Recent Advances in the Development of MMPIs and APNIs Based on the Pyrrolidine Platforms

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Abstract: Pyrrolidine scaffold has been widely used to design a variety of *N*-heterocyclic derivatives towards various targets. Amongst them, matrix metalloproteins (MMPs) and aminopeptidase N (APN) represent two kinds of important metalloproteinase targets which have been proved to be tightly related to tumor proliferation, invasion, metastasis and angiogenesis. As a result, their respective inhibitors, namely MMP inhibitors (MMPIs) and APN inhibitors (APNIs), have been systematically studied in our group for many years. Recent advances in the elucidation of MMPIs and APNIs based on the pyrrolidine platforms are briefly reviewed in this paper.

Keywords: MMPIs, APNIs, pyrrolidine, design, anticancer.

1. INTRODUCTION

1.1. MMP and MMPI

The matrix metalloproteinases (MMPs), also called matrixins, are a family of structurally related calcium- and zinc-dependent endopeptidases that are involved in the tissue remodeling and degradation of the macromolecular components in the extracellular matrix (ECM), such as collagens, laminins, elastins, fibronectins, gelatin, matrix glycoproteins, and the protein core of proteoglycan [1, 2]. The physiological function of MMPs consists of regulating the refreshment of ECM, keeping cells stable, tissue remodeling and repair, angiogenesis, embryonic development, apoptosis, ovulation, neural development, wound healing, cell adhesion and proliferation as well [3, 4]. Under normal physiological conditions, these enzymes are usually minimally expressed and well-regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). There are a total of four TIMPs (TIMP-1, -2, -3, and -4) and these four protein inhibitors are able to control the proteolytic activity of all MMPs and mediate the stability of cells.

MMPs are excreted by a spectrum of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes. These enzymes are expressed as zymogens, which are subsequently processed by other proteolytic enzymes, such as serine proteases, furin, plasmin and so on, to generate the active forms. Hence, MMPs are tightly regulated at both the transcriptional level and the protein activation level. However, in the presence of specific stimuli exemplified by cytokines and growth factors, MMPs are up-regulated destroying the balance between MMPs and TIMPs, resulting in the activation of MMPs and an excessive degradation of ECM components, which are believed to contribute to a wide array of pathological conditions related to tumor metastasis, angiogenesis, cardiovascular disease, osteoarthritis, rheumatoid arthritis, chronic periodontitis, pulmonary emphysema, skin ulceration, atherosclerosis, central nervous system disease, type I diabetes, myocarditis and dilated cardiomyopathy, coronary artery disease, multiple sclerosis, congestive heart failure, and so forth [5-11]. Accordingly, effective controlling MMP activity(s) using natural or synthetic small-molecule inhibitors (MMPIs) represents an attractive and potential area of drug development to intervene in the MMP-related disorders [12, 13]. Thus, MMPs have now been considered as a promising target for the treatment of these disorders.

Up to now, it has been reported that the mammal MMPs gene family consists of at least 26 structurally related members that fall into five groups according to their primary structure and function, substrate specificity, as well as their cellular sources: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases A and B (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, and MMP-12), membrane type (MT)-MMPs (MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP), as well as nonclassified MMPs [14].

Among these members, certain MMPs are involved in innate immunity and host defense against cancer, others stimulate tumor growth and malignant transformation, promote metastasis and angiogenesis, while others exert antiangiogenic effects [15]. Moreover, certain MMPs exert anticancer effect in the process of carcinogenesis and tumor-promoting effects at later stages of tumorigenesis. Similarly, certain MMPs exert cancer-promoting effects in some tumors and anticancer effects in other tumors. Accordingly, at present, although numerous natural or synthetic MMPIs have been investigated with regard to suppression of tumor progression and improvement of prognosis in patients with different types of cancer, including ovarian cancer, breast cancer, malignant glioma, pancreatic cancer, non-small cell lung

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caner (NSCLC), advanced bladder carcinoma, *etc*, which has been actively pursued and widely tested in preclinical or clinical trials, most of these clinical trials of MMPIs have yielded a consistent number of disappointing results and/or limited success, especially in the cancer pathology area, thus they are as yet unavailable for use as therapeutic drugs [16, 17].

Analysis of the reasons, on one hand, most developed MMPIs currently are broad-spectrum inhibitors, and they manipulate an assortment of physiological processes, leading to lack of selectivity. In consequence, even with tremendous improvements in the development of MMPIs over the past few decades, simple and effective drugs for inhibiting individual MMPs have not yet emerged. Till to now, antibiotic doxycycline still remains the only FDA-approved MMPI [18]. It is, therefore, anticipant to exploit specific MMPIs that are highly selective for certain MMPs associating with certain physiological conditions for a positive therapy [19]. Additionally, the worse clinical outcome can partly attribute to our limited understanding of the multiple roles of MMPs in diseases (especially in cancer), the full repertoire of MMP substrates, and hence the *in vivo* functions of MMPs, resulting in a variety of unforeseen side effects. For example, one clinical limitation of many MMPIs is their usage triggers a prevalent and dose-limiting side effect known as musculoskeletal syndrome (MSS) which is characterized by musculoskeletal pain, joint stiffness, inflammation, and tendonitis [20].

On the other hand, although most of MMPIs in clinical trials gave unsatisfactory results, many researchers attributed these unsatisfactory results to the inappropriate designing of clinical trials. This is because designing a clinical trial program for the development of effective MMPIs in cancer is rather complex. MMPs are especially important in the early stage of cancer progression, and thus strategies for future MMPI trials should be reconsidered. As most of synthesized MMPIs are not cytotoxic agents and, therefore, do not kill tumor cells as such, a traditional cytotoxic development strategy would not only be inappropriate and time-wasting, it would be potentially unfavorable for the success of this kind of agents. As a result, the critical examination of previous results has prompted serious re-evaluation of MMP-inhibition strategies focusing the attention of future research on the identification of specific MMP targets in tumors at different stages of tumor progression, both in order to improve efficacy and reduce the side effect profile [21, 22].

1.2. Strategies to Achieve the Selectivity of MMPIs

Clinical trials of MMPIs giving disappointing results have led to intensive discussions on how to conquer the drawbacks of broad-spectrum MMPIs, and gain new classes of inhibitors characterized by improved potency and, above all, high selectivity against the specific MMP subtypes involved in the targeted pathology in order to minimize side effects. Thereby, currently more efforts have been directed to the design of specific inhibitors towards certain MMPs for selective usage. To achieve this goal, Whittaker M *et al*. have offered us some useful clues [12]. They pointed out that an effective MMPI requires a zinc-binding group (ZBG, *e.g*. carboxylic acid, hydroxamic acid, sulfhydryl, mercapto, *etc*) as functional group capable of chelating the active site zinc(II) ion, at least one functional group which provides a hydrogen bond interaction with the enzyme backbone and, one or more substrate-like side chains which undergo effective van der Waals interactions with one of the subsites of the enzyme active sites, such as S_1' and S_2' pockets. Based on these clues, three possible strategies can be involved in the new compound design.

