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RESEARCH ARTICLE

Design, synthesis and preliminary activity evaluation of novel 3-amino-2-hydroxyl-3-phenylpropanoic acid derivatives as aminopeptidase N/CD13 inhibitors

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Abstract

Aminopeptidase N (APN/CD13) over expressed on tumour cells, plays a critical role in tumour invasion, metastasis and tumour angiogenesis. In this article, we described the design, synthesis and preliminary activity studies of novel 3-amino-2-hydroxyl-3-phenylpropanoic acid derivatives as APN inhibitors. The *in vitro* enzymatic inhibitions on APN from porcine kidney showed that compound **7e** had the most potent inhibitory activity against APN with the IC_{50} value to 1.26 ± 0.01 μM, which is better than that of bestatin (IC_{50} = 2.55 ± 0.11 μM). In addition, compound **7e** also showed better inhibitory activity against APN on human ovary clear cell carcinoma cell ES-2 than bestatin with the IC_{50} value to 30.19 ± 1.02 μM versus 60.61 ± 0.1 μM. Compound **7e** could be used as the lead compound in the future for anti-cancer agent research.

Keywords: Aminopeptidase N inhibitors, 3-amino-2-hydroxyl-3-phenylpropanoic acids derivatives, structure-based drug design

Introduction

Aminopeptidase N (APN; EC 3.4.11.2) is a zinc-dependent type II membrane-bond ectopeptidase and belongs to the M1 family of MA clan of peptidase[1,2]. This enzyme consists of 967 amino acids with a short N-terminal cytoplasmic domain, a single transmembrane part and a large cellular ectodomain containing the active site[3,4], which can preferentially release neutral or basic amino acids from the N-terminal of peptides[5,6]. APN is widely distributed in the body of mammalian. It can be expressed on the surface of different cells such as myeloid progenitors and monocytes, epithelial cells of the intestine and kidney, synaptic membranes in the central nervous system, fibroblasts, endothelial cells, epithelial cells[7–10] and so on. APN is highly expressed on tumour cells and plays a critical role in tumour invasion, metastasis and angiogenesis. Therefore, the design and synthesis of APN inhibitors may be a clinical significance for the discovery of anti-cancer agents.

Bestatin (AHPA-Leu), a well-known APN inhibitor, was first isolated from the culture filtrate of *Streptomyces olivoreticuli* in 1976[11], and now it is widely used in clinical practise for the treatment of adult acute non-lymphocytic leukemia. Since then, many APN inhibitors such as probestin[12], prebestin[12], AHPA-Val[13] and amatatin[14] have been developed. Judging from the structure of these compounds, we can see that they all contain the key unit 3-amino-2-hydroxy acyl scaffold, which is important for their biological activity for that it can chelate the Zn^{2+} in the active site of metal-dependent enzymes[15].

The interaction of bestatin with the active site of APN showed that the hydroxyl group and carbonyl group of bestatin belong to the zinc-binding group (ZBG) to chelate the Zn^{2+} in the active site of APN. The phenyl group can insert into the S1 pocket of APN and the Leu residue can insert into the S1′ pocket, while the free amino group can interact with Glu 350. In our previous work, we have designed a series of novel chloramphenicol amine derivatives as APN inhibitors, but the result was not satisfied, most compounds showed less activity than bestatin[16], the most active compound **13b** is a pseudotripeptide

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with the IC_{50} value to 7.1 μM, which is still less than that of bestatin (IC_{50} = 3.00 μM). Recently, our group has synthesised a series of novel L-isoserine derivatives as APN inhibitors, most of these compounds showed moderate activities, the most active compound **14b** (IC_{50} = 12.2 μM) showed still less inhibitory activity toward APN than that of bestatin (IC_{50} = 7.3 μM)[17,18].

