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Novel hydroxamic acid-related phosphinates: Inhibition of neutral aminopeptidase N (APN)

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Abstract—Here we describe the inhibitory activity toward neutral aminopeptidase of three new families of phosphinate inhibitors related in structure to hydroxamic acids. These compounds, even as racemic mixtures, are good inhibitors of APN and show strong structure activity relationship (SAR) depending on the substituents in P1 and P1′ positions.

Matrix metalloproteinases (MMPs) are considered as excellent target for new anticancer therapeutic.[1] A number of small molecular weight MMP inhibitors have entered clinical trials in humans. However, the results of these trials have been extremely disappointing. The first generation of MMP inhibitors exhibited poor bioavailability while second generation compounds caused some serious side effects including pain, inflammation or lack of efficacy.[2] It was estimated that more than 90% of MMP inhibitors contain a hydroxamic acid as the zinc-binding group (ZBG).[3] Hydroxamic acids are rapidly hydrolyzed in vivo generating a carboxylic acid, which is less effective as ZBG, and hydroxylamine, a well-known carcinogenic compound.[4] However, hydroxamic acid moiety in a MMP inhibitor molecule always leads to a better inhibitor than other ZBGs as carboxylate, thiolate, phosphonate or phosphinate. According to ligand field theory, the optimal zinc geometry is trigonal bipyramid. Hydroxamic acid residue acts as bidentate ligand forming thermodynamically favored five-member ring with zinc atom inside. The oxygen-carbonyl and oxygen-hydroxyl distance to zinc atom is about 2 Å.[5] The phosphonate/phosphinate form four-member ring, thermodynamically not favored, where the oxygen atoms are at different distances, usually around 2 and more than 3 Å, therefore they should be considered as monodentate ligand of zinc atom.[6] Relatively short distance of the second oxygen atom to zinc atom could be a simple consequence of short distance of the first one and does not originates from strong coordination bond formation. To overcome these limitations we have designed a few new families of phosphinates with extra ligand at the second right-hand side of molecules, able to bind zinc atom in the active side of MMPs. In contrast to classical phosphonate/phosphinate inhibitors such molecules could form perfect five-member ring and bind zinc in bidentate manner. In this speculative, right now, model one ligand originated from phosphorus-oxygen, when the second one is the alpha substituent on the right-hand side of phosphinate moiety. The hydroxamate inhibitors use right-hand side of MMP's binding pockets and these limited interactions with the specificity determination pockets of enzymes could be responsible for limited specificity. Phosphinates shown in Figure 1 could be extended to the size of required specificity in both directions of the active side. However, we need to emphasize that a different binding to the zinc (bidentate-like hydroxamate moiety) could require a different than substrate sequence of amino acids of these new phosphinate molecules to provide maximum inhibitory potency.

Our first target is inexpensive, commercially available aminopeptidase N (APN, CD13). This zinc-dependent egzopeptidase is transmembrane metalloprotease involved in many diseases (rheumatoid arthritis, chronic pain, sclerosis, and tumor progression). Elevated levels of APN were observed in such tumor cells like prostate, melanoma, gastric or pancreas, what makes this enzyme possible marker in clinical prognosis. Moreover, some

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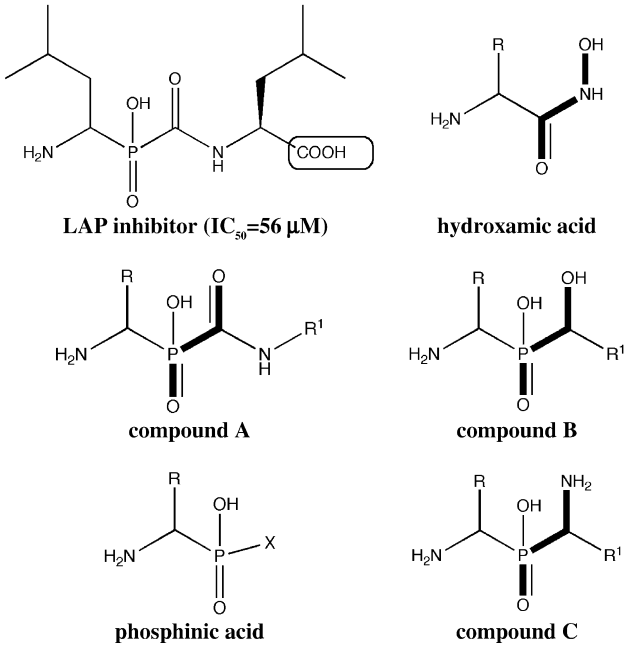


