Structure-Based Approach for the Discovery of Bis-benzamidines as Novel Inhibitors of Matriptase

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Matriptase, a trypsin-like serine protease, which may be involved in tissue remodeling, cancer invasion, and metastasis. Potent and selective matriptase inhibitors not only would be useful pharmacological tools for further elucidation of the role of matriptase in these processes but also could have therapeutic potential for the treatment and/or prevention of cancers. We report herein the structure-based approach for the discovery of bis-benzamidines as a novel class of potent matriptase inhibitors. The lead compound, hexamidine (**1**), inhibits not only the proteolytic activity of matriptase, (K_i = 924 nM) but also of thrombin K_i = 224 nM). By testing several available analogues, we identified a new analogue (**7**) that has a K_i = 208 nM against matriptase and has only weak inhibitory activity against thrombin (K_i = 2670 nM), thus displaying a 13-fold selectivity toward matriptase. Our results demonstrated that structure-based database screening is effective in the discovery of matriptase inhibitors and that bis-benzamidines represent a class of promising matriptase inhibitors that can be used for further drug design studies. Finally, our study suggested that there is sufficient structural differences between matriptase and its closely related serine proteases, such as thrombin, for the design of potent and selective matriptase inhibitors.

Introduction

Local invasion and metastasis of cancers have been proposed to require imbalanced or unregulated expression of proteases, such as metalloproteases and urokinase-type plasminogen activator (uPA), at invading edges of carcinoma cells.[1−5] In recent years, increasing efforts have been applied to the development of potent and selective inhibitors of these proteases as potential anticancer therapeutic agents.[6−8] Indeed, a number of metalloprotease inhibitors are now in clinical trial for the treatment of cancer.[7]

We have recently characterized a novel, integral membrane serine protease, matriptase (GenBank accession number AF118224), and its cognate inhibitor HAI-1 (hepatocyte growth factor activator inhibitor 1).[9−11] In contrast to most other protease−inhibitor systems, both matriptase and HAI-1 are selectively expressed by cultured breast epithelial cells and cancer cells, but not by fibroblasts or fibrosarcoma cells. In addition to the C-terminal serine protease domain, the N-terminal noncatalytic region of matriptase contains two tandem repeats of a CUB (*C*1r/s, *U*egf, and *B*one morphogenetic protein-1) domain and four tandem repeats of a low-density lipoprotein receptor domain that are likely to be involved in protein−protein interaction.[10] Immunofluorescent staining of cultured breast cancer cells demonstrated that matriptase is concentrated on the cell peripheries at pseudopodia and on membrane ruffles in spreading cells.[9] Of particular interest, matriptase has recently been shown to activate hepatocyte growth factor and single-chain urokinase plasminogen activator.[5,12] Recently, a large-peptide inhibitor of matriptase, ecotin, has been shown to retard the growth of PC-3 prostate cancer tumors in nude mice, further suggesting that matriptase may play a role in cancer progression.[13] Taken together, these data suggest that matriptase may be a central regulator of cell migration and cancer invasion and may provide a novel cancer-associated protease target for design of new anticancer drugs.

We are interested in the discovery and development of potent and selective small-molecule inhibitors of matriptase. A potent, selective, nonpeptide, and drug-like small-molecule matriptase inhibitor not only will be useful as a pharmacological tool to further elucidate the biological functions of matriptase but also may have the therapeutic potential for treatment of cancer through stopping invasion and metastasis. Although large-peptide matriptase inhibitors, such as ecotin, have been reported, drug-like, small-molecule matriptase inhibitors are currently not available. Herein, we report our discovery of bis-benzamidines as a class of matriptase inhibitors through structure-based database search.

