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REVIEW ARTICLE

Recent Advances of Hepsin-Targeted Inhibitors

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Abstract: Hepsin is a type II transmembrane serine protease (TTSP) that plays a crucial role in cell growth and development. Hepsin is highly expressed in prostate cancer (PCa) and associated with its progression and metastasis. Therefore, it has been considered as an attractive biomarker of PCa. Recently, low molecular weight inhibitors targeting hepsin have been developed. Based on the key chemical scaffold, they can be classified into four classes: Indolecarboxamidines, benzamidines, peptide-based analogs, and 2,3-dihydro-1*H*-perimidines. In this review, we discuss design strategy, structure-activity relationship (SAR), and binding mode of the four classes of hepsin inhibitors.

Keywords: Hepsin, prostate cancer, type II transmembrane serine protease, structure-activity relationship (SAR), amidine, peptides.

1. INTRODUCTION

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-related death among men in the USA [1-6]. When PCa is localized clinically, current treatment regimens such as surgery, chemotherapy, testosterone suppression, and radiation are effective in treating PCa. However, once PCa is metastasized to other organs such as bone and lymph nodes, it is difficult to control and is almost uniformly fatal [7, 8].

Hepatocyte growth factor (HGF) plays a key role in cancer progression. It is secreted by tumor-associated fibroblasts [9] as an inactive pro-HGF form. Trypsin-like serine proteases including hepsin, matriptase, and HGF activator (HGFA) cleave pro-HGF to produce HGF [10-18]. HGF binds to its cognate receptor tyrosine kinase MET which leads to the activation of downstream signaling pathways of cell survival, proliferation, and invasion of malignant cells [19]. Inhibition of HGF activation has been investigated as a potential therapeutic intervention of tumors. In cancer cells, HGF-activating proteases including hepsin, matriptase, and HGFA are upregulated, thus converting pro-HGF into active HGF [20-23]. Inhibitors of these proteases might block the cleavage of pro-HGF and inactivate HGF/MET signaling pathways. Moreover, endogenous inhibitors of HGF activation, HAI-1/2, inhibit the action of hepsin, matriptase, and HGFA [17, 18, 24, 25]. Elevated HGF level and reduced HAIs are closely associated with poor survival of patients with advanced cancers [26-32].

Type II transmembrane serine protease (TTSP) is a subgroup of trypsin-like serine protease. It is a membrane-anchored protein with an *N*-terminal cytosolic domain, a hydrophobic membrane-spanning region, and an extracellular domain. Hepsin belongs to the TTSP family that includes matriptase, enteropeptidase, corin, human airway trypsin-like protease (HAT), and TMPRSS2 [33]. Originally identified in the liver, hepsin is predominantly expressed in normal human liver and kidney [34]. It is also highly expressed in PCa, ovarian cancer, and renal cell carcinomas [35-37]. In particular, it is overexpressed in PCa as compared with benign prostatic hyperplasia (BPH) [22, 38-41]. Hepsin mRNA levels are elevated in about 90% of prostate cancers, with 10-fold higher levels in metastatic prostate cancers than that in normal prostate or BPH [21, 39, 42, 43]. It has been demonstrated that hepsin is correlated with prostate cancer progression and metastasis

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in vivo [44]. Hepsin plays a key role in promoting the metastasis of primary non-metastasizing prostate tumors to the bone, liver, and lung at *in vivo* animal studies [35, 36, 45, 46]. Hepsin is also amplified in other cancers including ovarian, lung, and breast cancers [47]. Furthermore, hepsin is involved in the disruption of basement membrane components and cell motility [44]. Therefore, hepsin has been considered as a potential biomarker of PCa metastasis and prognostic factor for predicting PCa progression [39, 48].

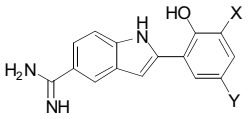
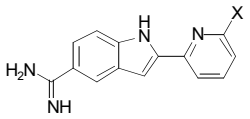
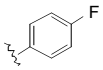
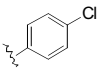
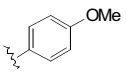
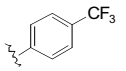
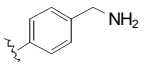
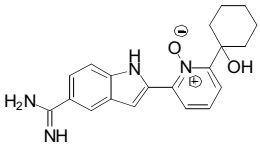
There have been a number of reports on the role of hepsin in cancer progression and metastasis up to date [35, 40, 44-46, 49, 50]. In this review, we focus on recent development of low molecular weight (M.W.) hepsin inhibitors despite recent progress in antibody-based hepsin inhibitors [51, 52]. Based on the core scaffold structure, low M. W. hepsin-targeted inhibitors are classified into four classes: Indolecarboxamidines, benzamidines, peptide-derived analogs, and 2,3-dihydro-1*H*-perimidines.

To compare the relative *in vitro* hepsin activity of inhibitors, the values of K_i and IC_{50} are used throughout this paper. In general, fluorescent enzyme assays of hepsin are performed by using Boc-Gln-Ala-Arg-AMC or PyroGlu-Pro-Arg-*p*NA as chromogenic substrate in order to determine the IC_{50} values. K_i values are calculated using the Cheng and Prusoff equation ($K_i = IC_{50}/(1 + [S]/K_m)$) [53]. The final chromogenic substrate concentration for the assays was at K_m (about 150~200 μM) for hepsin. It is recommended to refer to the original articles to obtain details of K_i and IC_{50} values.

2. INDOLECARBOXAMIDINES

The physiological substrate-binding sites of TTSPs consist of successive residues before the scissile amide bond (S4, S3, S2, S1) and those after it (S1', S2', S3', S4') [11, 54]. Hepsin substrates exhibit strong preference for Arg at P1 position. They favor Asn, Thr, or Leu at P2, Lys or Gln at P3, and Lys or Pro at P4 position [11]. In P1'-position, Ile or Val is a preferred amino acid residue. Hepsin forms a catalytic triad by three amino acid residues (Ser, His, and Asp) which are responsible for the hydrolysis of amide bond between P1 and P1'. Ramachandra and co-workers reported that 2-pyridine-2-yl/aryl-1*H*-indole derivatives substituted with amidine moiety for binding of S1 pocket are hepsin inhibitors [55]. During focused high throughput screening (HTS) of amidine-containing molecules, 1*H*-indole-5-carboxamidine was found to be a weak hepsin inhibitor with a K_i value of 500 μM [56, 57]. This compound was reported to have weak inhibition activity against serine proteases such as plasmin, urokinase, thrombin, and trypsin in the early 1980s [58]. The binding mode of 1*H*-indole-5-carboxamidine was similar to that of 2-(2-hydroxyphenyl)-1*H*-benzoimidazole-5-carboxamidine which was crystallized with hepsin (PDB ID: 1P57) [59]. Based on modeling studies [59], it was assumed that the amidine group of 1*H*-indole-5-carboxamidine shares similar binding poses with other reported amidine-based serine protease ligands by interacting with amino acids Asp-189 and Gly-219 in the S1 subpocket [60, 61]. As shown in Table **1**, compound **1** substituted with phenolic group at C-2 position of the indole ring showed 14-fold improved hepsin affinity (K_i = 35 μM) as compared to 1*H*-indole-5-carboxamidine itself. Compound **2** with an isopropyl group at X position of Class A (Table **1**) showed 7-fold stronger hepsin inhibition than compound **1**. Compound **3** with a chloro group at Y position showed similar hepsin inhibitory activity compared to compound **2**. Modifications of compound **1** by introducing piperidine at the X-position resulted in enhanced interaction with the S1' site of hepsin [56]. Compound **4** exhibited improved hepsin-binding affinity with a K_i value of 1.4 μM. However, it showed undesirable metabolic stability (MLM stability: 38% in 20 min, 22% in 40 min). Replacement of phenol moiety of compound **4** with a pyridine ring (compound **12**) increased its hepsin affinity (K_i = 0.6 μM) with improved metabolic stability in liver microsomes, implying that the OH group of the phenol moiety in compound **4** is associated with its interaction with hepsin and metabolism by liver. Replacement of the piperidine ring of **12** with a *tert*-hydroxyl cyclohexyl ring (compound **13**) resulted in increased binding affinity for hepsin (K_i = 0.1 μM). Compound **14** with a fluoro group showed reduced hepsin affinity compared to compound **13**, implying the importance of the OH group in compound **13**. X-ray crystal structure of hepsin in complex with compound **13** elucidated that the *tert*-hydroxyl group could interact with His-57, one of key residues in the catalytic triad of serine protease. However, the nitrogen of the pyridine ring was located ~4.5 Å away from the catalytic residue Ser-195. To achieve optimal interaction with Ser-195, *N*-oxide group was introduced in compound **13**, resulting in the potent hepsin inhibitor **15** with high selectivity for hepsin over other serine proteases such as matriptase, trypsin, and Factor Xa [56]. Compounds **13** and **15** inhibited the invasion and migration in hepsin-expressing LNCaPC42B4 cell lines, indicating that they are potentially lead compounds for the development of therapeutic agents for PCa.