Figure 1. Structures of leading compounds and synthesized inhibitors of aminopeptidase N.

data suggest that aminopeptidase N (CD13) may participate in programmed cell death (apoptosis) of cancer cells. These features make APN an interesting target in the design of specific inhibitors of this enzyme.[7]

Recently, we have described the general methods of synthesis of two families of potential chelating compounds, namely bis-α-aminoalkylphosphinic acids (Fig. 1, compound **C**) and α_1-aminoalkane-α_2-hydroxyalkanephosphinic acids (Fig. 1, compound **B**) which possess the designed features of phosphinates related to hydroxamic acids.[8,9]

Here, we reported the family of amino acid-related carbamoylphosphinic acids. Only one example of such compounds was previously described by Gianoussis as poor (K_i = 56 μM) inhibitor of leucine aminopeptidase LeuPLeu (10). We have modified it by removing the carboxyl group from the structure (Fig. 1, compound **A**), what could improve the binding properties by decreasing a total charge of the molecule. All of the synthesized compounds were in the form of racemic mixtures and were obtained according to the methods described earlier. Finally, a set of various substituents were tested in order to optimize side chains in P1 and P1′ positions of inhibitors of APN (Table 1).

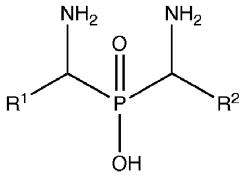
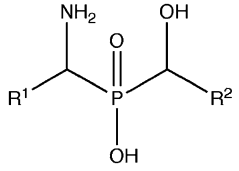
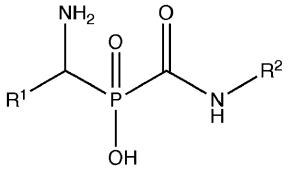
The most effective of the bis-α-aminoalkylphosphinic acids (**1**–**11**) were bis-α-(1-amino-1-phenylmethane) phosphinic acid **5** (IC_{50} = 0.6 μM), α_1-(1-amino-1-phenylmethane)-α_2-(1-amino-3-phenylpropane)-phosphinic acid **6** (IC_{50} = 0.71 μM), and bis-α-(1-amino-3-phenylpropane)phosphinic acid **7** (IC_{50} = 0.83 μM). All of these compounds possess highly hydrophobic, aromatic substituents in both—P1 and P1′ positions. This observation is in agreement with the data obtained earlier for the other phosphinic and phosphonic inhibitors of APN and general requirements in the other classes of inhibitors of this enzyme, where bulky substituents were in most cases preferred.

Moreover in the case of compounds **1**–**6**, where R^1 substituent is phenyl and in R^2 position are different groups, it is possible to see strong structure activity relationship (SAR). Substantial increase of hydrophobicity causes substantial increase of inhibitory activity. Interestingly, taking in to consideration all the tested bis-α-aminoalkylphosphinic acids (**1**–**11**) we can see that the best inhibitors possess in both positions (P1, P1′) aromatic substituents, middle class are compounds with one aromatic substituent, and the worst are those with both aliphatic groups in the side chain. Considering overall better inhibitory effect in our experiments of inhibitors with –H group (glycine analogues) over more hydrophobic $-CH_3$ (alanine analogues), we can probably explain it by the amount of active molecules in a measured mixture (1 of 2 in the case of glycine analogues and 1 of 4 in the case of alanine analogues).