The first is the use of MMP structures as a basis for inhibitor design. In fact, the increasing availability of high-resolution X-ray crystal structures for many members of this protein family makes MMPs ideally suited for structure-based design approaches, which are now routinely used in this area. Most of the currently available 3D structures for some MMP family members are presented as the complexes of the catalytic MMP domains with various known peptidic, peptidomimetic or non-peptidic inhibitors. Specifically, the enzyme binding site consists of a primary hydrophobic S_1' pocket whose size and shape is obvious differences between various family members. Besides, there are shallow S_2'and S_3' pockets that are primarily solvent exposed. And else, the unprimed S subsites of the MMPs are also shallow and solvent exposed [23, 24]. These resolved structures impel the structure-based drug design and the improvement of high-affinity ligands, which might be elaborated into potential drugs [25, 26]. Besides, the availability of combinatorial chemistry and computational resources, as well as the QSAR studies by using COMFA, GRID and CoMSIA approaches has accelerated the inhibitors development. All these studies provide useful information in elucidating the mechanisms of chemical-biological interactions for certain MMPs.

The second is the design of subtype selective MMPIs by optimizing the interactions between the inhibitors and the enzyme to achieve improved anticancer therapy. By molecular dynamics and docking-type techniques, Terp GE *et al*. provided us some useful information about the structural differences of MMP family members and their interactions with MMPIs. They proposed that in order to gain the selectivity among the MMP enzymes, the S_1' subsite is the key domain to be considered where obvious differences between MMP family members are found. Because the S_1' pockets are surrounded by a loop, which is of different length and amino acid composition in the individual MMP subtypes, as displayed in Fig. (**1**), therefore, there is a possibility of exploiting the resulting differences in the structures for selectivity purposes [27].

The third strategy involves the usage of a suitable ZBG due to the fact that the mechanism by which MMPs cleave their substrates directly involves the catalytic zinc ion. This approach is, undoubtedly, still the most popular method to date to obtain potent MMPIs. Early typically identified ZBGs include hydroxamate, reversed hydroxamate, thiolate, phosphinate, carboxylate, phosphonate, this is followed by the derivatives of these ZBGs, such as hydrazide, sulfonylhydrazide, *N*-hydroxyurea, mercaptosulfide, etc, and the newly exploited nitrogen-based ZBGs, heterocyclic bidentate ZBGs [28]. Now, new trends to achieve selective MMPIs include the development of new allosteric non-zinc binding inhibitors, devoid of ZBGs [29].

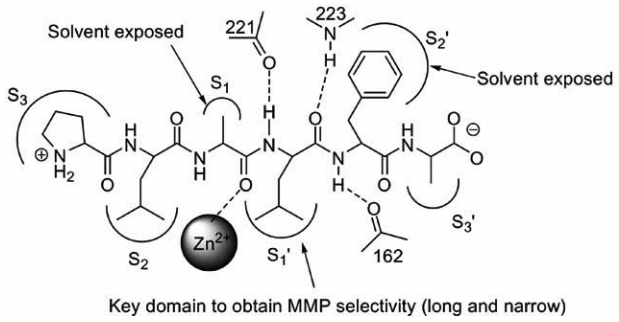


Fig. (1). Diagram of possible zinc-binding effect, the hydrogen bonding between a substrate and the MMP backbone, and the binding pockets.

1.3. APN and APNI

As a zinc-dependent type II membrane-bound endopeptidase, aminopeptidase N (APN; EC 3.4.11.2), also known as CD 13, has been named as microsomal aminopeptidase, aminopeptidase M, alaniney aminopeptidase, particle-bound aminopeptidase, p146, p161, or gp150. It is a 150-kDa myeloid cell surface glycoprotein belonging to the M1 family of the MA clan of peptidases, which preferentially releases neutral and basic amino acids from the *N*-terminal end of peptides [30]. APN/CD13 expresses on various cell surfaces, especially of hemopoietic system cells including granulocyte, fibroblast, and many endotheliocytes, as well as epithelial cells of kidney proximal convoluted tubule and intestina parva brush border. High expression of APN has been found in myeloid progenitors and monocytes, epithelial cells of the intestine and kidney, synaptic membranes in the central nervous system (CNS), fibroblasts, endothelial cells, placenta and also tumor cells. Therefore, APN/CD13 plays a variety of roles, including roles in inflammatory and immunological responses, signal transduction, antigen processing, neuropeptide and cytokine degradation [31, 32].

It is particularly noticeable that APN/CD13 plays a critical role in tumor progression by regulating complicated processes involving cell-cell contact, proliferation of tumor cells, the degradation of ECM, tumor invasion, metastasis and, angiogenesis [33]. Among all these processes, the degradation and neovasculation are of vital importance, meanwhile, the degradation of immunoactive peptides such as interleukin 8 (IL-8) leads to the impairment of body immunosystem, which is conversely in favor of the invasion of tumor cells. Additionally, tumor cells which over-express APN, *e.g*. melanoma cells, acute lymphocytic leukemic cells, as well as urological cancer cells, are highly motile and capable of migration through ECM [34, 35]. Moreover, angiogenesis emanating from microvascular endothelial cells plays a central role in tumor growth, invasion and metastasis [36]. In case the mRNA of the APN/CD13 was knocked out, the level of angiogenesis decreased dramatically [37]. On the other hand, APN inhibitors (such as CD13 monoclonal antibodies or bestatin) could significantly block induced-retinal neovascularization in mice and in chorioallantoic membrane angiogenesis *in vitro* [38, 39].

Given APN is over-expressed in various tumor cell surfaces as a protein hydrolyase and plays a pivotal role in the regulation in tumorgenesis process, it has been suggested as an attractive target for anticancer drug design. Accordingly, it is useful to develop potential APNIs to block its enzymatic activity for medical and therapeutic utilization [40].

1.4. Strategies to Design Potential APNIs

Similar with MMPIs, the principle approaches utilized for the design of small molecular weight synthetic APNIs is the substrate-based design method and most molecules designed are transition state analogues. This method relies on the understanding of the enzyme mechanisms and also the information from the x-ray crystallographic studies.