Table 1. Inhibitory activities of 1*H*-indole-5-carboxamidine analogs against hepsin.

 Class A			 Class B		
Compounds	**Class**	**X**	**Y**	**Hepsin inhibition**	**Refs.**
1	A	H	H	35 [a]	[55]
2	A		H	4.9 [a]	[55]
3	A		Cl	4.8 [a]	[55]
4	A		H	1.4 [a]	[55]
5	A	H		8.9 [b]	[62]
6	A	H		5.9 [b]	[62]
7	A	H		23 [b]	[62]
8	A	H		7.3 [b]	[62]
9	A	H		14 [b]	[62]
10	A	H		9.3 [b]	[62]
11	A	H		70 [b]	[62]
12	B			0.6 [a]	[55]
13	B			0.1 [a]	[55]
14	B			0.4 [a]	[55]
15				0.1 [a]	[55]

([a] K_i in μM, [b] IC_{50} in μM)

Byun and his co-workers reported 1*H*-indole-5-carboxamidine derivatives (compounds **5**-**11** in Table **1**) substituted with aryl ring at the Y position of class A [62]. As the biphenyl group of this series was projected onto the surface of hepsin, a large conjugate of compound **10** with a PSMA-binding scaffold retained the binding affinity for hepsin [62].

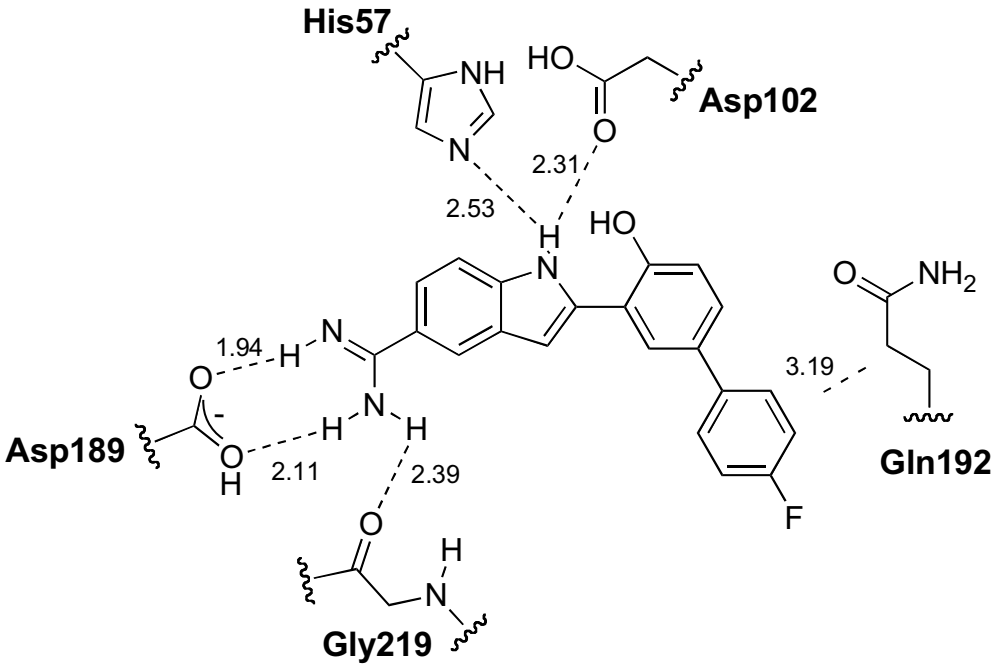


Fig. (1). Schematic depiction of key interactions between hepsin and compound **6**.

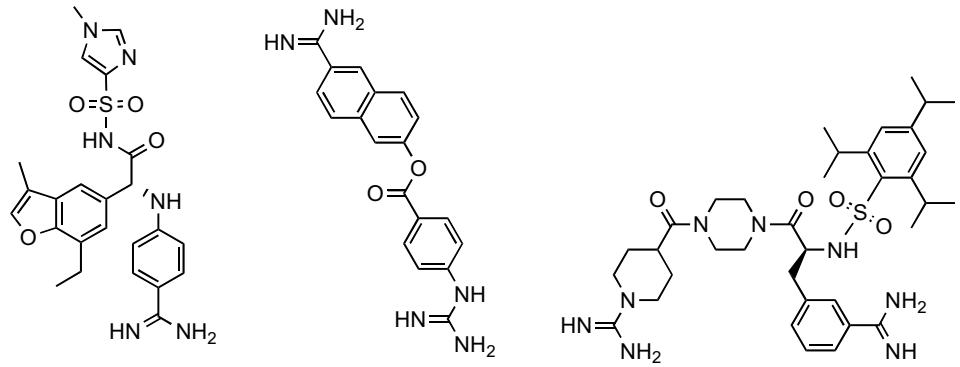
Modeling studies of compounds **5-11** with hepsin crystal structure (PDB ID: 1O5E) showed that the amidine group provides the key interaction with Asp-189 of the active site of hepsin [63]. When the amidine group was replaced with nitrile or oxadiazole group, the hepsin inhibitory activities were completely abolished. As shown in Table **1**, the biphenyl analogs **5-11** had hepsin-inhibitory activities, with IC_{50} values ranging from 5.9 to 70 μM. Compound **5** with the unsubstituted biphenyl group was moderately potent. Introduction of F, OCH_3, and CF_3 at the *para*-position also showed similar hepsin-binding affinity. However, compound **11** with a thiophene at the Y position showed the decreased hepsin inhibitory activity while compound **10** showed similar activity to compound **5**. Even the larger, bulkier heterobivalent compound targeting both hepsin and PSMA exhibited potent hepsin-inhibiting activity (IC_{50} = 2.8 μM) as monovalent hepsin inhibitors **5–10**, indicating that the bulky group could be conjugated with 1*H*-indole-5-carboxamidine with retaining hepsin affinity. The docked pose of compound **6** interacted with key amino acids Asp-189 and His-57 as shown in Fig. (**1**). The 4-fluorobiphenyl group was located in the open space without interacting with any amino acid residue within 2.5 Å.

