Determinants of the inhibition of a Taiwan habu venom metalloproteinase by its endogenous inhibitors revealed by X-ray crystallography and synthetic inhibitor analogues

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Venoms from crotalid and viperid snakes contain several peptide inhibitors which regulate the proteolytic activities of their snake-venom metalloproteinases (SVMPs) in a reversible manner under physiological conditions. In this report, we describe the high-resolution crystal structures of a SVMP, TM-3, from Taiwan habu (Trimeresurus muro-squamatus) cocRYstallized with the endogenous inhibitors pyroGlu-Asn-Trp (pENW), pyroGlu-Gln-Trp (pEQW) or pyroGlu-Lys-Trp (pEKW). The binding of inhibitors causes some of the residues around the inhibitor-binding environment of TM-3 to slightly move away from the active-site center, and displaces two metal-coordinated water molecules by the C-terminal carboxylic group of the inhibitors. This binding adopts a retro-manner principally stabilized by four possible hydrogen bonds. The Trp indole ring of the inhibitors is stacked against the imidazole of His143 in the S⁻¹ site of the proteinase. Results from the study of synthetic inhibitor analogues showed the primary specificity of Trp residue of the inhibitors at the P⁻¹ site, corroborating the stacking effect observed in our structures. Furthermore, we have made a detailed comparison of our structures with the binding modes of other inhibitors including batimastat, a hydroxamate inhibitor, and a barbiturate derivative. It suggests a close correlation between the inhibitory activity of an inhibitor and its ability to fill the S⁻¹ pocket of the proteinase. Our work may provide insights into the rational design of small molecules that bind to this class of zinc-metalloproteinases.

Keywords: snake-venom metalloproteinase; Trimeresurus muro-squamatus; endogenous tripeptide inhibitor; TNFα converting enzyme; retro-binding mode.

Venoms secreted from the glands of crotalid and viperid snakes are able to elicit shock, intravascular clotting, systemic and local hemorrhage, edema and necrosis upon victimized preys following snakebites [1]. The major complication arising from snake envenomation is hemorrhagic effects, which are generally thought to result from the structural destruction of capillary basement membranes via proteolytic degradation by snake-venom metalloproteinases (SVMPs) [2,3]. In order to avoid auto-digestion of the venom gland itself from its secreted metalloproteinases after in vivo generation of venom proteases, several strategies are presumably employed by snakes to regulate the proteolytic activities of SVMPs in their venom secretions. These include the following. (a) SVMPs in crude venoms might exist originally as a large multidomain precursor, in which the central zinc-metalloproteinase domain is flanked by an N-terminal propeptide and a C-terminal disintegrin-like domain [4]. A cysteine residue in a conserved PKMCGV region of the propeptide is believed to bind to the catalytic zinc ion in the inactive proenzyme, prior to activation by a cysteine-switch mechanism [5]. (b) Venom secretions contain several endogenous small peptides, e.g. pyroGlu-Asn-Trp and pyroGlu-Gln-Trp [6]. They could selectively bind to SVMPs, thereby partially inhibiting their proteolytic activities [7,8]. (c) A variety of crude snake venoms have been reported to have citrate at high concentration, in the range 30–150 mM, which is thought to play a role of chelating the active-site zinc ion of SVMPs, thus keeping their activities low [9]. Interestingly, many proteinase inhibitors (commonly called hemorrhagin neutralizing factors) were purified from the blood sera of some mammals and snakes, e.g. oprim from Didelphis virginiana [10], DM43 from Didelphis marsupialis [11], HSF from Trimeresurus flavoviridis [12], BJ46a from Bothrops jararaca [13], and TMI from Trimeresurus muro-squamatus [14]. These plasma inhibitors could act by noncovalently binding to SVMPs, and thus, neutralizing their hemorrhagic activities, and endowing these animals with resistance to envenomation by crotalid and viperid snakebites.