In the second tested group of inhibitors—α_1-aminoalkane-α_2-hydroxyalkanephosphinic acids, similarly as in the case of bis-α-aminoalkylphosphinic acids, the most active inhibitors possess in P1 and P1′ positions hydrophobic substituents. Among them, α_1-(1-amino-1-phenylmethane)-α_2-(1-hydroxy-3-phenylpropane) phosphinic acid **16** (IC_{50} = 0.24 μM), α_1-(1-amino-2-methylpropane)-α_2-(1-hydroxy-3-phenylpropane) phosphinic acid **17** (IC_{50} = 0.5 μM), and α_1-(1-amino-1-phenylmethane)-α_2-(1-hydroxy-1-phenylmethane) phosphinic acid **15** (IC_{50} = 0.55 μM) were the most active toward APN. Also in the case of this family of compounds, the comparison of inhibitors where R^1 substituent is phenyl and in R^2 position are different groups, it is possible to see SAR confirming preference of highly hydrophobic side groups in the active center of APN. Interestingly, in the case of compound **17**, which possesses aliphatic valine in the R^1 position we were able to see as good inhibitory effect as in the case of compound **15** with phenyl group in R^1 position. This is probably due to the shape of side chain of valine, which can rotate in limited manner mimicking bulky aromatic residue, and thus fitting well to the S1 pocket of APN. This effect cannot be observed in none of the tested by us bis-α-aminoalkylphosphinic or α_1-aminoalkane-α_2-hydroxy alkanephosphinic acids, where one methylene group longer residue of leucine was incorporated increasing free rotation of side chain. Usually this resulted in decrease of inhibitory activity toward APN in the tested compounds.

The latter group of the tested inhibitors (Fig. 1, compound **A**), namely carbamoylphosphinic acids, has shown little different structural preferences of side chain compared to previous two groups of phosphinates. The most active were α_1-(1-amino-3-methylbutane)-α_2-(carbamoylphenyl-methane) phosphinic acid **21** (IC_{50} = 0.53 μM) and α_1-(1-amino-2-methylpropane)-α_2-(carbamoylphenylmethane) phosphinic acid **23** (IC_{50} =

Table 1. Detailed structures and IC_{50} for all investigated inhibitors of APN

Compound			Complement inhibition IC_{50}[a,b], μM
	R^1	R^2	
1	$-C_6H_5$ (Phenylglycine)	–H (Gly)	42
2	$-C_6H_5$ (Phenylglycine)	$-CH_3$ (Ala)	46
3	$-C_6H_5$ (Phenylglycine)	$-CH_2CH_2CH_3$ (nor-Val)	17
4	$-C_6H_5$ (Phenylglycine)	$-CH_2CH(CH_3)_2$ (Leu)	4
5	$-C_6H_5$ (Phenylglycine)	$-C_6H_5$ (Phenylglycine)	0.6
6	$-C_6H_5$ (Phenylglycine)	$-CH_2CH_2$Phe (hPhe)	0.71
7	$-CH_2CH_2$Phe (hPhe)	$-CH_2CH_2$Phe (hPhe)	0.83
8	$-CH_2CH(CH_3)_2$ (Leu)	–H (Gly)	76
9	$-CH_2CH(CH_3)_2$ (Leu)	$-CH_3$ (Ala)	155
10	$-CH_2CH(CH_3)_2$ (Leu)	$-CH_2CH_2CH_3$ (nor-Val)	50
11	$-CH_2CH(CH_3)_2$ (Leu)	$-CH_2CH(CH_3)_2$ (Leu)	50
			
