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Identification of very potent inhibitor of human aminopeptidase N (CD13)

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ABSTRACT

In this Letter we describe broad comparision studies toward rat, pig, and human aminopeptidase N (CD13) orthologs using phosphinate inhibitors related in structure to hydroxamic acids. This SAR approach yielded a very potent inhibitor of human aminopeptidase N: α_1-amino-3-phenylpropyl(α_2-hydroxy-3-phenylpropyl)phosphinic acid with an IC_{50} = 60 nM.

Aminopeptidases (AMPs) are exopeptidases responsible for removing N-terminal amino acids by sequential cleavage of one residue at a time from target substrates. They are found both in eukaryotes and prokaryotes and participate in most of the major proteolytic events in the cell. Due to their versatile function aminopeptidases can have broad or narrow substrate specificity.

Among them aminopeptidase N (APN, CD13, EC 3.4.11.2) has raised interest due to its substantial role in physiologic mechanisms and pathological disorders. It plays a crucial role in signal transduction,[1] inflammation,[2] extracellular matrix degradation,[3] chemokines and cytokines processing,[4,5] antigen presentation,[6] immunological responses,[7] phagocytosis, and adhesion.[3] APN is present in many hematopoietic as well as non-hematopoietic cell types while its overexpression is frequently observed in case of cancer and inflammatory diseases.[8] In cancer cell aminopeptidase N play an important role in tumor growth and metastasis through its participation in apoptotic and angiogenetic events. Recent study show that radiation treatment increases APN activity in HeLa cell line and that combination of aminopeptidase inhibitor (bestatin) and radiation leads to activation of apoptotic pathway and tumor growth inhibition.[9]

In clinical studies on patients with pancreatic and non-small cell lung cancer significant correlation between APN expression in cancer cells and the increase of intratumor microvessel density as well as shorter survival time of APN-positive patients were suggested.[10,11] These features make aminopeptidase N not only an interesting target for anticancer therapy, but also a potential prognostic marker and excellent target for design of inhibitors.

Phosphonic and phosphinic acid derivatives are well known metalloprotease inhibitors. Phosphinates were reported earlier as very good inhibitors of aminopeptidases, but most of the studies published to date were performed using porcine ortholog.[12,13] Because APN orthologs in mammals have very similar substrate specificity, but differs in activity,[14] we sought to validate our inhibitor design by testing against rat, pig and human enzymes. Phosphinates act as noncovalent ligands binding to the zinc ion presented in the active center of APN. Their binding properties can be greatly improved by proper selection of side chains substituents that interact with appropriate pockets of the enzyme. Previously we described synthetic methodologies for α-aminoalkyl-(N-substituted) thiocarbamoylphosphinic acids and α_1-(aminoalkyl)-α_2-(hydroxyalkyl)phosphinates using mild Arbuzov reactions through addition of phosphinic acid ester to isothiocyanates or aldehydes, respectively[15–18] (Fig. 1). Preliminary porcine kidney APN inhibition studies suggested that these compounds are good scaffolds for more detailed SAR studies.

Here we report broad SAR studies of these two classes of phosphinate inhibitors and their activity towards human, rat, and porcine kidney aminopeptidase N.[19] All the compounds were obtained as racemic mixtures using previous methodologies and differ only by their side chain substituents. The most potent inhibitor among all of the tested α-aminoalkyl-(N-substituted) thiocarbamoylphosphinic acids (Table 1 and **1**–**32**) towards human APN was compound **29** with IC_{50} = 0.47 μM. Additionally, its activity is three fold higher against human aminopeptidase N. Similar result was obtained for derivative **28** with IC_{50} values of 0.56 μM (human APN). Compound **31** was fourfold stronger as an inhibitor of the human enzyme (IC_{50} = 0.64 μM) in comparison to rat and porcine