Results and Discussion

Homology Modeling. The X-ray structure of human thrombin, entry 1hxe (Figure 1A) from the Protein Data Bank,[14,15] was used as template for building the 3D structure of the protease domain of matriptase using homology modeling. It was shown that when the sequence identity/similarity between the modeled protein and the template is between 30% and 40%, the expected main-chain rms deviation between the modeled and the experimental structures for the protein is about 1.5 Å

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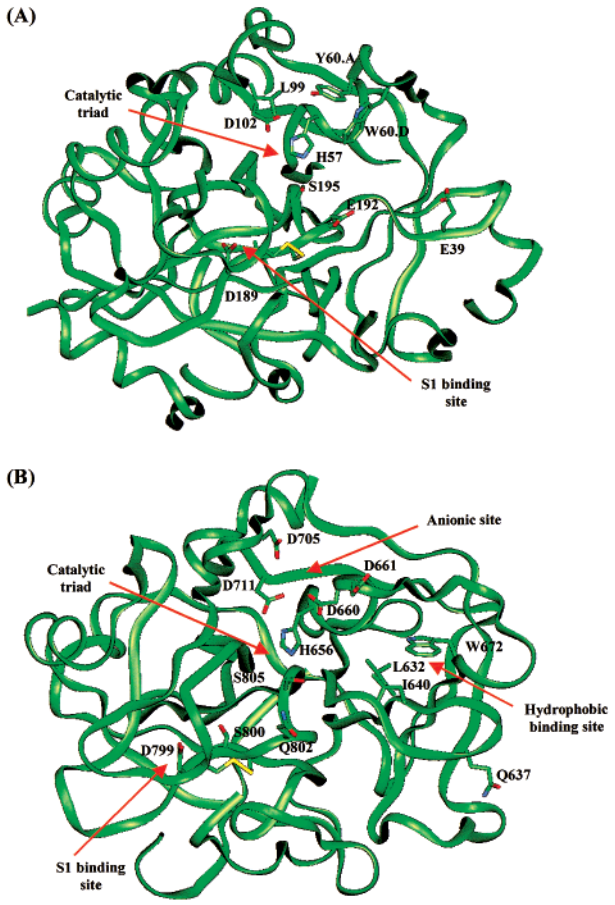


Figure 1. A: Active site residues in thrombin, entry 1hxe in the Protein Data Bank. B: Active site of matriptase, as obtained after homology modeling and refinement using MD simulation in water.

for 80% of residues.[16,17] Since the sequence identity and similarity between thrombin (Figure 1A) and matriptase are 34% and 53%, respectively, and both enzymes belong to the same protease family, it is expected that the 3D structure of matriptase can be modeled accurately. Figure 1B shows the modeled structure of the protease domain of matriptase. By analogy to thrombin, the serine protease domain of matriptase has a catalytic triad positioned on the surface, marked by Ser805, His656, and Asp711 corresponding to Ser195, His57, and Asp102, respectively in thrombin. Consistent with the observation that matriptase prefers substrates with an Arg or Lys as P1 residue,[10,12] a negatively charged residue, Asp799, is located at the bottom of the S1 binding site (Figure 1B). This residue corresponds to Asp189 in thrombin. Ser800 in the S1 binding site in matriptase differs from the corresponding Ala190 in thrombin. Close to the S1 site, Gln802 and Gln637 in matriptase correspond to charged Glu192 and Glu39, respectively, in thrombin (Figure 1). Anionic site residues Asp705, Asp660, and Asp661 in matriptase differ from the corresponding Trp96, Tyr60.A, and Trp60.D, respectively in thrombin. This shows that this site in matriptase is charged while it is neutral in thrombin. A putative hydrophobic S1′ binding site in matriptase is marked by Leu632, Ile640, and Trp672, as shown in Figure 1B, that is similar to the hydrophobic pocket formed by Leu33, Leu41, and Leu64 in thrombin, Figure 1A.