Together with the matrixins (vertebrate collagenases, or denoted as matrix metalloproteinases, MMPs), serralysins (large bacterial zinc-endopeptidases) and astacins, SVMPs
are grouped in a superfamily of metzincin which exhibits some typical structural features, such as the Met-turn and active-site consensus HExxHxGxxH sequence [15–17]. Some organisms and mammalian tissues recently have been reported to contain a number of multidomain proteins, which are related to the fertilization, neurogenesis and inflammation processes [18–20]. They are generally called ADAMs (a disintegrin-like and metalloproteinase domain) with the same central catalytic domain as SVMPs and MMPs, especially at the active-site structure [21,22]. A well known example is the TACE, also known as ADAM 17, responsible for the release of a major proinflammatory cytokine, tumor necrosis factor-α (TNFα), from its membrane-anchored precursor into extracellular space [23,24]. The crystal structure of the catalytic domain in TACE was reported and revealed a characteristic polypeptide fold containing a catalytic zinc environment resembling that of the SVMP family [22]. Moreover, two SVMPs isolated from the venoms of Bothrops jararaca and Echis carinatus laekeyi, respectively, were shown to be able to release the active TNFα at the envenoming site [25], corroborating the structural similarities between SVMPs and TACE as mentioned above. Before the TACE structure was solved, adamalysin II had been considered to be a good starting model in SVMP family for the rational design of drugs against TACE-involved inflammatory diseases. Based on the crystal structure of adamalysin II and modeled on an endogenous venom tripeptide, several peptidic inhibitors were synthesized, such as Furroyl-Leu-Trp (pol647) and its cyclic and phosphonate derivatives [26–28].

In our laboratory, the crystal structure of a snake-venom metalloproteinase TM-3 from Trimeresurus macropsocausmatus was solved and refined to 1.35 Å resolution [29]. It is more similar to TACE than adamalysin II in terms of the disulfide configurations and the S1-pocket dimension. Currently, some macrocyclic and succinate-based hydroxamic acids have been reported to directly block the release of TNFα in vitro and in vivo by inhibiting the activity of TACE [30,31]. However, most designs for inhibitors were of the type that mimicks the structural features of substrate binding described for MMPs, or through the screening of libraries of MMP inhibitors in-house [32–34]. Investigations of the SVMP structures along with the retro-binding characteristics of their endogenous peptide inhibitors would offer an alternative for the rational design of inhibitors against TACE.

Previously, we had purified three endogenous tripeptide inhibitors from the venoms of Taiwan habu (Trimeresurus macropsocausmatus), including a newly identified tripeptide, pyroGlu-Lys-Trp [35]. In this report, we describe the crystal structures of TM-3 complexed with the inhibitors pENW, pEQW and pEKW. Based on these high-resolution crystal structures, we have also made a detailed comparison of the binding affinity and inhibitory activity of more than 10 chemically synthesized inhibitor analogues for TM-3.

**MATERIALS AND METHODS**

**Materials**

4-(2,4'-Dimethoxyphenyl-Fmoc-amino methyl)phenoxyl-resins and Fmoc-amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland). The substrate FITC (fluorescein isothiocyanate)-labeled casein (FITC-casein, 38 μg FITC per mg protein) was procured from Sigma (St Louis, MO, USA). The membranes (Centricon, YM-10) for ultrafiltration and concentration was obtained from Millipore (Amicon bioseparation, Bedford, MA, USA).

**Preparation of inhibitor analogues and proteinase inhibition assays**

Inhibitor analogues were synthesized using 4-(2,4'-dimethoxyphenyl-Fmoc-amino methyl)phenoxyl-resins and Fmoc-amino acid derivatives by an automatic peptide synthesizer (Applied Biosystems, Foster City, CA, USA). At the end of synthesis cycles, peptides on the resin were cleaved off by a solvent mixture of trifluoroacetic acid and ethanediol, and solvent was evaporated to dryness. The resins were then washed with cold ether and the peptides were extracted with 5% acetic acid. Combined solutions were lyophilized to offer an alternative for the rational design of drugs against TACE-involved inflammatory diseases.

