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Novel β-dicarbonyl derivatives as inhibitors of aminopeptidase N (APN)

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ABSTRACT

Most zinc metalloproteases are over-expressed in tumor cells and play a critical role in the genesis, development, and metastasis of tumors. Novel zinc binding groups (ZBGs) represent a novel strategy to obtain optimal potency and selectivity for zinc metalloproteases inhibitors. Here we described the design, synthesis, and biological studies of novel β-dicarbonyl derivatives as aminopeptidase N (APN/CD13) inhibitors. The results demonstrated that some compounds exhibited moderate to good inhibitory activities against APN with compound **5c** being the most potent, suggesting that **5c** could serve as new lead for the future APN inhibitor development. The results further confirm our design rationale of β-dicarbonyl moiety as a new ZBG, which may provide a new direction for the design and discovery of zinc metalloproteases inhibitors as new anti-tumor agents.

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Most of zinc metalloproteases are significantly important biological and medical markers for the development of cancer. For example, matrix metalloproteinases (MMPs), a family of calcium- and zinc-dependent endopeptidases, have been proved to play crucial roles in tumor proliferation, invasion, angiogenesis, and metastasis;[1] Zn^{2+}-dependent HDACs, especially class I and class II isozymes are associated with tumor proliferation, angiogenesis, migration, resistance to chemotherapy and preventing apoptosis and differentiation;[2] the aminopeptidase N (APN/CD13, EC 3.4.11.2) has been identified as a target for inhibition of tumor vascularization and growth.[3,4] Accordingly, it is meaningful to develop zinc metalloproteases targeted agents for tumor imaging and therapy. One zinc binding group (ZBG) which can coordinate with zinc ion in the active site of enzyme is indispensable to a successful zinc metalloproteases inhibitor. Therefore, novel ZBGs are urgently needed to obtain optimal potency and selectivity zinc metalloproteases inhibitors.

APN explored in depth by our laboratory is used as the target to search for new ZBG. It is a widely expressed type II membrane-bound metalloprotease that plays a key role in tumor angiogenesis, growth, and metastasis. Since 1976, several natural inhibitors of APN have been reported, for example, **Bestatin**,[5] Probestin,[6] Amastatin,[7] Actinonin,[8] Phebestin,[9] Lapstatin,[10] AHPA-Val,[11] Leuhistin,[12] **Curcumin**[13] and so on. Amongst these inhibitors, **Curcumin**, which IC_{50} was 10 μM compared with 2.5 μM of **Bestatin**, is a special natural APN inhibitor without 3-Amino-2-hydroxy-4-phenylbutyric acids (**AHPA**) scaffold. However, hydrazinocurcumin, a synthetic analog of **Curcumin** with the central β-dicarbonyl moiety substituted by pyrazole, did not present the enzyme inhibitory activity,[13] we envisioned that the β-dicarbonyl scaffold may be able to chelate with the zinc ion which is the crucial catalytic factor in active site and could potentially serve as a starting point for the development of novel APN inhibitors with anti-tumor activity. To examine this hypothesis, a series of novel β-dicarbonyl derivatives were synthesized, (Fig. 1) the activity results and analysis of structure activity relationship (SAR) were also shown in this letter.

Syntheses of target compounds were outlined in Scheme 1. Optically pure Boc-D-Phe was condensed with Meldrum's acid to obtain the key intermediate compound **2**. Alcoholysis or aminolysis of **2** with corresponding alcohols or amino acid methyl esters yielded β-dicarbonyl derivatives **3a**–**e**.[14] Removal the Boc protecting group of compounds **3a**–**d** produced **4a**–**e**. Additionally, the methyl esters of **3c**–**e** were hydrolyzed and then the Boc groups were removed to yield corresponding acids **5a**, **5c**–**e**.[15]

The target compounds were evaluated for their inhibitory activities toward APN/CD13 and HDACs as previously described.[16] HDACs are zinc metalloproteinase as well as APN and associated closely with the invasion and metastasis of tumors. The difference between them is that HDACs mainly exist in nucleus while APN is an exopeptidase. In order to identify the selectivity of the target compounds against the two enzymes, the target compounds were assayed for the inhibitory activities against APN and HDACs, all the inhibition results were summarized in Tables 1 and 2.

