies to the situation of both standard and tight-binding inhibition. For the different peptidases tested, evidence for slow-binding behavior was not observed.

Inhibitor Synthesis. Solid-phase syntheses were performed manually. The side chains of the protecting groups used were Asp(tBu) and Arg(Pmc). The phosphinic pseudodipeptide Boc-(*R*,*S*)-Glu(OAd)(PO(OAd)-CH_2)-(*R*,*S*)-LeuOEt was prepared as recently described (*24*). Saponification of the C-terminal ethyl ester group gave the protected phosphinic pseudodipeptide Boc-(*R*,*S*)-Glu(OAd)((PO(OAd)-CH_2)-(*R*,*S*)-LeuOH, which was incorporated as a block during the solid-phase synthesis (*2*). The first Fmoc amino acids were attached to the 2-chlorotrityl resin, as previously described (*36*). The coupling of Boc-(*R*,*S*)-Glu(OAd)((PO(OAd)-CH_2)-(*R*,*S*)-LeuOH was achieved using 1.5 equiv of this block. Fully protected peptides were cleaved from 2-chlorotrityl resin with a mixture of glacial acetic acid, trifluoroethanol,

Table 1: Inhibition Constants of Aminophosphinic Peptides for APA, APN, NEP, and ACE

enzymes	**1**, GluΨ(PO_2CH_2)Leu	**2**, GluΨ(PO_2CH_2)Leu-Asp	**3**, GluΨ(PO_2CH_2)Leu-Leu	**4**, GluΨ(PO_2CH_2)Leu-Ala	**5**, GluΨ(PO_2CH_2)Leu-Arg	**6**, AlaΨ(PO_2CH_2)Leu-Ala
APA	194 nM	5.7 nM	37 nM	0.8 nM	30 nM	0.9 μM
APN	8 μM	7 μM	43 μM	31 μM	8 μM	25 nM
NEP	38 μM	50 nM	1320 nM	62 nM	3160 nM	29 nM
ACE	>100 μM[b]	4 μM	144 μM	53 μM	6 μM	5.6 μM

[a] Values for K_i were determined as described in Materials and Methods, where the assay conditions for each enzyme are given. [b] No inhibition at this inhibitor concentration.

and dichloromethane (1:1:3) for 2 h. Solutions of protected peptides were dried in vacuo. Protective groups were removed by the action of trifluoroacetic acid containing 5% H_2O, 5% thioanisol, 5% phenol, 2.5% ethanedithiol, and 32.5% dichloromethane. Solutions of deprotected peptides were concentrated in vacuo. The residues were dissolved in H_2O and the solutions were repeatedly extracted with cold diethyl ether and then lyophilized. Peptides were purified by preparative reverse-phase HPLC (Vydac, 218TP1022 column). Because of the presence of both S and R configurations of the pseudoglutamyl and pseudoleucyl residues in the phosphinic block, each peptide synthesis afforded a mixture of four diastereoisomers. At this stage, because of the high polarity of the glutamyl aminophosphinic peptides, separation of these four diastereoisomers was not possible. These peptides were, therefore, tested as a mixture of four diastereoisomers. Peptide purities were checked by analytical reverse-phase HPLC (Vydac, 218TP104 column) and mass spectroscopy.

RESULTS AND DISCUSSION

Phosphinic tripeptides containing a pseudoglutamyl residue in their P_1 position behave as highly potent inhibitors of APA (Table 1). The lower potency of the phosphinic dipeptide (compound **1**), as compared to the inhibitory potencies displayed by the phosphinic tripeptides (compounds **2**, **3**, **4**, **5**), highlights the contribution of the $P_{2'}$ position in enzyme−inhibitor interactions. As frequently observed in the case of peptide−ligand interactions, the higher affinity of the tripeptides probably results from the occurrence of intermolecular backbone H-bonding between the inhibitor and the enzyme. In addition to these interactions, the nature of the side chain in the $P_{2'}$ position also plays a role in inhibitor potency (compounds **2**, **3**, **4**, **5**). The few substitutions analyzed in this study suggest that the $S_{2'}$ subsite of APA exhibits a preference for small residues, such as alanine or aspartic acid. An influence of the $S_{2'}$ occupancy for inhibitor potency, as observed for APA, has also been reported in the case of APN peptide inhibitors (*23, 37*).

