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Design of the First Highly Potent and Selective Aminopeptidase N (EC 3.4.11.2) Inhibitor

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Abstract. A series of phosphinic compounds mimicking the transition state of substrates hydrolysed by aminopeptidase N (EC 3.4.11.2) were synthesized. These new compounds have potent inhibitory activities with Ki values in the nanomolar range. These derivatives behave as the most potent APN inhibitors designed to date.

Keywords : Enzyme inhibitors / Phosphinic acids and derivatives / Resolution.

APN, a monomeric or homodimeric type II membrane-bound zinc exopeptidase, is widely distributed in mammalian tissues including the central nervous system and is particularly abundant at the level of the kidney, the intestine and the lung [1]. *In vivo*, this enzyme is involved in the metabolism of angiotensin III in the brain and peripheral organs [2], in the degradation of nociceptin [3] and in the inactivation of enkephalins, in association with neutral endopeptidase NEP [4]. Furthermore, APN has been proved to be identical to a human lymphocyte surface cluster differentiation antigen CD13 [5], and to behave as a receptor for coronaviruses TGEV and 229E in pigs and humans [6,7]. APN has been also reported to play an important role in the invasion of metastatic tumors *in vitro* [8,9]. All these findings make this enzyme an interesting target for possible therapeutic applications, which require the development of potent and selective inhibitors.

APN exhibits a broad specificity for peptides with a N-terminal neutral or basic amino acid such as alanine, arginine or leucine. Sequence comparisons and site-directed mutagenesis experiments [10-12] have suggested that this enzyme has the same mode of zinc coordination as thermolysin [13]. In APN, the zinc is coordinated by three residues, two histidines found in a consensus sequence ^{383}HEXXH^{387}, and a glutamate (E^{406}) bound 18 residues C-terminal to the second histidine. The glutamate of the consensus sequence acts as a general base in catalysis. But, until now, little is known about the structure and the mechanism of action of this enzyme.

Natural products such as bestatin [14], amastatin [15], MA-387A and B [16] and actinonin [17] have been found to act as inhibitors of APN. Bestatin and amastatin have been the most extensively studied. Amastatin

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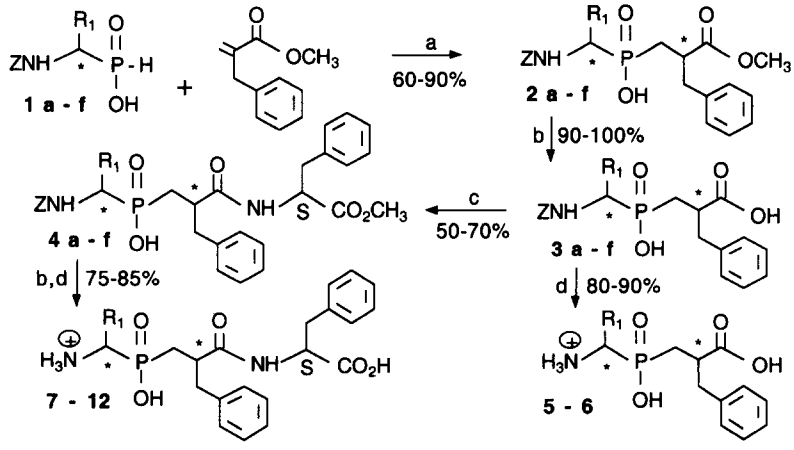
showed the greatest efficiency on APN with IC_{50} around 80 nM, but this compound presents a poor selectivity versus other aminopeptidases. Synthetic APN inhibitors have been developed from analogues of amino acids including aminohydroxamates [18], ß-aminothiols [19], aminoboronic acids [20], aminophosphonic acids [21] and aminoaldehydes [22]. Among them, only ß-aminothiols [19] are both selective and relatively potent inhibitors with Ki values around 10^{-8} M. In order to improve APN inhibition, mercapto analogues of bestatin [23] or diaminothiols [24] have been prepared. Unfortunately these compounds did not significantly increase APN recognition. Furthermore, peptide analogues bearing ketomethylene or hydroxyethylene bonds [25] or α-ketoamide groups [26] were shown to have IC_{50}s in the 10^{-6} M range. Non peptide inhibitors have been also prepared [27,28] with the aim to obtain compounds with increased stability and better bioavailability. However their Ki values were found in the 10^{-7}-10^{-8} molar range and their activities *in vivo* have not been reported. In order to design potent and selective APN inhibitors, we use the concept of "transition state" analogues capable to interact with the S_1, S_1' and S_2' subsites of the active site [29] and endowed with a phosphinic group as zinc ligand. Inhibitors of endopeptidases matrix metalloendopeptidases [30], containing a phosphinic acid as zinc ligand have been already described, but until now this type of molecules with a free amino group for aminopeptidase inhibition was not reported. In a first time, α-aminophosphinic acids bearing a hydrophobic side chain interacting with the S_1 subsite were synthesized and found to inhibit APN in the 10^{-7} molar range. Coupling of these α-aminophosphinic acids with an analogue of the dipeptide Phe-Phe, which has been shown to recognize efficiently the S_1' and S_2' subsites of APN [31], provided new "phosphino"peptides which have the most potent inhibitory activities towards APN described until now.