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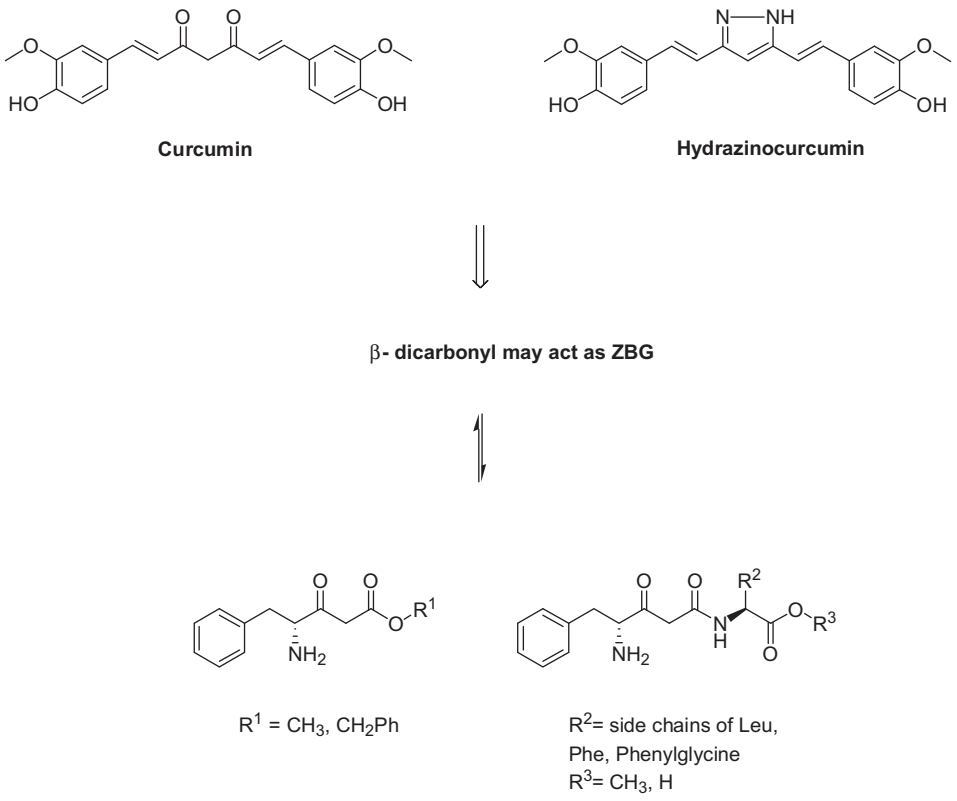
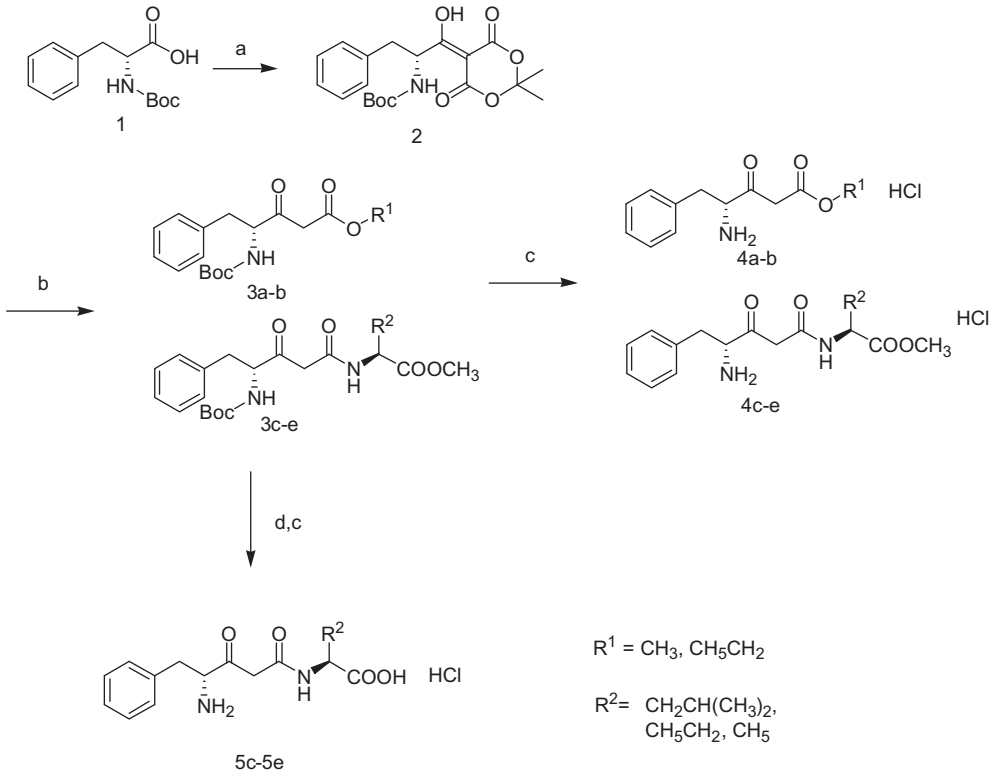
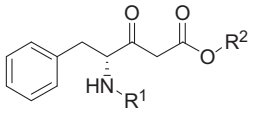


