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NON - THEMATIC REVIEW

Specifically targeting cancer proliferation and metastasis processes: the development of matriptase inhibitors

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Abstract
Matriptase is a type II transmembrane serine protease, which has been suggested to play critical roles in numerous pathways of biological developments. Matriptase is the activator of several oncogenic proteins, including urokinase-type plasminogen activator (uPA), hepatocyte growth factor (HGF) and protease-activated receptor 2 (PAR-2). The activations of these matriptase substrates subsequently lead to the generation of plasmin, matrix metalloproteases (MMPs), and the triggers for many other signaling pathways related to cancer proliferation and metastasis. Accordingly, matriptase is considered an emerging target for the treatments of cancer. Thus far, inhibitors of matriptase have been developed as potential anti-cancer agents, which include small-molecule inhibitors, peptide-based inhibitors, and monoclonal antibodies. This review covers established literature to summarize the chemical and biochemical aspects, especially the inhibitory mechanisms and structure-activity relationships (SARs) of matriptase inhibitors with the goal of proposing the strategies for their future developments in anti-cancer therapy.

Keywords Cancer · Invasion · Migration · Matriptase · Inhibitor · Structure-activity relationship

1 Introduction

Cancer, a kind of tumor with strongly invasive and metastatic ability and high lethality induced by abnormal cell growth, is one of the main causes of preventing the improvement of life expectancy worldwide. According to a recent report on the global burden of cancer [1], 18.1 million new cancer cases and 9.6 million cancer-caused deaths were recorded in 2018. It has been evidenced that many members of the type II transmembrane serine protease (TTSP) family, the largest group of membrane-anchored serine proteases, are highly and selectively expressed in various cancer cells [2, 3]. The genetic loss-/gain-of-function animal models have demonstrated that the dysregulated expression of TTSPs is connected to cancer initiation and progression [4]. Therefore, TTSPs are considered potential targets for cancer therapy [3].

The TTSP family includes over 180 human serine proteases. Based on phylogenetic analysis and different combinations of domains in backbone regions, they are classified into four subfamilies in vertebrates: matriptase, hepsin/transmembrane protease/serine (TMPRSS), human airway trypsin-like (HAT)/differentially expressed in squamous cell carcinoma (DESC), and corin [2, 5, 6]. Among TTSPs, matriptase is the most widely studied member due to its abnormal expression in tumor tissues.

In 1993, matriptase was first isolated as a novel protease from human breast cancer cells (T47D) and assigned as a gelatinase by Shi et al. [7]. Later, it was independently isolated as a serine protease from prostate cancer cells and human breast milk in 1999 [8–10]. To emphasize the ability of the degradation of extracellular matrix (ECM), the terminology "matriptase" was used to name this protease. Subsequent studies have confirmed that matriptase closely correlates to cancer progression and plays an essential role in the metastatic process of cancer cells. The association of matriptase with cancer

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development is likely attributed to that it is an upstream activator for key cancer-related enzymes, such as urokinase-type plasminogen activator (uPA), matrix metalloproteases (MMPs), which degrade extracellular matrix [11–21]. In this review, the chemical and biochemical aspects and structure-activity relationships (SARs) of matriptase inhibitors in recent years is summarized, aiming at facilitating the design and structural modification of matriptase-based inhibitors as novel anti-cancer agents.

2 Matriptase

Matriptase, also known as MT-SP1, TADG-15, suppressor of tumorigenicity 14 (ST14), or epithin, mainly expressed in epidermis, salivary gland, thyroid, stomach, kidney, prostate, ovaries, etc. [22–24]. It consists of 855 amino acids. Matriptase contains a C-terminus trypsin-like serine protease domain (SPD), four tandem cysteine-rich domains homologous to low-density lipoprotein receptor (LDLR), two tandem repeats of C1r/Cls, urchin embryonic growth factor and bone morphogenic protein 1 (CUB) domains, and a single sea urchin sperm protein, enterokinase, agrin (SEA) domain (Fig. 1a) [8, 13, 14, 25, 26]. The SPD of matriptase belongs to the S1 serine protease family and is structurally similar to thrombin and hepsin. Its substrate-binding pocket is dominantly charged in negative and can be decomposed into eight subsites, namely S4–S1 and S1′–S4′ (Fig. 1a). Correspondingly, the group bonded at the subsite is referred to as P4–P4′, respectively. Matriptase shares the same catalytic mechanism by a catalytic triad, consisting of H57 (base), D102 (electrophile), and S195 (nucleophile) and a highly conserved activational cleavage motif Arg^{15}-Val^{16}-Val-Gly-Gly (Fig. 1a) [8, 27, 28]. Similar to other trypsin-like proteases, matriptase has an S1 pocket with a negative-charged D189 at the bottom, which shows the preference of the accommodation of positive-charged P1 residues (arginine or lysine) in substrates or inhibitors [8, 13]. Besides, the most striking feature of matriptase SPD is the unusually long and oriented 60-loop [29] (Fig. 1a). The length of the 60-loop is the same as that in thrombin, forming a protruding irregular β-hairpin loop stabilized *via* hydrogen bonds between the main chain of D60A and the carboxylate moiety of D60B (numbered according to the chymotrypsin numbering). This unique loop is rotated away from the active site, making the cavity more spacious which might contribute to the wide substrate specificity of matriptase. In contrast, the 60-loop from other serine proteases, e.g., factor XIa (FXIa), plasmin, uPA, hepsin, and trypsin are shorter than matriptase by 4–5 residues, which indicates a larger internal binding domain in matriptase. Biochemical assays further suggested that the 60-loop of matriptase is a rate-limiting factor for the enzyme-substrate combination [29, 30]. In this review, the term "matriptase" specifically indicates the catalytic domain if there is no particular explanation.

After being synthesized and translocated to the plasma membrane, matriptase can be activated by two endoproteolytic cleavages, after G149 at the SEA domain and R614 at the catalytic domain. Matriptase is also physiologically regulated by its cognate inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1) by forming a non-covalent complex [8, 13, 31, 32]. Shedding with HAI-1 from the cell membrane, the activated matriptase induces the extracellular matrix degradation which would cause tissue remodeling. Experiments identified that the pro-forms of hepatocyte growth factor (HGF), uPA receptor-bound pro-uPA, prostasin, and protease-activated receptor 2 (PAR-2) are all the potential substrates of matriptase [14, 33–37]. The activation of HGF, uPA, and PAR-2 further triggers the downstream signaling events related to cancer invasion and metastasis, such as the tyrosine-protein kinase MET (c-MET) signaling pathway, plasmin, and MMP-induced extracellular matrix degradation (Fig. 1b). In breast, prostate, and some other cancers, matriptase is found to be highly expressed and correlates with the tumor grade and stage. The matriptase/HAI-1 ratio in cancer cells is larger than that in normal cells, which indicates the imbalance between matriptase and its endogenous inhibitor contributing to tumorigenesis, migration, and invasion [38]. Moreover, other cancer-related proteins, including CUB domain-containing protein 1 (CDCP1/SIMA135/TRASK), vascular endothelial growth factor receptor 2 (VEGFR2), and insulin-like growth factor-binding protein-related protein-1 (IGFBP-rP1) [39, 40] are also proposed substrates of matriptase. Hence, the development of potent matriptase inhibitors is of great clinical significance for cancer treatments.

Up to now, 21 resolved matriptase crystal structures of hominine origin have been deposited in the RCSB PDB database (https://www.rcsb.org/). Among them, there are three apo matriptase structures and 18 holo structures of matriptase-inhibitor complexes. These crystal structures provide vital information for the study of the physiological and pathophysiological functions of matriptase and the inhibitor design and development. The key information of the representative structures is summarized in Table 1.

3 Inhibition of matriptase

As a key node on the invasion and metastasis pathway of cancer cells, matriptase is a potential target for anti-cancer therapy. It is of great scientific and practical significance to develop matriptase-specific small-molecule inhibitors to suppress tumor invasion and metastasis. Since the discovery of