Recently, the 3D-structures of APN have been investigated by the x-ray crystallographic studies on the co-crystal complex of the enzyme and various inhibitors [41-43]. In general, peptidomimetics that incorporate zinc binding groups and interact with the enzyme subsites, S_1 and/or S_1' pocket, represent the most common structural features of effective inhibitors. Accordingly, the interactions of the APNI (bestatin) with the active sites of *Escherichia coli* APN can be illustrated by a simplified model, as shown in Fig. (**2**). The binding model has several characteristics: (i) Beside the catalytic center zinc (II) ion of APN, there are three hydrophobic binding domains, which are called S_1, S_1' and S_2' pocket, respectively. It should be emphasized that among these three subsites, at least two (S_1 and S_1') are necessary for a potent inhibitor, and one or more side chains should be introduced to insert into these pockets to generate effective interactions [44]. For bestatin, the phenyl ring enters into the S_1 pocket while isopropyl residue interacting with the S_1' pocket. (ii) The 2-hydroxyl group and 1-carbonyl group of amide act as ZBG to coordinate with the catalytic zinc ion and; (iii) the salt bond formed between the amino group of bestatin and Glu350 strengthens the binding interactions.

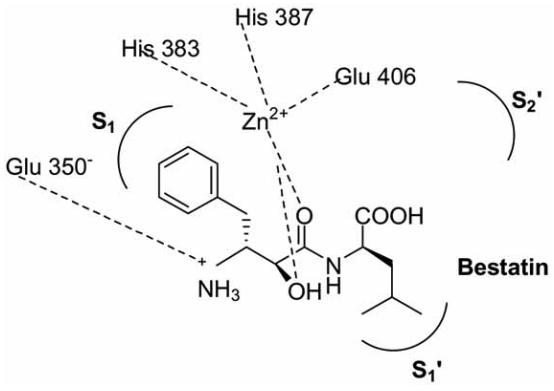


Fig. (2). Schematic representation of the interaction of bestatin with *Escherichia coli* APN.

These reported crystal models and studies give insight to structural requirements for designing more potent analogues as potential APNIs. Furthermore, since APN/CD13 is also a zinc-dependent metalloproteinase, the search for suitable ZBGs is another approach to achieve the potent APNIs.

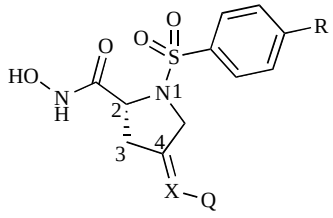
2. MMPIS AND APNIS BASED ON THE PYRROLIDINE PLATFORM

Pyrrolidine scaffold represents an important class of biologically active drug molecules, which has attractive attention of medicinal chemists and been widely used to design a variety of *N*-heterocyclic derivatives towards various targets. A variety of studies have been carried out on this useful heterocyclic system as a pharmacophore. This article offers an overview of the major development in research into MMPIs and APNIs, particularly introduces the pyrrolidine-based compounds.

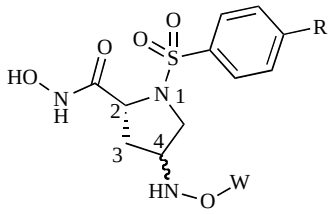
2.1. MMPIs Based on the Pyrrolidine Platform

Given sulfonamide functional group plays an important role in MMP inhibitory activities by forming effective hydrogen bonding as well as properly accommodation to the active binding pocket of the enzyme [45, 46], Cheng M *et al*. [47] synthesized a spectrum of sulfonamide pyrrolidine-based MMP inhibitors (**1& 2**) starting from *cis*-hydroxy-D-proline with the purpose of oxidizing the C-4 center to form an sp^2 center. As a result, SAR studies revealed a spectacular potency enhancement in these synthesized compounds. It is noticeable that compounds containing a functionalized oxime moiety or an exomethylene at C-4 provided satisfactory results, with <10 nM potencies towards MMP-3, and <100 nM potencies towards MMP-1. Additionally, substitutions on the sulfonamide portion of the molecules demonstrated a pronounced enhancement, which might be considered as the useful information for further modification to generate selectivity profiles. Moreover, these compounds were used in the construction of some useful 3D-QSAR models to guide novel inhibitor design [48].

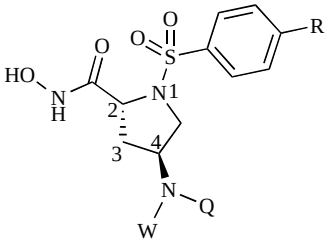
Inspired by the above 3D-QSAR models, Natchus MG *et al*. [49] prepared a range of aminopyrrolidine-based hydrox-



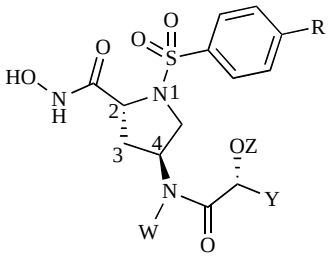
1: R=OMe, OEt, O^{n}Bu, O^{n}Pr, OPh, O$(CH_2)_2$OMe, OC_6H_4F, etc.
X=N, CH, Q= OH, OMe, OEt, O^{t}Bu,etc.



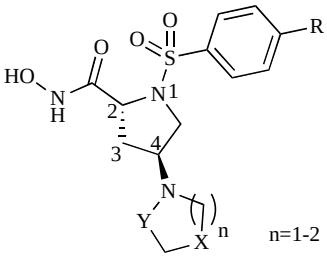
2: R=OMe, O^{n}Bu, OPh, O$(CH_2)_2$OMe.
W=CH_3, ^{t}Bu



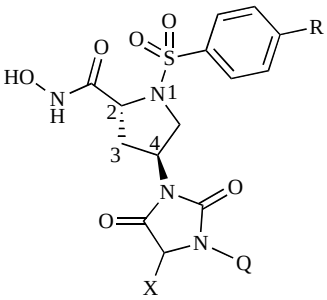
3: R=OMe, O^{n}Bu, O^{n}Pr, O-4-Pyr.
W=H, Me, $(CH_2)_2CH_3$, CH_2-3-Pyr
Q= H, $(CH_2)_2CH_3$, $(CH_2)_2Ph$, SO_2CH_3, etc.