I. CHEMISTRY

The general scheme for the synthesis of inhibitors is reported in Figure 1. The benzyloxycarbonyl (Z) protected phosphinic acids **1 a-f** were prepared following previously reported methods [32] and were



a, BSA • b,1N NaOH or 1N LiOH • c, Phe-OCH_3/BOP/DIEA • d, BBr_3 • *, mixture of R and S isomers

Figure 1. Scheme for the synthesis of the APN inhibitors

condensed with methyl 2-benzyle acrylate in presence of N,O-bistrimethylsilylacetamide, yielding **2 a-f**.

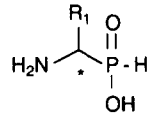
The successive hydrolysis of the methyl ester (compounds **3**) and cleavage of the Z protective group gave the dipeptide analogues **5** and **6**.

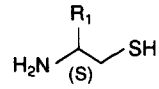
The coupling of **3 a-f** with phenylalanine methyl ester led to **4 a-f** and a two steps deprotection gave compounds **7** to **12**. These latter were obtained as mixtures of four stereoisomers due to the presence of two unresolved asymmetric carbons. Preparative HPLC yielded generally two fractions, each containing two isomers (A+B) and (C+D). They were firstly tested on APN without further separation.

II. RESULTS AND DISCUSSION

In a first step, the inhibitory activities of various α-aminophosphinic acids containing a hydrophobic side chain were measured on pig kidney APN (purchased from Boehringer) and compared with those of the corresponding ß-aminothiols (Table 1). It is interesting to note that the inhibitory activities of α-aminophosphinic acids are very dependent on the nature of the R_1 side chain, the best results being obtained with compounds containing phenylethyl or ethyl-S-methyl side chains with Ki values of $1.3x10^{-7}$ M and $4x10^{-7}$ M respectively (Table 1). Unexpectedly the less active compound contains a methyl side chain mimicking the alanine residue considered as the "model-substrate" of APN. For the corresponding ß-aminothiols, the inhibitory potencies are almost the same whatever the nature of R_1, with Ki values from 1.1 to $3x10^{-8}$, in agreement with previous results [19], giving compounds which are 30 to 400 fold most active than the α-aminophosphinic acids. However, this ratio is certainly overestimated since the stereodependance of APN towards its inhibitors is very important [19] and the ß-aminothiols were tested as pure stereoisomers while the α-aminophosphinic acids were under racemic forms.

Table 1. Inhibitory potencies of analogues of phosphinic acid and ß-amino thiols for APN.