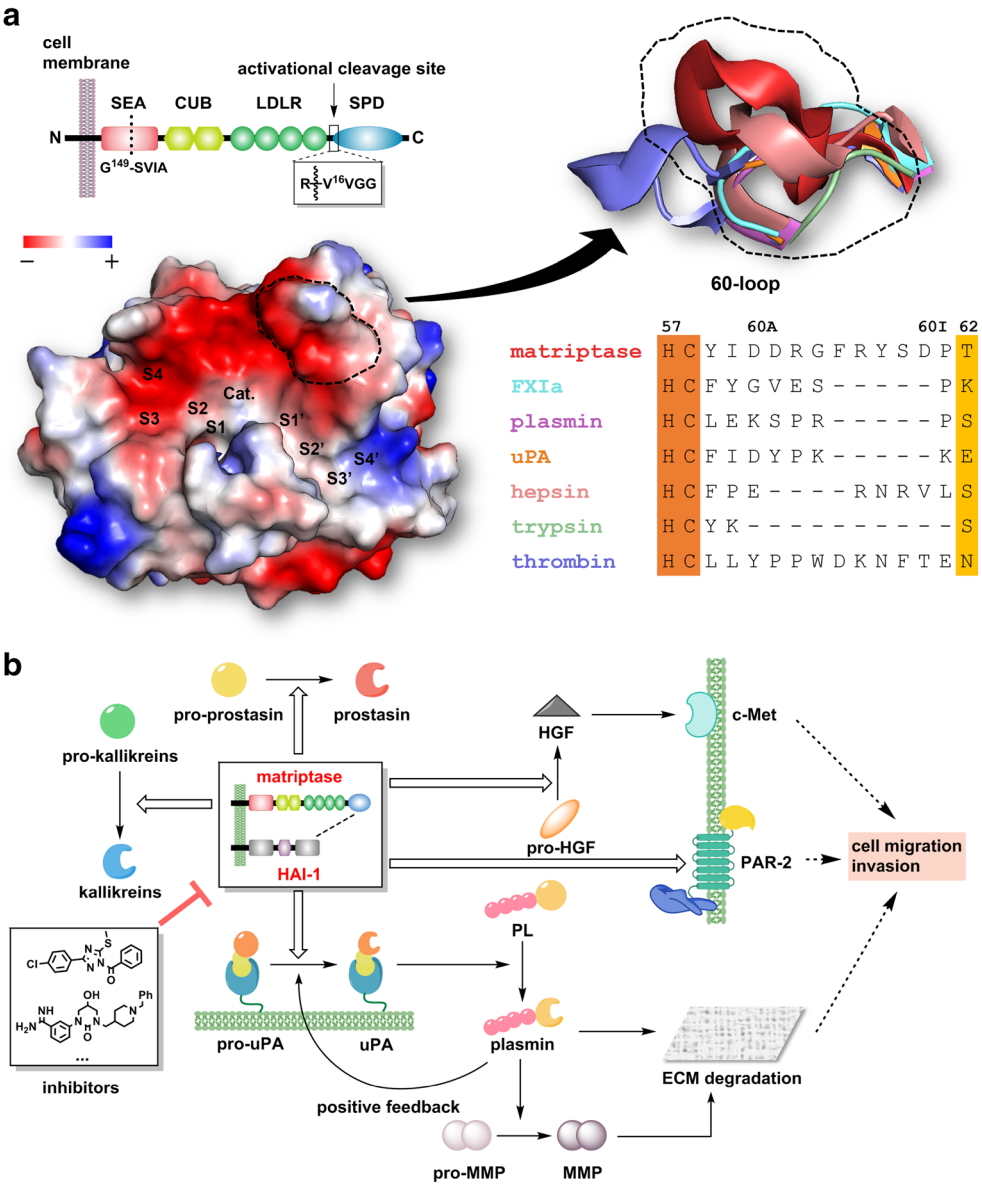


Fig. 1 Structural features of matriptase (**a**) and the activation cascade triggered by matriptase (**b**). The electrostatic potential of the matriptase SPD (PDB ID: 4JZI) is depicted with PyMOL. The superimposition of 60-loop is based on the crystal structures of matriptase (red, PDB ID: 4JZI), FXIa (aquamarine, PDB ID: 5TKS), plasmin (violet, PDB ID: 3UIR), urokinase-type plasminogen activator (uPA, orange, PDB ID: 4H42), hepsin (salmon, PDB ID: 1P57), trypsin (palegreen, PDB ID: 4WWY), and thrombin (slate, PDB ID: 6CYM)

matriptase, various inhibitors including small-molecule, peptide-, and antibody-based ones have been reported. In this section, we summarized the discoveries of structure, binding modes for matriptase, and inhibitory activities of some representative inhibitors, respectively.

3.1 Small-molecule inhibitors

According to the chemical skeleton characteristics, the reported small molecules with matriptase inhibitory activity can be divided into seven categories, including bis-benzamidines, sulfonylated 3-amidinophenylalanines, 1,2,4-triazole derivatives, cyanodipheylarylamides, 2-aryl/pyridine-2-yl-1H-indole derivatives, coumarin derivatives, and tetrahydropyrimidin-2 (1*H*)-one analogs.

3.1.1 Bis-benzamidines inhibitors

In 2001, Wang's group at Georgetown University Medical Center first reported hexamidine (**1**), a bis-benzamidine compound, with matriptase inhibitory activity ($K_i = 924$ nM), using virtual screening of the National Cancer Institute (NCI) database on a matriptase model constructed by homology modeling [50]. The docking model of the complex showed that the amidine at one end of **1** bound to the negatively charged D189 at the bottom of the S1 site *via* a salt bridge, while the distal amidine extended into the anionic site to interact with D96 or D60A (Fig. 2). The linker connecting the two benzamidine occupied the catalytic triad, and its length and rigidity played important roles in inhibition.

According to the binding mode of benzamidine with the matriptase catalytic domain obtained from the co-crystal

Table 1 Information of matriptase crystal structures from RCSB Protein Data Bank (PDB)

No.	PDB ID	Sequences[a]	Ligand	Note
1 [29]	1EAX, 1EAW	V16–V244	BPTI, benzamidine	The first binding mode of benzamidine in S1 subsite provides important information for the following small-molecule inhibitor development.
2 [41]	4JYT, 4JZ1, 4JZI	A: V16–V244	Pyridyl(bis(oxy))dibenzimidamide inhibitor	The typical small-molecule inhibitor with benzamidine group
3 [42]	4O97, 4O9V	A: V16–V244	Phenyl(bis(oxy))dibenzimidamide inhibitor	Phenyl substitution still remains inhibitory activity.
4 [43]	4R0I	A: V16–V244	*O*-(3-carbamimidoylphenyl) serine derivative	The amino acid scaffold is tolerant.
5 [44]	2GV6, 2GV7	V16–V244	(3-Carbamimidoylphenyl)propanoic acid derivative	Guanidine moiety is beneficial for inhibition.
6 [45]	4IS5	V16–V244	–	–
7	5LYO	A: A603–V855; B: G601–A613, G617–V855; C: A603–V855	–	–
8 [45]	4ISL, 4ISN, 4ISO	A: V16–V244	HAI-1	The Kunitz domain of endogenous inhibitor
9 [46]	3P8F, 3P8G	A: V16–V244	SFTI-1, benzamidine	The first co-crystal structure of SFTI-1 in complex with matriptase, illuminating structural foundation of peptide-based matriptase inhibitor design.
10 [47]	3BN9	V16–V244	Fab E2	The standard mechanism antibody-based inhibitor.
11 [48]	3NCL	V11–G153, T161–V258	4-Carbamimidoylbenzyl phosphonate analog	The probe molecule binding to the active pocket with the highest resolution
12 [49]	3NPS, 3SO3	V16–V244	Fab S4, Fab A11	Two potent antibody-based inhibitors exhibit different inhibitory mechanisms.

[a] The sequence is numbering refer to that of chymotrypsin except 5LYO

structure, Ramachandra's group [41] discovered pyridyl bis(oxy)benzamidine based on structure-guided design strategies. Combined with WaterMap calculations, the structure modification indicated that the naphthyl substitution improves the inhibitory naturally as in **2** (K_i = 40 nM, Fig. 2) and meanwhile, retains the selectivity to matriptase. The crystallographic binding mode of **2** revealed that the van der Waals interaction of naphthyl at S1′ subsite is a vital factor for its higher activity, and the selectivity comes from the unfavorable clash of the naphthyl with residues at non-conserved 60-loop regions from factor Xa (FXa) and thrombin.

Following analysis on the crystal structure composed of pyridyl bis(oxy)benzamidine and matriptase by Goswami et al. [42] suggested that the pyridyl nitrogen atom of **2** does not form any contact with matriptase, and there is sufficient space around the 4- and 5-position of the central pyridyl for further optimization. Hence, 1,3,5-trisubstituted benzenes (**3**) were designed as selective, potent matriptase inhibitors (Fig. 2). The crystallographic binding mode of **3** revealed that Q175 forms polar interaction with adjacent P173 to stabilize itself in a rotameric state, and as a result, the amino nitrogen atom of 4-aminocyclohexyl of **3** hydrogen bonds with the carbonyl of Q175. Additionally, a robust CH–π contact is formed between the cyclohexyl and W215. According to the bioactivity evaluation in severe combined immune deficiency (SCID) mice, **3** inhibited the median primary tumor growth obviously without any toxicity effect at the test dosage.