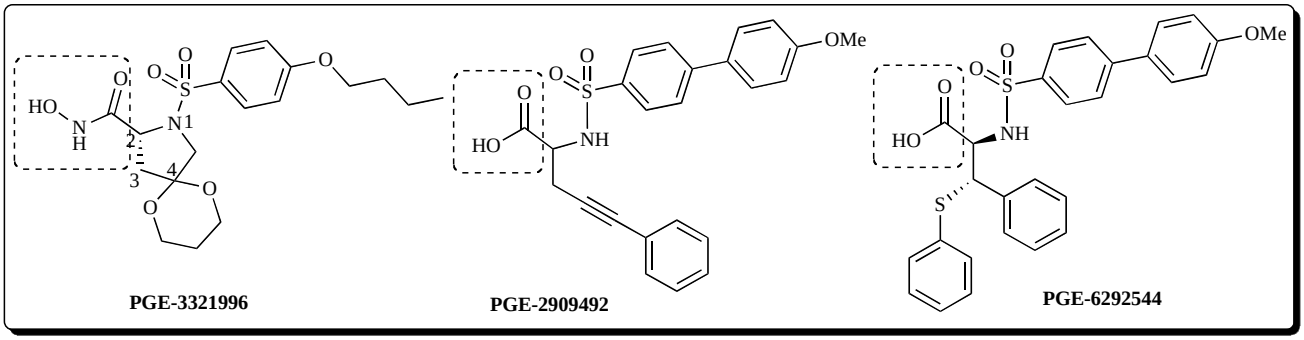
4: R=OMe, O^{n}Bu, O^{n}Pr.
W=H, ^{n}Pr; Z=H, CH_2Ph
Y=CH_3, CH_2Ph, $(CH_2)_2Ph$, etc



5: R=OMe, O^{n}Bu, O^{n}Pr, OPh, etc
X=O, CH_2, SO_2.
Y=CH_2, SO_2.
5a: n=2, X=Y=CH_2, R=OPh,
IC_{50}(MMP-1)=17 nM, IC_{50}(MMP-3)=8 nM, IC_{50}(MMP-13)<0.4nM



6: R=OMe, OEt, O^{n}Bu, O^{n}Pr, OPh,
O-4-Pyr, O$(CH_2)_2OCH_3$, etc
X=H, SCH_3, $(CH_3)_2$.
Q=CH_3, H, $CH_2CH{=}CH_2$, $(CH_2)CH_3$.

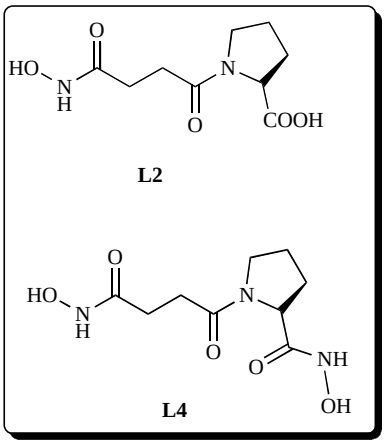


amate MMP inhibitors (**3~6**) derived from functionalized 4-aminoprolines, successively they were submitted to assay the inhibitory activities against five MMPs in order to obtain specific MMP inhibitors with minimal side effects. Spectacularly, compound **5a** exhibited broad-spectrum MMPs inhibitory activity with sub-nanomolar potency for certain MMPs, such as MMP-1 (IC_{50} = 17 nM), MMP-3 (IC_{50} = 8 nM), MMP-13 (IC_{50} < 0.4 nM).

Similarly, these pyrrolidine-based hydroxamate compounds (**3~6**) were also carried out the QSAR studies by Gupta SP *et al*. [50] with the anticipation of correlating their inhibition potencies. It revealed that the correlations obtained for the inhibitions of all the enzymes studies, *i.e*. MMP-1, MMP-2, MMP-3, MMP-7, and MMP-13 as well, were not so uniform. Generally speaking, in most cases, the substituents at the amide nitrogen may be conductive to the activity, though the whole amide group may be sterically unfavorable. Furthermore, the R-substituents at the phenyl moiety have been found to have a beneficial effect. Additionally, such advantageous effect can also be observed coupled by the involvement of the electronic sulfonyl group (SO_2) through its sulfur atom or both oxygen atoms in some statical interactions with the enzymes in most of cases.

Since MMPs are zinc-containing endopeptidases, hydroxamate or carboxylate functionality as effective ZBGs are commonly used in many potential MMPIs. To search for effective MMPIs for the treatment of osteoarthritis, Janusz MJ *et al*. [51] compared a hydroxamic acid pyrrolidine-based MMPI (**PGE-3321996**) and two carboxylate-based MMPIs (**PGE-2909492** & **PGE-6292544**) *via* investigation their corresponding potencies against various MMPs, pharmacodynamic properties and *in vivo* efficacy in a model of cartilage degeneration. The study unveiled the pyrrolidine-based **PGE-3321996** was efficacious in this model with high plasma protein binding (79%), even though it displayed inferior pharmacokinetic property in comparison with the other two compounds. Accordingly, **PGE-3321996** might be considered to be chemically modified for further development of specific MMP-13 inhibitors for the treatment of osteoarthritis.

In comparison with the carboxylate functionality, hydroxamate group has higher affinity with the metalloenzymes like MMPs and its inhibitory mechanism has been well documented. In 2003, Santos MA *et al*. prepared a series of Pro- and Phe-succinyl hydroxamate derivatives, *e.g*. pyrrolidine-based compounds **L2** & **L4**, which were tested as effective MMP inhibitors with nanomolar protease inhibitory activity. Interestingly, both the mono-hydroxamate and di-hydroxamate compounds presented very similar inhibitory properties against an array of MMPs, implying that only one hydroxamate group can interact with the catalytic metal ions [52]. To examine the role of the second hydroxamate group in the mechanism involved in the inhibitory process, namely in the metal coordination modes at the pH physiological conditions, as well as the effect of having a different peptide residue in the metal-complexation effectiveness, a systematic study of the complexation behavior of these succinylhydroxamate derivatives with Cu^{2+}, Ni^{2+} and Zn^{2+} was investigated by Tegoni M *et al*. [53]. The results manifested that the complexation of the mono-hydroxamate derivatives with Ni^{2+} and Zn^{2+} is very similar to that of simple mono-hydroxamic acids. Moreover, for all three metal ions, formation of 1:2 (M:L) species have been found. It is noteworthy that both types of ligands present some similar behavior at physiological pH, namely in terms of zinc affinity and zinc coordination mode, thus supporting the fact that no significant differences emerged from the inhibitory activity of both the groups of compounds against the studied zinc-containing MMPs.