3. BENZAMIDINES

3.1. Amidino-phenylalanine Benzamidine

The second low M.W. hepsin inhibitors are benzamidine derivatives developed by Janetka and co-workers [64]. The initial compound as a promising trypsin-like serine protease was **Hgfac-221**. It was developed as an inhibitor of Factor VIIa by Genentech (Fig. **2**). **Hgfac-221** inhibited matriptase, HGFA, and hepsin with K_i values of 7180, 7100, and 553 nM, respectively [64]. One of matriptase inhibitor, so-called **CJ-672**, was utilized as a starting point in the design of benzamidine-based hepsin inhibitors. **CJ-672** was a potent matriptase inhibitor (K_i = 14 nM) and crystallized with matriptase (PDB ID: 2GV7) with a resolution of 2.2 Å [65]. Trypsin-like serine proteases including hepsin, matriptase, and HGFA cause hydrolysis of specific substrates that have basic amino acids such as Arg or Lys at the S1 pocket of active site [66]. The Asp-189 residue in the S1 site contributes to substrate specificity. It has preference for positively-charged Arg or Lys in the P1 portion of substrates. **Nafamostat** had strong inhibition activity against hepsin, matriptase, and HGFA by forming arylacyl-enzyme intermediate. [67].

Because hepsin, matriptase, and HGFA share structural similarity for biological substrates, benzamidine-derived matriptase or HGFA inhibitors also inhibited hepsin equipotently in most cases [65, 68]. However, some compounds showed better binding affinity for hepsin over HGFA or matriptase. As shown in Table **2**, among piperidinyl benzamidine sulfonamide analogs **16-20**, compound **18** with *N*-phenylethylcarboxamidyl group substituted at the piperidine ring and 4-methylphenyl group at the sulfonylamide portion



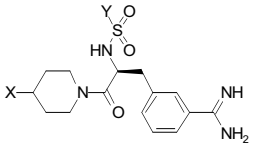
Hgfac-221
Matriptase K_i 7,180 nM
HGFA K_i 7,100 nM
Hepsin K_i 553 nM

Nafamostat
Matriptase K_i 0.02 nM
HGFA K_i 25 nM
Hepsin K_i 0.53 nM

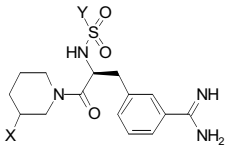
CJ-672
Matriptase K_i 4.3 nM
HGFA K_i 17,000 nM
Hepsin K_i 1,580 nM

Fig. (2). Benzamidine-containing trypsin-like serine protease inhibitors. K_i values were adapted from [64].

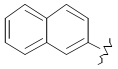
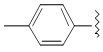
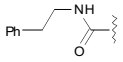
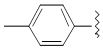
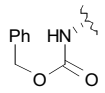
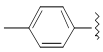
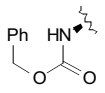
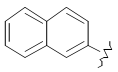
Table 2. Inhibitory activities of piperidinyl benzamidine sulfonamides against hepsin.



Class A



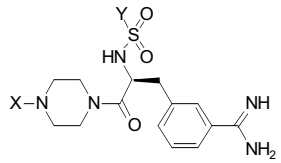
Class B

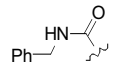
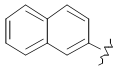
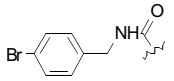
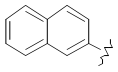
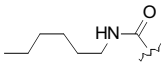
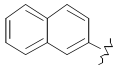
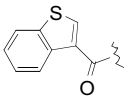
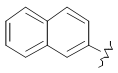
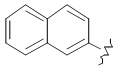
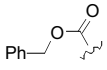
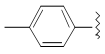
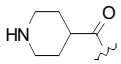
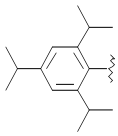
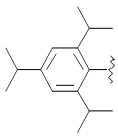
Compds.	Class	X	Y	K_i (μM)	Refs.
16	A			3.3	[64]
17	A			4.7	[64]
18	B			0.67	[64]
19	B			2.3	[64]
20	B			2.6	[64]

exhibited strong inhibitory activity against hepsin with a K_i value of 670 nM. However, the K_i values of compound **18** against HGFA and matriptase were higher than 2 μM.

Among piperazinyl benzamidine sulfonamide analogs **21-28**, compound **22** with bromobenzyl and naphthyl groups showed potent and selective binding affinity for hepsin, with a K_i value of 48 nM. However, its K_i values for HGFA and matriptase were 16.2 and 4.6 μM, respectively [64]. Except for compounds **18** and **22**, the other piperidinyl and piperazinyl compounds showed moderate binding affinity for hepsin with K_i values ranging from 1.0 to 7.4 μM as shown in Tables **2** and **3**. Janetka *et al.* explained that the differential affinities of these compounds against the three trypsin-like serine proteases (hepsin, matriptase, and HGFA) were due to the S3' subpocket diversity. In the composition of S3' subpocket amino acid residues, hepsin is occupied by positively charged basic amino acids Arg-62 and Pro-60 while HGFA and matriptase are occupied by neutral His-60 and acidic Asp-60, respectively. The selective binding mode of compound **22** to

Table 3. Inhibitory activities of piperazinyl benzamidine sulfonamides against hepsin.



Compds.	X	Y	K_i (μM)	Refs.
21			1.0	[64]
22			0.048	[64]
23			1.2	[64]
24			3.5	[64]
25			7.0	[64]
26			2.4	[64]
27			7.4	[64]
28			7.4	[64]

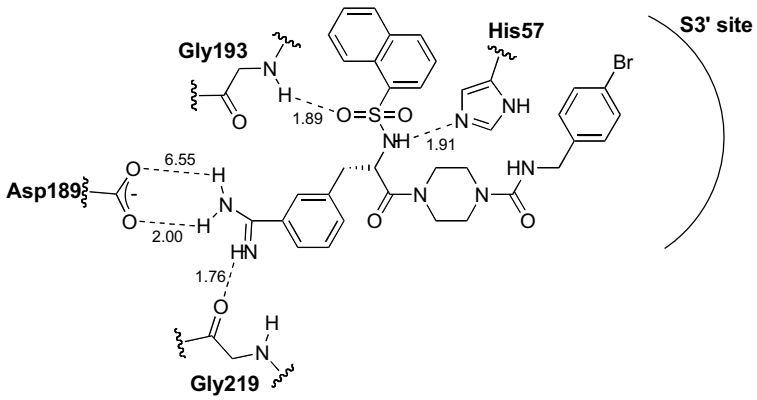


Fig. (3). Key interactions between hepsin and compound **22**.

hepsin over matriptase and HGFA could be explained by the maximal interaction of the 4-bromobenzyl group with the S3' subpocket generated by Pro-60 (Fig. **3**).

Compound **27** derived from **CJ-672** showed 11-fold decrease in hepsin affinity compared to **CJ-672**. These results indicated that selectivity of benzamidine sul-

fonamides for hepsin could be differentiated by the interaction between inhibitor and the three proteases in the S3' pocket. The decrease in hepsin affinity of compound **27** can be explained by repulsive force between Arg-62 of S3' site and free amine group of terminal piperidine ring.

