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Structure-based virtual screening and biological evaluation of potent and selective ADAM12 inhibitors

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Abstract—We describe a series of potent and selective inhibitors of ADAM12 that were discovered using computational screening of a focused virtual library. The initial structure-based virtual screening selected 64 compounds from a 3D database of 67,062 molecules. Being evaluated by a cell-based ADAM12 activity assay, compounds **5**, **11**, **14**, **16** were further identified as the potent and selective inhibitors of ADAM12 with low nanomolar IC_{50} values. The mechanism underlying the potency and selectivity of a representative compound, **5**, was investigated through molecular docking studies.

ADAMs (a disintegrin and metalloprotease) are the type 1 transmembrane metalloproteases that are involved in cell–cell adhesion, surface proteolysis and transmembrane signaling. The extracellular portion of a typical ADAM protein contains an amino-terminal secretion signal, prodomain, metalloprotease, disintegrin, cysteine-rich, and epidermal growth factor-like domains.[1,2] Cardiac hypertrophy is an adaptive response to an increase in blood pressure. Despite initial benefits, it eventually leads to heart failure and thus is an independent risk factor for cardiovascular disease.[3,4] Recently, ADAM12 was shown to play an important role in the development of cardiac hypertrophy by shedding heparin-binding epidermal growth factor.[5] Therefore, inhibition of ADAM12 could be a potent therapeutic strategy for cardiac hypertrophy and congestive heart failure. The hydroxamate-based ADAM12 inhibitors, OSUB7-6,[6] OSUB8-1,[6] and KB-R7785,[5] and general matrix metalloprotease (MMP) inhibitors, PD166793[7] and GM6001,[8] have been used to inhibit ADAM12 in both in vitro and in vivo experiments. However, these inhibitors have broad spectra to MMPs. We report here the identification of potent and selective ADAM12 inhibitors through structure-based virtual screening and a cell-based ADAM12 assay.

We first built the 3D structure of ADAM12 with the aid of the published structures of reference proteins whose sequences have over 30% similarity to ADAM12. Successfully used as reference proteins in this study were adamalysin (*Crotalus adamanteus*; PDB code, 4aig), acutolysin-C (*Agkistrodon acutus*; PDB code, 1qua), and acutolysin-C (*Agkistrodon acutus*; PDB code, 1bud). Modeling of ADAM12 was initially performed by using HOMOLOGY module of INSIGHT2000,[9] and further refined by using Discover Ver 2.98 of INSIGHT2000. A pharmacophore map for catalytic site of ADAM12 was generated by using PharmoMap/PharmoScan (Fig. 1).[10] Since ADAM12 is a Zn^{2+}-containing metalloenzyme, Zn^{2+}-binding moieties are prerequisite for potent inhibitors against the enzymatic activity. Therefore, we prepared a focused virtual library, which comprised 67,062 compounds containing a Zn^{2+}-binding moiety such as carboxyl, hydroxyl sulfur, or hydroxamate. Virtual screening of this library for the ADAM12 pharmacophore using PharmoMap/PharmoScan system resulted in 1217 compounds as initial hits,

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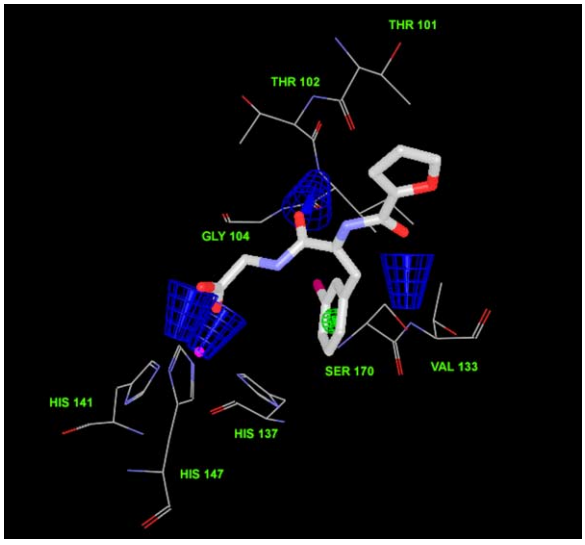


Figure 1. Interaction between the pharmacophore of the Zn^{2+}-binding site of ADAM12 and a selected compound, **5**. Blue conical shape and green circle represent hydrogen bond donor and hydrophobic core, respectively.

which were further refined by consensus scoring function comprised of Ligscore, PLP, JAIN, PMF, and LUDI.[11] Applying a criterion of top 30% of each score, 64 compounds were finally selected for biological testing. Figure 1 shows one of the selected compounds, **5**, bound to the pharmacophore of ADAM12.