Recently, the number of high-resolution X-ray crystal structure of MMP-inhibitor complexes has dramatically increased [25]. It has been reported that most MMPIs shared two dominant structure features: (1) a effective ZBG, such as hydroxamate and carboxylate, *etc*, that capable of chelating with the catalytic zinc ion; (2) hydrophobic extensions which undergo configurationally changes to protrude into active binding domains of the specific enzymes, for which purpose detailed understanding of the binding domains is indispensa-

ble [54]. Moreover, in different MMP subtypes, besides the catalytic activity center zinc(II) ion, there are two hydrophobic domains, which are called S_1' pocket and S_2 pocket, respectively [55]. Amongst these two pockets, the structural differences between MMP family members mainly occur in the S_1' subsite, which has been considered for the development of small-molecule drugs for selectivity purposes [56, 57].

As observed from X-ray crystal structures, different MMP subtypes present different characteristics for the S_1' pocket, in specific, the S_1' pocket is long and narrow for MMP-2, MMP-3, MMP-8; and short and narrow for MMP-1, MMP-9 [58-60]; while deeper for MMP-12, MMP-13 [61, 62]. Alternatively, one of effective therapeutic strategy to combat the dysregulation of MMPs is the exploitation of drugs to well accommodate specific binding domains.

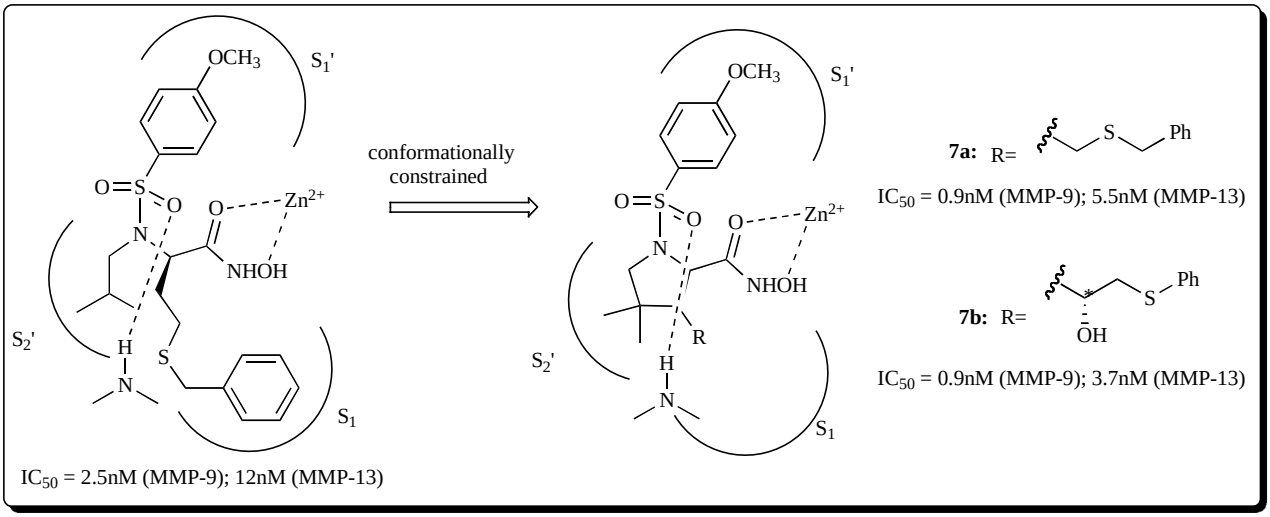
In an effort to discover novel MMPIs with high selectivity toward specific MMP subtypes, Hanessian S *et al.* [63] exploited a spectrum of conformationally constrained pyrrolidine analogues (**7**) starting from commercially available D-pyroglutamic acid, with the purpose of probing the binding interactions with the active domains of MMPs. This structural modification has been found to exhibit a predictable pattern of potencies. Although enzymatic assay gave modest selectivity toward various MMP subclasses, all of these pyrrolidine analogues gave nanomolar activities, suggesting the importance of binding to the active domains for both activity and selectivity. It is noticeable that **7a** & **7b** display enhanced MMP-9 & MMP-13 inhibitory potencies in comparison with the unconstrained acyclic precursor, in which all of the targeted compounds (**7**) and its precursor deployed similar binding modes with the enzymes. This information provided us useful clues for further design of improved compounds with higher affinity.

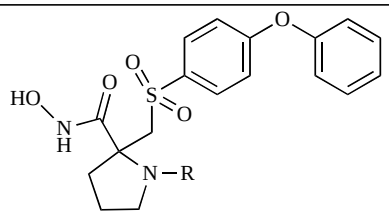
In 2006, Later Fernandez M *et al.* [64] successfully developed linear and nonlinear QSAR models grounded in the above mentioned compounds by using 2D autocorrelation descriptors, providing useful molecular information with respect to the ligand specificity for MMP active binding sites. Specifically speaking, the anchoring pattern of compounds in MMP active sites is guided by spatial and hydrophobic-related effects. Moreover, the electronic effects have a poor contribution, but enhance their importance in MMP-1 and MMP-3 inhibition.

Considering that the clinically observed musculoskeletal side effect of broad-spectrum inhibitor marimastat is mainly due to its inhibition of MMP-1, Becker DP *et al*. developed an array of praline-derived α-alkyl-α-amino-β-sulphone hydroxamates, with the anticipation of showing potency for selective inhibition of MMP-2 and MMP-13, not the MMP-1 [65]. It resulted that low nanomolar potency was acquired with selectivity of MMP-2 and MMP-13 versus MMP-1. Since the racemic hydroxamate (**19**) displayed excellent MMP-13 inhibition with relatively potent selectivity towards MMP-1 (MMP-1/13=308), it was further separated into two enantiomers *via* chiral chromatography method. It is interesting that the eutomer (**8a**) was found to be more potent with improved MMP-13 & MMP-2 inhibition (<0.1 nM), as well as higher selectivity in sparing MMP-1 (>3000), while the distomer (**8b**) gave impaired potency both on the enzymatic inhibitions and the selectivity.

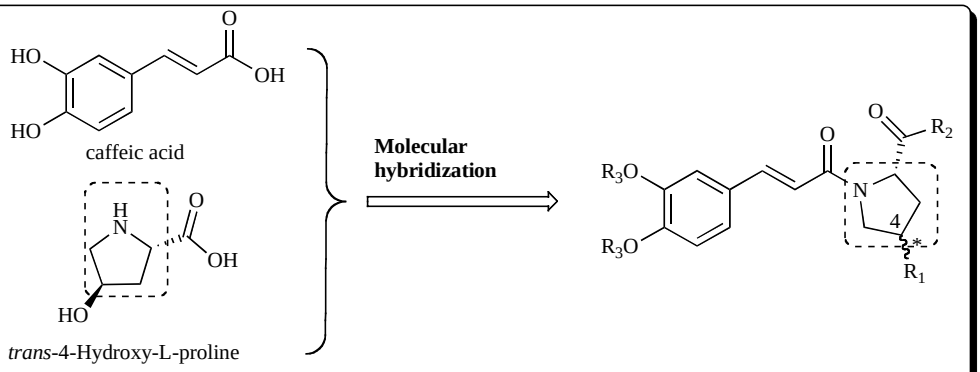
In our laboratory, great efforts have been devoted on rational drug design based on the pyrrolidine scaffold for many years. In the efforts to dig out effective MMPIs, we noted that among the mammal MMP structurally related members, MMP-2 (gelatinase) is found to have high correlation with malignant tumors [66]. As a consequence, we mainly concentrated on the investigation of effective and specific MMP-2 inhibitors [67].