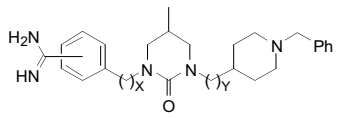
3.2. 2-Oxotetrahydropyrimidin-1(2*H*)-yl Benzimidamides

Recently, Galemmo and co-workers reported that 2-oxotetrahydropyrimidin-1(2*H*)-yl benzimidamide is a novel scaffold for the development of hepsin inhibitors [69]. They developed phenylamidine cyclic urea analogs **29-34,** which exhibited strong inhibition activities against hepsin, matriptase and HGFA. In this series, the amidine group is the key motif that has strong interaction with Asp-189 in the S1 pocket. First, they investigated whether the position of the amidine group in the phenyl ring had an effect on the three proteases (hepsin, matriptase, and HGFA). Overall, compounds **29** and **31** with amidine group at *meta*-position showed higher hepsin-inhibiting activities than the corresponding *para*-compounds **30** and **32** as summarized in Table **4**. Direct conjugation of phenylamidine ring with cyclic urea resulted in the increase of hepsin-inhibiting activity compared to that of benzylamidine ring (compound **29** *vs.* compound **31**). Methylene linker (compound **33**) between cyclic urea and the piperidine ring had the best effect to obtain hepsin-inhibiting activity compared to direct coupling (compound **29**) or ethylene spacer (compound **34**). The *N*-benzylpiperidine group contributed to hepsin affinity of the parent compound by providing additional binding with Trp-215 in the S4 subpocket. When cyclic urea structure of compound **29** was transformed to acyclic compound **35**, hepsin-inhibiting activity was decreased by about 16-fold, indicating that the cyclic urea is one of key moieties for hepsin binding [69]. Compound **33**, named as **SRI 31215** in Table **4**, exhibited strong potency against other proteases (matriptase K_i = 0.53 μM, HGFA K_i = 0.48 μM). As **SRI 31215** inhibited fibroblast-induced HGF/MET signaling pathways in tumor cells, **SRI31215** treatment led to the accumulation of pro-HGF [70]. This compound was compared to (2-oxo-1,3-diazepan-1-yl)benzimidamide which inhibits factor Xa [71, 72].

Compounds **36-47** with substituents at the X-position in the central cyclic urea ring are summarized in Table **5**. Structural optimization of **SRI31215** was performed based on binding mode information obtained from molecular docking studies and X-ray crystal structures of hepsin, matriptase, and HGFA: (1) strong ionic interaction between the amidine group and Asp-189 in the S1 subpocket; (2) hydrogen-bonding involvement of the urea core; and (3) lipophilic interaction generated by the *N*-benzylpiperidine group [69].

Modification of methyl group at the X position did not make an effect on hepsin binding and all of the compounds **36-47** showed similar hepsin-inhibiting activities with K_i values ranging from 0.21 to 1.12 μM (Table **5**) [69]. However, this structural modification increased off-target selectivity. In particular, compound **36** with OH group at the X-position of the 6-membered

Table 4. Inhibitory activities of other 2-oxotetrahydropyrimidine against hepsin.



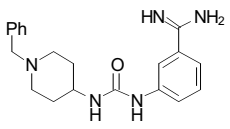
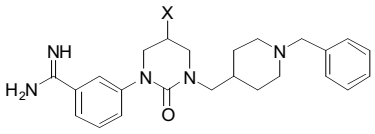
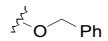
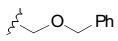
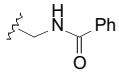
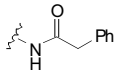
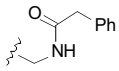
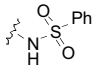
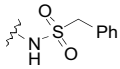
Compounds	Substitutions		K_i (μM)	Refs.
29	X = 0, Y = 0	*meta*	3.4	[69]
30	X = 0, Y = 0	*para*	61.2	[69]
31	X = 1, Y = 0	*meta*	15.1	[69]
32	X = 1, Y = 0	*para*	20.8	[69]
33 (SRI 31215)	X = 0, Y = 1	*meta*	0.54	[69]
34	X = 0, Y = 2	*meta*	2.12	[69]
35			53.1	[69]

Table 5. Inhibitory activities of 2-oxotetrahydropyrimidin-1(2*H*)-yl benzimidamide against hepsin.



Compounds	X	K_i (μM)	Refs.
SRI 31215	-CH_3	0.54	[69]
36	-OH	0.21	[69]
37	-CH_2OH	0.69	[69]
38	-OMe	0.76	[69]
39	-CH_2OCH_3	0.92	[69]
40		0.76	[69]
41		0.56	[69]
42		1.1	[69]
43		1.12	[69]
44		0.31	[69]
45		0.72	[69]
46		0.67	[69]
47		0.53	[69]

cyclic urea ring showed 4-fold selectivity over Factor Xa [70]. Compound **44** also exhibited potent hepsin-inhibiting activity with 5-fold selectivity against Factor Xa. The docked pose of compound **36** in hepsin (Fig. **4**) confirmed that the amidine of 2-oxotetrahydropyrimidin-1(2H)-yl benzimidamide analogs participated in ionic interaction with the carboxylate side chain of Asp-189. The carbonyl oxygen of the central cyclic urea ring contributed to hydrogen-bonding with Gly-216. In addition, N-benzylpiperidine contributed to the hydrophobic interaction with Trp-215. The only (S)-enantiomer of **SRI 31215** was found in the co-crystal structure with hepsin (PDB ID: 1Z8G). **SRI 31215** was used to study the inhibition of HGF-activation pathway. It showed high stability in mouse and human microsomes with < 70% of hepatic clearance and a half-life of 5.8 hr according to an *in vivo* mouse study [69]. It has been shown to be capable of overcoming HGF-dependent resistance to EGFR inhibitors in cancer treatment [70]. **SRI 31215** is currently considered as an effective inhibitor of fibroblast-induced HGF/MET activation.

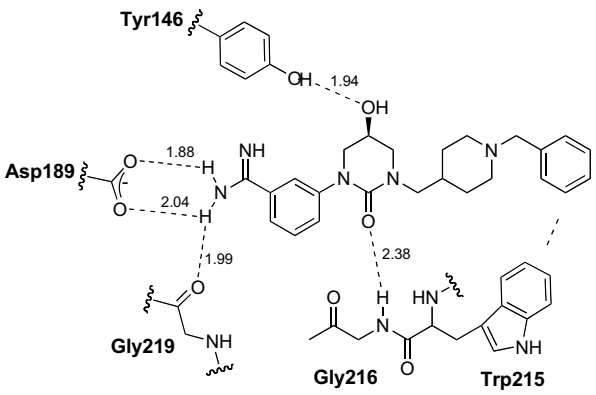
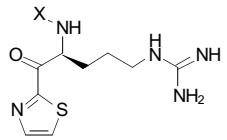


Fig. (4). Key interactions between hepsin and compound **36**.

Table 6. Inhibitory activities of tetrapeptide derivatives against hepsin.



Compounds	X	K_i (nM)	Refs.
48 (pro-HGF-based)	Ac-KQL-	0.22	[73]
48a	Ac-KHL-	0.41	[73]
48b	Ac-WQL-	0.21	[73]
48c	Ac-KQF-	0.58	[73]
48d	Ac-KFL-	2.10	[73]
48e	Ac-RQL-	0.28	[73]
48f	Ac-SQL-	0.34	[73]
48g	Ac-KRL-	0.57	[73]
48h	Ac-WRL-	0.21	[73]
49 (pro-MSP-based)	Ac-SKL-	1.20	[73]
49a	Ac-SHL-	0.60	[73]
49b	Ac-WKL-	0.55	[73]
49c	Ac-SKF-	8.5	[73]
49d	Ac-NKL-	1.40	[73]
49e	Ac-SRL-	0.68	[73]
49f	Ac-TKL-	0.61	[73]
49g	Ac-SWL-	1.20	[73]
49h	Ac-RKL-	0.47	[73]