**Crystallization of TM-3**

TM-3 was isolated from the venom of Taiwan habu (Trimeresurus macropsocausmatus) and purified to high homogeneity as described previously [37]. Crystals were obtained using the crystallization screening kits of Hampton Research (Laguna Niguel, CA, USA). Finally, 4 μL mother liquid [0.1 M CdCl2, 0.1 M sodium acetate and 30% (v/v) poly(ethylene glycol) 400 at pH 4.6] was mixed with 3.5 μL TM-3 (10.5 mg/mL) and 0.5 μL of the synthetic inhibitor, followed by cocryocrystallization at 4 °C using hanging-drop vapor diffusion method. Crystals started to appear with their dimensions reaching 0.6 × 0.8 × 1.6 mm within 1 week. The concentration of inhibitors used are: pENW, 114.3 μM; pEQW, 107.1 μM; pEKW, 101.6 μM.

**Data collection, processing and structure refinement**

Data for the pENW-bound and pEKW-bound TM-3 crystals were collected on beamline 17B2 of the Synchrotron Radiation Research Center in Hsinchu, Taiwan, whereas that of pEQW-bound form was obtained from the Spring-8 on beamline 38B1, Hyogo, Japan. All data collections were accomplished at −150 °C (see Table 1). Data were processed and scaled by employing the programs DENZO and SCALEPACK, respectively, or directly using the program HKL2000 [38]. The difference Fourier maps were phased with the refined structure of unbound TM-3 [29]. Manual rebuilding and computational refinement were performed by employing the program o [39] and CNS [40] running on an SGI Octane or O2 workstations. The parameters for ideal protein geometry from Engh & Huber [41] were used for the refinements, and the stereochemical quality of the refined structures was checked with the program PROCHECK [42]. In addition, well-ordered water molecules were located and included in the model. Both R-factor and Rfree were used to monitor the progress of structural refinement.
Table 1. Data collection and refinement statistics. All refinement and calculation of R-factor were done by CNS [40] using all reflections.

<table>
<thead>
<tr>
<th></th>
<th>TM-3 + pENW</th>
<th>TM-3 + pEQW</th>
<th>TM-3 + pEKW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal data</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>( a = b ) (Å)</td>
<td>61.151</td>
<td>61.082</td>
<td>61.220</td>
</tr>
<tr>
<td>( c ) (Å)</td>
<td>131.193</td>
<td>127.593</td>
<td>128.086</td>
</tr>
<tr>
<td>Space group</td>
<td>P4₁2₁2</td>
<td>P4₁2₁2</td>
<td>P4₁2₁2</td>
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<tr>
<td>Resolution (Å)</td>
<td>1.37</td>
<td>1.50</td>
<td>1.45</td>
</tr>
<tr>
<td>No. of observations</td>
<td>121 022 (30–1.37 Å)</td>
<td>273 510 (20–1.50 Å)</td>
<td>96 550 (30–1.45 Å)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>49 996</td>
<td>38 788</td>
<td>42 718</td>
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<tr>
<td>Completeness (%)</td>
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<td>97.9</td>
<td>96.8</td>
</tr>
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<td>in the outmost shell</td>
<td>88.1 (1.42–1.37 Å)</td>
<td>99.4 (1.55–1.50 Å)</td>
<td>98.5 (1.50–1.45 Å)</td>
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<td>Average I/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in the outmost shell</td>
<td>2.4</td>
<td>5.7</td>
<td>2.2</td>
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<tr>
<td>( R_{merge} ) (%)</td>
<td>6.0</td>
<td>7.2</td>
<td>6.5</td>
</tr>
<tr>
<td>R-value/no. of atoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total nonhydrogen atoms</td>
<td>15.7/2288</td>
<td>18.6/2074</td>
<td>17.5/2112</td>
</tr>
<tr>
<td>protein</td>
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<td>14.5/1616</td>
<td>13.2/1616</td>
</tr>
<tr>
<td>heavy atom</td>
<td>22.5/11</td>
<td>25.6/10</td>
<td>25.0/10</td>
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<tr>
<td>water</td>
<td>30.9/630</td>
<td>34.7/416</td>
<td>32.9/454</td>
</tr>
<tr>
<td>inhibitor</td>
<td>8.8/31</td>
<td>16.7/32</td>
<td>11.8/32</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>residues in most favored regions</td>
<td>171 (91.0%)</td>
<td>169 (89.9%)</td>
<td>167 (88.8%)</td>
</tr>
<tr>
<td>additional allowed regions</td>
<td>16 (8.5%)</td>
<td>18 (9.6%)</td>
<td>20 (10.6%)</td>
</tr>
<tr>
<td>generously allowed regions</td>
<td>1 (Cys118, 0.5%)</td>
<td>1 (Cys118, 0.5%)</td>
<td>1 (Cys118, 0.5%)</td>
</tr>
</tbody>
</table>