As mentioned above, the active center of matriptase contains several binding subsites that can be targeted by the cationic group of dibasic inhibitors, complicating the rational molecular design. Furtmann et al. proposed to introduce symmetry in bi- and tri-benzamidine inhibitors to limit the conformational space aiding in structure-based design [51]. Through this strategy, the meta-substituted oxyethylene derivative **4** with good inhibitory potency against matriptase (K_i = 38.4 nM) was designed. Comparative analysis on the binding

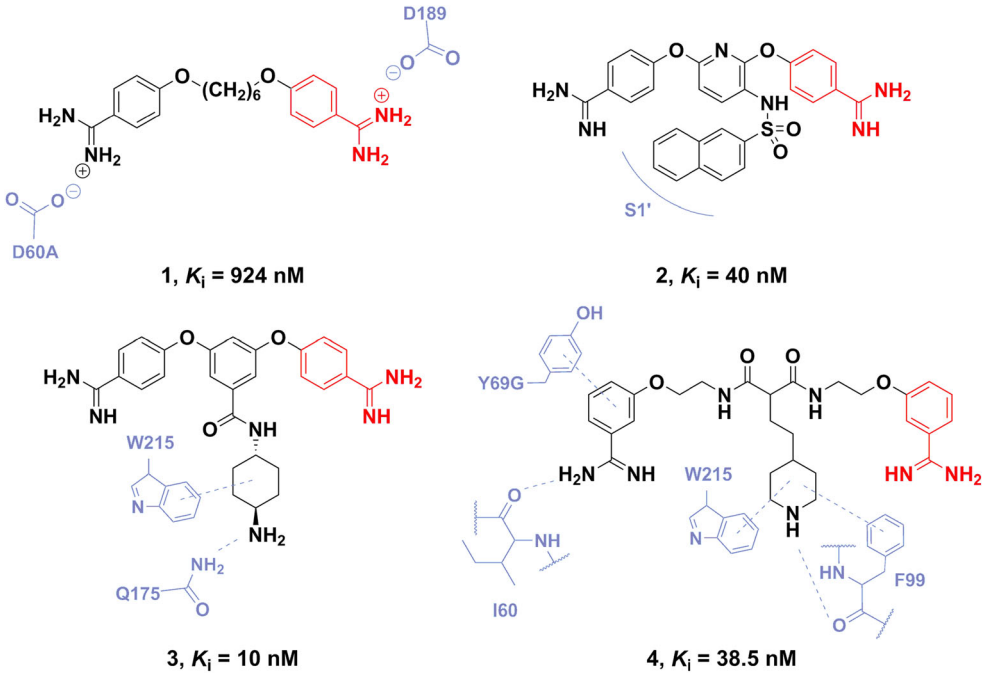


Fig. 2 Chemical structures of bis-benzamidine inhibitors and their interactions with matriptase active site residues

mode of **4** predicted by molecular docking and the one of similar pyridyl bis(oxy)dibenzimidamide inhibitor resolved by crystallography indicated that the enhanced inhibitory activity of **4** may be probably because the 2-(piperidin-4-yl)-ethyl branch occupies the S3/S4 pockets and forms a hydrogen bond with F99, while being in a suitable position to develop simultaneous cation–π interactions with F99 and W215. Besides, the distal benzamidine is directed into the third binding area above the S2 pocket and might form a hydrogen bond with I60 as well as being well-positioned for π–π stacking interactions to Y60G.

3.1.2 Sulfonylated 3-Amidinophenylalanines

3-Amidinophenylalanines were originally derived from a class of *S* configuration of uPA inhibitors. Compound **5** showed potent inhibitory activity against matriptase with a K_i value of 57 nM and was then used as a lead compound for subsequent structural modification [44]. The crystal structure of amidinophenylalanine derivatives bound to matriptase showed that the inhibitors occupy the active site *via* a Y-shaped conformation [44]. Molecular modeling suggested that the interaction of S2/S4 subsites with the sulfone group could be further enhanced through the substitution of bisaryl-3-sulfonyl (**6**) [44] which was supported by the high potency of **6** (K_i = 0.08 nM). However, the selectivity of **6** decreased due to the structural similarity with thrombin inhibitors [52].

It is noteworthy that the multibasic characteristic of **6** limits its oral bioavailability. To improve the oral bioavailability, the 2′,4′-dichlorobiphenyl moiety was introduced and led to compound **7** [53]. The introduction of 2′,4′-dichlorobiphenyl made **7** a dibasic inhibitor with a K_i value of 2 nM. The potency of **7** was attributed to its halogen-π interaction with W215 and polarized halogen-carbonyl contact with Q217 at S3/S4 binding pockets. The halogen-substituted bisaryl-3-sulfonyl derivatives had a high inhibitory activity not only on matriptase but also on thrombin (< 50 nM). In order to further improve the selectivity, Steinmetzer et al. [53] introduced urea into the C-terminal (of the phenylalanine scaffold) amide, based on the fact that the 60-loop of matriptase is more open than thrombin. Indeed, the inhibitory potency against matriptase was increased nearly 4500 times to thrombin. This may be due to the lack of suitable basic groups binding with the β-alanyl residue at the N-terminal of the compounds in thrombin S3/S4-binding pockets and the rigidity of cyclohexyl urea which prevents proper binding below 60-loop of thrombin. After further screening, the first selective monobasic matriptase inhibitor **8** (K_i = 2.7 nM) was obtained [53].

For the C-terminal optimization, in most cases, the activity of guanidine substituted compounds is stronger than that of other secondary amino group substituted compounds [54]. The eliminations of the C-terminal basic group decreased the inhibitory activity on matriptase. Using 4-piperidylbutanoic acid with opposite charge to replace the guanidine group, the activity of the inhibitor decreased obviously and the hydrophilicity of the molecule was also reduced [54]. Moreover, the oral bioavailability of the methylated one (prodrug strategy) was not significantly improved by the intragastric administration in mice as well. [54]

Ramachandra's group employed the fragment-linking method to discover *O*-(3-carbamimidoylphenyl)-L-serine amides (**9**) as matriptase inhibitor [43]. Structurally, these

inhibitors can be considered derivatives of phenol substituted to extend the P1 group of amidinophenylalanine. The co-crystal structure of **9** bound to matriptase suggested that the hydrophobic naphthyl of the inhibitor is enclosed between F99 and W215. To explore the effect of hydrophobic groups on inhibitory activity, 2,4,6-tri-isopropylphenyl was utilized to replace the naphthyl (**10**). The improved activity of **10** indicated that the tri-isopropylphenyl can better bind to the S4 subsite. In addition, inhibitor **10** significantly inhibited primary tumor growth in a DU-145 prostate cancer xenograft model *in vivo* [43] (Fig. 3).

3.1.3 1,2,4-Triazole derivatives

When screening for small, drug-like kallikrein inhibitors, Tan et al. identified 1,2,4-triazole derivative **11** (Fig. 4) as a covalent inhibitor against matriptase with IC_{50} value of 420 nM [55]. The 1,2,4-triazole moiety is a potential leaving group, and the carbonyl of the *N*-acyl triazole is able to be nucleophilically attacked with the hydroxyl group of S195. According to molecular docking calculations, the pyridine/phenyl and benzoyl rings of the inhibitor may occupy the S1 and S2 subsites, respectively. Due to the presence of F99 in matriptase rather than H99 in other trypsin-like enzymes, the benzoyl ring may preferentially bind to the S2 subsite *via* non-polar interactions [55].

3.1.4 Cyanodipheylarylamides

Cyanodipheylarylamides were first identified as a novel, specific, non-covalent kallikrein inhibitors after screening on the ChemBridge commercial compound library with a multistep protocol by Tan et al. [56]. Considering the fact that positively charged groups tend to cause a difficulty for oral administration and membrane penetration, they mainly focused on the low-affinity compounds without such well-known cationic groups in hitting set. This risky attempt dug out some novel chemical skeletons for matriptase inhibitors. According to the SAR analysis, the hydroxyl and negatively charged $-OCH_2COO^-$ substitutions were beneficial for matriptase inhibition (**12**, $IC_{50} = 75$ μM). Besides, compounds **13** and **14** with new scaffold also exhibited slight potency against matriptase. However, both inhibitors lack specificity. Thus, further structural optimization is needed to increase selectivity.

3.1.5 2-aryl/pyridine-2-yl-1H-indole derivatives

To discover the potent and selective inhibitors of hepsin, Goswami et al. [57] reported 2-aryl/pyridine-2-yl-1*H*-indole