4. PEPTIDE-BASED ANALOGS

4.1. Tetrapeptides

Janetka and his co-workers have developed multi-peptide derivatives (compounds **48** and **49**) by mimicking natural substrates pro-HGF and pro-MSP of HGFA, matriptase, and hepsin, and TMPRSS2 (Table **6**) [73]. Electrophilic carbonyl groups were introduced to the P1' site in the development of mechanism-based serine protease inhibitors [73, 74]. Pro-HGF, the known natural substrate of HGFA, contains Lys-Gln-Leu-Arg at the N-terminal portion and Val-Val-Asn-Gly at the C-terminal portion of the cleavage site. Compound **48** (Ac-KQLR-kt) was designed and synthesized by capping the terminal amine of Lys with acetyl group and introducing ketothiazole (kt) at the carboxylic acid of Arg in the sequence of P1' site of Pro-HGF [73]. Compound **48** exhibited strong inhibiting activities against the three proteases (HGFA, hepsin, and matriptase) with K_i values in nanomolar range (hepsin: 0.22 nM; matriptase: 0.92 nM; HGFA: 53 nM). However, synthetic pro-MSP peptide analog **49** (Ac-SKLR-kt) showed decreased hepsin-inhibiting activity by 5-fold compared to analog **48** (hepsin: 1.2 nM; matriptase: 58 nM; HGFA: 81 nM) [73]. Although variation of amino acids in P4 and P3 regions significantly affected their inhibitory activities against HGFA and matriptase, hepsin-inhibitory activities were retained with K_i values ranging from 0.22 to 8.5 nM. All tetrapeptide analogs except compound **49c** showed stronger inhibitory activities for hepsin than for HGFA or matriptase. In particular, compounds **48b** (Ac-WQLR-kt) and **49a** (Ac-SHLR-kt) showed higher selectivity for hepsin by > 100-fold than for HGFA or matriptase, indicating that they could be utilized for further structural modification. Docking studies of compound **48** with hepsin (PDB ID: 1Z8G) [75] showed strong interaction between the ligand and the active site of hepsin. Arg in P1 region interacted with Asp-189 and Gly-219 *via* ionic and hydrogen-bonding (Fig. **5**), whereas Leu in

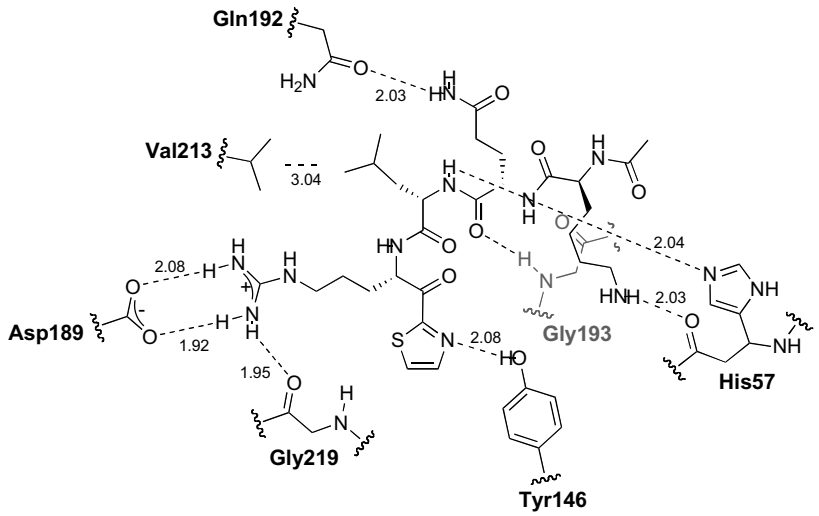


Fig. (5). Key interactions between hepsin and compound **48**.

P2, Gln in P3, and Lys in P4 region interacted with Val-213, Gln-192, and His-57, respectively. In addition, the ketothiazole ring contributed to the strong hydrogen-bonding interaction with Tyr-146 [76].

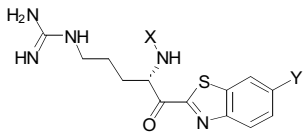
Recently, Janetka *et al.* synthesized and evaluated a variety of tetrapeptide-derived hepsin inhibitors substituted with a ketobenzothiazole (kbt) moiety [74]. Replacement of kt with kbt (compound **50**) increased the hepsin-inhibiting activity by > 2-fold based on fluorogenic enzyme inhibition assay (compound **48** *vs* compound **50**). Parallel results were obtained after similar modification (compound **49** *vs* compound **52**). However, introduction of aromatic amino acids (compounds **55** and **57**) at both P2 and P4 sites decreased the hepsin-inhibiting activity by > 5-fold compared to compounds **50** and **52**, indicating that S2 and S4 sites of hepsin prefer amino acids with aliphatic group at side chains rather than those with aromatic group. Nonetheless, all kbt-substituted tetrapeptides increased their selectivity for hepsin over thrombin or Factor Xa [74]. Based on SAR data of tetrapeptides with non-substituted kbt, SKLR and KRLR sequences (compounds **53** and **58**) were selected for further structural modification (compounds **61a-62d**). Although substitution of the Y-position on benzothiazole ring increased HGFA-inhibiting activities for KQLR or SKLR analogs, their hepsin-inhibiting activities were affected slightly as shown in Table **7**. Substituents of kbt ring was projected in the S3' subpocket of hepsin and made interactions with Asn-209. In particular, the carboxylic acid **60c** increased their activities against hepsin (K_i = 0.08 nM) and HGFA (K_i = 6.1 nM). However, it decreased inhibition for matriptase (K_i = 7.6 nM) compared to its parent compound **56** (K_i values: 0.11 nM for hepsin, 7.5 nM for HGFA, and 1.2 nM for matriptase).

These results might be explained by the composition of amino acids in the S3' subpocket. Hepsin and HGFA possess Asn and His, respectively, while matriptase has Asp in the subpocket, which might result in electrostatic repulsion with carboxylic acid group of compound **60c**.

4.2. Di- and Tripeptides

Based on the results of tetrapeptide analogs, Byun and co-workers designed and developed truncated peptides, particularly dipeptides (Table **8**) [76]. They tried to investigate three aspects: (1) Leu-Arg sequence found in compounds **48** and **49** is a minimum requirement for hepsin inhibition, (2) the absolute configuration change of C-terminal Arg from (S) to (R) affects hepsin affinity, and (3) the ketothiazole ring at the C-terminus can be replaced with other aromatic rings. In docking studies of truncated peptide analogs with hepsin, dipeptide **61** and tripeptide **63** showed binding patterns similar to compound **48**. In particular, the side chain of Arg residue in the truncated analogs **61** and **63** was projected deeply into the S1 pocket, making a salt bridge with the carboxylate group of Asp-189. This feature was retained in the analog substituted with benzothiazole (Fig. **6**). Based on molecular modeling prediction, Ac-QLR-kt (tripeptide **63**) with Lys removed at the *N*-terminus of compound **48** showed potent hepsin-inhibiting activity with a K_i value of 1.04 nM, similar to compound **48**. However, the hepsin-inhibiting activity of dipeptide **61** was decreased (K_i = 22.4 nM) while its selectivity against matriptase was increased. Interestingly, mono-peptide **64** (Ac-R-kt) lost

Table 7. Inhibitory activities of tetrapeptide derivatives against hepsin.