Taxol, a well-known anti-cancer drug, also has the 3-amino-2-hydroxy acyl scaffold ((2R, 3S)-3-amino-2-hydroxy-3-phenylpropanoic acid residue). Compared with L-isoserine, (2R, 3S)-3-amino-2-hydroxy-3-phenylpropanoic acid is much more similar with the residue of bestatin (2S, 3R)-3-amino-2-hydroxy-4-phenylbutyl acid except for the chirality and one carbon. There is a phenyl group in C_3 of (2R, 3S)-3-amino-2-hydroxy-3-phenylpropanoic acid, which may be beneficial for APN interaction. So, we used bestatin and **14b** as the lead compound, (2R, 3S)-3-amino-2-hydroxy-3-phenylpropanoic acid as the key scaffold, by coupling with different amino acids or other functional groups, and we got a series of novel bi-peptide and tri-peptide derivatives with 3-amino-2-hydroxy-3-phenylpropanoic acid scaffold . Considering the best ZBG hydroxamate group may be good for APN inhibitors, we also converted the carboxyl group of some compounds into hydroxamate group (Figure 1).

Results and discussion

Chemistry

The target compounds were synthesised via the route outlined in Schemes 1 and 2. The commercial (2R, 3S)-3-amino-2-hydroxyl-3-phenylpropanoic acid hydrochloride **1** was protected by $(Boc)_2O$ to get the key intermediate compound **2**. Compound **2** can be coupled with different methyl ester of amino acids or methyl ester of dipeptides by classical EDCI/HOBt method to provide compounds **3a**–**3o**. The esters of compounds **3a**–**3o** were hydrolysed to carboxylic acid by $NaOH/H_2O$ in methanol, and then converted into the target compounds **5a**–**5o** by deprotecting the Boc group. Compounds **7a**–**7h** containing hydroxymate group were obtained from compounds **3a**–**3h** by reacting with NH_2OK in methanol and then cleaving the Boc group.

As shown in **Scheme 2**, compounds **9a**–**9d** were obtained from compound **4a** by coupling it with different substituted organic aromatic amines and then cleaving the Boc group.

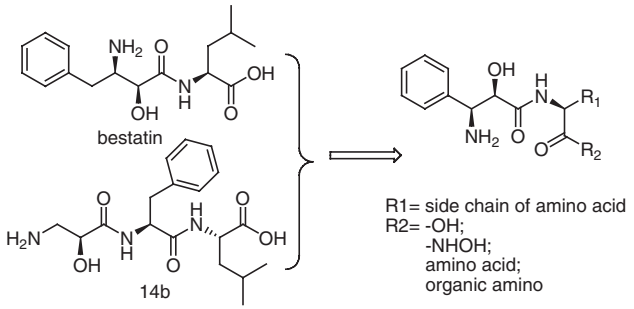
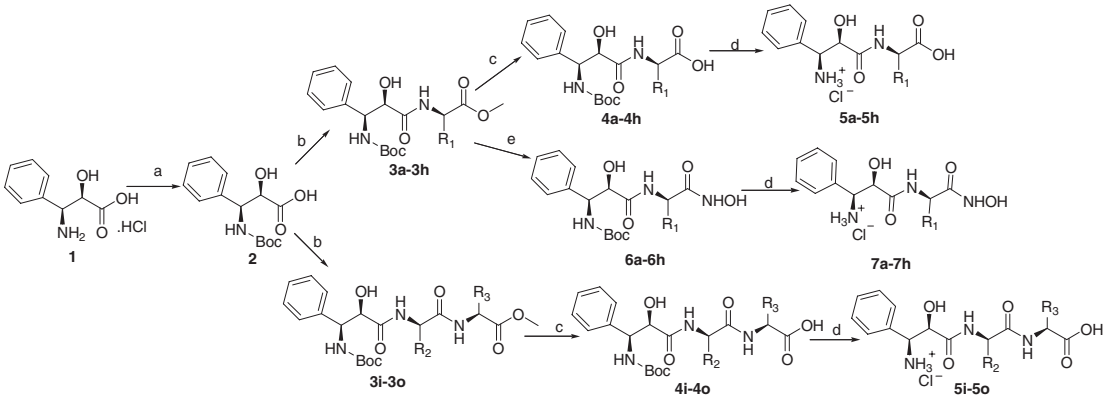
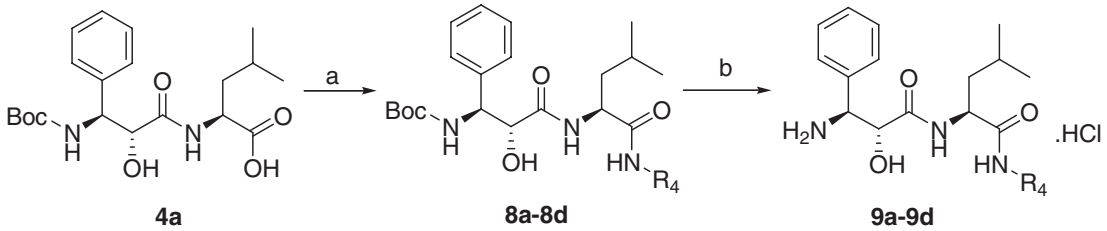