It has been proved that collagens are the main substrates of gelatinases (MMP-2 and MMP-9), while *trans*-4-hydroxy-L-proline is an essential component of collagens. *Trans*-4-hydroxy-L-proline is thereof used as a basic scaffold to design MMP-2 inhibitors, which might specifically interact with the enzyme in a competitive way [25]. Additionally, caffeic acid is proved to inhibit gelatinases [68]. According to the principle of hybridization in medicinal chemistry, Li YL *et al.* [69] reported a range of caffeoyl pyrrolidine derivatives (**9**) *via* combination of pyrrolidine scaffold with caffeoyl fragment, which has been proved to demonstrate





8 (racemic): R=propargyl, IC_{50}(MMP-13)=1.3nM; IC_{50}(MMP-2)=0.2nM; IC_{50}(MMP-1)=400nM; MMP-1/13=308
8a (eutomer): R=propargyl, IC_{50}(MMP-13)=<0.1nM; IC_{50}(MMP-2)=<0.1nM; IC_{50}(MMP-1)=300nM; MMP-1/13>3000
8b (distomer): R=propargyl, IC_{50}(MMP-13)=60nM; IC_{50}(MMP-2)=19.3nM; IC_{50}(MMP-1)=>10,000nM; MMP-1/13>167



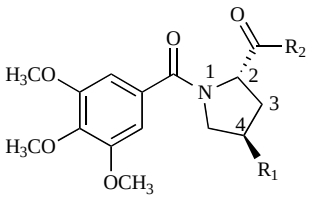
9: R_1=OH, OSO_2CH_3, NH_2, N_3, $NHCOC_2H_5$,$NHCOCH_2C_6H_5$, etc; R_2=MeO, NHOH; R_3=Me, Ac, H. IC_{50} = 6.7-731.4 nM
9a: R_1=$NHCOCH=CHC_6H_3$(OMe)-4'(*E*); R_2=NHOH; R_3=Me IC_{50} = 6.7nM; inhibitory rate 35.05%)
9b: R_1=$NHCOCH_2C_6H_5$; R_2=NHOH; R_3=Me (IC_{50} = 11.5 nM; inhibitory rate 69.25%)
9c(LY52): R_1=$NHCOC_5H_4$N-3'; R_2=NHOH; R_3=Me (IC_{50} = 9.0 nM; inhibitory rate 55.70%)

outstanding MMP-2 inhibitory activity [70, 71]. Enzymatic assay revealed that the targets compounds displayed inhibitory activities with IC_{50} value from 6.7 to 731.4 nM. Further SAR studies revealed longer and more flexible side chain linked to the pyrrolidine ring at C4 position caused higher activity. What's more, aromatic heterocycle and sulfamide in the same position produced enhanced potencies. In addition, compounds with free phenol hydroxyl group presented higher activity in comparison with the methylated counterparts, suggesting the importance of phenol hydroxyl functionality in the interaction with the enzyme *via* the possible hydrogen bonding interaction. *In vivo* anti-metastasis evaluation showed that most of the tested compounds manifested remarkable antitumor activities (inhibitory rate > 35%) without any obvious toxic effects. Although compound **9a** gave the highest enzymatic inhibition, it showed impaired *in vivo* activity in comparison with other counterparts. Nevertheless, compound **9b** & **9c** (**LY52**) gave relatively coincidental *in vitro* and *in vivo* activities, which might be promising leads.

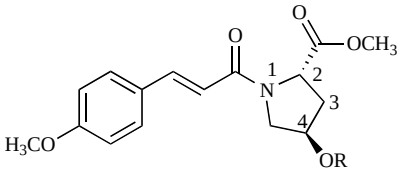
Further pharmacological studies on these two leads discovered that **LY52** (**9c**) could significantly block the proteolytic activity of MMP-2 and suppress human ovarian carcinoma cell line SKOV3 invasion *in vitro*. Furthermore, a dramatically inhibition of pulmonary metastasis of Lewis lung carcinoma cells was observed in LY52-administrated mice [72-74]. Therefore, we can deduce that LY52 might suppress invasion and metastasis of carcinoma cells *via* inhibition of MMP-2 proteolytic activities, making it worthy to carry out further research.

In our ongoing efforts to identify more potent MMP-2 inhibitors, a variety of pyrrolidine-based LY52 analogues were successively reported hereby. Most of them are designed according to the "combination principles". Consequently, many active fragments with potential pharmaceutical values, such as gallic acid [75] and cinnamic acid [76], are incorporated into *trans*-4-hydroxy-L-proline backbone to form new integrated structural patterns (**10-13**). Some of the resulting compounds, *e.g.* **10a-13a**, exhibited comparable or more potent MMP-2 inhibitory activities (0.9-9.7 nM) than the positive control LY52 with IC_{50} value of 9.0 nM.

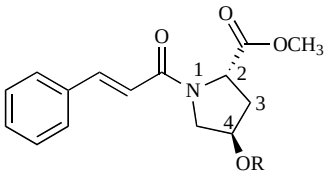
More exciting findings were observed from series of sulfonamide hydroxamates (**14-16**). Given the structural feature of reported MMP inhibitors CGS27023A and prinomastat, Cheng XC *et al.* [77-80] incorporated the sulfonyl group and proper hydrophobic substituents into *trans*-4-hydroxy-L-proline backbone in order to obtain more effective inhibitors through improving the enzyme-inhibitor binding interactions. The optimization of pyrrolidine scaffold was mainly concentrated on the N1, C2 and C4 orientations. As expected, the resulting pyrrolidine derivatives (**14-16**) exhibited strong inhibition against MMP-2, confirming the strategies to design potential MMP-2 inhibitors based on pyrrolidine scaffold. Further *in vitro* assay of these compounds