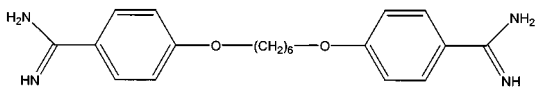
Table 1. Initial Screening of Compound Inhibitors for Matriptase

behavior	number of compounds
over 95% inhibition	15
90−94% inhibition	4
70−89% inhibition	15
40−69% inhibition	13
below 39% inhibition	17
high absorbency	3
increase activity	3

Structure-Based 3D Database Screening. The refined structure of matriptase, obtained from molecular dynamics (MD) simulation, was used for structure-based screening of the NCI database.[18] Since the S1 site is considered to be the primary binding site in serine proteases, it is likely to be a good target site for inhibitor design.[6,19] In addition, two other putative binding sites, the anionic site and the hydrophobic S1′ site, were included in the docking site used for 3D database searching with the program DOCK.[20] Ligands were scored based on the DOCK energy score computed as a sum of the electrostatic, van der Waals, and ligand conformational energy. Since the S1 site of matriptase is negatively charged, the potential inhibitor candidates that target this site should be positively charged in water under physiologic conditions for optimal interactions. Using this hypothesis a total of 69 candidate compounds were selected for testing from the best-scoring 2000 compounds based upon the DOCK program.

Inhibitory Activity Screening. Table 1 shows the results from an initial inhibitory activity screening. Each of the 69 candidate compounds was tested at 75 μM for the inhibitory activity against matriptase: 47 compounds inhibited at least 40% of the protease activity and 15 of them inhibited more than 95% of the protease activity. The 15 compounds that exhibited more than 95% inhibition were analyzed further for their K_i values, as described in the Experimental Section.

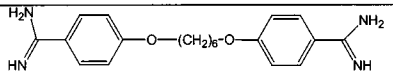
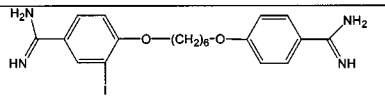
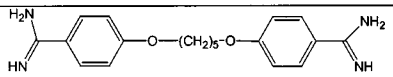
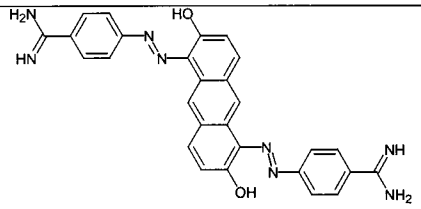
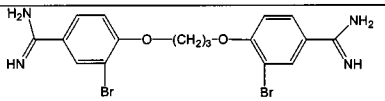
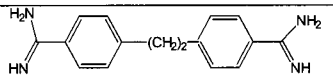
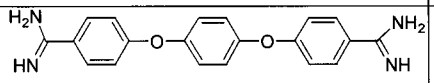
Bis-benzamidines as a Class of Potent Matriptase Inhibitors. One of the compounds that we have identified from our screening is hexamidine, compound **1**, a topical antiseptic. It inhibits matriptase with $K_i = 924$ nM, which makes it a good lead compound for further optimization. We therefore tested 7 closely related analogues that are available from the NCI database. Their chemical structures and K_i values for inhibition of matriptase enzymatic activity are summarized in Table 2. The K_i values of these compounds ranged from 191 nM to greater than 10 μM. Dixon plots of these inhibitors showed that they behaved as competitive inhibitors to the peptide substrate.



1 (hexamidine)

One important aspect in the design of protease inhibitors is their selectivity. For this reason compounds **1**, **2**, **5**, and **7**, with K_i values below 1 μM, were further evaluated for their selectivity against two other serine proteases, uPA and thrombin. Thrombin is a serine

Table 2. K_i Values Obtained for Bis-benzamidine Analogues of Hexamidine[a]

Cpd	Structure	K_i (nM)		
		Matriptase	uPA	Thrombin
1		924	14,400	224
2		191	1,980	796
3		1,160	N.T.	N.T.
4		4,500	N.T.	N.T.
5		535	1,570	946
6		> 10,000	N.T.	N.T.
7		208	1,950	2,670

[a] N.T. = not tested.

protease that plays a role in blood clotting, and its structure was used as the template to model the structure of matriptase. uPA is another serine protease that was proposed to play a role in cancer invasion and has a high sequence homology to matriptase.[4]