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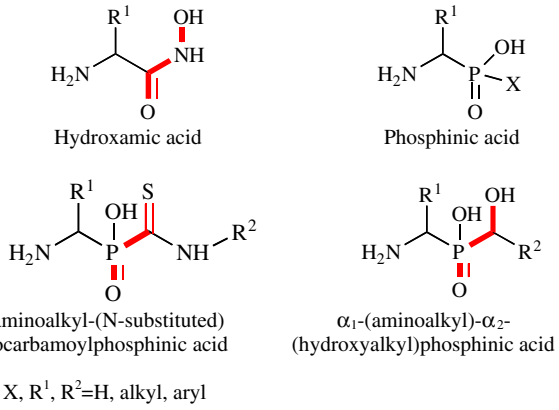


Figure 1. The general structures of hydroxamic acid and hydroxamic acid-related phosphinic peptidomimetics.

APN (IC_{50} = 2.65 μM). The most effective porcine aminopeptidase N inhibitor was **16** with IC_{50} = 0.25 μM. Moreover, this compound was the most potent rat APN inhibitor in the group along with **14** (IC_{50} = 0.62 μM and 0.61 μM). SAR relationships for the compounds showed that the best substituents in the P1 position are these with 2-phenylethyl and butyl groups. This is in agreement with recently published substrate specificity profiling of these enzymes, where compounds with these side chains were among the best substrates.[14] However, our results indicate that the structure of the S1 binding pocket of human aminopeptidase N has higher binding affinity for the 2-phenylethyl group than other two enzymes tested. The butyl substituent at P1 has similar binding characteristics for all three enzymes. Comparisons between analogues with butyl and propyl side chain in P1 clearly indicate a higher specificity of the S1 pocket towards longer chains, which also agrees with substrate specificity data. For example, the IC_{50} value for compound **17** (0.64 μM—human APN) is much better compared to compound **10** with shorter side chain (IC_{50} = 2.15 μM). Although alanine is a very good substrate for APN, in our study phosphinic alanine analogues were only medium inhibitors of the enzymes. These results are explained by the recent observation that transformation of substrates into some types of inhibitors for APN should not be based on k_{cat}/K_m, but rather K_m values (both hPhe and Ala are almost equally good substrates, but K_m value for hPhe is much lower than this observed for Ala).[14] Analysis of the P1′ substituents revealed that the presence of a carboxyl group, or even its esters, has a strong adverse effect on inhibitory activity of the compounds tested. Much better results were obtained for the analogue with a 4-metoxybenzyl moiety. An example of this principle is compound **17**, which is one of the best inhibitors we obtained. In addition, aliphatic chains in particular 3-methylthiopropyl (e.g., **29**) and propyl (e.g., **28**) were effective substituents at P1′.

In the second tested group of compounds: α_1-(aminoalkyl)-α_2-(hydroxyalkyl)phosphinic acids (**33**–**46**, Table 2), the most potent inhibitor of human aminopeptidase N was α_1-amino-3-phenylpropyl(α_2-hydroxy-3-phenylpropyl)phosphinic acid (**46**) with IC_{50} = 60 nM. This derivative was also a very good inhibitor of porcine APN (IC_{50} = 0.22 μM) as well as the best inhibitor of the rat enzyme (IC_{50} = 0.4 μM). As observed for the previous group of

Table 1
Structures and inhibitory activity of α-aminoalkyl-(N-substituted)thiocarbamoylphosphinic acid derivatives towards porcine, rat, and human aminopeptidase N