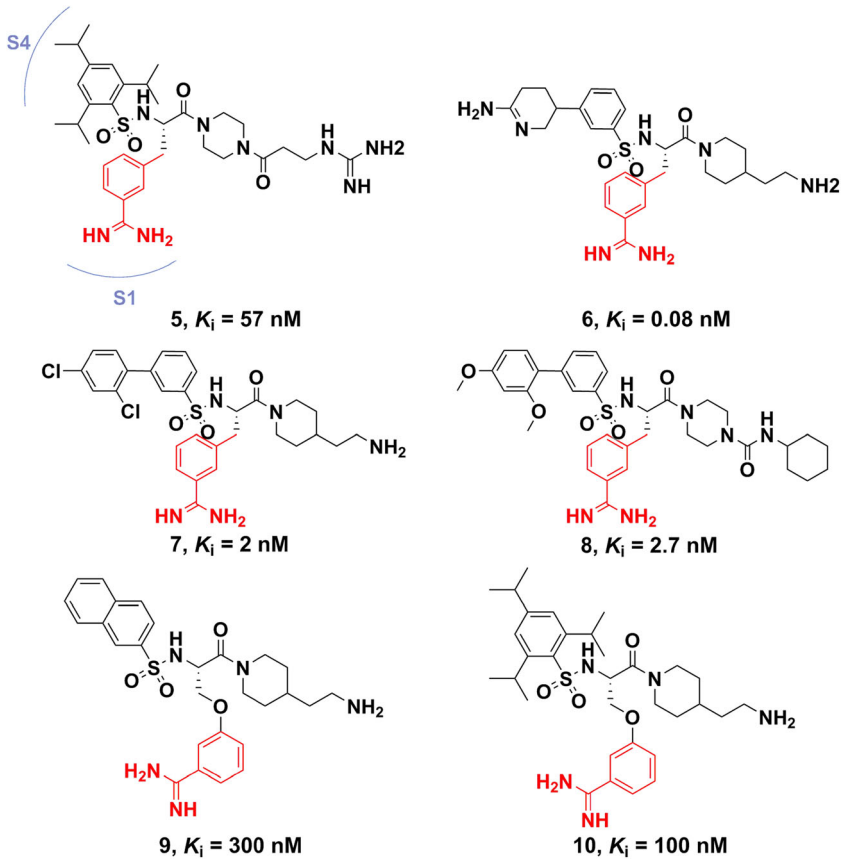


Fig. 3 Molecular structures of sulfonylated 3-amidinophenylalanine inhibitors

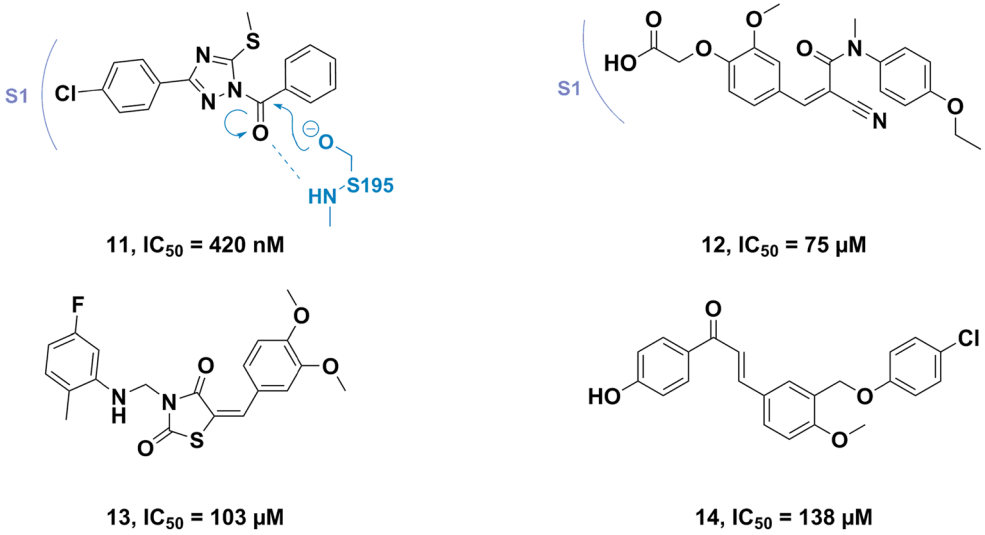


Fig. 4 Molecular structures of 1,2,4-triazole derivatives and cyanodipheylarylamide inhibitors

derivatives with moderate potency (micromolar inhibition constant) against hepsin. In this series, some of the derivatives inhibited matriptase with potency, e.g., compound **15** inhibited matriptase and hepsin with K_i values of 500 and 1400 nM, respectively. This may be due to the bulky piperidine group of the inhibitor which facilitates stronger van der Waals contact with S1′ pocket. Superimposition of the binding subsites of matriptase and hepsin indicated that the difference in potency is likely originated from the variation of residue at 99 where it is polar N99 in hepsin while hydrophobic F99 in matriptase. As a new chemical scaffold, 2-aryl/pyridine-2-yl-1*H*-indole is promising for further optimization to develop as potent and selective matriptase inhibitors.

3.1.6 Coumarin derivatives

Coumarin derivatives were developed as anti-tumor, anti-microbial, anti-inflammatory agents for its profound pharmacological activities. Tan et al. designed and synthesized a series of coumarin-3-carboxylate derivatives (Fig. 5) as the first suicide inhibitors of kallikreins and matriptase [58]. According to the chemical structure of coumarin-3-carboxylate ester, the protease active S195 could attack lactonic carbonyl to form the acyl-enzyme complex. After, the departure of the leaving group (i.e., $–CH_2Br$ in **16**) at the 6-position, a methylene quinone intermediate with high sensitivity to nucleophilic addition is generated and finally leads to inactivation of the protease. The 6-bromomethyl and aryl ester derivatives are the active and potent inhibitors against kallikreins and matriptase. The molecular docking model of **16** to matriptase revealed that the distance between the oxygen atom of the hydroxyl of S195 and the carbon atom of the carbonyl of the lactone was around 2.35 Å and the 6-bromomethyl directed toward H57, indicating a possible formation of covalent bonding ligand-protease complex.

3.1.7 Tetrahydropyrimidin-2(1H)-one analogs

Tetrahydropyrimidin-2(1*H*)-one analogs are the first non-peptide triplex inhibitors against matriptase, hepsin, and hepatocyte growth factor activator (HGFA) designed by Galemmo's group [59]. Based on the conserved structural features among the three serine proteases (i.e., D189 in the S1 subsite, G216 and W215 in the S4 subsite), the benzamidine, urea core, and lipophilic *N*-benzylpiperidyl were incorporated into the scaffold to form ionic interaction, hydrogen bond, and hydrophobic contact with the corresponding amino acids, respectively. SARs revealed that the cyclization of urea with six-membered ring improves the inhibitory activity (**17**). Compound **17** is the most potent inhibitor against matriptase in its analogs but much less potent for HGFA and FXa, indicating that it is a promising lead for the development of selective matriptase inhibitors.

3.2 Peptide-based and Peptidomimetic inhibitors

Peptide-based inhibitors are capable to occupy a large area of the active sites, and thus exhibit high selectivity to the targeted proteases. Moreover, due to the diversity of the 20 natural amino acids, the sequences of inhibitors can be engineered by changing the types of structurally critical residues to enhance their potency and selectivity. Furthermore, with the chemical decoration or the introduction of non-natural amino acids, the stability and pharmacokinetic properties of peptide-based inhibitors have been improved straightforwardly.

3.2.1 Sunflower trypsin inhibitor-1

Sunflower trypsin inhibitor-1 (SFTI-1), a potent serine protease inhibitor was first isolated in the late 1990s from the seed of the common sunflower *Helianthus annuus* by Luckett et al.

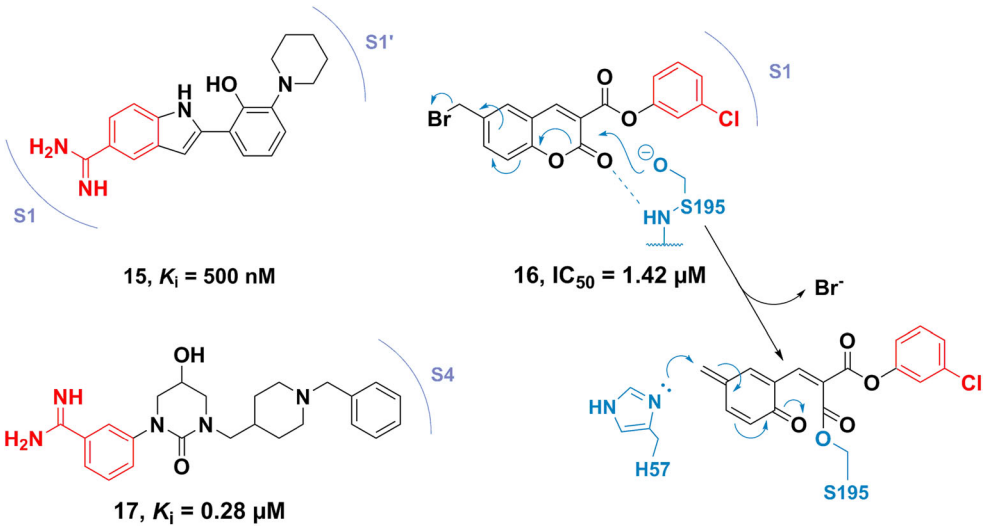
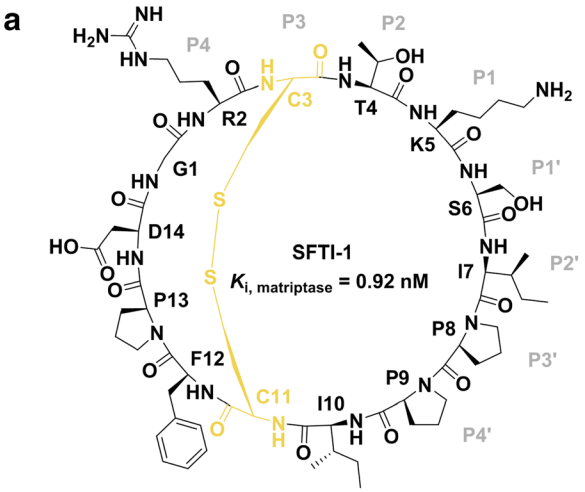


Fig. 5 Molecular structures of 2-aryl/pyridine-2-yl-1H-indole derivatives, coumarinic derivatives, and tetrahydropyrimidin-2(1*H*)-one analog inhibitors

[60]. It is a 14-mer bicyclic peptide, containing an antiparallel β-sheet with a single disulfide bridge between C3 and C11 (Fig. 6) [60, 61]. Long et al. [62] reported the synthesis and evaluation of the bioactivity of SFTI-1 against matriptase, in addition with the binding mode of SFTI-1 to matriptase *via* homology modeling and molecular docking. The experimental results showed that SFTI-1 selectively and efficiently inhibits the activity of matriptase, i.e., the K_i values for matriptase and thrombin are 0.92 and 5050 nM, respectively.