While **1** is 16-fold selective for matriptase over uPA, it is a 4-fold more selective inhibitor for thrombin over matriptase (Table 2). Compounds **1** and **2** have the same structure except for a 3-iodo substituent on one benzamidine phenyl ring. In contrast, **2** is a more selective inhibitor for matriptase; i.e., it displays 5- and 11-fold selectivity for matriptase over thrombin and uPA, respectively. This iodo substituent improved the potency for matriptase by approximately 5-fold, while it decreased the potency for thrombin by approximately 4-fold suggesting that iodo substituents on the benzamidine phenyl ring can improve the potency and selectivity of bis-benzamidines for matriptase. A structural comparison of compounds **1**, **3**, and **6** shows that the length of the linker between the two benzamidine groups plays a role for the inhibitory activity of bis-benzamidines. As the length of the linker decreases, the potency of the inhibitors decreases, which is consistent with our modeled structure of matriptase. Based upon our modeled matriptase structure, the distance between the S1 binding site and the anionic binding site is approximately 21 Å, similar to the length of **1** (20.9 Å) and **3** (19.3 Å) when they adopt a fully extended conformation. Interestingly, although compound **5** has a shorter linker than do **1** and **3**, it is approximately 2-fold more potent than **1** and **3** against matriptase. This suggests that 3,3′-dibromo substituents on the benzamidine phenyl rings improve the inhibitory potency of a compound. Compound **7** is the most rigid compound among **1**, **2**, **5**, and **7** and is also the most selective and one of the most potent inhibitors identified in this study. Despite its shorter linker **7** is as potent inhibitor as compound **2** against matriptase, but **7** has improved selectivity between matriptase and thrombin as compared to **2**. The conformational rigidity of **7** may play a role for its good potency and improved selectivity.

Docking Results. To gain a better understanding of the interactions between bis-benzamidines and matriptase or thrombin, we have docked compounds **1** and **7**, two potent inhibitors with reversed selectivity when tested for thrombin and matriptase. The goal of the docking study is to understand the structural basis of binding and selectivity of these ligands. Docking was done in two steps. First every compound was docked into the corresponding protein using the DOCK[20,21] program. During this docking the ligand was flexible while the protein structure was rigid. To take the flexibility of the protein into account, the complex structure was further optimized using MD simulation

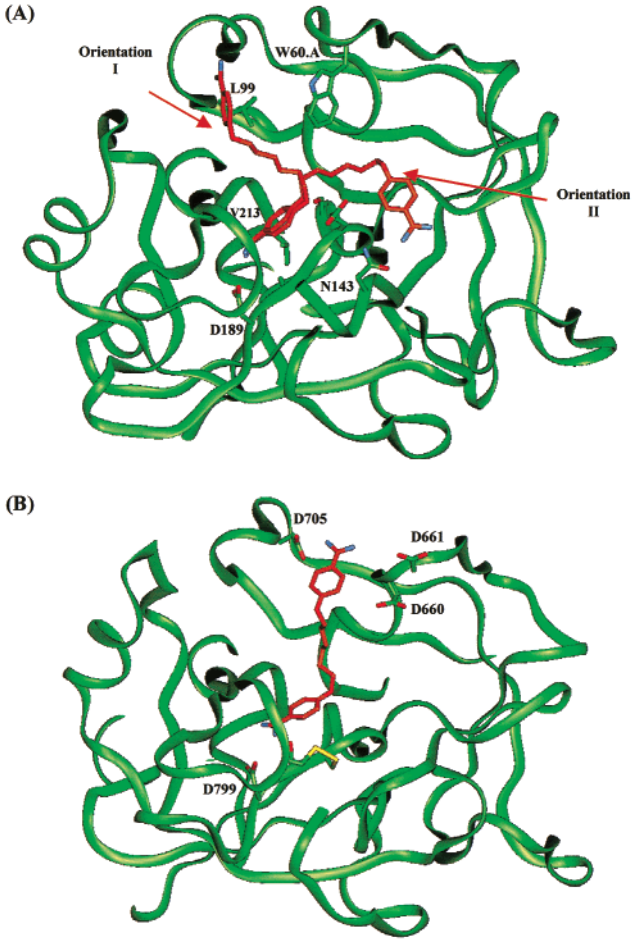


Figure 2. A: Different starting orientations of **1** in the active site of thrombin obtained after flexible ligand docking with the program DOCK. B: Orientation of **1** in the active site of matriptase obtained after flexible ligand docking with DOCK