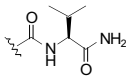
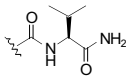
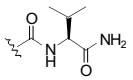
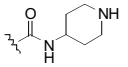
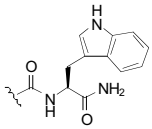
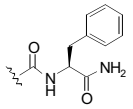
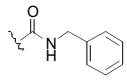
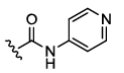
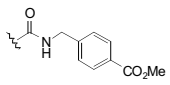
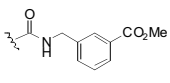
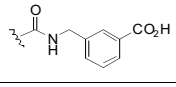
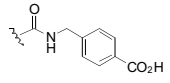
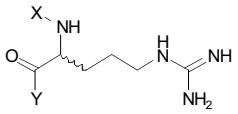
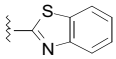
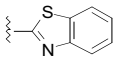
Compounds	X	Y	K_i (nM)	Refs.
50	Ac-KQL-	-H	0.09	[74]
51	Ac-KQL-		0.43	[74]
52	Ac-SKL-	-H	0.07	[74]
53	Ac-SKL-		0.22	[74]
54	Ac-WRL-	-H	0.17	[74]
55	Ac-FLF-	-H	1.40	[74]
56	Ac-KRL-	-H	0.11	[74]
57	Ac-WLF-	-H	0.35	[74]
58	Ac-KRL-		0.23	[74]
59a	Ac-SKL-		0.16	[74]
59b	Ac-SKL-		0.27	[74]
59c	Ac-SKL-		0.25	[74]
59d	Ac-SKL-		0.21	[74]
59e	Ac-SKL-		0.14	[74]
59f	Ac-SKL-		0.29	[74]
60a	Ac-KRL-		0.52	[74]
60b	Ac-KRL-		0.64	[74]
60c	Ac-KRL-		0.08	[74]
60d	Ac-KRL-		0.67	[74]

Table 8. Inhibitory activities of dipeptide and tripeptide derivatives against hepsin.



Compds.	X	Y	Chirality	K_i (nM)	Refs.
61	Ac-L-		*S*	22.4	[76]
61R	Ac-L-		*R*	21.5	[76]
62	Ac-L-		*S*	3.38	[76]
62R	Ac-L-		*R*	2.91	[76]
63	Ac-QL-		*S*	1.04	[76]
63R	Ac-QL-		*R*	2.88	[76]
64	Ac-			>10,000	[76]
Leupeptin	Ac-LL-	-H	*S*	61.0	[73]

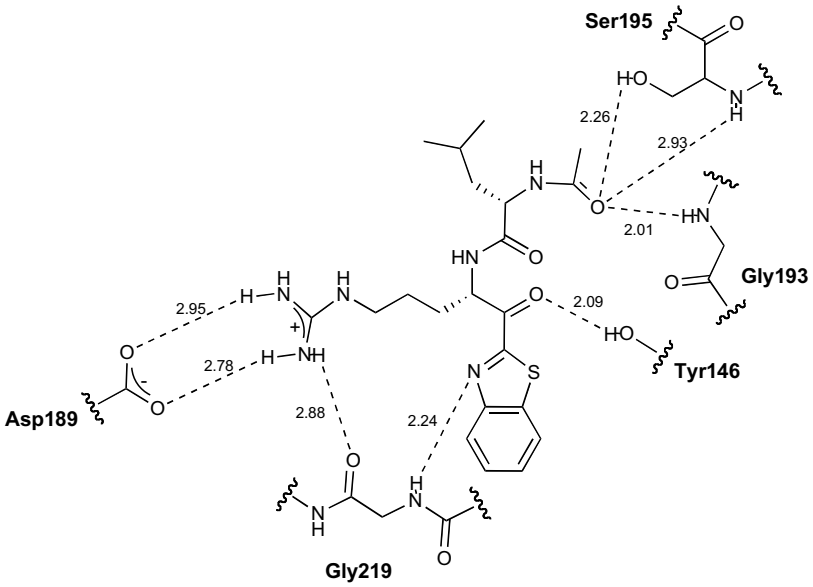
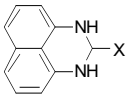
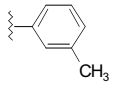
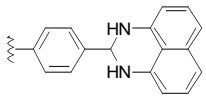
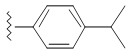
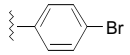
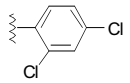
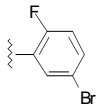
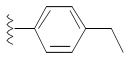


Fig. (6). Key interactions between hepsin and compound **62.**

completely its hepsin-binding activity, implying that dipeptide sequence is essential for hepsin affinity. Compounds **61R-63R** with (R)-configuration of α -carbon of Arg were as potent as their corresponding (S)-epimers **61-63**. Replacement of kt with kbt in LR sequence resulted in increased affinity for hepsin by 6-fold and selectivity for hepsin over matriptase by 5-fold. Compound **62** showed hepsin-inhibiting activity with K_i value of 3.38 nM. It had increased selectivity for hepsin over matriptase by > 60-fold [76]. Therefore, compounds **62** and **62R** could be potentially utilized as lead compounds for optimization of peptide-based hepsin inhibitors.

Table 9. Inhibitory activities of 2,3-dihydro-1*H*-perimidines against hepsin.



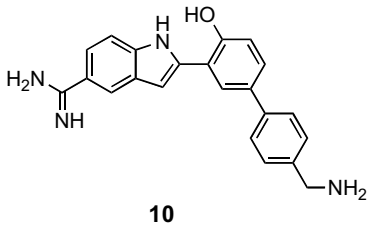
Compounds	X	IC_{50} (μM)	Refs.
65		0.35	[77]
66		0.48	[47]
67		0.72	[47]
68		0.33	[47]
69		0.84	[47]
70		0.35	[47]
71		0.38	[47]

5. 2,3-DIHYDRO-1*H*-PERIMIDINE DERIVATIVES

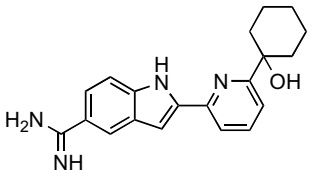
Compounds with 2,3-dihydro-1*H*-perimidine scaffold were also been evaluated as low M. W. hepsin inhibitors [47]. Initial compound **65** was identified from high throughput screening of ChemBridge compound library [77]. Vasioukhin and co-workers synthesized 25 perimidine analogs and evaluated their hepsin-inhibiting activities [47]. To evaluate these compounds, they used recombinant human hepsin originated from Drosophila S2 cells [78]. Among these compounds, seven compounds **65-71** (Table **9**) showed strong hepsin-inhibiting activities with IC_{50} values ranging from 0.33 to 0.84 μM. They also showed high selectivity for hepsin over matriptase. These compounds effectively inhibited the activity of full-length hepsin and pro-HGF cleavage at submicromolar concentrations. In particular, compound **68** substituted with 4-bromophenyl ring was the most potent hepsin inhibitor with an IC_{50} of 0.33 μM. It was further investigated at *in vitro* and *in vivo* experiments. Compound **68** did not show any acute toxicity when it was injected into mice at a maximal dose of 20 mg/kg. *In vivo* efficacy studies of compound **68** using LPB-Tag/PB-hepsin mouse model displayed a dose-dependent inhibition pattern. No metastasis to liver, lung, and bone was observed in mice fed with food containing 0.25% of compound **68** for 13 weeks [47]. Mice in the control group showed 66% of metastasis, mainly to the liver. However, PK parameters such as oral bioavailability of compound **68** were poor. According to *in silico* docking studies of compound **68** with hepsin (PDB ID: 1P57), it interacted with Ser-195, a key amino acid in the catalytic triad of serine protease. [47]. Although compounds of this series have minimal functional groups that can make hydrophilic interaction with hepsin active site, they showed strong hepsin-inhibiting activity, indicating that 2,3-dihydro-1*H*-perimidine scaffold has the potential to be used for structural modification for the purpose of developing hepsin inhibitors as therapeutic agents of prostate cancer.