\( R_{merge} = \sum_{hkl} \frac{||I(hkli)| - \langle I(hkli) \rangle|}{\sum_{hkl} |I(hkli)|} \)  
\( R_{working} = \sum_{hkl} \frac{|F(hkli)_{obs} - \langle F(hkli)_{calc} \rangle|}{\sum_{hkl} |F(hkli)_{obs}|} \)

The atomic coordinates of these crystal structures have been deposited at Research Collaboratory for Structural Bioinformatics ( RCSB) Protein Data Bank (accession numbers: pENW, 1KUG; pEQW, 1KUI; pEKW, 1KUK).

RESULTS AND DISCUSSION

Main features of the inhibitor-bound TM-3

The overall structures of inhibitor-bound TM-3 show no significant conformational change, as compared to that of TM-3 proteinase without inhibitor (Fig. 1A,B). The RMS deviations are 0.320, 0.299 and 0.294 Å for the backbone atoms of pENW-bound, pEQW-bound and pEKW-bound TM-3s, respectively. A slight movement is observed in some of the residues around the inhibitor-binding environment (see Fig. 1C). As shown, the S⁻¹-wall forming segment Ala168–Ile170 of TM-3 is shifted away from the active-site center after binding of inhibitors. The distance of His143C–Ala168C in the inhibitor-bound forms is about 7.17–7.29 Å in contrast to 6.86 Å in the unbound form. In addition, this inhibitor binding also causes the guanidino group of Arg106 to direct toward the surface of the proteinase molecule (Fig. 1C). The orientation of this guanidino group is quite different among the three inhibitor-bound forms.

The crystal structure of the unbound TM-3 [29] showed that the active-site zinc ion is replaced by a cadmium ion during the crystallization process. In this report, purified TM-3 was recrystallized with each of the three inhibitors using the same condition as unbound TM-3. The structures of inhibitor-bound TM-3 exhibit similar characteristics to that of the unbound form, including a comparable temperature factor of cadmium ion to its ligated His Nᵢ distances (see Table 2), and the distorted octahedral geometry of cadmium ion with six ligands. They suggest that the active-site metal ion of these three structures is also cadmium. The binding of inhibitor to TM-3 results in the replacement of two water molecules, i.e. Wat359 and Wat418, by two oxygens of the C-terminal carboxylic group of the inhibitor, which coordinate to the metal ion in an asymmetric bidentate manner (see Fig. 2). His143 Nᵢ and Wat416 are located at the vertexes of a distorted octahedron of cadmium ion at the active site, while His147 Nᵢ and His153 Nᵢ and the two C-terminal oxygens of the inhibitor lie on the octahedral base plane (Figs 2 and 3). In contrast to the substrate-based inhibitors, such as peptide hydroxamate and peptide thiol inhibitors for neutrophil collagenase (see Fig. 1D) [43,44], the backbone of these inhibitors occupy the primed substrate-binding region in a reverse direction (termed retro-binding). The orientations are parallel to βIV of the central β sheet, and antiparallel to the S⁻¹-wall forming segment Ala168–Ile170 (Fig. 3).