Figure 1. Novel aminopeptidase N inhibitors with 3-amino-2-hydroxy-3-phenylpropanoic acid scaffold.



Scheme 1. Reagents and conditions: (a) $(Boc)_2O$, NaOH, THF; (b) EDCI, HOBT, DCM, NH_2-AA-$COOCH_3$; (c) NaOH, CH_3OH; (d) 3N HCl-EtOAc; (e) NH_2OK, CH_3OH;



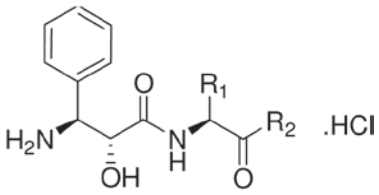
Scheme 2. Reagents and conditions: (a) EDCI, HOBT, DCM, NH_2R_4; (b) 3N HCl-EtOAc

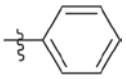
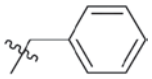
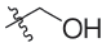
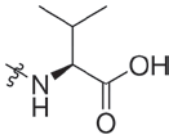
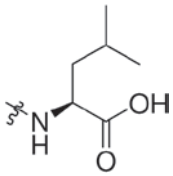
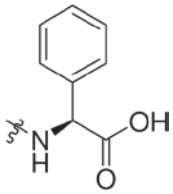
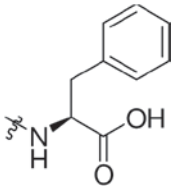
Preliminary *in vitro* evaluation and SAR discussion

The newly synthesised 3-amino-2-hydroxyl-3-phenyl-propanoic acid derivatives were assayed for the enzymatic inhibitions both on APN from porcine kidney (Microsomal; Sigma-Aldrich, St. Louis, MO, USA) and matrix metalloproteinases-2 (MMP-2). The results are listed in Table 1. Similar to APN, MMP-2 is also a zinc-dependent metalloproteinase, which is involved in the process of tumour invasion and metastasis[19]. The difference between them is that MMP-2 is an endopeptidase, while APN is an exopeptidase. All the target compounds were assayed for the inhibitory activities on APN and MMP-2 to observe the selectivity.

As shown in Table 1, the results showed that most compounds had moderate MMP inhibitory activity, which was consistent with our early report. But the result of APN inhibition was not satisfied, most compounds did not show expected APN inhibition, and some compounds

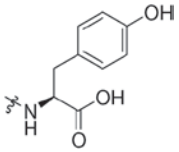
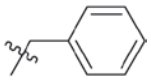
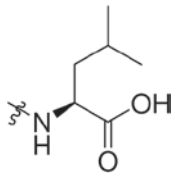
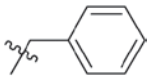
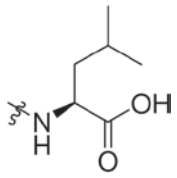
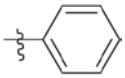
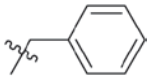
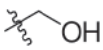
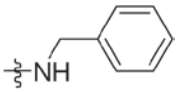
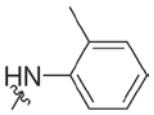
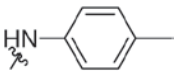
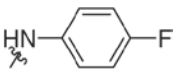
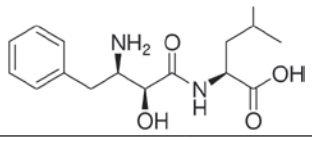
Table 1. The structures and *in vitro* APN inhibitory activities of the target compounds.