Since in vitro assays for ADAM12 activity were unavailable, we utilized a cell-based assay[12,13] to evaluate the potency of the selected 64 compounds.[14] Human placental alkaline phosphatase (AP) is anchored in the Golgi membrane of *Drosophila* S2 cells by being linked to a Golgi integral membrane protein, Golgi α-mannosidase II (GMII). Inserted between AP and GMII is an ADAM12 substrate sequence that is a 78 amino acid fragment of heparin-binding epidermal growth factor. This triple fusion protein is normally retained in the intracellular compartment. However, cleavage of the substrate site by ADAM12 renders AP to be secreted to the culture media, where it can be conveniently monitored and quantified by colorimetric measurement (Fig. 2A). Initially, the 64 compounds were tested at a concentration of 10 μM, of which eight compounds showed more than 50% inhibition of ADAM12 activity (Fig. 2B). Further detailed evaluations of these eight compounds eliminated four compounds as ineffective at nanomolar concentrations. On a contrary, the remaining four compounds, **5**, **11**, **14**, and **16**, were highly potent against ADAM12 (Fig. 2C). These compounds are more than 100 times effective than a widely used general metalloprotease inhibitor, GM6001. They all share a structural backbone, which is characterized by unusual dipeptide analog containing a rigid double bond between α and β carbons (Table 1). They were then tested for their efficacy against other ADAM proteases using in vitro assays with purified enzymes.[15] All of them were totally ineffective against ADAM9 and TACE (ADAM17), and slightly effective against ADAM10, while a non-selective MMP inhibitor, GM6001, effectively inhibited the activity of ADAM9, ADAM10, and TACE at nanomolar concentrations. This implies that our compounds are highly specific to ADAM12 (Table 1).

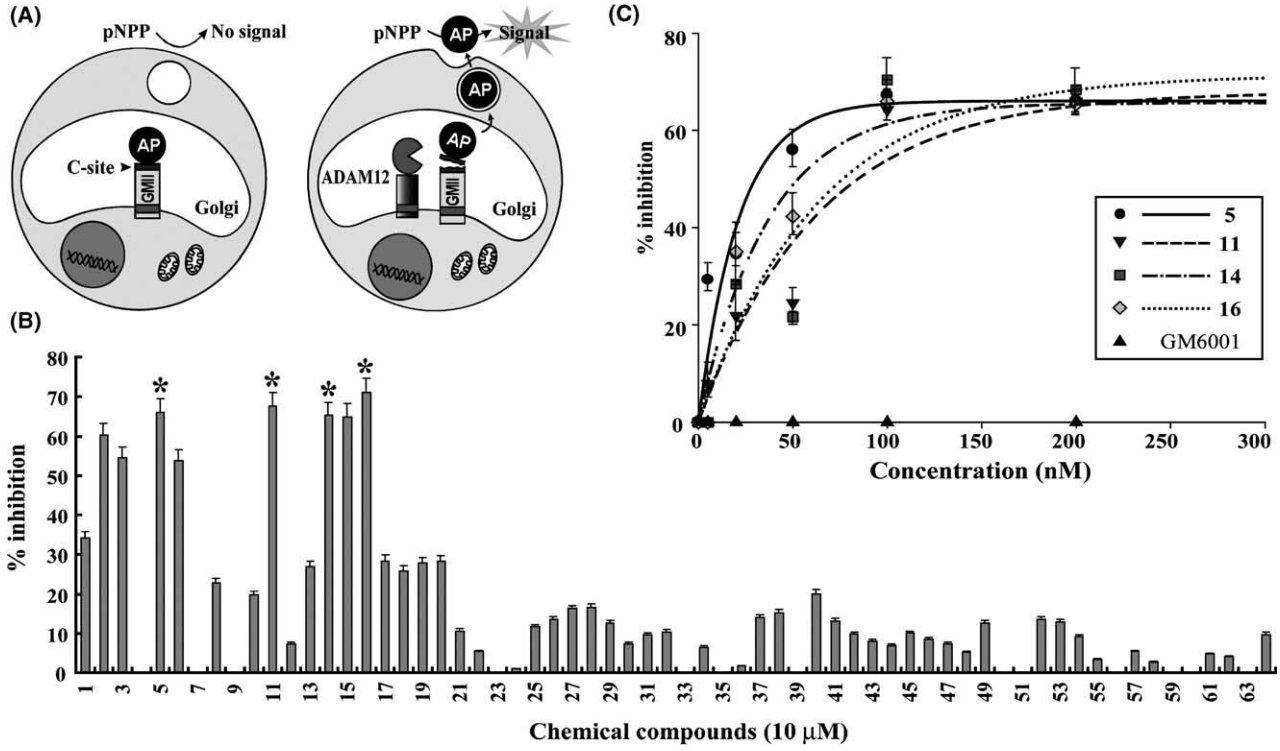
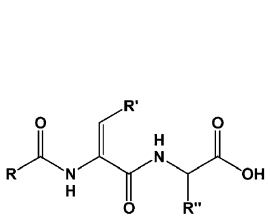
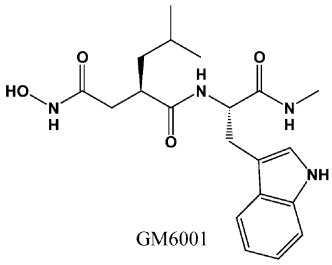