Figure 1. The new APNIs to examine the hypothesis that β-dicarbonyl moiety may act as ZBG.



Scheme 1. Reagents and conditions: (a) Meldrum's acid, EDCI·HCl, DMAP, CH_2Cl_2, 2 h; (b) corresponding alcohols or amino acid methyl esters, acetonitrile, 80 °C; (c) 3N HCl–EtOAC; (d) NaOH, MeOH, 1 h.

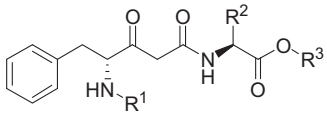
Table 1
The structures and inhibitory activities of the target compounds **3a**,**b** and **4a**,**b** against APN and HDACs



Compd	R^1	R^2	IC_{50}[a] (μM) APN	IC_{50}[a] (μM) HDACs
3a	Boc	CH_3	>1000	>1000
3b	Boc	CH_2Ph	>1000	>1000
4a	H	CH_3	984 ± 310	>1000
4b	H	CH_2Ph	711 ± 260	>1000
Bestatin			5.58 ± 0.55	>1000
SAHA				0.14 ± 0.13
Curcumin			15.50 ± 4.00	

[a] Mean values and standard deviations of triplicate experiments are given.

Table 2
The structures and inhibitory activities of the target compounds **3c–e, 4c–e and 5c–e** against APN and HDACs



Compd	R^1	R^2	R^3	IC_{50}[a] (μM) APN	IC_{50}[a] (μM) HDACs
3c	Boc	$CH_2CH(CH_3)_2$	CH_3	>1000	>1000
3d	Boc	CH_2Ph	CH_3	>1000	>1000
3e	Boc	Ph	CH_3	>1000	>1000
4c	H	$CH_2CH(CH_3)_2$	CH_3	5.04 ± 2.10	>1000
4d	H	CH_2Ph	CH_3	7.02 ± 1.30	>1000
4e	H	Ph	CH_3	6.03 ± 0.48	>1000
5c	H	$CH_2CH(CH_3)_2$	H	1.41 ± 0.25	>1000
5d	H	CH_2Ph	H	4.73 ± 0.17	>1000
5e	H	Ph	H	3.82 ± 0.45	>1000
Bestatin				5.58 ± 0.55	
SAHA					0.14 ± 0.13
Curcumin				15.50 ± 4.00	

[a] Mean values and standard deviations of triplicate experiments are given.