Compound	R1	R2	IC_{50}[a](μM) APN	MMP-2
5a	$-CH_2CH(CH_3)_2$	–OH	351.71 ± 0.70	214.87 ± 0.49
5b	$-CH(CH_3)_2$	–OH	NA[b]	287.42 ± 0.93
5c	$-CH_3$	–OH	342.42 ± 0.45	273.08 ± 0.25
5d	$-CH(CH_3)CH_2CH_3$	–OH	NA[b]	264.1 ± 0.5
5e		–OH	197.57 ± 11.02	254.11 ± 0.25
5f		–OH	NA[b]	497.15 ± 0.91
5g		–OH	NA[b]	407.25 ± 1.89
5h		–OH	NA[b]	561.79 ± 2.55
5i			590.39 ± 10.61	690.44 ± 1.92
5j			512.91 ± 23.91	424.18 ± 1.18
5k			NA[b]	185.90 ± 0.36
5l			NA[b]	NA[b]

(*Continued*)

Table 1. *(Continued).*

Compound	R1	R2	IC_{50}[a](μM) APN	MMP-2
5m			NA[b]	NA[b]
5n			104.53 ± 0.21	NA[b]
5o			NA[b]	NA[b]
7a	$-CH_2CH(CH_3)_2$	–NHOH	56.71 ± 0.01	64.60 ± 0.79
7b	$-CH(CH_3)_2$	–NHOH	76.95 ± 0.43	128.12 ± 0.14
7c	$-CH_3$	–NHOH	NA[b]	292.22 ± 2.08
7d	$-CH(CH_3)CH_2CH_3$	–NHOH	82.89 ± 2.67	207.11 ± 1.32
7e		–NHOH	1.26 ± 0.01	212.97 ± 1.35
7f		–NHOH	44.00 ± 2.32	249.42 ± 0.94
7g		–NHOH	172.87 ± 1.99	298.79 ± 1.48
7h		–NHOH	157.64 ± 0.58	292.35 ± 1.97
9a			NA[b]	NA[b]
9b			362.76 ± 0.04	NA[b]
9c			NA[b]	NA[b]
9d			NA[b]	NA[b]
Bestatin			2.55 ± 0.11	163.48 ± 1.13

[a]Values are means of three experiments and standard derivation.
[b][b]NA, no activity.

(such as **5b**, **5d**, **5f** and so on) even had no APN inhibitory activity except for the hydroxamic acid derivatives **7a**–**7h**. Of all these compounds, compound **7e** showed to be the best APN inhibitor with the IC_{50} value to 1.26 μM, which was much better than that of the positive control bestatin (IC_{50} = 2.55 μM). By analysing the results, we supposed that these compounds may not have the same interaction model with bestatin by using the hydroxyl group and carbonyl group to chelate the zinc ion but using the hydroxamic acid group, this hypothesis could be verified by the docking results (Figure 2). If the phenyl group of 3-amino-2-hydroxyl-3-phenylpropanoic acid could form

hydrophobic interaction with the S_1 pocket as bestatin, the distance of the hydroxyl group and carbonyl group to the zinc ion may be unsuitable because of the lack of one carbon, this may explain why compounds **5a**–**5o** and **9a**–**9d** had poor APN inhibitory activity. But compounds **7a**–**7h** showed very good APN inhibitory activity because of the introduction of the strong ZBG hydroxamic acid group.