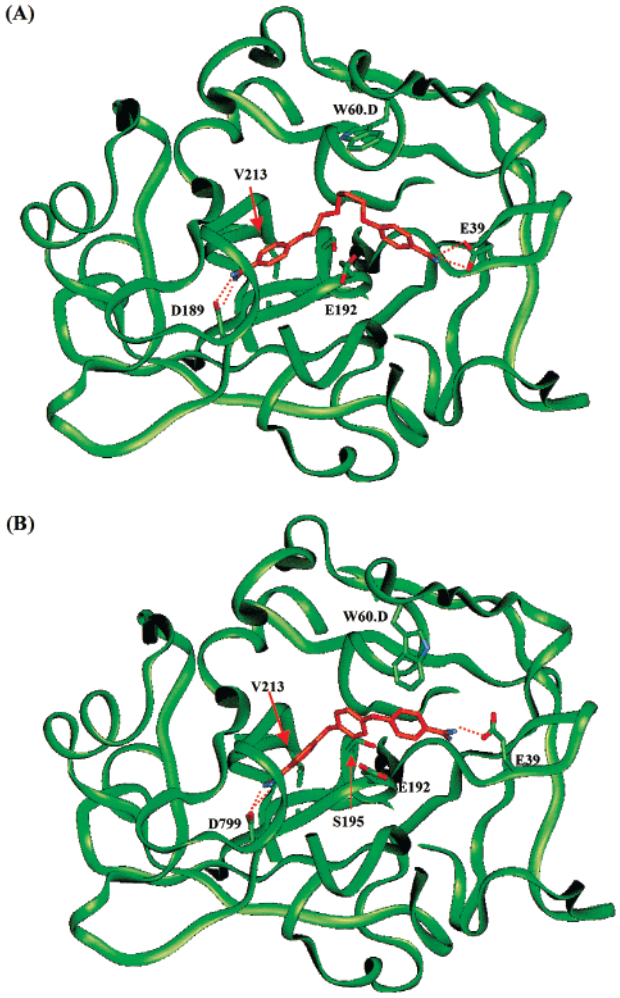


Figure 3. Lowest energy structure of the complex of compound **1** with (A) thrombin and (B) matriptase obtained after refinement using MD simulation with the generalized effective potential.

with a generalized effective potential[22] followed by conventional MD simulation. Docking with DOCK of **1** and **7** showed that these ligands adopt two different binding modes in thrombin (Figure 2A). In both binding modes, one benzamidine ring interacts with the negatively charged S1 binding site. The main difference between these two binding models is that in one binding mode (Figure 2A, orientation I) the second benzamidine fragment interacts with main-chain carbonyl oxygens in a region that corresponds to the anionic site in matriptase, while in the other binding mode (Figure 2A, orientation II), the second benzamidine fragment of the inhibitor interacts with Asn143. Since the automatic docking did not take into account the conformational flexibility of the protein, we investigated whether these two different binding models can converge to the same or similar binding model when the conformational flexibility of the protein is taken into account. For this purpose two parallel simulations were performed starting from the two different binding models of **1**. Using the MD simulation with the generalized effective potential,[22] after 1 ns, both simulations led to the same binding model of **1** in complex with thrombin, Figure 3A. Similarly, simulations of two different binding models for **7** obtained from the DOCK program led to a single converged binding model, Figure 3B. Based on the predicted binding models for **1** and **7** to thrombin, both inhibitors interact with the S1 site through a salt bridge to Asp189, hydrophobic interaction with Val213, and weak hydrogen bond between the amidino group of the inhibitor and the carbonyl oxygen of Ala190 (Figure 3A,B). While **1** interacts with Trp60.D through its linker, **7** interacts with Trp60.D through the second benzamidine fragment. While **1** forms bidentate hydrogen-bonding interactions with the carboxylate of Glu39, **7** forms only monodentate hydrogen-bonding interactions with the carboxylate of Glu39, because the rigid linker in **7** does not allow a favorable orientation of the amidino group to form a bidentate hydrogen-bonding interaction with Glu39. Bidentate hydrogen-bonding interactions between oppositely charged groups are shown to be stronger than monodentate interactions as observed for a series of thrombin inhibitors,[23] which may be one of the reasons why **7** is less potent than **1**.