To analyze the SAR of SFTI-1, Roller's group [63] mutated R2, K5, I10, and F12 with different proteinogenic or non-proteinogenic amino acids such as Cit2 (2-amino-5-ureidovaleric acid), hArg2, Orn5 (2,5-diaminopentanoic acid), hLys5, Bip12 (L-4-biphenylalanine), and modified the disulfide bond. The SAR indicated that R2 and K5 were crucial for the inhibitory activity and could not tolerate any modification. In addition, the selectivity of SFTI-1 to matriptase inhibition was fine-tuned by I10 of the inhibitor, located at a



Mutations & K_i values	
R2Cit	22500 nM
K5R	4750 nM
I10Q	2330 nM
F12Bip	1000 nM
olefin bridge	25 nM
monocyclic SFTI-1 [*1,14*]	0.7 nM
SDMI-1	11.0 nM
SDMI-2	6.2 nM
H-KRCTKSIPPRCH-NH_2	8.4 nM

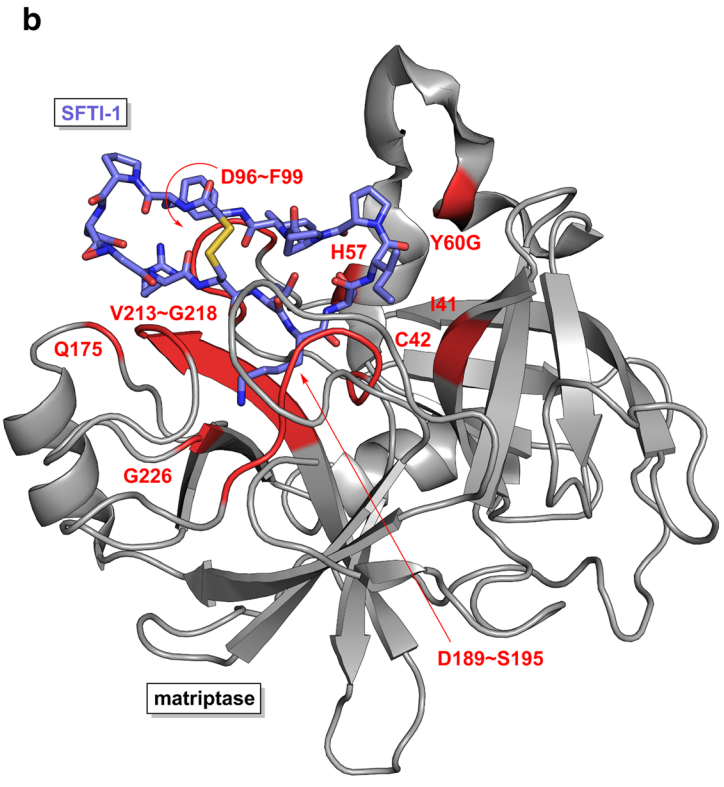


Fig. 6 Structure-activity relationship of SFTI-1 (**a**) and its binding mode with matriptase in the crystal structure (PDB ID: 3P8F). **b** The SFTI-1 is shown with slate stick model while matriptase with gray cartoon. The contact residues are highlighted in red

cavity in the proximity of the 60-loop of matriptase. Furthermore, the mutation of F12 decreased SFTI-1 binding affinity with matriptase. The disulfide bond tolerated only minor changes (i.e., olefin, ethylene, 1,5-, or 1,4-disubstituted 1,2,3-triazole bridge), which is crucial for its inhibitory potency and selectivity [63–66].

With the aid of molecular dynamics simulations, Empting's group [65, 67] demonstrated monocyclic SFTI-1 [1, 14] possessing slightly higher potency against relevant protease compared with its bicyclic counterpart ($K_i = 0.7$ nM). After three-step optimization (increments, combination, and truncation) of monocyclic SFTI-1 [1, 14], the same group showed that the I10R/F12H mutations (SDMI-1, $K_i = 11.0$ nM) and the truncation of conformational constraints at C-terminal P13 and D14 are beneficial for the improvement of inhibitory affinity (SDMI-2, $K_i = 6.2$ nM), due to the formations of additional hydrogen bonds with matriptase. Based on the open-chain derivative of SFTI-1, Empting et al. designed novel sequences and found that the mutation of G1K provided an additional addressable site ensuring the introduction of tailor-made functionalities, and meanwhile led to a potent matriptase inhibitor (H-KR**C**TKSIPPR**C**H-NH_2, $K_i = 8.4$ nM) [68]. Moreover, compared with the bicyclic variants, the truncated SDMIs provided similar inhibitory potency, which suggested that the C-terminal amino acids are not required for the efficient interaction with matriptase. The alanine scanning results further indicated that positions at 2, 4, 5, 6, and 14 of SFTI-1 were essential to act inhibitory activity [69].

In the subsequent work, Swedberg's group [70] elucidated that the P2′ residue of SFTI-1, I7, has a strong influence on inhibitory potency and selectivity. The I7D mutation of truncated non-cyclic peptide shows a 350-fold decrease in activity to trypsin, 196-fold selectivity over plasmin, compared with the wild-type SFTI-1.

3.2.2 Cystine-knot inhibitor

Cystine-knot peptides, also known as knottins, consisting of about 30 to 40 amino acids, are compacted by three disulfide bonds which form a mechanically interlocked structure. The cystine-knot motif displays an exceptional structural and thermal robustness [71]. The MCoTI-II (Fig. 7a), isolated from the *Momordica cochinchinensis* strongly inhibited matriptase ($K_i = 2.8$ nM) [69]. Cellular assays further suggested that MCoTI-II effectively inhibits the re-establishment of tight junctions and epithelial barrier function in MDCK-I cells, exactly as the function that matriptase performs in epithelial integrity regulation, without inhibition of matriptase-dependent proteolytic activation of prostasin [72]. According to the alanine scanning experiments, Quimbar and coworkers [69] found that most of the alanine mutations decrease the inhibitory activity of MCoTI-II against matriptase. However, V3A substitution in MCoTI-II maintained its potency to matriptase but not to trypsin. The V3R mutation further led to the enhanced potency with a K_i value of 290 pM, due to that the increase of buried surface area of MCoTI-II variant complexed with matriptase and the establishment of electrostatic contact with D189 at the S1 pocket in matriptase. Subsequently, MCoTI-II library design and screening studies found that some mutations at positions 1–3, 7–10, 24, and 25 are also beneficial for the improvement of the inhibitory potency against matriptase [73]. The open-chain MCoTI Var. 4 strongly inhibited matriptase with a K_i value of 0.83 nM [73]. In addition, another cystine-knot inhibitor SOTI–III (Fig. 7b), isolated from *Spinacia oleracea* was a less potent inhibitor of trypsin and had no inhibitory effect on matriptase. However, after double mutations at R29 and R32, the inhibitor (SOTI Var. 1) exhibited potent inhibition on matriptase with a K_i value of 28.9 nM [73].

3.2.3 Kempopeptin

Marine natural products are powerful resources for the discovery of lead compounds. Recently, Al-Awadhi and coworkers [74] reported that two new cyclic depsipeptides, kempopeptins B and C (Fig. 8), isolated from a cyanobacterium *Lyngbya* sp., had potent inhibitory activity against matriptase with K_i values of 1.83 and 0.28 μM, respectively. The molecular docking results indicated that the potency difference between the two inhibitors may be attributed to the larger size of bromine atom of kempopeptin B than the chlorine of kempopeptin C. The halogen atom of the former may point toward the binding pocket, leading to a steric hindrance and a reduction of the binding affinity to matriptase. In addition, kempopeptin bears a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety, and enzyme inhibition studies [74, 75] indicated that such moiety and its adjacent amino acid are closely related to the inhibitory selectivity to serine proteases (Fig. 8).