R_1	APN [a], Ki(M) [b]	
CH_3	$8.4 \pm 0.5x10^{-6}$	$2.8 \pm 0.1x10^{-8}$
$CH_2CH(CH_3)_2$	$1.2 \pm 0.1x10^{-6}$	$2.2 \pm 0.2x10^{-8}$
$(CH_2)_2SCH_3$	$4.0 \pm 0.5x10^{-7}$	$1.1 \pm 0.1x10^{-8}$
Ph	$4.8 \pm 0.5x10^{-6}$	$2.5 \pm 0.2x10^{-8}$
CH_2Ph	$9.6 \pm 0.2x10^{-7}$	$3.0 \pm 0.5x10^{-8}$
CH_2CH_2Ph	$1.3 \pm 0.1x10^{-7}$	$2.7 \pm 0.7x10^{-8}$

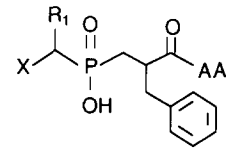
[a] APN activity, from pig kidney, was measured using Ala-p.NA as substrate. [b] Ki values are the mean ± SEM from three independent experiments performed in triplicate. [c] analogues of phosphinic acid are racemic mixtures. [d] ß-amino thiols are optically pure S isomers.

However, these compounds are more efficient than the corresponding aminoacid analogues such as α-aminohydroxamates or α-aminophosphates (not shown) suggesting that the phosphinic moiety would be an

interesting alternative to the thiol group for Zn chelation in the catalytic site of APN.

In a second step, the inhibitory potencies of compounds **5** and **6** which have been designed to interact with the S_1 and S_1' subsites of APN were measured (Table 2). The Ki values of these compounds (3.7 and 1.9×10^{-7} M) were only slightly increased as compared to those of the corresponding α-aminophosphinic acids (1.2×10^{-6} M and 9.6×10^{-7} M). However, according for the presence of two unresolved asymmetric centers in these compounds, it could be assumed that the occupancy of the S_1' subsite led to a gain in affinity of about one order of magnitude. These data confirms the efficiency of the phosphinic group as zinc ligand for APN inhibition, but also demonstrate the necessity of interacting with the S_2' subsite of APN for an optimal inhibition of this enzyme.

Table 2. Inhibitory potencies of phosphinic derivatives for APN.



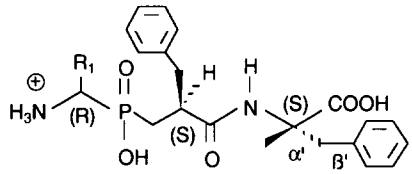
n		X	R1	AA	APN [b] (Ki (nM) [a])
5 [c]		NH_2	$CH_2CH(CH_3)_2$	OH	370 ± 80
6 [c]		NH_2	CH_2Ph	OH	190 ± 30
7	**(A+B)**	NH_2	CH_3	Phe	2.2 ± 0.2
	(C+D)				670 ± 90
8	**(A+B)**	NH_2	$CH_2CH(CH_3)_2$	Phe	3.2 ± 0.5
	(C+D)				90 ± 16
9	**(A+B)**	NH_2	$CH_2CH_2SCH_3$	Phe	6.3 ± 0.3
	(C+D)				370 ± 20
10	**(A+B)**	NH_2	Ph	Phe	3.8 ± 0.48
	(C+D)				320 ± 10
11	**(A+B)**	NH_2	CH_2Ph	Phe	2.9 ± 0.8
	(C+D)				200 ± 40
12	**(A+B)**	NH_2	CH_2CH_2Ph	Phe	2.3 ± 0.4
	(C+D)				360 ± 60

[a] Ki values are the mean ± SEM from three independent experiments performed in triplicate. [b] APN activity, from pig kidney, was measured using Ala-p.NA as substrate. [c] Compounds **5** and **6** are a mixture of four stereoisomers.