Docking of **1** and **7** into matriptase using the program DOCK resulted in only one orientation for both inhibitors, Figure 2B. Both binding models were further refined using the same MD protocol as for thrombin. The refined binding models for **1** and **7** are shown in Figure 4A,B. Based upon the predicted models, **1** and **7** interact with the S1 site of matriptase through a salt bridge with Asp799, hydrogen bond with Ser800 OH, and have hydrophobic interaction with Val 824, similar to the interactions with thrombin. However, **1** forms two

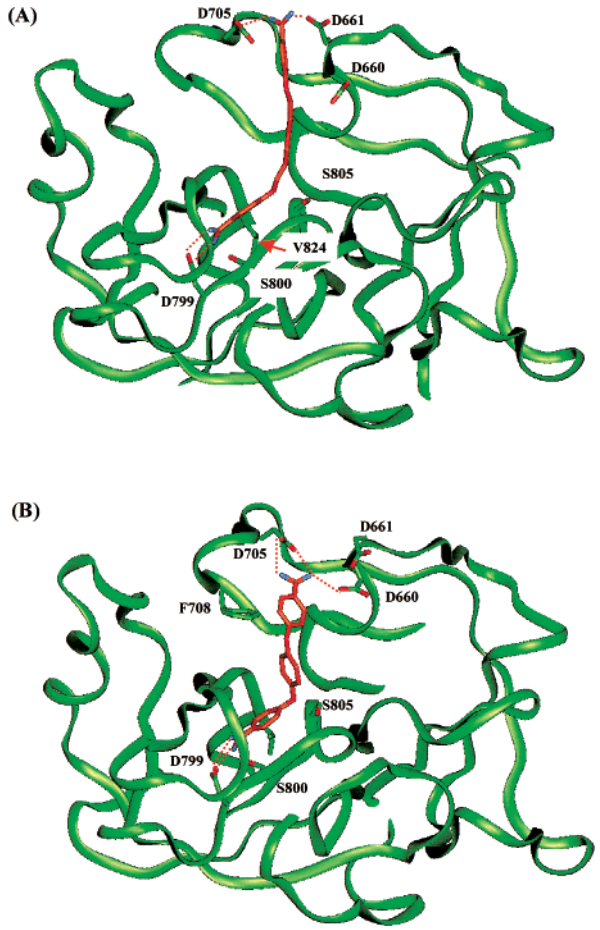


Figure 4. Lowest energy structure of the complex of compound **7** with (A) thrombin and (B) matriptase obtained after refinement using MD simulation with the generalized effective potential.

monodentate hydrogen-bonding interactions with Asp705 and Asp661 in matriptase that correspond to a bidentate hydrogen-bonding interaction with Glu39 in thrombin. However there is no hydrophobic interaction of the linker with matriptase hydrophobic residues, unlike that with thrombin. The lack of hydrophobic interactions of the linker in **1** with matriptase may be one important reason for its lower potency to matriptase as compared to thrombin. Due to its shorter linker, compound **7** was able to form a bidentate hydrogen-bonding interaction with Asp705 and a monodentate hydrogen-bonding interaction with Asp660. These interactions are stronger than that observed in thrombin, which may explain the higher potency of **7** to matriptase than to thrombin. Taken together, our docking studies provide an understanding of the structural basis of a class of novel inhibitors binding to matriptase and to thrombin and can offer a plausible explanation for the selectivity of two potent inhibitors.