3.2.4 Ecotin

Ecotin is an effective macrobiomolecular inhibitor of serine protease with a dimeric fold specificity, produced by *Escherichia coli* [76]. Ecotin can cover both conserved active site and non-conserved regions of target proteins due to its large contact area (Fig. 9). Thus, it often exhibits excellent inhibitory activity against serine proteases. Craik's group [77] used ecotin as a precursor to improve its selectivity to serine protease by mutagenizing all four contact loops (the 50, 60, 80, and 100 s). When the mutations were concentrated in the 60, 80, and 100 s, the inhibitory activity against matriptase increased, such as the MT-6 variant (W67R, G68W, D70Y, Y71I, V81R, M84K, M85R, A86G, R108S, and K112N). The inhibitory activity of the latter against matriptase was 66 pM, 4-fold over the wild-type ecotin, and its activity against FXa, FXIIa, and Pkal decreased by 2 to 4 magnitudes. Therefore,

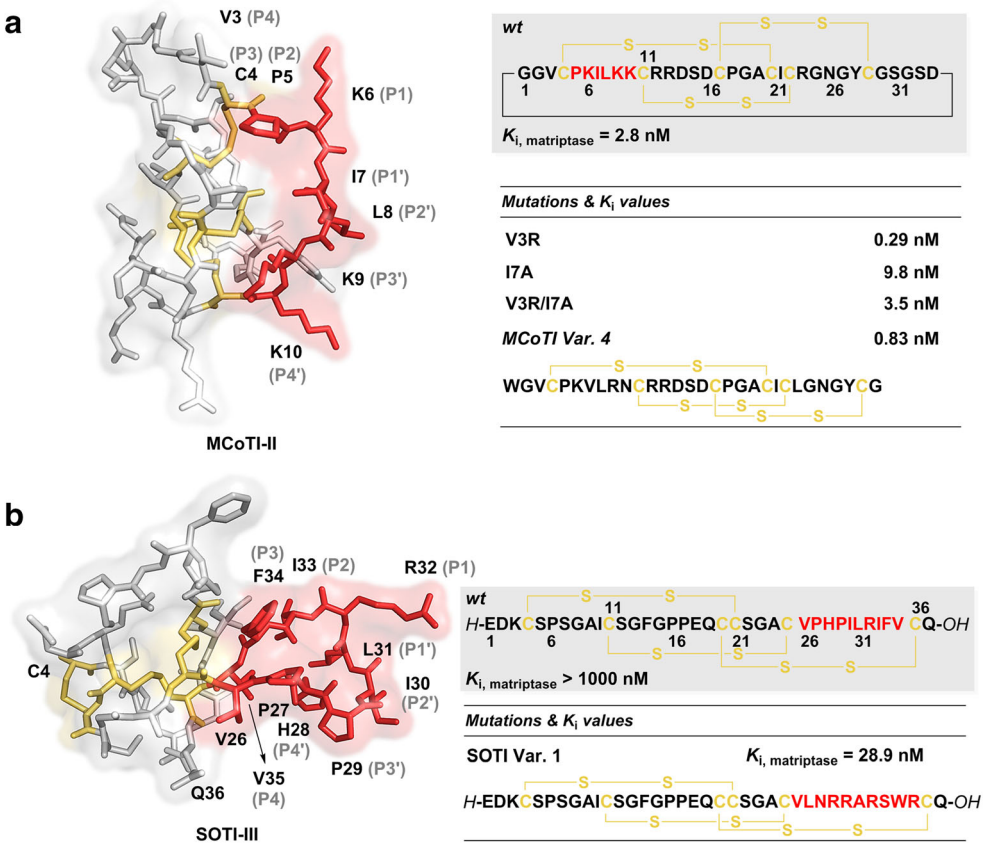


Fig. 7 Structures and structure-activity relationships of MCoTI-II (**a**, PDB ID: 4AOR) and SOTI-III (**b**, PDB ID: 4GUX)

optimization of ecotin-binding loop is an effective strategy for the development of specific matriptase inhibitors.

3.2.5 Eglin c

Originally, as an elastase polypeptide inhibitor consisting of 70 amino acids with a molecular mass of 8.1 kDa, eglin c was isolated and purified from the medicinal leech *Hirudo medicinalis* in the 1980s (Fig. 10) [78]. The P42-Y49 residues of eglin c correspond to the P4–P4′ sites, respectively, which determine the bioactivity of serine protease inhibitor. Désilets and colleagues [79] constructed an eglin c Y49X (P4′) library and employed it for the screening of matriptase inhibitor. The P42R/L45R-eglin c variant exhibited potent inhibitory activity toward matriptase with a K_i value of 26 nM in contrast with the non-active wild-type one. To further improve the potency and selectivity of eglin c, mutant on P4′ residue (Y49) was generated based on the above double variant. The triple mutant (P42R/L45R/Y49K-eglin c) exhibited improved inhibitory activity of 4-fold with a K_i value of 6.1 nM.

Further optimizations of the inhibitory loop of eglin c found that the L45R/Y49K-eglin c was the most potent and selective inhibitor against matriptase with a K_i of 4.5 nM and had little inhibitory activity to furin and human airway trypsin-like

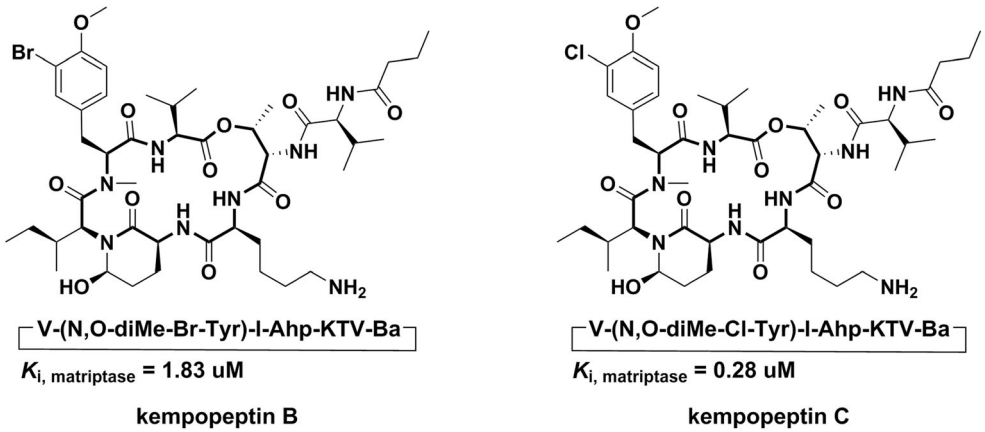


Fig. 8 Structures and K_i value of kempopeptins B and C

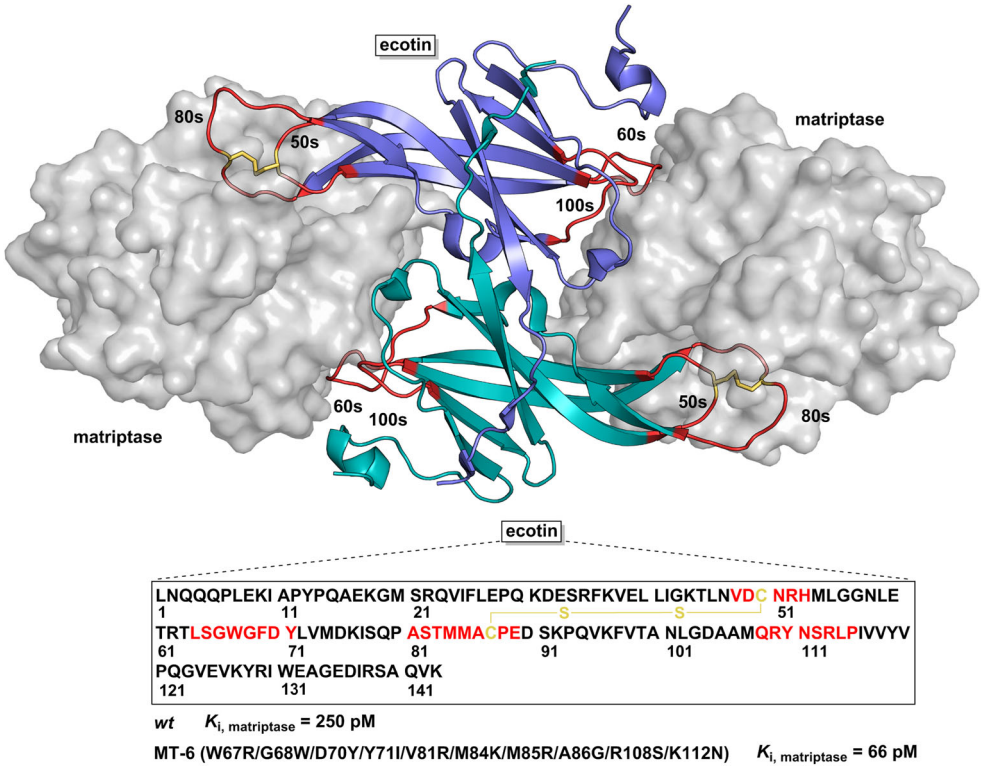


Fig. 9 The ecotin-matiptase complex model constructed *via* superimposing crystal structure of matriptase (PDB ID: 4JZI) onto the ecotin-FXIa complex structure (PDB ID: 1XX9)

protease [79]. Contrary with other tri-mutants, after incubated with matriptase over 5 h, L45R/Y49K exhibited virtually no cleavage, which indicated high stability of L45R/Y49K. Molecular modeling suggested that L45R (P1) mutation resulted in the favorable electrostatic interaction between R45 of the inhibitor and D189 of matriptase, which is more important in the stabilization of the enzyme-inhibitor complex than the hydrophobic contact with L45. Additionally, Y49K mutation allowed one more hydrogen bond with Q38 of matriptase to further enhance the stability of the complex.