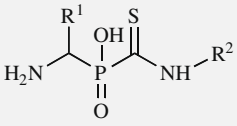
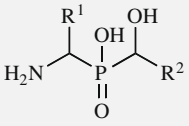
Compound			Aminopeptidase N inhibition (μM)		
	R^1	R^2	Porcine APN	Rat APN	Human APN
1	$-CH_3$	$-CH_2(CH_2)_2SCH_3$	2.45	3.45	2.52
2	$-CH_3$	$-CH_2C_6H_5$	1.9	1.45	2.2
3	$-CH_3$	$-CH_2CH_2C_6H_5$	5.2	8.7	5.8
4	$-CH_3$	$-CH_2(p\text{-}OCH_3\text{–}C_6H_4)$	>20	>20	>20
5	$-CH_3$	$-CH_2(p\text{-}COOCH_2CH_3\text{–}C_6H_4)$	>20	>20	>20
6	$-CH_2CH_3$	$-CH_2(CH_2)_2SCH_3$	1.65	2.5	1.6
7	$-CH_2CH_2CH_3$	$-CH_2(CH_2)_2SCH_3$	1.95	1.45	1.45
8	$-CH_2CH_2CH_3$	$-CH_2C_6H_5$	1.6	2.1	1.25
9	$-CH_2CH_2CH_3$	$-CH_2CH_2C_6H_5$	1.35	2.55	3.6
10	$-CH_2CH_2CH_3$	$-CH_2(p\text{-}OCH_3\text{–}C_6H_4)$	2.15	2.45	2.15
11	$-CH_2CH_2CH_3$	$-CH_2(p\text{-}COOCH_2CH_3\text{–}C_6H_4)$	>20	>20	>20
12	$-CH_2CH_2CH_3$	$-CH_2(p\text{-}COOH\text{–}C_6H_4)$	>20	>20	>20
13	$-CH_2CH_2CH_3$	$-CH_2(o\text{-}COOCH_3\text{–}C_6H_4)$	>20	>20	>20
14	$-CH_2(CH_2)_2CH_3$	$-CH_2(CH_2)_2SCH_3$	0.36	0.61	0.77
15	$-CH_2(CH_2)_2CH_3$	$-CH_2C_6H_5$	0.62	1.1	1.5
16	$-CH_2(CH_2)_2CH_3$	$-CH_2CH_2C_6H_5$	0.25	0.62	0.71
17	$-CH_2(CH_2)_2CH_3$	$-CH_2(p\text{-}OCH_3\text{–}C_6H_4)$	0.46	1.05	0.64
18	$-CH_2(CH_2)_2CH_3$	$-CH_2(COOH)\text{–}CH_2C_6H_5$	>20	>20	>20
19	$-CH_2(CH_2)_3CH_3$	$-CH_2(o\text{-}COOCH_3\text{–}C_6H_4)$	>20	>20	>20
20	$-CH(CH_3)_2$	$-CH_3$	10.15	21.35	14.6
21	$-CH(CH_3)_2$	$-CH_2(CH_2)_4CH_3$	1.7	5.05	1.2
22	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	4.8	8.95	15.35
23	$-CH_2CH(CH_3)_2$	$-CH_3$	>20	10.8	>20
24	$-CH_2CH(CH_3)_2$	$-CH_2(CH_2)_4CH_3$	1.4	1.7	0.96
25	$-CH_2CH(CH_3)_2$	$-CH_2C_6H_5$	2.8	5.1	8.2
26	$-CH_2CH(CH_3)_2$	$-CH_2CH_2C_6H_5$	4.2	5.4	>20
27	$-CH_2CH(CH_3)_2$	$-CH_2(o\text{-}COOCH_3\text{–}C_6H_4)$	>20	>20	>20
28	$-CH_2CH_2C_6H_5$	$-CH_2CH_2CH_3$	1.45	1.35	0.56
29	$-CH_2CH_2C_6H_5$	$-CH_2(CH_2)_2SCH_3$	1.55	1.65	0.47
30	$-CH_2CH_2C_6H_5$	$-CH_2C_6H_5$	2.3	2.5	1.3
31	$-CH_2CH_2C_6H_5$	$-CH_2(p\text{-}OCH_3\text{–}C_6H_4)$	2.65	2.65	0.64
32	$-CH_2CH_2C_6H_5$	$-CH_2(p\text{-}COOCH_2CH_3\text{–}C_6H_4)$	>20	>20	11.4

Table 2
Structures and IC_{50} values for the inhibition of porcine, rat, and human aminopeptidase N by α_1-(aminoalkyl)-α_2-(hydroxyalkyl)phosphinic acid derivatives