Structural characteristics of inhibitor binding

The \( P^{-1} \) (binding to \( S^{-1} \) site) Trp residue of the inhibitors. As shown in Figs 2A and 3, the indole ring of Trp in the inhibitors, which occupies the \( S^{-1} \) site of TM-3, is
stacked with the imidazole ring of His143, similar to some cases reported in the literature [45,46]. The distance between both rings is 3.2–3.9 Å (3.54 Å on average). In addition, the indole N\textsubscript{e} atom is anchored to the carbonyl oxygen of Ser167 by a hydrogen bond (the distance is about 2.80–2.99 Å) as shown in Fig. 3B.

The binding of inhibitors to TM-3 also causes the bottom of the S\textsuperscript{1} specificity pocket to be slightly extended (Fig. 4A,B). This is attributed to a shift of the relatively bulky side chain of Gln174 away from the pocket center. However, although the S\textsuperscript{1} pocket is not completely filled by the Trp side chain, the volume of this pocket is far smaller than those of adamalysin II and atrolysin C complexed with a peptidic inhibitor (Fig. 4C,D) [26,47]. This is due to a deeper hole formed at the S\textsuperscript{1} site of adamalysin II and atrolysin C, reminiscent of the deep S\textsuperscript{1} pocket of the two-disulfide SVMPs [29]. According to the adamalysin II model, two ordered water molecules remain at the S\textsuperscript{1} pocket after binding of a Trp-containing peptide inhibitor [26]. However, these water molecules are not observed in our crystal structures, indicating that some structural differences may exist among SVMPs from different snake species.

The P\textsuperscript{2} Asn, Gln and Lys residues of the inhibitors. As shown in Fig. 5, the Asn, Gln and Lys residues of the inhibitors are stabilized at the S\textsuperscript{2} site of TM-3 by three possible hydrogen bonds: (a) The side-chain amide or amino nitrogens of Asn, Gln and Lys are hydrogen-bonded to the carbonyl oxygen of Arg106. (b) The N-terminal nitrogens of these three residues are hydrogen-bonded to the carbonyl oxygen of Asn107. (c) The C-terminal carbonyl oxygens of these residues are hydrogen-bonded to the N-terminal nitrogen of Ile109. In addition, the side chain of Lys residue also contacts extensively with the alkyl part of Arg106 (the distance is about 4.1 Å, see Fig. 5C), via nonpolar interactions.

Table 2. Coordination geometry of the active-site cadmium ion.

<table>
<thead>
<tr>
<th>Bond lengths (Å)</th>
<th>+ pENW</th>
<th>+ pEQW</th>
<th>+ pEKW</th>
<th>Uncomplexed</th>
</tr>
</thead>
<tbody>
<tr>
<td>His143–Cd</td>
<td>2.28</td>
<td>2.30</td>
<td>2.31</td>
<td>2.27</td>
</tr>
<tr>
<td>His147–Cd</td>
<td>2.21</td>
<td>2.24</td>
<td>2.19</td>
<td>2.19</td>
</tr>
<tr>
<td>His153–Cd</td>
<td>2.18</td>
<td>2.19</td>
<td>2.37</td>
<td>2.31</td>
</tr>
<tr>
<td>Inh. O\textsubscript{c1}–Cd</td>
<td>2.26</td>
<td>2.30</td>
<td>2.13</td>
<td></td>
</tr>
<tr>
<td>Inh. O\textsubscript{c2}–Cd</td>
<td>2.46</td>
<td>2.49</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>Wat359–Cd</td>
<td>2.46</td>
<td>2.49</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>Wat418–Cd</td>
<td>2.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.24</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
The $P^{-3}$ pyro-Glu residue of the inhibitors. The pyro-Glu of these inhibitors located at the $S^{-3}$ site is surrounded by Asn107, Ile109, Val169 and Ile170 as shown in Fig. 3. No plausible hydrogen bond is detected, though the pyro-ring nitrogen is near the amide oxygen of Asn107. The alkyl part of pyro-ring is oriented to contact with the hydrophobic side chain of Ile109 and Ile170 (distances are about 4.1–4.7 Å), making a good fit at the $S^{-3}$ site by hydrophobic interactions.