3.2.6 Peptidomimetic inhibitors

Mimicking the structures of peptide inhibitors with chemical libraries is a powerful strategy for the discovery of lead compounds. Application of such strategy resulted in the development of peptidomimetic matriptase inhibitors with enhanced activity, selectivity, and stability [80–85]. CVS-3983, designed by Galkin and coworkers is the first reported selective and potent peptidomimetic inhibitor of matriptase (K_i = 3.3 nM). It significantly suppressed the growth of aggressive prostate cancer in nude mice models [80]. However, CVS-3983 failed in preventing activation of prostasin, a downstream physiological substrate of matriptase, in HaCaT human keratinocytes, which indicated targeting the free active matriptase only might limit the potential utility of SPD inhibitors to control matriptase function [86]. Later, Marsault's group [81] designed a tetrapeptide based on the P4–P1 positions (Arg-Gln-Ala-Arg) of the activation peptide sequence of matriptase by adduction of a serine trap, ketobenzathiazole

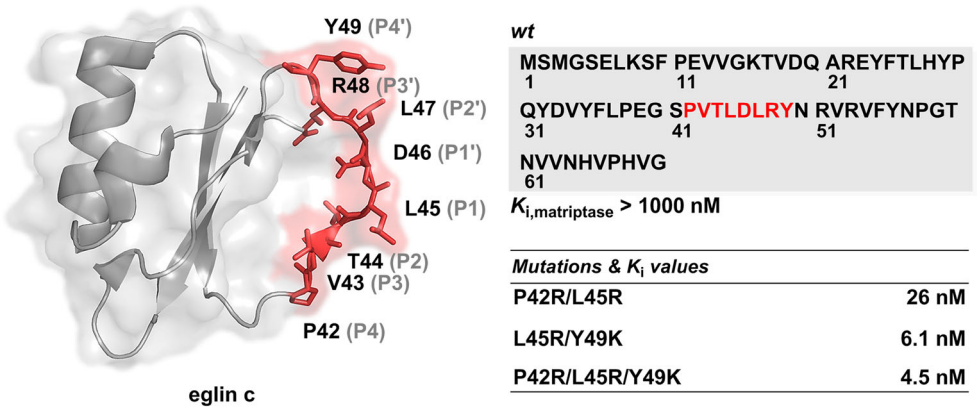


Fig. 10 Structure and structure-activity relationship of eglin c (PDB ID: 4H4F)

(kbt) group at C-terminal. The kbt group was functioned to form a reversible covalent bond with the active serine residue in the catalytic triad of matriptase. Molecular docking results indicated that the arginine at P1 was stabilized by a network of hydrogen bonds, the alanine at P2 laid over F99 separating the S2 and S4 pockets, the residue at P3 bridged over the arginine at P4 to interact with Q192, and the P4 arginine interacted with D217 and Q175. The Arg-Gln-Ala-Arg-kbt displayed excellent inhibitory potency to matriptase with a K_i value of 11 pM as well as high selectivity relative to other trypsin-like proteases, such as matriptase-2, hepsin, thrombin, and furin. With the aid of binding-free energy calculations and the druggability analysis, Arg-Gln-Ala-Arg-kbt-based single mutants, Arg-Gln-Pro-Arg-kbt and Ser-Gln-Ala-Arg-kbt were also identified as potent and selective matriptase inhibitors, i.e., their K_i values were 61 and 92 pM against matriptase, and they were 128- and 378-fold more selective for matriptase over matriptase-2, respectively [82] (Fig. 11).

To target three cancer progression and metastasis-related proteases (matriptase, HGFA, and hepsin), Janetka's group [87] designed acetyl (Ac) and ketothiazole (kt)-containing HGFA peptidomimetic inhibitors, Ac-Lys-Arg-Leu-Arg-kt, Ac-Ser-Lys-Phe-Arg-kt, and Ac-Ser-Trp-Leu-Arg-kt. These peptidomimetics inhibited matriptase (K_i = 1.1, 3.0, and 69 nM, respectively) and hepsin with similar potencies. Biochemical assays further demonstrated that they were capable to block c-MET phosphorylation and related cancer cell signaling [87]. The same group [83] then reported Ac-Lys-Gln-Leu-Arg-kbt and Ac-Ser-Lys-Leu-Arg-kbt as triplex inhibitors against HGFA, matriptase, and hepsin, which blocked the c-MET and tyrosine kinase receptor (recepteur d'origine nantais (RON)) signaling pathway. Based on the scaffold of Ac-Lys-Gln-Leu-Arg-kt, Kwon et al. [84] evaluated the truncated forms of the peptidomimetics as hepsin inhibitors, and it was found that the minimal inhibitory unit was the dipeptide Leu-Arg. The Ac-Leu-Arg-kbt inhibited hepsin and matriptase with K_i values of ~ 3.0 and ~ 200 nM, respectively (Fig. 12).

Recently, Steinmetzer's group [85] developed two new D-hPhe/Asp-hTyr-Ala-4-amidinobenzylamide peptidomimetic inhibitors for matriptase. They were derived from a poorly selective thrombin and FXa inhibitor, benzylsulfonyl-D-Arg-Pro-4-amidinobenzylamide using molecular modeling methods. Both peptidomimetics inhibited the activity of matriptase with K_i values of nanomolar level; however, they still had modest potencies with trypsin and thrombin (Fig. 13).

3.3 Antibodies

Craik's group [88] screened a single chain fragment variable (scFv) library and obtained two potent anti-matriptase antibodies, scFv E2 and S4, with K_i values of 50 and 590 pM, respectively. Further inhibitory mechanism studies *via* alanine scanning of the loops around active site [89] revealed that both

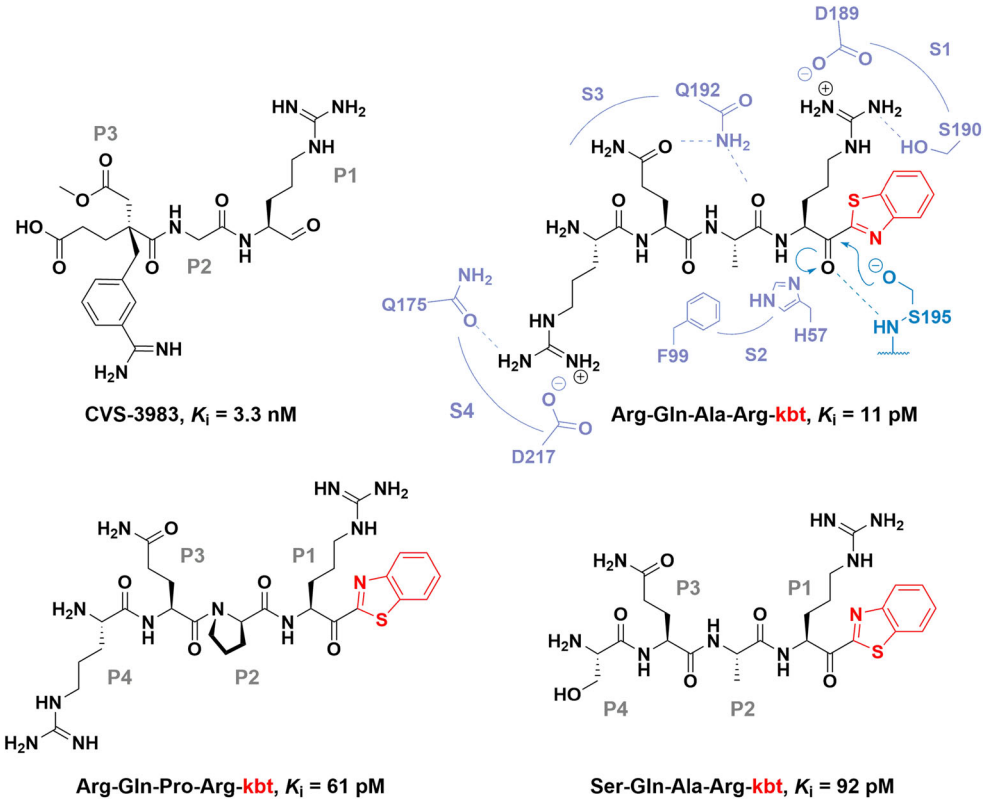


Fig. 11 Structures of CVS-3983 and Arg-Gln-Ala-Arg-kbt-based peptidomimetic inhibitors