Conclusion

We have identified bis-benzamidines as a class of matriptase inhibitors through structure-based database search. The lead compound, hexamidine (**1**) has K_i = 924 nM in inhibiting matriptase. Testing available analogues of the lead compound (**1**) led to the identification of **2** and **7** that are better inhibitors of matriptase with K_i = 191 and 208 nM, respectively. Compound **7** is the most selective compound for matriptase among the compounds tested. It has a selectivity of 9- and 13-fold between matriptase and uPA and between matriptase and thrombin, respectively. Our limited SAR and docking studies showed that the length of linker between the two benzamidine groups, the conformational rigidity of the linker, as well as the substituent(s) on the benzamidine ring(s) play important roles for the activity and selectivity. Differences in the relative position to the S1 site of the anionic site in matriptase versus thrombin can be used to design matriptase-selective inhibitors. The discovery of these small-molecule and nonpeptide matriptase inhibitors provides us with valuable pharmacological tools to further elucidate the biological function of matriptase. Structure-based design and chemical modifications toward improving potency and selectivity of the discovered lead compounds are currently underway and will be reported in due course.

Experimental Section

Homology Modeling and Structure Refinement. The sequence for matriptase was obtained from sequencing data. Templates for homology modeling were obtained by searching the Protein Data Bank,[14] using the program BLAST.[24] The structure of thrombin, entry 1hxe with 34% identities, 53% similarity and 6% gaps, was used as a template for modeling matriptase structure using the program MODELLER.[16] The structure obtained from homology modeling was further refined using the MD program CHARMM.[25] Hydrogen atoms were assigned to the modeled structure using the program HBUILD[26] from CHARMM. The protein was then solvated by inserting it in a 30 Å sphere of water and by deleting solvent molecules with heavy atoms that are at less than 2.5 Å from protein heavy atoms. The MD simulation was done using the all-atom parameter set from QUANTA3.2/CHARMm[27] force field, a constant dielectric $\epsilon = 1$, and constant temperature $T = 300$ K. The leapfrog method with 1-fs time step was applied for numerical integration. Long-range electrostatic forces were treated with the force switch method in a switching range of 8−12 Å. van der Waals forces were calculated with the shift method and a cutoff of 12 Å. The nonbond list was kept to 14 Å and updated heuristically. Solvent waters were kept from evaporating by using a spherical miscellaneous mean field potential as implemented in CHARMM. The solvated protein was energy minimized using 250 cycles of steepest descent and 500 cycles of adopted-basis Newton Raphson methods. This was followed by 100-ps MD simulation.

The structure of thrombin, entry 1hxe from the Protein Data Bank, was prepared for docking in the same way as the structure of matriptase.

Structure-Based Database Search. The refined structure of matriptase obtained from homology modeling, as described in the previous section, was used as the target in a structure-based 3D database search. The program DOCK[28] was used for computer-aided database screening to identify potential inhibitors. Shape and binding energy scoring functions were used to screen and rank the potential ligands. Filters were used to eliminate molecules that have more than 10 flexible bonds, to avoid considering overly flexible molecules, and also discarded molecules with fewer than 10 or more than 50 heavy atoms.

The screening of the large NCI database was done on a Silicon Graphics Indigo2 R10000 workstation. The docking was done in two stages, with the ligand flexibility being considered in both. In the first stage, two minimization cycles, with 50 iterations maximum, were considered for each compound from the database. The best-scoring 10 000 molecules were considered in the second stage, when 100 minimization cycles and 100 maximum iterations per cycle were carried out in order to refine the position of the ligand and its score. The top 2 000 compounds were then considered for selecting potential inhibitors for matriptase by inspection to determine if they contain

ionizable groups that will bind to the S1 site and the anionic site. After these screenings, 69 compounds were selected for further biochemical testing.