Compound			Aminopeptidase N inhibition (μM)		
	R^1	R^2	Porcine APN	Rat APN	Human APN
33	$-CH_3$	-*p*-$CH(CH_3)_2$–C_6H_4	1.2	3.5	0.87
34	$-CH_2CH_3$	$-CH_2CH_2C_6H_5$	1.3	2.4	0.6
35	$-CH_2CH_2CH_3$	$-CH_2C_6H_5$	0.95	1.2	2.0
36	$-CH_2(CH_2)_2CH_3$	-(*p*-OH–C_6H_4)	5.8	6.2	9.3
37	$-CH_2(CH_2)_2CH_3$	$-CH(CH_3)C_6H_5$	0.22	0.46	0.86
38	$-CH_2(CH_2)_2CH_3$	$-CH_2CH_2C_6H_5$	0.21	0.52	0.22
39	$-CH(CH_3)_2$	$-CH_2CH_2C_6H_5$	0.5[a]	1.1	0.25
40	$-CH_2CH(CH_3)_2$	$-CH_2CH_2C_6H_5$	1[a]	0.92	0.42
41	$-C_6H_5$	$-CH_3$	48[a]	15.2	18.2
42	$-C_6H_5$	$-CH_2CH(CH_3)_2$	12[a]	4.7	4.4
43	$-C_6H_5$	$-C_6H_5$	0.55[a]	0.46	1.5
44	$-C_6H_5$	$-CH_2CH_2C_6H_5$	0.24[a]	2.2	0.73
45	$-CH_2C_6H_5$	$-CH_2CH_2C_6H_5$	0.66	1.6	0.93
46	$-CH_2CH_2C_6H_5$	$-CH_2CH_2C_6H_5$	0.22	0.4	0.06

[a] Value reported previously.[18]

inhibitors, 2-phenylethyl group at P1 seems to be optimal for the human enzyme. Further confirmation is given by comparison of inhibitory activity between analogues **45** and **46**. The structures of these compounds differ by the presence of a single methylene group in the substituent at P1. Compound **46** is 15-fold more potent as an inhibitor of human APN compared to **45** (IC_{50} = 0.06 μM vs 0.93 μM). Similar observations apply to derivatives with butyl chain and their analogues with shorter or branched substituents at the P1 position. The most potent norleucine phosphinic analogue is compound **38** with IC_{50} = 0.22 μM for human APN, 0.21 μM for the porcine enzyme and 0.52 μM for APN from rat. The exception here is compound **39** with IC_{50} = 0.25 μM towards human aminopeptidase N. The most effective moiety at P1′ is the 2-phenylethyl group, while hydrophilic *p*-hydroxyphenyl (**36**) and methyl (**41**) were much weaker substituents.

We have demonstrated inhibitory activity of several hydroxamic acid-related phosphinic derivatives toward aminopepidase N from porcine, rat and human sources. The investigated compounds are good inhibitors of all three enzymes. Comparison between the two groups of inhibitors did not indicate the superiority for any of the ligands tested here. The best inhibitors are these with hydrophobic side chains in both P1 and P1′, which is in agreement with previously published data as well as substrate specificity of tested APN. However, in this Letter for the first time we have performed broad SAR studies for three mammalian orthologs of APN. Of particular importance was identification and validation of potent inhibitor for human ortholog of APN, enzyme for which inhibitors are nowadays especially desired.

Acknowledgments

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19. Inhibitors were screened toward appropriate APNs at 37 °C in the 100 mM Tris–HCl, pH 7.5. Buffer was prepared at 20 °C. Enzymes were preincubated for 30 min at 37 °C with selected phosphinate inhibitor before adding substrate (Ala-ACC, final concentration equal to 50 μM) to the wells of a 96-well plate reader operating in the kinetic mode. Enzyme assay conditions were as follows: (100 μL reaction), eight different inhibitor concentrations and enzymes at 0.4–5 nM. Release of ACC fluorophore was monitored continuously with excitation at 355 nm and emission at 460 nm. The final DMSO concentration was 1%. All IC_{50} values are means of two experiments, standard deviation is ±20%. All compounds are racemic mixtures.