Figure 2. (A) Schematic diagram of the cell-based assay for ADAM12. (B) Initial in vivo evaluation of the 64 compounds that were selected by virtual library screening. (C) Inhibition of ADAM12 activity with the four potent inhibitors.

Table 1. Cell-based and in vitro assays





Compd	R	R′	R″	Cell-based assay	In vitro assay		
				ADAM12	ADAM9	ADAM10	TACE
5	Furyl	3-Br-Ph	H	16.7 nM	ND	>10.5 μM	ND
11	Ph	2-F-Ph	*i*-Pr	40.5 nM	ND	>10.5 μM	ND
14	Furyl	Furyl	Me	42.3 nM	ND	10.5 μM	ND
16	5-Br-furyl	4-Br-Ph	Me	24.8 nM	ND	ND	ND
GM6001				5.93 μM	1.0 nM	8.1 nM	7.5 nM

ND, not determined (IC_{50} values were not determined, because the compounds were not active up to 50 μM).

To understand the mechanism underlying the highly selective inhibition of ADAM12 by the selected compounds, we compared the docking modes of a representative compound, **5**, in the active sites of ADAM12 and TACE. HOMOLOGY and AFFINITY modules[16] of INSIGHT2000 were used for the modeling and docking studies. Docking protocol was based on Monte Carlo and Simulated Annealing methodologies. All default parameters were used and 1000 minimization steps were applied to the final structure and the active subset of both receptors was defined as residues 3 Å from the inhibitor, **5**. Zn^{2+} in the active site of ADAM12 was inserted manually at the initial position, which was about 2.5 Å away from the three nitrogens of histidines in the active site. Since the potential of Zn^{2+} could not be assigned in CVFF force field in INSIGHT2000, Zn^{2+} was replaced with Na^{+}, which can be recognized by CVFF force field.[17] The partial charge and formal charge parameter was then set to +2 and the final position of Zn^{2+} was determined by energy minimization, while the position of ADAM12 structure was fixed. The TACE structure co-crystallized with a peptide inhibitor was obtained from PDB data bank (PDB code: 1BKC).[17] To compare the docking structure of **5** to both ADAM12 and TACE, the active sites of ADAM12 and TACE were superimposed based on the sequence alignment. Although the compositions of amino acids in the active sites are highly conserved and the total binding energy is similar (−78 kcal/mol for **5**-ADAM12 complex and −53 kcal/mol for **5**-TACE complex), the binding modes of **5** in the active sites of ADAM12 and TACE were significantly different. Consistent with the proper docking of **5** in the pharmacophore of Zn^{2+}-binding site of ADAM12 (Fig. 1), **5** fitted into the active site of ADAM12 with the conserved Zn^{2+}-binding motif and hydrophobic interactions (Fig. 3A). However, **5** could not be docked in the active site of TACE by being repelled from the active site upon the calculation of energy minimization (Fig. 3B). Especially, 3-bromobenzyl group in hydrophobic site was dislocated from the binding pocket. This striking difference in the docking mode of **5** appears to be caused by the distinct hydrophobic cavity sizes in the active sites of ADAM12 and TACE. The hydrophobic pocket of TACE, consisting of Ala439, His405, and Val402 residues (yellow with brown diagonal lines in Fig. 3C and D), was relatively smaller than that (blue in Fig. 3C and D) of ADAM12. The hydroxamic acid inhibitor[18] of TACE containing Leu side chain instead of the corresponding bulky bromobenzyl group in **5** was well tolerated in both of the pockets (Fig. 3D). Therefore, the hydrophobic interaction as well as the Zn^{2+}-carboxy group interaction might serve as an essential factor for the specific inhibition of ADAM12 by **5**.