Comparing compounds **7a**–**7h**, we can know that the APN inhibitory activities were different with the substitutions in R_1. Compounds **7a** and **7b** with the Leu and Val in R_1 had better APN inhibitory activity than the others (such as **7c**, **7d**, **7g** and **7h**), this may suggest that the Leu and Val in R_1 is good for APN inhibition. Compound **7e** with phenylglycine in R_1 had the best APN inhibition with the IC_{50} value to 1.26 ± 0.01 μM. Compound **7f** with phenylalanine in R_1 also had good APN inhibition but not better than compound **7e** with the IC_{50} value to 44.00 ± 2.32 μM. Comparing with compounds **7e** and **7f**, we can know that the length of the α-carbon atom to the aromatic ring may be important for APN inhibition. Compound **7g** with a hydroxyl group in the *para*-position of the phenyl group had less inhibitory activity than compound **7f**, this may suggest

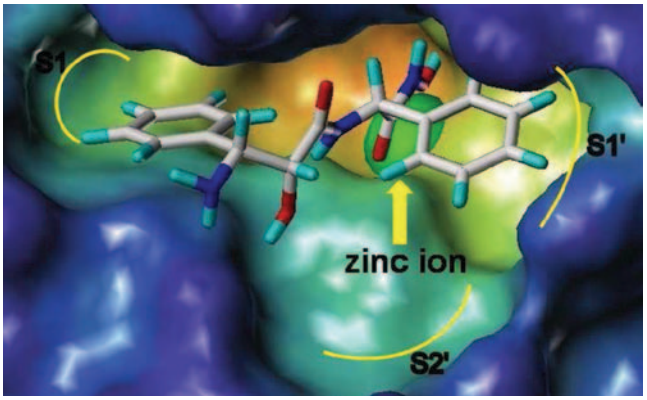


Figure 2. The FlexX docking result of **7e** with the active site of aminopeptidase N (PDB: 2DQM).