As shown in Tables 1 and 2, it is worthy to note that most of β-dicarbonyl derivatives displayed a better enzymatic inhibition towards APN than that of HDACs, with IC_{50} values lying in micromole level. These results, to a certain extent, validated our design strategy for novel potential APNIs. The results may be explained as follows. In one hand, APN is an exopeptidase which can hydrolyze the neutral or basic amino acids from the N-terminal of the peptide, such as Phe, Tyr, Ala, and Leu. The designed compounds all have the Phe residue which could be well recognized by APN, so the compounds may be suitable as APN inhibitors. On the other hand, the length between hydrophobic group and ZBG of compounds which is suitable for APN is too short to the slender channel of HDACs. Thereby the following structure–activity relationships were mainly focused on APN inhibition.

Among these inhibitors, generally speaking, compounds with free amino group showed better activity than compounds with Boc protecting group, suggesting that the free amine is still the successful optimization for the inhibition of APN. And it must be emphasized that the existence of free amine group can endow the compounds with higher affinity toward the enzyme, which is consistent with reference and indicates the importance of amino group in the recognition of inhibitors to APN. Comparing **4a** and **4b**, we can find that the activity of benzyl ester is better than that of methyl ester, which may be due to the system of the aromatic ring enhancing the interaction with the **S1′** hydrophobic region of the APN. Based on the above result, we introduced amino acids to replace the benzyl ester of **4b** in order to increase the interaction with the hydrophobic region, the activities of these compounds (**4c–e** and **5c–e**) confirming our design. Comparing **4c–e** with **5c–e**, we could find that the carboxylic acid derivatives present better activities than those corresponding methyl ester compounds. This may be due to the fact that the carboxylic acid can increase the water solubility of compounds and interact with **S2′** pocket by hydrogen bonding. Comparing compounds **5c–e,** of which the R^2 groups were different amino acid residues, the APN inhibitory activities were different owing to the residues. The data shown in Table 2 suggested that the preferred substitutions against APN were, in decreasing order, L-leucine, L-phenylglycine and L-phenylalanine residues, which was consistent with the result from ureido derivatives we reported earlier.[17]

The most active compound is **5c**, which has a better inhibitory activity (IC_{50} = 1.41 ± 0.25 μM) than positive control **Bestatin** (IC_{50} = 5.58 ± 0.55 μM) and **Curcumin** (15.50 ± 4.00 μM). Compound **4c**, **5d** and **5e** also dispaly moderate activities (IC_{50} = 5.04, 4.73, 3.82 μM, respectively). The only difference of the structure of **Beststin** and **5c** is the hydroxyl group and carbonyl group in **Bestatin** replaced by β-dicarbonyl (Fig. 2). While it is interesting to note that the hydroxyl group and carbonyl group act as the **ZBG** of **Bestatin**, which indicate that the β-dicarbonyl moiety may interact with APN by chelating the zinc. Therefore, β-dicarbonyl moiety can act as ZBG.

To further characterize the affinity of these compounds for Zn in vitro, a spectrophotometry study was performed.[18] In the absence of zinc acetate, **5c** showed absorption maxima at 244.0 nm which shifted to approximately 266.6 nm when as little as Zn^{2+} was added. Addition of Zn^{2+} also increased the peak height by 0.38 OD units. (Fig 3) The results suggested our compounds can

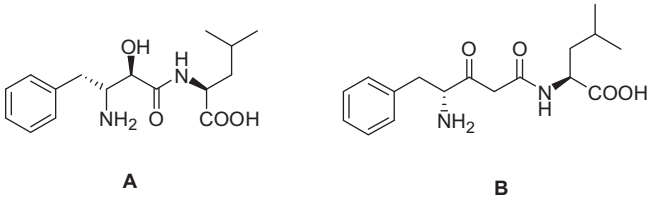


Figure 2. The structure of compound **Bestatin** (A) and **5c** (B).