Design and comparison of synthetic inhibitor analogues of TM-3

Previously, we had purified the three above-mentioned tripeptide inhibitors, $p$ENW, $p$EQW and $p$EKW, from the venom of Taiwan habu in small amounts [35]. These small peptide inhibitors were useful for elucidating the inhibition mechanism of snake-venom metalloproteinases by endogenous inhibitors, as well as providing an initial model for the rational design of inhibitors against disease-related ADAMs and MMPs, such as TACE. By solid-phase peptide synthesis, we have prepared these three endogenous tripeptides plus more than 10 inhibitor analogues with substitutions of native peptides $p$ENW and $p$EKW by $\alpha$-amino acids at various positions, which are designed for binding to various putative substrate-binding subsites of SVMP (Table 3).

Results from the detailed comparison of these synthetic inhibitors show that the inhibition activity of $p$EKW is slightly stronger than that of $p$ENW and $p$EQW, consistent with our previous report [35]. This may be due to the exclusion of an additional water molecule from the $S^{-2}$ site of TM-3 by the Lys side chain that is in contact with the alkyl part of Arg106, resulting in an increase of entropy (Fig. 5C).

The $P^{-1}$ position of inhibitor. The $S^{-1}$ pocket of TM-3 primarily prefers to bind a bulky tryptophan residue. As shown in Table 3, the inhibition activity of $p$ENF and $p$ENL dropped by approximately 50-fold as compared to that of the wild type $p$ENW, though van der Waals dimension of the Trp indole ring is only 1.37 and 1.62-fold larger than the phenyl group of Phe and the side chain of Leu, respectively [48]. In addition, $p$ENG analogue showed almost no activity in spite of the intact pyroglutamate and asparagine residues. This glycine mutant would increase conformational flexibility, so its low activity could also be due to an entropic effect. Our results point to the importance and the high specificity of tryptophan residue in the binding of inhibitors to TM-3. This is attributed to the stacking of Trp indole ring against the imidazole side chain of His143 in TM-3 and the specific hydrogen bond between the indole $N_e$ atom and the carbonyl oxygen of Ser167. Thus, nature chooses tryptophan as the main component in the endogenous inhibitors to compete with Phe or Leu in the proteinous substrates for the $S^{-1}$ pocket of SVMPs, because SVMPs usually hydrolyze their substrates at the N-terminal side of Leu and Phe residues [49].

In order to increase the dimension and hydrophobicity of the inhibitor at the $P^{-1}$ position, two analogues, $p$ENLW and $p$ENWL, were synthesized and shown to be weaker inhibitors than the native tryptophan-containing tripeptides, strengthening the requirement of a strict size limitation for inhibitors to bind $S^{-1}$ site.
This may be attributed to the fact that the Asp residue of equal activity, respectively, compared to the native Sp pocket are shown. Both figures were prepared using GRASP.

The P⁻² and P⁻³ positions of inhibitor. pEDW and pEAW, two tripeptide inhibitors designed to probe the S⁻² site (Table 3), are found to be ≈eightfold weaker and equal activity, respectively, compared to the native pENW. This may be attributed to the fact that the Asp residue of pEDW fails to form a hydrogen bond to the carbonyl oxygen of Arg106, as the side-chain carboxylic group of Asp (pKa = 3.65) is deprotonated in our assay system (pH 8.0). On the other hand, the small Ala residue does not experience steric hinderance for the inhibitor binding to TM-3.