12	$-C_6H_5$ (Phenylglycine)	$-CH_3$ (Ala)	48
13	$-C_6H_5$ (Phenylglycine)	$-(CH_2)_3CH_3$ (nor-Leu)	28
14	$-C_6H_5$ (Phenylglycine)	$-CH_2CH(CH_3)_2$ (Leu)	12
15	$-C_6H_5$ (Phenylglycine)	$-C_6H_5$ (Phenylglycine)	0.55
16	$-C_6H_5$ (Phenylglycine)	$-CH_2CH_2$Phe (hPhe)	0.24
17	$-CH(CH_3)_2$ (Val)	$-CH_2CH_2$Phe (hPhe)	0.5
18	$-CH_2CH(CH_3)_2$ (Leu)	$-(CH_2)_3CH_3$ (nor-Leu)	3
19	$-CH_2CH(CH_3)_2$ (Leu)	$-CH_2CH_2$Phe (hPhe)	1
			
20	$-CH_2CH(CH_3)_2$ (Leu)	$-C_6H_5$ (Phenylglycine)	35
21	$-CH_2CH(CH_3)_2$ (Leu)	$-CH_2$Phe (Phe)	0.53
22	$-CH(CH_3)_2$ (Val)	$-C_6H_5$ (Phenylglycine)	160
23	$-CH(CH_3)_2$ (Val)	$-CH_2$Phe (Phe)	0.93
24	$-CH(CH_3)_2$ (Val)	$-CH_2CH_3$	n.i.
25	$-C_6H_5$ (Phenylglycine)	$-(CH_2)_5CH_3$	n.i.

[a] Values are means of two experiments, standard deviation is ±30%. Substrate (L-leucine-p-nitroanilide, $C_{fin.} = 0.05$ mM), n.i., no inhibition at concentration <300 μM. APN $C_{fin.} = 2$ μg/ml, no incubation time.

[b] All values for racemic mixtures.

0.93 μM). However, these carbamoylphosphinates are much better inhibitors than those reported earlier.[10]

Preliminary kinetic inhibition data for all the tested families of phosphinates suggest that the tested compounds inhibit APN in competitive fashion. The plot in Figure 2 for derivative **23** confirms competitive nature of the investigated here inhibitors. Thus, competitive inhibition proves the interaction with the active site of enzyme, however the binding to the zinc in bidentate manner if occurs cannot be determined by kinetic experiments and needs to be established by crystallographic studies.

In summary, we have demonstrated three new families of inhibitors of metalloproteases of phosphinate origin with the structural features of hydroxamic acids. These compounds are good inhibitors of neutral aminopeptidasae and reveal strong structure activity relationships. This can be an indicator for the design

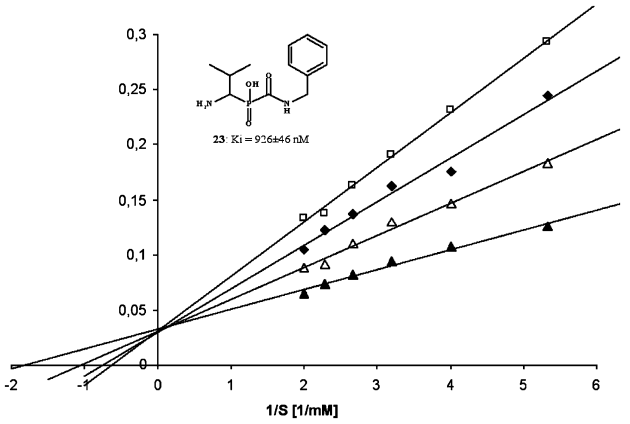


Figure 2. Lineweaver-Burk plot of competitive inhibition of compound **23**.

of more potent inhibitors of APN and other metalloproteases based on modification of P1 and P1′ elements. Moreover, this feature can be used for the design and synthesis of specific activity based probes (ABP), which can give insight into the activity and function of these enzymes as recently demonstrated using hydroxamic acids.[11] Investigation of the inhibition of MMPs by these new families of inhibitors is in progress.

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