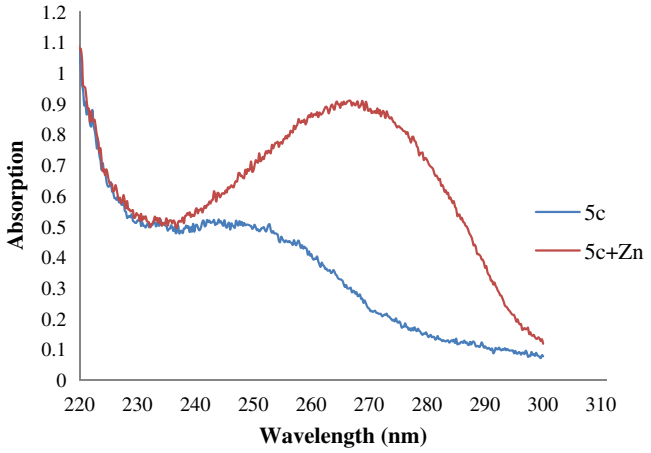


Figure 3. Absorption spectra of **5c** in the presence and absence of zinc.

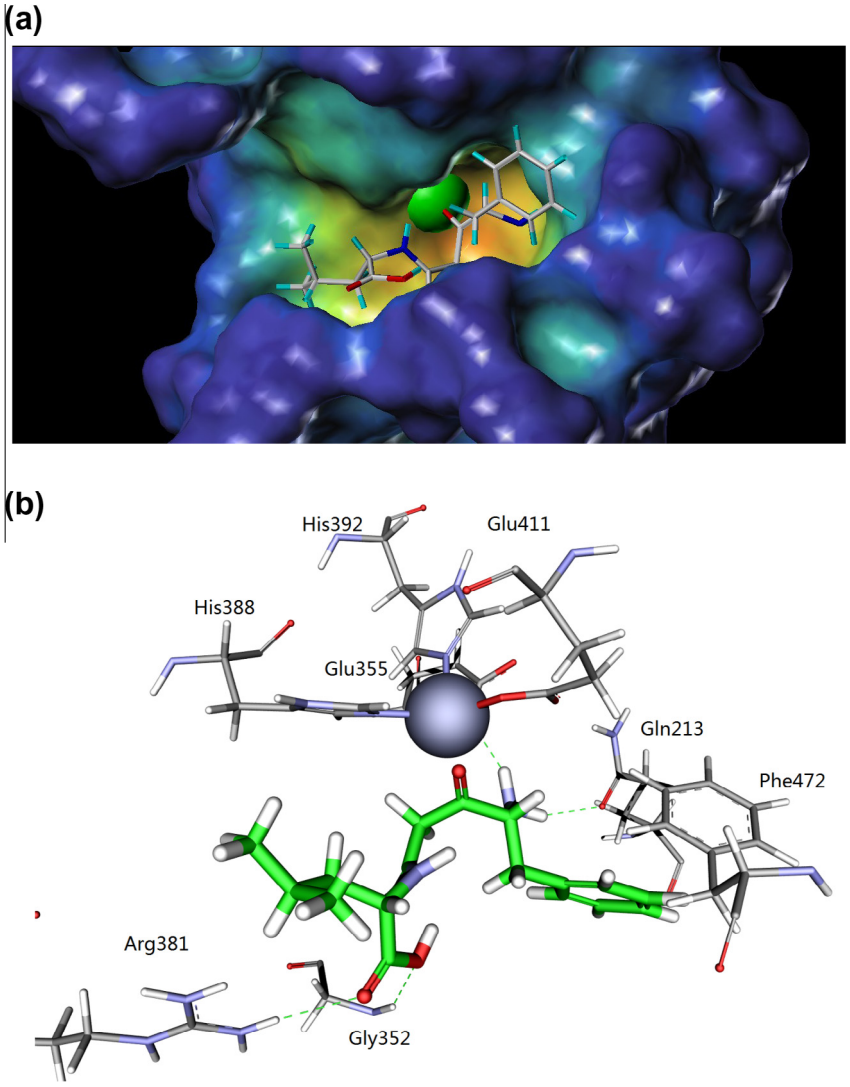


Figure 4. (a) The FlexX docking result of **5c** with APN (PDB code: 4YFR). (b) The docking result of **5c** shown by LIGPLOT.

chelate with Zn^{2+} in vitro which provided strong evidence for our assumption.