Molecular Modeling of the Best-Scoring Ligands. Compounds with K_i values less than 1 μM against matriptase were also docked into thrombin using DOCK with the second protocol as shown above. The orientation of the ligand obtained after docking was used as starting orientation in MD refinement, which was done on a Beowulf cluster of PCs in our laboratory. The active site of the protein−inhibitor complex was solvated by centering the molecule on Ser805/Ser195. A 20 Å radius TIP3P[29] water sphere centered in the origin was then added for solvation. Water molecules closer than 2.5 Å to any protein or inhibitor heavy atom were deleted. The solvated complex was energy minimized using 250 cycles of steepest descent and 500 cycles of adopted-basis Newton Raphson methods. This was followed by 1-ns MD refinement using the generalized effective potential implemented in CHARMM by our group.[22] The temperature of the simulation was 300 K and a 1-fs time step was used for numerical integration of the equation of motion. Long-range electrostatic forces were treated with the force switch method in a switching range of 8−12 Å. van der Waals forces were calculated with the shift method and a cutoff of 12 Å. The nonbond list was kept to 14 Å and updated heuristically. For ligand optimization an annealing protocol was used with the maximum q value[22] for calculating the generalized effective potential of 1.0005; this value was reached from the starting $q = 1$ after 10-ps simulation, followed by 10-ps simulation during which the q value was decreased to 1, which corresponds to MD, and 30-ps MD simulation. This cycle was repeated for the entire length of the simulation. A harmonic restraining force, with a force constant of 0.5 kcal/Å^2, was applied on the protein main-chain atoms that are within 20 Å of the catalytic triad Ser805/195. Residues that were farther than 20 Å from the active site Ser were fixed. This was followed by 1-ns regular MD simulation at 300 K without restraining force on the protein main-chain atoms. During this simulation residues that were farther than 20 Å from the catalytic triad Ser were also fixed. We used the same setup as for the generalized effective potential with the exception that $q = 1$ during the entire length of the simulation.

Materials. Active matriptase was purified from human milk as will be described later. Active urokinase-type plasminogen activator (uPA) was purified by aminobenzamidine-Sepharose 6B (Amersham Pharmacia, Piscataway, NJ) from a partially purified uPA from human urine. Bovin β-trypsin, bovine thrombin, and fluorescent peptide substrates *N*-*tert*-butoxycarbonyl (*N*-*t*-Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), *N*-*t*-Boc-Leu-Gly-Arg-AMC, and *N*-*t*-Boc-Leu-Arg-Arg-AMC were purchased from Sigma (Sigma Chemical Co., St. Louis). Small-molecule inhibitors were obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Other chemicals are all reagent grade.

Purification of Active Form Matriptase. Activated matriptase, in a complex with its endogenous inhibitor HAI-1, was purified from human milk by immunoaffinity chromatography and maintained in its uncomplexed status in glycine buffer pH 2.4, as described previously.[11] Matriptase and HAI-1 were further separated by 10% SDS−PAGE. The proteins were stained by zinc stain kit (Bio-Rad, Hercules, CA). Gels containing the 70-kDa active matriptase were sliced out and eluted using Electro-Eluter (Bio-Rad, Hercules, CA) under nondenaturing conditions (Tris-glycine buffer, pH 8.3). Purified, active matriptase was then stored at −80 °C in acidic solution.

Determination of Inhibitory Activity. Inhibitory activity of compounds against each protease was measured at room temperature using fluorescent substrate peptides in 100 mM Tris-HCl (pH 8.5), containing 100 μg/mL bovine serum albumin. To a cuvette containing 170 μL of buffer were added 10 μL of enzyme solution and 10 μL of inhibitors. After preincubation, 10 μL of substrate was added, and the solution was mixed well by shaking the cuvette. The residual enzyme activity was then determined by following the change of fluorescence released by hydrolysis of the fluorescent substrates in a fluorescent spectrophotometer (HITACHI F4500), with excitation at 360 nm and emission at 480 nm. Peptide *N*-*t*-Boc-Gln-Ala-Arg-AMC was used as a substrate for matriptase and trypsin, peptide *N*-*t*-Boc-Leu-Gly-Arg-AMC was used as a substrate for uPA, and peptide *N*-*t*-Boc-Leu-Arg-Arg-AMC was used as a substrate for thrombin.

Kinetic Screening of Compound Inhibitors. The inhibitory activity of each compound was first investigated by using a fixed (75 μM) concentration both of inhibitor and matriptase. Compounds that exhibited inhibition were then subjected to a further analysis for their K_i values using Dixon plotting. We recorded the rate of hydrolysis in duplicate in the presence of 6−7 different concentrations of each inhibitor. A straight line of the concentration of inhibitor versus the reciprocal values of the rate of hydrolysis was plotted with SigmaPlot software. Two lines were obtained from two unsaturated substrate concentrations; the X value of the intersection of these lines gives the value of $-K_i$.

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