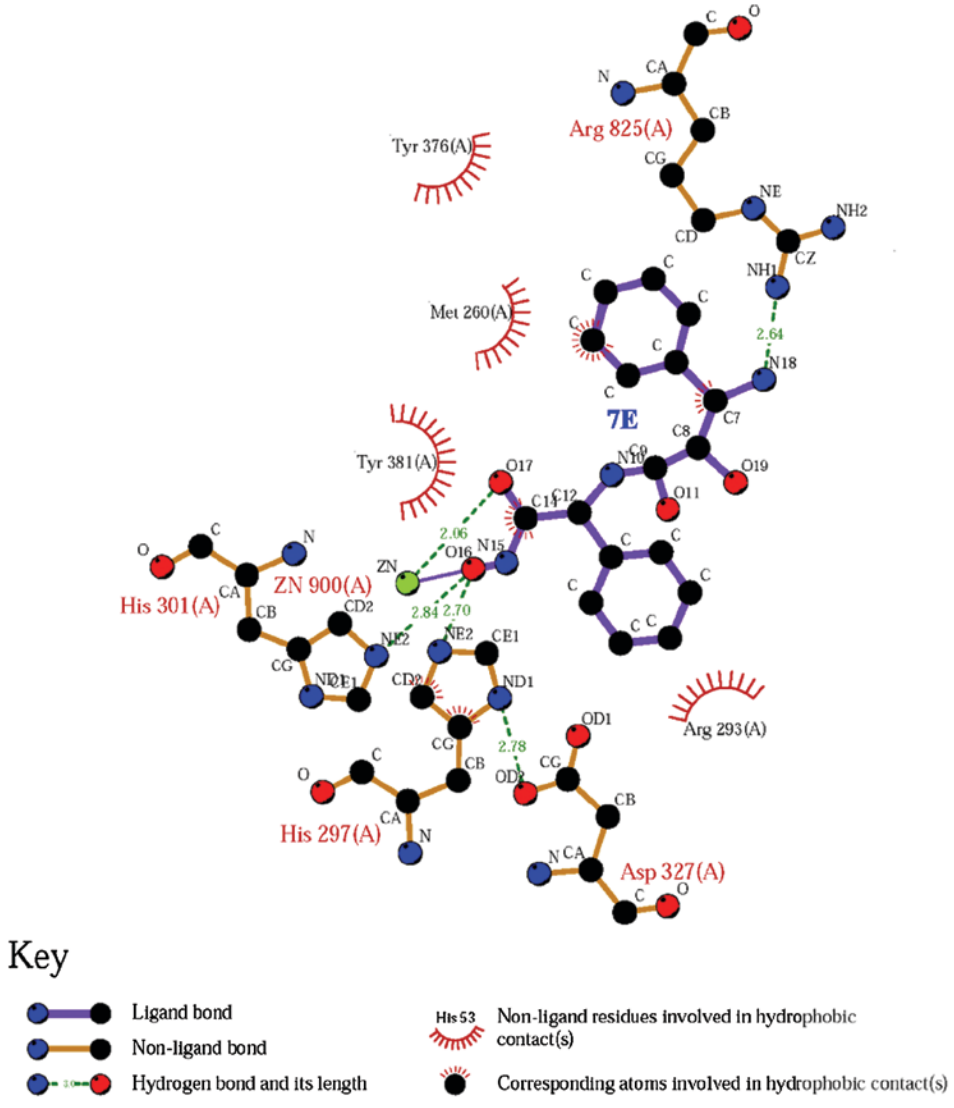


Figure 3. The docking result of **7e** with aminopeptidase N showed by LIGPLOT.

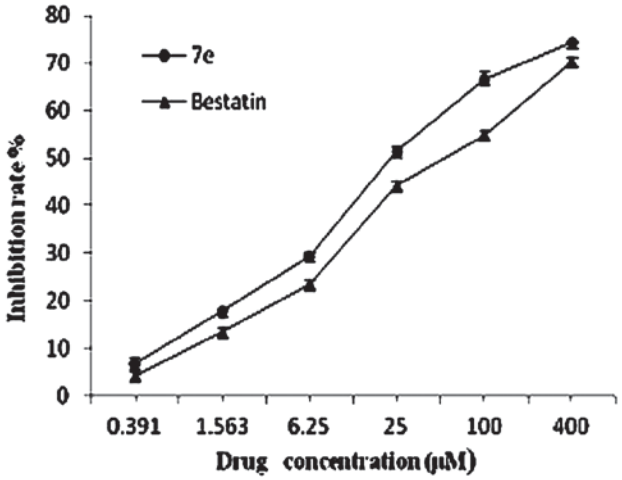


Figure 4. Inhibition of aminopeptidase N (APN)/CD13 activity on ES-2 induced by **7e** or bestatin. OVCA cells (1×10^5) seeded in 96-well plates were exposed to various concentrations of **7e** or bestatin at 37°C, and the substrate L-leucine-p-nitroanilide was added. APN/CD13 activity was estimated by measuring absorbance at 405 nm using a microplate reader after 60 min of incubation. The inhibition rate was calculated by comparing to the control (without drug exposure). The bars indicate means ± standard deviation ($n = 3$).

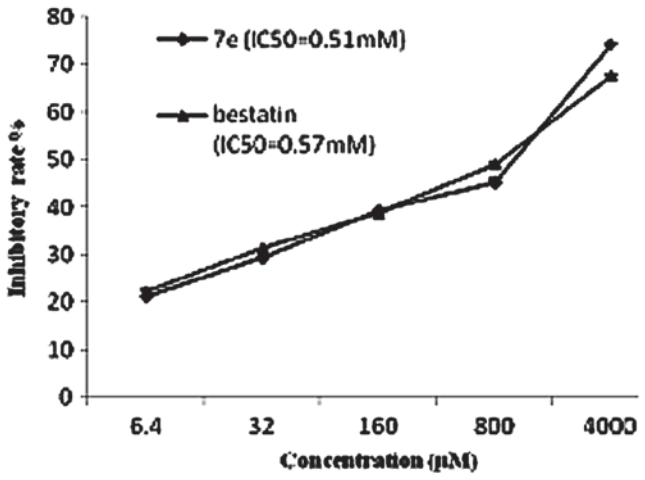


Figure 5. *In vitro* anti-proliferative activities of compound **7e** and bestatin against ES-2 cells. The bars indicate means ± standard deviation ($n = 3$).

that the additional hydroxyl group may be unsuitable for APN interaction.