Aiming to investigate the interaction between the target compounds and APN, the most active compound **5c**, was chosen to be constructed using a Sybyl/Sketch module and optimized via Powell's method by the Tripos force field with convergence criterion set at 0.05 kcal/(Å mol), and assigned with the Gasteiger–Hückel method. The docking study of **5c** and the active site of APN were performed using Sybyl/FlexX module. The active site was defined as 10.0 Å radius circles around **Bestatin** in the co-crystal structure (PDB code: 4FYR). Other docking parameters utilized in the program were remained default. The docking result was shown in Figure 4a. As diagramed in Figure 4a, the β-dicarbonyl group can chelate with the zinc ion which is the crucial catalytic factor in active site. The phenyl group of Phe moiety can insert into **S1** pocket as the same with **Bestatin**; the hydrophobic residues of L-Leu can plunge into the **S1′** pocket of APN as the same with **Bestatin**. In addition, a 2D pattern of detailed binding mode was created as well (Fig 4b). The phenyl group of compound **5c** can form hydrophobic interaction with Phe 472 of S1 pocket, while, the free amine group and carboxylic acid group can form hydrogen bonds with Gln 213, Glu 355 and Gly 352, Arg 381, respectively.

In summary, one series of novel β-dicarbonyl derivatives as APN inhibitors had been designed, synthesized and evaluated for the biological activity. The preliminary results showed that most of the target compounds represented moderate to good inhibition and selectivity against APN. The most potent compound, **5c**, exhibited a better activity profiles than Bestain which could be served as new lead for further structure optimization in the future APNIs research. These results suggest that β-dicarbonyl moiety could act as ZBG, which provides a new direction for the design and discovery of zinc metalloproteases inhibitors as new antitumor agents.

Acknowledgments

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15. *General procedure for the preparation target compounds*: To a solution of **1** (2.65 g) in CH_2Cl_2 (50 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl) (2.88 g), Meldrum's acid (2.8 g), and DMAP (1.2 g) at 0 °C. After being stirred for 2 h, the mixture was poured into 1 M hydrochloric acid and extracted with diethyl ether three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated to give crude **2**. This crude and corresponding alcohols or amino acid methyl esters were dissolved in acetonitrile (50 ml). After being heated at 80 °C for 3 h, this mixture was evaporated. The residue was chromatographed over silica gel to give compound **3**. To the stirring solution of **3** (0.5 g) in 10 ml anhydrous ethyl acetate was added dropwise 5 ml 3 N EtOAc–HCl and after 2, 3 h, compounds **4** were obtained in the form of hydrocholoride salt. Filter quickly and dry the cake in vaccum to obtain dried white solid with high yield. Compound **3** (0.5 g) and 1 N NaOH (4 ml) were add to 4 ml methanol, after 1 h the solution was acidified with 1 N HCl to pH 2, extracted with EA (30 ml ×3), and subsequently the solvent was removed under reduced pressure. Then the boc group was removed as above to obtain compound **5**. *Compound **5c***: yield 30.7%. Mp 120.8–122.2 °C; ESI-MS *m*/*z* $[M+1]^+$ 321.4; ^1H NMR (300 MHz, DMSO-d_6): δ 8.59–8.55 (m, 5H), 7.35-7.23 (m, 5H), 4.49 (m, 1H), 4,25 (m, 1H), 3.85–3.77 (m, 2H), 3.11–2.98 (m, 2H), 1.54 (m, 1H), 1.35 (m, 2H), 0.86 (m, 6H).
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