Representative inhibitors for the four classes (indolecarboxamidines, benzamidines, peptide-based

1. Indolecarboxamidines

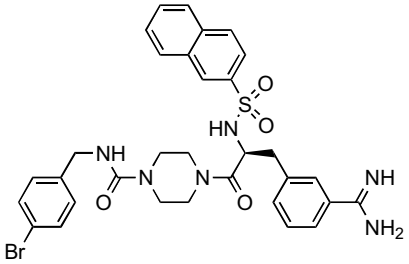


10
Hepsin IC_{50} = 9.3 μM

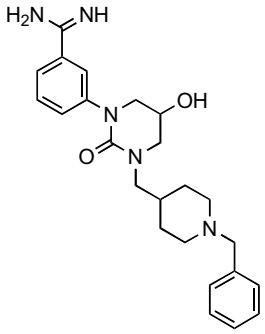


13
Hepsin K_i = 0.1 μM

2. Benzamidines

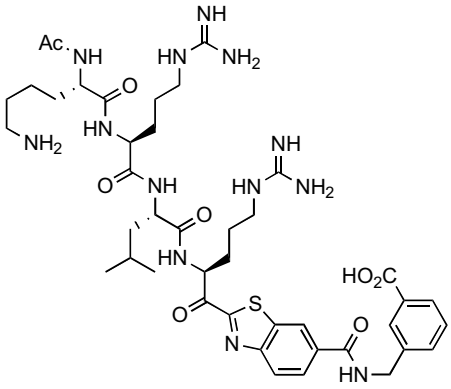


22
Hepsin K_i = 0.048 μM

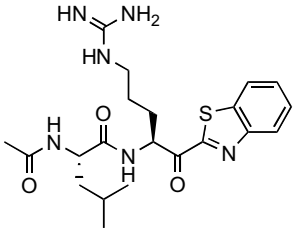


36
Hepsin K_i = 0.21 μM

3. Peptide-based analogs

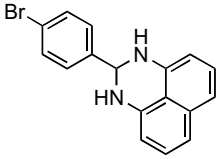


60c
Hepsin K_i = 0.08 nM



62
Hepsin K_i = 3.38 nM

4. 2,3-Dihydro-*1H*-perimidines



68
Hepsin IC_{50} = 0.33 μM

Fig. (7). Summary of representative compounds of the four classes of hepsin-targeting inhibitors.

analogs, and 2,3-dihydro-1*H*-perimidines) are summarized in Fig. (**7**). For indolecaroxamidine analogs, introduction of 2-hydroxyphenyl (compound **10**) or 2-pyridinyl group (compound **13**) was well tolerated for binding to hepsin. Compound **10** with aminomethyl group was successfully conjugated with a large PSMA-binding moiety and sulfoCy7, implying that hepsin-targeted imaging probes can be developed by linking

indolecaroxamidine analog with optical dyes [62]. Compounds **22** and **36** are potent hepsin inhibitors in the class of benzamidine. They displayed strong hepsin-inhibiting activities by taking advantage of additional binding in S3' subpocket. Peptide-based analogs were the most potent hepsin inhibitors based on K_i and IC_{50} values. The K_i value of tetrapeptide-derived **60c** was at subnanomolar range. Ketobenzothiazole group at the C-terminus of peptide-based analogs enhanced hepsin-binding affinity compared to ketothiazole group. Although truncation of tetrapeptides into dipeptides decreased the *in vitro* hepsin-binding affinity, compound **62** derived from Leu-Arg was quite potent. 2,3-Dihdyro-1*H*-Perimdine analogs displayed a decrease in potency *in vitro* compared to peptide- and benzamidine-based analogs. However, compound **68** exhibited promising *in vivo* results by inhibiting metastasis to liver, lung, and bond in PB-hepsin mouse model.

Hepsin is associated with breachment of basement membrane and degradation of extracellular matrix molecules, the first step in the initiation of metastasis of primary tumor cells [44]. High expression of hepsin in epithelial cells of prostate of transgenic mice altered basement membrane structure and caused metastasis in the liver, lung, and bone [44], suggesting that hepsin inhibitors might be able to block the metastasis of primary prostate cancer and other malignancies. Hepsin is a cell surface protease with an active site in the extracellular region. A PSMA-hepsin conjugate labeled with a bulky optical dye showed access to the active site of hepsin at an *in vivo* preliminary study [62], indicating that it might be possible to develop diagnostic agents by targeting hepsin.

CONCLUSION

Over the last decade, low M. W. hepsin inhibitors having diverse scaffolds have been developed. Peptide-based and benzamide sulfonylamidyl analogs were found to be potent inhibitors for hepsin with IC_{50} values at one digit nanomolar concentration. Although potent hepsin inhibitors have been developed with strong *in vitro* hepsin-inhibiting activities, more advanced studies such as *in vivo* therapeutic or diagnostic agents should be performed to optimize parameters for clinical use. Increasing evidences suggest that hepsin plays a key role in metastasis of aberrant cancers. Although several compounds targeting hepsin have successfully entered animal studies, more efforts are required to optimize the structure of current potent compounds and to improve their physicochemical properties and selectivity profiles for clinical application.

LIST OF ABBREVIATIONS

BPH	=	Benign prostatic hyperplasia
HAI	=	HGF activator inhibitor
HAT	=	Human airway trypsin-like protease
HGF	=	Hepatocyte growth factor
HGFA	=	Hepatocyte growth factor activator
HPN	=	Hepsin
kbt	=	ketobenzothiazole
kt	=	ketothiazole
M.W.	=	Molecular weight
MLM	=	Mouse liver microsomes
MSP	=	Macrophage stimulating protein
PCa	=	Prostate cancer
PSMA	=	Prostate-specific membrane antigen
SAR	=	Structure-activity relationship
TMPRSS2	=	Transmembrane Protease, Serine 2
TTSP	=	Type II transmembrane serine protease

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Ferlay, J.; Autier, P.; Boniol, M.; Heanue, M.; Colombet, M.; Boyle, P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann. Oncol.,* **2007**, *18*(3), 581-592.

[2] Jemal, A.; Clegg, L.X.; Ward, E.; Ries, L.A.; Wu, X.; Jamison, P.M.; Wingo, P.A.; Howe, H.L.; Anderson, R.N.; Edwards, B.K. Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival. *Cancer,* **2004**, *101*(1), 3-27.

[3] Greenlee, R.T.; Hill-Harmon, M.B.; Murray, T.; Thun, M. Cancer statistics, 2001. *CA Cancer J. Clin.,* **2001**, *51*(1), 15-36.

[4] Jemal, A.; Bray, F.; Center, M.M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *CA Cancer J. Clin.,* **2011**, *61*(2), 69-90.

[5] Jemal, A.; Thomas, A.; Murray, T.; Thun, M. Cancer statistics, 2002. *CA Cancer J. Clin.,* **2002**, *52*(1), 23-47.

[6] Siegel, R.; Ward, E.; Brawley, O.; Jemal, A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J. Clin.,* **2011**, *61*(4), 212-236.

[7] Kasten, B.B.; Liu, T.; Nedrow-Byers, J.R.; Benny, P.D.; Berkman, C.E. Targeting prostate cancer cells with PSMA inhibitor-guided gold nanoparticles. *Bioorg. Med. Chem. Lett.,* **2013**, *23*(2), 565-568.

[8] Chang, A.J.; Autio, K.A.; Roach, M. 3rd; Scher, H.I. High-risk prostate cancer-classification and therapy. *Nat. Rev. Clin. Oncol.,* **2014**, *11*(6), 308-323.

[9] Bhowmick, N.A.; Neilson, E.G.; Moses, H.L. Stromal fibroblasts in cancer initiation and progression. *Nature,* **2004**, *432*(7015), 332-337.

[10] Forbs, D.; Thiel, S.; Stella, M.C.; Sturzebecher, A.; Schweinitz, A.; Steinmetzer, T.; Sturzebecher, J.; Uhland, K. *In vitro* inhibition of matriptase prevents invasive growth of cell lines of prostate and colon carcinoma. *Int. J. Oncol.,* **2005**, *27*(4), 1061-1070.