Compound **7e** showed the most inhibitory activity toward APN. To investigate the interaction of these compounds with APN, the docking studies of the representative compound **7e** were carried out via Sybyl/Sketch module and optimised using Powell's method with the Tripos force field with convergence criterion set at 0.05 kcal/ (Å mol) and assigned with Gasteiger–Hückel method. The docking study performed using Sybyl7.3/FlexX module, the residues in a radius of 7.0 Å around Bestatin in the co-crystal structure (PDB code: 2DQM) were selected as the active site. Other docking parameters implied in the program were kept default. The docking result was shown in Figure 2. As shown in Figure 2, 3-amino-2-hydroxyl-3-phenylpropanoic acid scaffold of compound 7e inserted into the S1 pocket, and the hydroxymate group chelated with the zinc ion in the active site, while the phenyl group of L-phenylglycine moiety interacted with the S1′ pocket. For a further and detail understanding of the binding mode of **7e** with APN, a two-dimensional picture was also created with the program LIGPLOT, which was shown in Figure 3; we can see that the phenyl group of 3-amino-2-hydroxyl-3-phenylpropanoic acid could form hydrophobic interaction with the S_1 pocket formed by Arg^{825}, Tyr^{376}, Met^{260} and Tyr^{381}. The free amino group could form hydrogen bond with Arg^{825} at the distance of 2.64 Å. The phenyl group of L-phenylglycine moiety could form hydrophobic interaction with the S1′ pocket formed by Arg^{293}, Asp^{372} and His^{297}. The two oxygen atoms of hydroxamic acid could chelate with the zinc ion of APN. Additionally, the hydroxyl group of hydroxamic acid could also form hydrogen bond with His^{297} and His^{301} at the distance of 2.70 Å and 2.84 Å.

Although the computed information partially supported our assumption, the exact binding mode of the 3-amino-2-hydroxyl-3-phenylpropanoic acid derivatives with APN should be obtained from further X-ray crystal studies.

APN is highly expressed on the surface of human ovary clear cell carcinoma cell ES-2[20]; to study the inhibitory activity of the target compounds toward human APN, we also tested the *in vitro* enzymatic inhibitions of compound **7e** toward APN on ES-2. The results showed that compound **7e** is also a dose-dependent APN inhibitor in the concentration range of 0.39–400 μM (Figure 4). The IC_{50} value of compound **7e** is 30.19 ± 1.02 μM, which is still better than that of bestatin (IC_{50} = 60.61 ± 0.1 μM).

Additionally, the effects of compound **7e** on the proliferation of human ovary clear cell carcinoma cell ES-2 compared with bestatin were further assessed by using MTT assay, which were shown in Figure 5. The results showed that the anti-proliferative effects of compound **7e** against ES-2 cell were slightly better than that of bestatin with the IC_{50} value of 0.51 ± 0.08 mM versus 0.57 ± 0.04 mM.

Conclusion

In summary, we have described the synthesis and properties of a series of novel (2R,3S)-3-amino-2-hydroxyl-3-phenylpropanoic acid derivatives as APN inhibitors. Because of the lack of one carbon as compared with 3-amino-2-hydroxyl-4- phenylbutyl acid, most of the target compounds did not show expected APN inhibitory activities, but the introduction of hydroxamate group to the compounds could greatly improve the inhibitory activities. The most effective compound **7e**, not only had better inhibitory activity against APN both from porcine kidney and human but also showed

slightly better anti-proliferative effects toward ES-2 cells than that of bestatin. Compound **7e** could be used as a lead compound for further development of small molecular peptidomimetic APN inhibitors. Though the preliminary enzymatic results are not very encouraging, it still provides useful clues for further APN inhibitors exploitation.

Supplementary data

Supplementary data (Experimental details, and spectroscopic data for all the compounds and procedures for the activity assays were provided.).

Declaration of interest

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