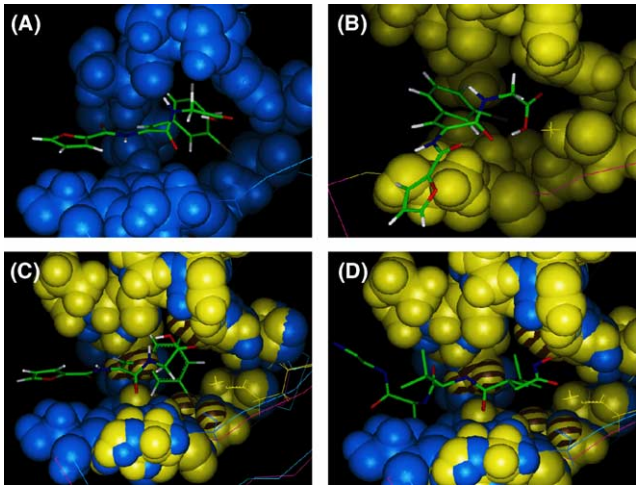


Figure 3. Superimposition of the space filling models of ADAM12 and TACE (blue, ADAM12; yellow, TACE; brown diagonal lines, Ala439, His405, and Val402). (A) Binding mode of **5** in ADAM12. (B) Binding mode of **5** in TACE. (C) Comparison of the hydrophobic pockets of ADAM12 and TACE bound with **5**. (D) Binding mode of a TACE inhibitor in TACE.

In this study, we were able to discover highly potent and selective inhibitors of ADAM12 through virtual library screening and a cell-based assay for ADAM12. The compounds, **5**, **11**, **14**, and **16**, inhibited the proteolytic

activity of ADAM12 in a cell-based assay with low nanomolar IC_{50} values, but not those of ADAM9, ADAM10, and TACE in in vitro assays. These compounds have the same backbone structure characterized by unusual dipeptide analog containing a double bond between α and β carbons. Although these compounds per se may not be used as efficient ADAM12 inhibitors in vivo owing to the presence of peptide bonds, they could be served as excellent lead compounds for the development of efficient ADAM12 inhibitors. Our data also suggest that the pharmacophore of ADAM12 was properly constructed, thus could be useful for further virtual library screening or refinement of the lead compounds.

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13. Generation of pHS/GMII-AP was described elsewhere. A DNA fragment encoding ADAM12 cleavage site (amino acids 84–162 of HB-EGF) was amplified, and inserted into the *Hind*III/*Nhe*I site of pHS/GMII-AP, resulting in pHS/GMII-HB-EGF-AP. The full-length ADAM12 and catalytic site deletion mutant ADAM12 (△205 → 413) coding sequences were amplified and inserted into the *Xho*I/*Bam*HI site of pMK33/pMtHygor, resulting in pMT/wtADAM12 and pMT/Δ mutant ADAM12, respectively. Therefore, the substrate and protease constructs are under the control of *Drosophila hsp70* and *metallothionein* promoters, respectively.
14. The compounds were purchased from ChemBridge, Inc. (San Diego, CA).
15. The purified ADAM9, ADAM10, and TACE, and their fluorescent peptide substrate (Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH2) were purchased from R&D systems Inc. 10–200 ng of purified enzymes were incubated with 10 μM peptide substrate in a 100 μL reaction mixture (25 mM Tris/HCl, 2.5 μM $ZnCl_2$, 0.005% Brij 35, pH 9.0) for 4 h at 37 °C. The cleavage of the substrate was measured with a fluorometer (Molecular Devices) at excitation and emission wavelengths of 320 nm and 405 nm, respectively. Dose-dependent inhibition of ADAM12 was analyzed using SigmaPlot (SPPS Inc.).
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