The N-terminal pyro-ring of the inhibitor probably contributes to the required hydrophobicity of P⁻³ position as judged by the sixfold weaker activity of ENW than pENW. This is consistent with the previous observation that the pyro-Glu bound to the S⁻³ site of TM-3 is hydrophobically held by Ile109 and Ile170. Furthermore, the residues at the P⁻¹ and P⁻² positions of inhibitors are not interchangeable with each other, i.e. inhibition is relatively position-specific, as indicated by the low potencies of pENW and pEWK (Table 3).

Structural comparison of pENW-(TM-3) with the peptidic inhibitor-complexes of atrolysin C, TACE and HNC

Batimastat (BB-94) is well known to be a potent inhibitor of matrix metalloproteinases with IC₅₀ values in the low nanomolar range [50]. In vivo, it is capable of effectively blocking or delaying the growth of some human tumor cells by intraperitoneal administration [51]. The 2.0-A crystal structure of atrolysin C complexed with batimastat showed that the thiophene group of batimastat deeply inserts into the deep S⁻¹ site of atrolysin C, reaching near the bottom of this hydrophobic pocket (see Figs 4D and 6B) [47]. This deep insertion is probably related to the high potential of batimastat in inhibiting the activities of matrix metalloproteinases. The thiophene ring corresponds to a clockwise rotation of about 70° as compared with the Trp indole ring of pENW. The phenyl group of batimastat is located between the primed S⁻¹ and S⁻² sites of atrolysin C, close to the position of the Asn side chain of pENW in our structure (compare Fig. 6B with A). The isobutyl group of batimastat is directed toward the S⁻³ site of atrolysin C. However, it is too short to make favorable contacts, unlike the pyro-Glu residue of our pENW. In addition, the terminal methylamide group of batimastat is employed to ligate the active-site zinc ion. Four hydrogen bonds were identified to impart additional significance to the orientation of individual groups to account for the enhanced binding of batimastat to atrolysin C.

We have compared our pENW-(TM-3) structure with the complex of TNFα converting enzyme (TACE, or named ADAM 17) and a substrate-based hydroxamate inhibitor (Fig. 6C) [22]. The P⁻¹ isobutyl group of this inhibitor fits into the neck of hydrophobic S⁻¹ site (Fig. 4E), presumably, mimicking the binding of the P⁻¹ Val77 in pro-TNFα to the TACE active site. Interestingly, the remaining volume of the S⁻¹ pocket following such a binding is larger than that of our pENW-(TM-3) structure, due to its poor utilization of the S⁻¹ site by the isobutyl group (Fig. 4E). The P⁻² t-butyl group, like the Asn side chain of pENW, extends away from the active-site cleft. In contrast, TACE has a large S⁻³ pocket, but is only partially filled by the P⁻³ Ala residue of the inhibitor. By close comparison of Fig. 6C with 6A, this hydroxamate inhibitor has an extensive diaminoethyl group at the C-terminus, pointing to the surface of the enzyme. More recently, a class of macrocyclic TACE inhibitors were
synthesized by linking the P\(^{1}\) and P\(^{2}\) residues of acyclic anti-succinate-based hydroxamic acids [31]. It is of interest to note that a Gly residue at the P\(^{3}\) site of inhibitors was identified as a critical structural component to achieve a good potency. Coupled with a morpholylamide group at the P\(^{4}\) site, it could effectively inhibit the TNF\(\alpha\) release in human whole blood assays (IC\(_{50}\) = 0.067 \(\mu\)M).