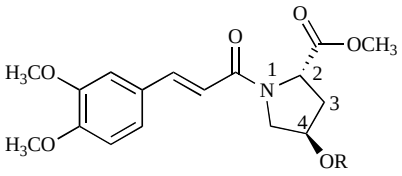
10: R_1=OH, OSO_2CH_3, NH_2, N_3, $NHCOC_2H_5$,$NHSO_2CH_3$, etc; R_2=MeO, NHOH; IC_{50} (MMP-2)= 0.9-1000 nM
10a: R_1=$NHCOCH=CHC_6H_3(OMe)_2$-3',4'; R_2=NHOH; IC_{50}(MMP-2)=0.9nM; inhibitory rate 57.42%



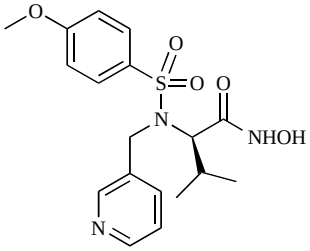
12: R=H, $COCH_3$, COC_6H_5, etc; IC_{50}(MMP-2)= 7.8-439.8 nM
12a: $R=COCH=CHC_6H_4$-OCH_3-*p*; IC_{50}(MMP-2)= 7.8 nM



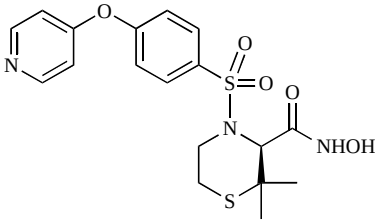
11: R=H, $COCH_3$, COC_6H_5, etc; IC_{50}(MMP-2)= 5.2-259.5 nM
11a: $R=COCH=CHC_6H_5$; IC_{50} (MMP-2)= 5.2 nM



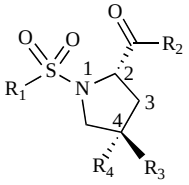
13: R=H, $COCH_3$, COC_6H_5, etc; IC_{50} (MMP-2)= 9.7-320.2 nM
13a: $R=COCH=CHC_6H_3(OCH_3)_2$-3',4';IC_{50} (MMP-2)= 9.7 nM



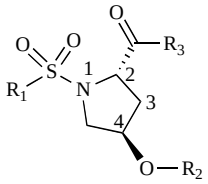
CGS-27023A



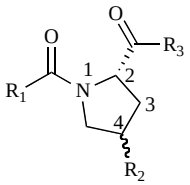
Prinomastat



14: R_1=CH_3, C_6H_5, *p*-$CH_3C_6H_4$; R_2=OCH_3, NHOH; R_3=OH, C=O, O-$(CH_2)_n$-O (n=2,3,4); R_4=H, IC_{50} (MMP-2)=3-9500 nM
14a: R_1=*p*-$CH_3C_6H_4$; R_2=NHOH; R_3=O-$(CH_2)_2$-O; IC_{50} (MMP-2)=3 nM
14b: R_1=*p*-$CH_3C_6H_4$; R_2=NHOH; R_3=O-$(CH_2)_3$-O; IC_{50} (MMP-2)=6 nM
14c: R_1=*p*-$CH_3C_6H_4$; R_2=NHOH; R_3=O-$(CH_2)_4$-O; IC_{50} (MMP-2)=8 nM
14d: R_1=C_6H_5; R_2=NHOH; R_3=O-$(CH_2)_2$-O; IC_{50} (MMP-2)=8 nM



15: R_1=CH_3, C_6H_5, *p*-$CH_3C_6H_4$; R_2=H, C_6H_5CO, $C_6H_5SO_2$, CH_3SO_2, etc; R_3=NHOH, OCH_3, IC_{50} (MMP-2)=2-8900 nM
15a: R_1=*p*-$CH_3C_6H_4$; R_2=H; R_3=NHOH; IC_{50} (MMP-2)=2 nM
15b: R_1=C_6H_5; R_2=H; R_3=NHOH; IC_{50} (MMP-2)=4 nM
15c: R_1=*p*-$CH_3C_6H_4$; R_2=*p*-$CH_3C_6H_4SO_2$; R_3=OCH_3; IC_{50} (MMP-2)=7 nM



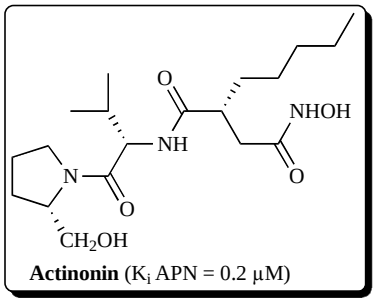
16: R_1=CH_3, $CH=CHC_6H_3(OCH_3)_2$-3',4'(*E*), etc; R_2=OH, $NHCOC_6H_5$, $NHCOC_5H_4$N-3', etc; R_3=NHOH, OCH_3, OH, etc, IC_{50} (MMP-2)=1-6100 nM
16a (*S*): R_1=CH_3; R_2=$NHCOC_6H_2(OCH_3)_3$-3',4',5'; R_3=NHOH; IC_{50} (MMP-2)=1 nM
16b (*S*): R_1=CH_3; R_2=NHCOCH=CHC_6H_5(*E*); R_3=NHOH; IC_{50} (MMP-2)=4 nM
16c (*S*): R_1=CH_3; R_2=$NHCOC_5H_4$N-3'; R_3=NHOH; IC_{50} (MMP-2)=5 nM

on various cell cultures and animal models are underway and will be reported in the near future.

2.2. APNIs Based on the Pyrrolidine Platform

Some of the most intriguing APNIs found to date are natural products. According to the provided binding mode between bestatin and APN, a number of natural products with remarkable APN inhibitory activities have been isolated from bacterial cultures.

Actinonin, a well-known natural inhibitor of APN and peptide deformylase, is previously isolated as pseudopeptide antibiotic that inhibits collagenase at micromolar concentration and its inhibition towards APN was found to be competitive with the substrate [81]. In addition, it was also a strong inhibitor for some MMPs, with a K_i of 300 nM for its adduct with MMP-1 [82].



Actinonin (K_i APN = 0.2 μM)

Probestin (produced by *Streptomyces azureus* MH663-2F6) [83], MR387A and MR387B (produced by *Streptomyces neyagawaensis* SL-387) [84] are modified or reformed compounds based on the bastatin. It revealed that the introduced pyrrolidine carboxylic acid portion enters S_2' pocket, suggesting the interaction with S_2' pocket may make significant sense to the enhancement of enzymatic inhibition.