In a third step, compounds **7** to **12** containing a C terminal phenylalanine in P_2' position were tested and in each case the mixture of two stereoisomers (A+B) showed nanomolar inhibitory potencies. A direct comparaison between **5** and **8** or **6** and **11** indicated that the 100 fold increased activities observed with **8** and **11** was due to the S_2' subsite occupancy. Two other results could also be underlined : i) there is no direct relationship between the inhibitory potency of the α-aminophosphinic acids reported in table 1 and those of the phosphinic peptides reported in Table 2. For instance, compound **7**, which contain a methyl group in position R_1, is one of the most active inhibitor, while the corresponding α-aminophosphinic acid is the less efficient of the series with a Ki value of 8×10^{-6} M. The inverse situation occurs with the methionine side

chain ; ii) For all the compounds tested, the mixture of isomers (A+B) is significantly more active than (C+D), showing a very large importance of the absolute configuration of the asymmetric carbons. Consequently to isolate the most potent isomers, a complete separation was performed for compounds **7** and **11**. The recristallization of the (+) α-methylbenzylamine salts of the α-aminophosphinic acid **1a** ($R = CH_3$) and **1e** ($R = CH_2Ph$) led to the R isomers [32]. Following the synthetic pathway reported in Figure 1, these pure isomers led *in fine* to the formation of compounds **7** and **11** as a mixture of isomers B and D which differed only by the absolute configuration of the second asymmetric carbon. Isomers B and D were easily separated by preparative HPLC. The isomers **7B** and **11B** were the most active (Table 3) and their stereochemical assignment (R,S,S) were determined by ^1H NMR spectrocopy [33].

Table 3. Inhibitory potencies of optically pur phosphinic derivatives for APN, APA and APB.



		Ki (M) [a]		
n	R_1	APN [b]	APA [c]	APB [d]
7B	CH_3	$0.6 \pm 0.05 \times 10^{-9}$	$1.3 \pm 0.3 \times 10^{-7}$	$> 10^{-5}$
11B	CH_2Ph	$1.5 \pm 0.1 \times 10^{-9}$	$4.0 \pm 0.3 \times 10^{-5}$	$> 10^{-5}$

[a] Ki values are the mean ± SEM from three independent experiments performed in triplicate. [b] APN activity was measured using Ala-p.NA as substrate. [c] APA activity was measured using GluNA as substrate. [d] APB activity was measured using Arg p.NA as substrate.

To determine the selectivity of these inhibitors towards the targetted enzyme, they have been tested on other Zn^{2+} metallopeptidases of the same family. Selectivity factors from 200 to 10,000 were obtained versus APA and higher than 10,000 versus APB (Table 3). These results are consistent with the preference of these latter enzymes for acidic or basic residues in position S_1 respectively. The best inhibitors have also been tested on two endopeptidases NEP and ACE. Indeed, accounting for the hydrophobic character of their active sites, the synthesized phosphinic compounds could inhibit efficiently these physiological enzymes. However, selectivity factors from 100 to 10,000 were obtained, showing that either the presence of the free amino group of **7B** and **11B** is unfavourable for NEP or ACE recognition or the hydrophobic side chains of the inhibitors do not optimally interact with the two endopeptidases. High selectivity factors (> 100) were also obtained with the cytosolic leucine aminopeptidase LAP whose mechanism of action is different from that of gluzincins.

Due to their high affinity towards APN and their selectivities versus other metalloenzymes, the phosphinic inhibitors could be used for a complete charaterization of the biochemical and pharmacological

properties of APN and the synthesis of the radiolabelled analogues of **7B** is now underway in our laboratory.

In conclusion, we have developed a new series of potent and selective APN inhibitors, designed as transition state analogues interacting with the S_1, S_1' and S_2' subsites of the enzyme. This approach has led to the most potent inhibitors on APN reported to date, with Ki values in nanomolar range. Moreover these compounds could be modified to generate new dual inhibitors of neutral endopeptidase NEP and APN [34], because the critical point in designing dual inhibitors is to optimize the recognition of APN.

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