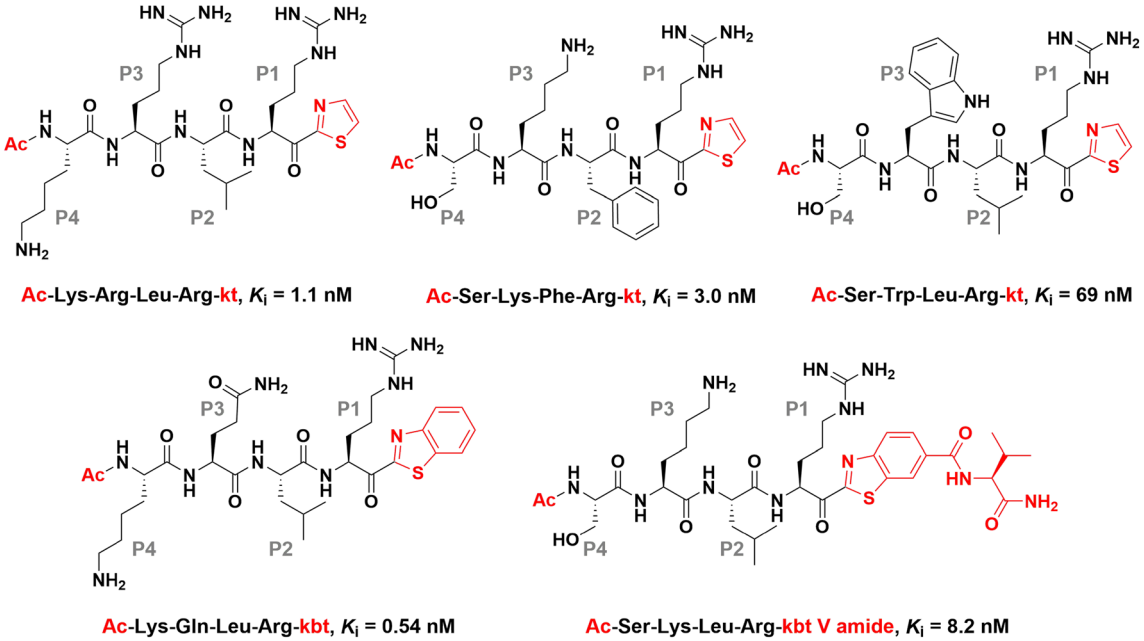


Fig. 12 Structures of Ac-Arg-Gln-Ala-Arg-kbt variants

antibodies interacted with the residues flanking the active site, which formed a unique binding epitope. Especially, E2 exhibited as a standard mechanism (also known as canonical or Laskowski mechanism) inhibitor of matriptase, that is, like SFTI-1, eglin c, etc., E2 also functions by being extremely slowly hydrolysable substrates which blocking the active site. The co-crystal structure of Fab E2 in complex with matriptase showed that the third complementarity determining region loop on the heavy chain (CDR-H3) of Fab E2 bound to the active site of matriptase through a catalytically non-competent manner. Specifically, the guanidino group of the RH100b (Kabat numbering scheme) of Fab E2 formed a hydrogen bond with S190 in the S1 pocket and indirect contact with D189 *via* a water molecule, which is different from the binding mode of benzamidine-based inhibitors of matriptase. Interestingly, they found that replacing the scFv E2 with fragment antigen-binding (Fab) scaffold not only improved the inhibitory potency (K_i = 15 pM) but also led to a distinct mechanism [47].

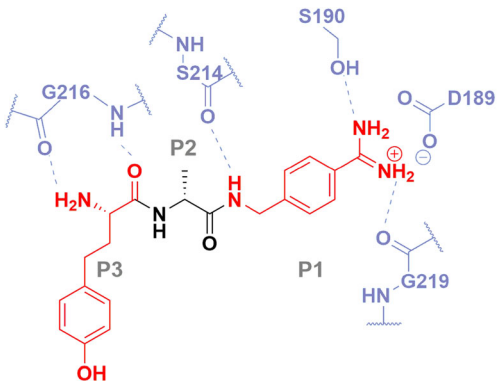


Fig. 13 Structures of D-hTyr-Ala-4-amidinobenzylamide

Craik and colleagues [49] also reported a Fab antibody-based matriptase inhibitor, A11 with K_i values of 720 pM. Although the buried area of CRD-H3 loop of Fab A11 in the binding site cleft was ~40% less than that of Fab E2 and S4, the RH99 residues of CRD-H3 loops from Fab E2, S4, and all formed stable salt bridges with Q175 in the S3 subsite of matriptase. Considering the dysregulation of matriptase with HAI-1, the increasing proteolytic activity of matriptase might be exploited for imaging purpose. They found the fluorescently labeled E2 and A11 could be applied for the malignant tumor detection [90].

Finally, what should not be neglected is that the matriptase's endogenous inhibitor, HAI-1 has excellent activity against tumor growth and metastatic nodule formation. Therefore, engineering HAI-1 with antibody could be a shortcut in the inhibitor development. Much recently, Mitchell and coworkers [91] designed a potent matriptase inhibitor engineering the Kunitz domains 1 (KD1) and 2 (KD2) of HAI-1 fused with Fc domain of an antibody. The KD1-KD2/1-Fc inhibited matriptase with a K_i value of 70 pM. This work provided a smart strategy for the design of antibody-based matriptase inhibitor.

4 Conclusion

The strong invasion and metastatic ability of cancer is an essential factor leading to its high recurrence rate. Matriptase is an upstream target in the degradation of extracellular matrix

by tumor cells and is also one of the most interesting cases for illustration of the mechanistic challenge on the strategy of drug development. Inhibition of its activity can significantly reduce cancer invasion and metastasis. Since the first report, matriptase inhibitors have been extensively studied, including small-molecule, polypeptide, and antibody inhibitors.

As far as the reported small-molecule inhibitors are concerned, most of them are competitive inhibitors, and some of them contain benzamidine moiety which binds D189 in the S1 subsite to improve molecular selectivity. Polypeptide and protein inhibitors are mainly represented by SFTI-1. After mutation and side-chain modification, single-chain SFTI-1 derivatives with excellent activity and potential for development are obtained. In addition, as a kind of specific small-molecule inhibitors, peptidomimetic inhibitors could enhance the selectivity with the P1 arginine, and the modification of the "serine trap" kb or kbt group is beneficial for improving the inhibitory activity. The antibody-based matriptase inhibitors are also competitive inhibitors, and the most potent inhibitors with picomolar K_i values can be obtained by fusion strategy with Kunitz domain of cognate inhibitor HAI-1. Among these inhibitors, peptide- and antibody-based inhibitors have higher potency and selectivity than small-molecule ones due to their larger contact area and better recognition with matriptase, especially the latter seems to provide a new avenue to overcome the cross-inhibition problem due to the high structural homology of the SPD. However, from the perspective of molecular pharmaceuticals, the bioavailability and metabolic problems of peptides and antibodies are often lower than those of traditional small-molecule inhibitors [92]. Also, dosage forms of small-molecule inhibitors are easier to design, and the cost of production is usually lower than biological agents.

In this review, the discovery, structural features, and SARs of matriptase inhibitors were summarized. According to the co-crystal structures of matriptase-inhibitor complex, most of the reported inhibitors bind to S1–S4 and S1′ subsites, some of which can form polar interactions with 60-loop, but few of them bind to S2′/S3′ subsite. Essentially, the unique structure of 60-loop and its highly negative potential characteristics in the active pocket of matriptase provide a promising basis for the design of specific inhibitors with improved the selectivity to matriptase. The binding pattern between matriptase and the cognitive inhibitor HAI-1 KD1 suggests that the interactions with 60-loop are beneficial to increase the selectivity and inhibitory potency of KD1. In fact, in the design of inhibitors of SPD such as uPA and thrombin, there is no shortage of reports on improving selectivity and activity by binding to 60-loop [93–96]. However, as for matriptase inhibitors, this factor seems to receive less attention. Therefore, when designing a matriptase inhibitor by a substituent, in addition to considering whether it can form interactions with some key residues, the influence on the molecular electrostatic potential should also be concerned. Moreover, the binding mode of peptide phosphonate inhibitor reported by Brown et al. [48] provides a structural basis for designing inhibitors that may bind to S2′/S3′ subsite. We also found that the skeleton diversity of matriptase inhibitors is too weak. To address this issue, the scaffold hopping, bioisosterism and other techniques can be applied. Based on phytochemistry, marine organisms, or microbial metabolites, more different chemical skeletons are often provided. It is worth noting that after zymogen activation, the active matriptase is temporally coupled with the inhibition of the nascent active matriptase by HAI-1. While a small proportion of nascent active matriptase is rapidly shed into the extracellular milieu and escapes from the inhibition of HAI-1, these escapees can be inhibited by the abundant serine protease inhibitors in the interstitial fluids. Coupling the activation of matriptase zymogen with matriptase substrate activation may lead to new breakthroughs in the development of matriptase inhibitors. The zymogen activation targeting inhibitors reported by Xu et al. [86] is a good case. By molecular design for the structure domain outside the catalytic activity domain of matriptase, unforeseen gains may be obtained.

Unfortunately, as of now, no matriptase inhibitors have been entered clinical trials. One likely reason is that the higher off-target effects of serine protease inhibitors caused by the conserved structures across SPD domain are not limited to matriptase inhibitors. Although some molecules demonstrated inhibitory potency against matriptase hundreds fold higher than that against other proteases in the S1 family, such as hepsin, trypsin, thrombin, etc., they still exhibit nanomolar inhibitory activities on some of these non-target proteins. Accordingly, successful development of anti-protease drugs, especially the ones targeting the catalytic domains, has been slowing down. The clinical failure of the MMP inhibitors is largely due to this issue [97]. Another important reason can be attributed to the fact that matriptase is widely expressed in various organs [22–24] and are critical for the organ developments, such as epidermis, stomach, kidney, etc. The matriptase-deficient mice could not survive in the embryo period [13]. Therefore, although the inhibition of tumor invasion and metastasis against matriptase has achieved good results in experimental studies, the accompanied pathological incidences are still unclear. That might be the reason why matriptase is not a favorable target for pharmaceutical companies. These two issues indicate that while designing specific matriptase inhibitors, we should also consider the means of reducing the adverse effects. More studies on the biochemical functions and mechanisms are needed. As long as these issues are solved, matriptase inhibitors would become a sharp weapon in anticancer therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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