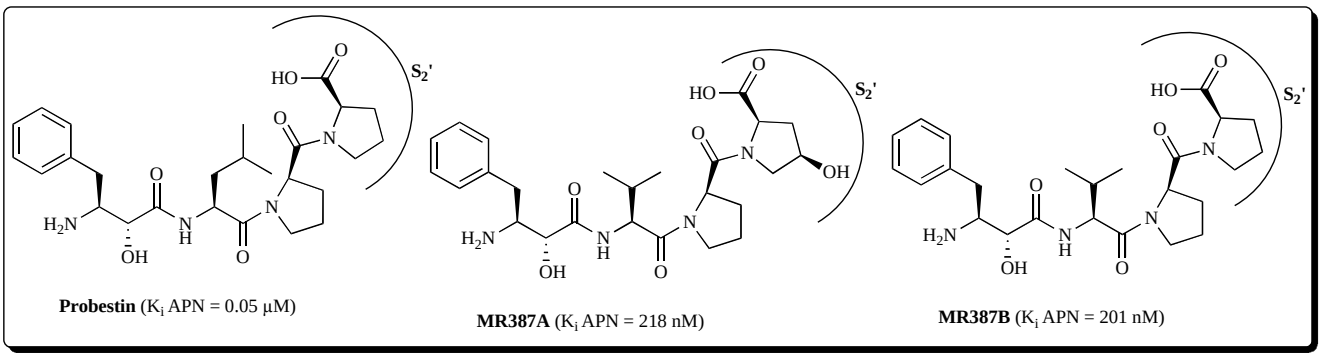
Since both MMPs and APN/CD13 are zinc-dependent metalloproteinase involved in the process of tumor invasion and metastasis, in our lab, the synthesized pyrrolidine-based derivatives mentioned above (**9-16**) were, therefore, performed on both of MMPs and APN so as to identify the selectivity of target compounds. The results demonstrated that among these evaluated compounds, **10b**-**10d** gave the most potent APN/CD13 inhibitory activities.

The methylated gallic acid can interact with the S_1 pocket, while the substituents at C4 position insert into the S_1' domain, and the carbonyl group at C2 position acts as ZBG to coordinate with the catalytic zinc ion of the enzyme.

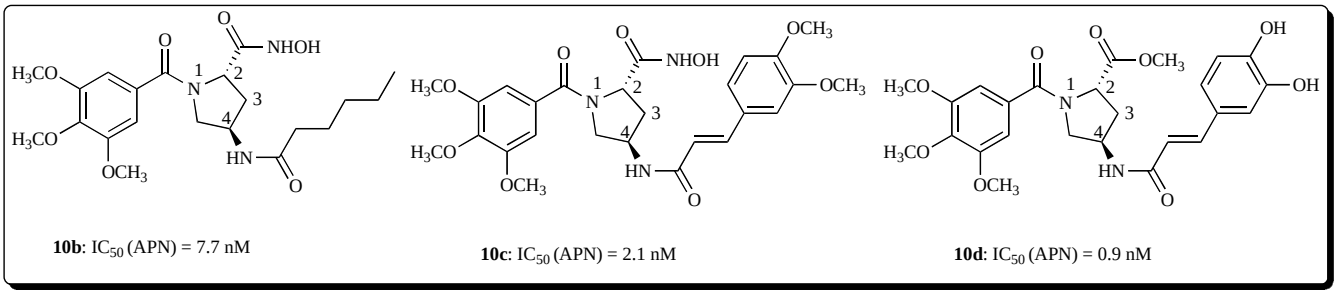
3. CONCLUSION AND PERSPECTIVE

We reviewed the recent progress in the pyrrolidine-based derivatives targeting two kinds of zinc-dependent metalloproteinase, MMPs and APN/CD13, as well as the strategies to design potent MMPIs and APNIs. It is especially noticeable that inhibitors targeting to this class of enzymes are likely to be cytostatic rather than cytotoxic so there is expectation that they may be better tolerated than current cytotoxics and more potent than conventional clinical drugs [85]. Consequently, the development of both MMPIs and APNIs has become an area of intense interest in both academic and pharmaceutical industry in recent years.

From the description of current status of MMPIs, we can conclude that although a number of MMPIs have progresses into preclinical or clinical trials for cancer, rheumatoid arthritis, and osteoarthritis, the vast majority of them are broad-spectrum compounds with obvious side effects (such as MSS etc). So far, progression of these broad-spectrum MMPIs through these trials has to hamper. Accordingly, current focus in the MMPIs field is directed toward the achievement of selective inhibition of MMPs involved in a given pathology.



Probestin (K_i APN = 0.05 μM) **MR387A** (K_i APN = 218 nM) **MR387B** (K_i APN = 201 nM)



10b: IC_{50} (APN) = 7.7 nM **10c**: IC_{50} (APN) = 2.1 nM **10d**: IC_{50} (APN) = 0.9 nM

As to the APNIs, it has proved that potential APNIs can offer effective anticancer therapy by limiting angiogenesis without direct killing of tumor cells and enhancing the immunological responses of lymphocytes generates enormous excitement among medicinal researchers. In addition, APNIs may have therapeutic potential towards other unmet medical needs such as diabetic nephropathy, rheumatoid arthritis and viral infections [86, 87]. However, like MMPIs, despite a number of new families of natural or synthetic APNIs have been developed, few compounds have reached advanced clinical trials, implying the development of APNIs is also a challenging goal. Though we can use the computer aided drug design to mimic the three-dimensional structure of human APN, there is no information about NMR spectroscopy and real x-ray crystallography structure of human APN. Moreover, current strategy to design APNIs is focused on the substrate-based design and the available SAR studies only revolved around the proposed amino acids located in the active site of the target proteins. Hence, there is still a lot of work to be conducted in the future which includes the determination of the crystal structure of human APN, as well as the elucidation of the active site structure.

Our group and others have been exploiting pyrrolidine scaffold-based MMPIs and ANPIs for a number of years. This scaffold has some advantages, for example, it can be readily prepared from commercially available materials and synthetically available in optically pure form, which has been widely used to design a variety of *N*-heterocyclic derivatives towards various targets.

At present, a rational drug design (RDD) has been involved in the development of both MMPIs and APNIs. Additionally, the crystallographic studies, molecular modeling and computational analysis, as well as the in-depth understanding of the enzyme mechanism may provide opportunities and encouragement for future studies of potential MMPIs and APNIs.

ACKNOWLEDGEMENT

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ABBREVIATIONS

MMPs = Matrix metalloproteins

APN = Aminopeptidase N

MMPIs = MMP inhibitors

APNIs = APN inhibitors

ECM = Extracellular matrix

TIMPs = Tissue inhibitors of metalloproteinases

NSCLC = Non-small cell lung caner

ZBG = Zinc-binding group

CNS = Central nervous system

IL-8 = Interleukin 8

MSS = Musculoskeletal syndrome

RDD = Rational drug design

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