In addition, a unique inhibition mechanism was observed in the binding of a barbiturate inhibitor to human neutrophil collagenase (HNC, or termed MMP-8) [46]. Compared with the structure of pENW-(TM-3), this inhibitor appears more compact, using its phenyl and piperidine rings to point to the primed S\(^{1}\) and S\(^{2}\) sites of HNC, respectively (Fig. 6D). The third rigid barbiturate ring of this inhibitor chelates the catalytic zinc ion, and contributes two hydrogen bonds for the inhibitor binding. The P\(^{1}\) phenyl ring, almost identical in orientation to the Trp indole ring of pENW, is stacked against the imidazole ring of a Zn-coordinated His residue, similar to our observation in this report. However, in contrast to the thiophene ring of the above mentioned batimastat, it is too short to make a deep insertion (Fig. 4F). In fact, the large S\(^{1}\) pocket of HNC is only half occupied following the insertion of this phenyl group. This might be the primary reason to account for the significant difference of inhibitory effects between batimastat (IC\(_{50}\) = 10 nM) and this barbiturate inhibitor (IC\(_{50}\) = 1.7 \(\mu\)M) on the activity of HNC [46,47].

**CONCLUSION**

We report the high-resolution crystal structures of TM-3 cocrystallized with three endogenous tripeptide inhibitors. The binding of inhibitors to TM-3, adopting a retro-manner, cause some of the residues around the inhibitor-binding environment to slightly move away from the active-site center. The C-terminal carboxylic group of the inhibitors chelates the active-site cadmium ion in an asymmetric bidentate manner, resulting in the replacement of two water molecules, i.e. Wat359 and Wat418, originally present in the structure of unbound TM-3. The S\(^{1}\) pocket of TM-3 appears more shallow as compared with those of the two-disulfide SVMPs isolated from American diamondback rattlesnakes [26,45]. Three principal interactions that stabilize the binding of inhibitors to TM-3 are as follows. (a) The Trp indole ring of the inhibitors is stacked against the imidazole ring of His143 in the S\(^{1}\) pocket of the proteinase. (b) The middle residue of the tripeptide inhibitors are stabilized at the S\(^{2}\) site of TM-3 by three possible hydrogen bonds. (c) The pyro-ring of these inhibitors is...
snuggly held at the S\(^{-3}\) site of TM-3 by hydrophobic interactions. Results from the comparisons of the synthetic inhibitor analogues show that the P\(^{-1}\) Trp residue of the inhibitors is primarily specific for binding to TM-3. The side chain of the middle residue in the inhibitor contributes an important hydrogen bond for the stabilization of inhibitor binding, but other residues with low steric hinderance are equivalently favorable. The P\(^{-3}\) position of the inhibitors probably prefers a hydrophobic residue. These data are consistent with our structural observations.

In addition, the comparisons of our structure and some of other inhibitor-bound metalloproteinases suggest a close relationship between the inhibitory activity of an inhibitor and its ability to fill the S\(^{-1}\) pocket of the proteinase. The inhibitor-enzyme hydrogen bonds impart additional significance to the orientation and stabilization of the inhibitor binding. Consistent with this, in our recent studies [29], the structure of human neutrophil collagenase (HNC) appeared to have a deep S\(^{-1}\) pocket, similar to those of the two-disulfide adamaslyn II and atrolysin C. Consistently, the potent atrolysin C inhibitor batimastat (IC\(_{50} = 6\) nM) was also effective to inhibit the activity of HNC (IC\(_{50} = 10\) nM). In contrast, TM-3 and the TACE are presumably less susceptible to batimastat, because the S\(^{-1}\) pockets of both structures are too shallow to make proper insertion by the thiophene ring. Conversely, a good TM-3 inhibitor may be more effective towards TACE than HNC or atrolysin C because of the similar depth/dimension of the S\(^{-1}\) pocket between TM-3 and TACE. On the other hand, the shallow S\(^{-1}\) pocket of TACE is not fully occupied by the isobutyl group of a hydroxamate inhibitor as indicated in this report. The indole group of tryptophan or its modified derivatives are likely the better candidates of the P\(^{-1}\) residue of a potential TACE inhibitor, owing to their abilities to make a favorable insertion and a precise stacking with the TACE active site. Our work along this line may be helpful to form a firm basis for the rational design of inhibitors against TACE-related disorders.

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