The dramatic drop in inhibitor potency observed between compounds **4** and **6** demonstrates the critical role of the P_1 pseudoglutamyl residue in a tight inhibitor−APA interaction. This result is consistent with the cleavage specificity exhibited by APA on peptide substrates. Another important consequence of the presence of a glutamyl side chain in aminophosphinic peptides concerns the selectivity of these inhibitors for APA. In fact, all these phosphinic derivatives behave as poor inhibitors of APN (Table 1). Strikingly, thiol derivatives designed to interact with the S_1 subsite of APA (see Figure 1) are not highly potent inhibitors of APA (*31, 38, 39*). Introduction in the P_1 position of these thiol derivatives of several kinds of acidic side chains does not improve their potency and only slightly affects their affinity (*39*). These results are in contrast to the efficiency of such thiol derivatives in potently blocking zinc aminopeptidases such as APB, APN, and leucine aminopeptidase (K_i values in the nanomalor range) (*22, 40, 41*). Moreover, gluthiol inhibits APA and APN with the same potency. These results, as well as the potency and the specificity of the glutamyl aminophosphinic peptide inhibitors reported in this study, may suggest that gluthiol inhibitor does not interact with the APA active site as anticipated.

These inhibitors failed to block ACE potently. On the other hand, depending on the nature of the residue at the $P_{2'}$ position, some compounds in this study behaved as rather potent inhibitors of NEP (compound **6**). The unexpected finding that free aminophosphinic peptides can potently block NEP has been reported for compounds with alkyl or aromatic residues in the P_1 position (*23*). The potency of compounds **2** and **4** toward NEP, despite the presence of a pseudoglutamyl side chain in the P_1 position of the inhibitor, suggests that the S_1 subsite in NEP has a very broad specificity. This property made it possible to develop mixed inhibitors of NEP and APN (*23*). As far as selective inhibition of APA is concerned, the present study suggests that the systematic substitution of the $P_{2'}$ position should be a strategy in developing inhibitors able to differentiate APA from NEP, with a higher selectivity.

The complete in vitro differentiation of APA from APN with the glutamyl aminophosphinic peptide inhibitors raises the possibility of selectively blocking APA in vivo, even at high inhibitor doses without significant APN inhibition. Thus, formation of angiotensin III, from conversion of angiotensin II by APA, should be prevented, while the degradation of angiotensin III by APN should be unaffected. Recently, EC33 ((*R,S*)-3-amino-4-thiol-butylsulfonate) was reported to selectively block APA in vivo after intracerebroventricular injection (*14*). EC33, as compared to APA, is 2 orders of magnitude less potent toward APN, but only displays a K_i value of 0.29 μM for APA (*39*). Thus, this compound is much less potent than the inhibitors reported in the present study.

For many years, intense effort has been devoted to the development of phosphinic peptide chemistry. Solid-phase synthesis, allowing either parallel or combinatorial chemistry to be performed, was a major improvement in preparing such compounds (*42*). This has led us to discover many highly potent and selective inhibitors of zinc-metalloproteases (*36, 43*). Recently, the first inhibitor able to discriminate the two active sites of ACE was found in a phosphinic peptide library (*33*). Interestingly, despite the presence of peptide bonds in this compound, all the inhibitor i.v. dose injected in the rat

was recovered in urine with the inhibitor intact, thus showing that this phosphinic peptide inhibitor is not metabolized in vivo (*33*). In addition, aminophosphinic inhibitors, with hydrophobic residues in the P_1 position, have been observed to block brain APN after i.v. injection (100 mg/kg dose) (*23*). This result, which demonstrates that aminophosphinic peptides are able to cross the blood−brain barrier, suggests that the present aminophosphinic inhibitors of APA might also selectively block brain APA after i.v. injection. Thus, apart from their potency and selectivity, as transition-state analogues, phosphinic peptides can be used for in vivo experiments. As mentioned in this study, the synthesis of phosphinic blocks containing polar side chains could be a difficult task. Nevertheless, taking into consideration the interesting properties of this class of zinc-metalloproteinase inhibitors, development of phosphinic peptide chemistry should be still encouraged.

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