[11] Herter, S.; Piper, D.E.; Aaron, W.; Gabriele, T.; Cutler, G.; Cao, P.; Bhatt, A.S.; Choe, Y.; Craik, C.S.; Walker, N.; Meininger, D.; Hoey, T.; Austin, R.J. Hepatocyte growth factor is a preferred *in vitro* substrate for human hepsin, a membrane-anchored serine protease implicated in prostate and ovarian cancers. *Biochem. J.,* **2005**, *390*(Pt 1), 125-136.

[12] Kataoka, H.; Hamasuna, R.; Itoh, H.; Kitamura, N.; Koono, M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. *Cancer Res.,* **2000**, *60*(21), 6148-6159.

[13] Lee, S.L.; Dickson, R.B.; Lin, C.Y. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J. Biol. Chem.,* **2000**, *275*(47), 36720-36725.

[14] Owen, K.A.; Qiu, D.; Alves, J.; Schumacher, A.M.; Kilpatrick, L.M.; Li, J.; Harris, J.L.; Ellis, V. Pericellular activation of hepatocyte growth factor by the transmembrane serine proteases matriptase and hepsin, but not by the membrane-associated protease uPA. *Biochem. J.,* **2010**, *426*(2), 219-228.

[15] Parr, C.; Watkins, G.; Mansel, R.E.; Jiang, W.G. The hepatocyte growth factor regulatory factors in human breast cancer. *Clin. Cancer Res.,* **2004**, *10*(1 Pt 1), 202-211.

[16] Szabo, R.; Rasmussen, A.L.; Moyer, A.B.; Kosa, P.; Schafer, J.M.; Molinolo, A.A.; Gutkind, J.S.; Bugge, T.H. c-Met-induced epithelial carcinogenesis is initiated by the serine protease matriptase. *Oncogene,* **2011**, *30*(17), 2003-2016.

[17] Kawaguchi, M.; Kataoka, H. Mechanisms of hepatocyte growth factor activation in cancer tissues. *Cancers,* **2014**, *6*(4), 1890-1904.

[18] Lin, J.; Deng, H.F.; Jin, L.; Pandey, P.; Quinn, J.; Cantin, S.; Rynkiewicz, M.J.; Gorga, J.C.; Bibbins, F.; Celatka, C.A.; Nagafuji, P.; Bannister, T.D.; Meyers, H.V.; Babine, R.E.; Hayward, N.J.; Weaver, D.; Benjamin, H.; Stassen, F.; Abdel-Meguid, S.S.; Strickler, J.E. Design, synthesis, and biological evaluation of peptidomimetic inhibitors of factor XIa as novel anticoagulants. *J. Med. Chem.,* **2006**, *49*(26), 7781-7791.

[19] Comoglio, P.M.; Giordano, S.; Trusolino, L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat. Rev. Drug Discov.,* **2008**, *7*(6), 504-516.

[20] Ernst, T.; Hergenhahn, M.; Kenzelmann, M.; Cohen, C.D.; Bonrouhi, M.; Weninger, A.; Klaren, R.; Grone, E.F.; Wiesel, M.; Gudemann, C.; Kuster, J.; Schott, W.; Staehler, G.; Kretzler, M.; Hollstein, M.; Grone, H.J. Decrease and gain of gene expression are equally discriminatory markers for prostate carcinoma: a gene expression analysis on total and microdissected prostate tissue. *Am. J. Pathol.,* **2002**, *160*(6), 2169-2180.

[21] Magee, J.A.; Araki, T.; Patil, S.; Ehrig, T.; True, L.; Humphrey, P.A.; Catalona, W.J.; Watson, M.A.; Milbrandt, J. Expression profiling reveals hepsin overexpression in prostate cancer. *Cancer Res.,* **2001**, *61*(15), 5692-5696.

[22] Luo, J.; Duggan, D.J.; Chen, Y.; Sauvageot, J.; Ewing, C.M.; Bittner, M.L.; Trent, J.M.; Isaacs, W.B. Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. *Cancer Res.,* **2001**, *61*(12), 4683-4688.

[23] Welsh, J.B.; Sapinoso, L.M.; Su, A.I.; Kern, S.G.; Wang-Rodriguez, J.; Moskaluk, C.A.; Frierson, H.F. Jr.; Hampton, G.M. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. *Cancer Res.,* **2001**, *61*(16), 5974-5978.

[24] Ye, J.; Kawaguchi, M.; Haruyama, Y.; Kanemaru, A.; Fukushima, T.; Yamamoto, K.; Lin, C.Y.; Kataoka, H. Loss of hepatocyte growth factor activator inhibitor type 1 participates in metastatic spreading of human pancreatic cancer cells in a mouse orthotopic transplantation model. *Cancer Sci.,* **2014**, *105*(1), 44-51.

[25] Kawaguchi, M.; Takeda, N.; Hoshiko, S.; Yorita, K.; Baba, T.; Sawaguchi, A.; Nezu, Y.; Yoshikawa, T.; Fukushima, T.; Kataoka, H. Membrane-bound serine protease inhibitor HAI-1 is required for maintenance of intestinal epithelial integrity. *Am. J. Pathol.,* **2011**, *179*(4), 1815-1826.

[26] Hoshiko, S.; Kawaguchi, M.; Fukushima, T.; Haruyama, Y.; Yorita, K.; Tanaka, H.; Seiki, M.; Inatsu, H.; Kitamura, K.; Kataoka, H. Hepatocyte growth factor activator inhibitor type 1 is a suppressor of intestinal tumorigenesis. *Cancer Res.,* **2013**, *73*(8), 2659-2670.

[27] Saleem, M.; Adhami, V.M.; Zhong, W.; Longley, B.J.; Lin, C.Y.; Dickson, R.B.; Reagan-Shaw, S.; Jarrard, D.F.; Mukhtar, H. A novel biomarker for staging human prostate adenocarcinoma: overexpression of matriptase with concomitant loss of its inhibitor, hepatocyte growth factor activator inhibitor-1. *Cancer Epidemiol. Biomarkers Prev.,* **2006**, *15*(2), 217-227.

[28] Oberst, M.D.; Johnson, M.D.; Dickson, R.B.; Lin, C.Y.; Singh, B.; Stewart, M.; Williams, A.; al-Nafussi, A.; Smyth, J.F.; Gabra, H.; Sellar, G.C. Expression of the serine protease matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. *Clin. Cancer Res.,* **2002**, *8*(4), 1101-1107.

[29] Zeng, L.; Cao, J.; Zhang, X. Expression of serine protease SNC19/matriptase and its inhibitor hepatocyte growth factor activator inhibitor type 1 in normal and malignant tissues of gastrointestinal tract. *World J. Gastroenterol.,* **2005**, *11*(39), 6202-6207.

[30] Nakamura, K.; Abarzua, F.; Kodama, J.; Hongo, A.; Nasu, Y.; Kumon, H.; Hiramatsu, Y. Expression of hepatocyte growth factor activator inhibitors (HAI-1 and HAI-2) in ovarian cancer. *Int. J. Oncol.,* **2009**, *34*(2), 345-353.

[31] Hamasuna, R.; Kataoka, H.; Meng, J.Y.; Itoh, H.; Moriyama, T.; Wakisaka, S.; Koono, M. Reduced expression of hepatocyte growth factor activator inhibitor type-2/placental bikunin (HAI-2/PB) in human glioblastomas: implication for anti-invasive role of HAI-2/PB in glioblastoma cells. *Int. J. Cancer,* **2001**, *93*(3), 339-345.

[32] Morris, M.R.; Gentle, D.; Abdulrahman, M.; Maina, E.N.; Gupta, K.; Banks, R.E.; Wiesener, M.S.; Kishida, T.; Yao, M.; Teh, B.; Latif, F.; Maher, E.R. Tumor suppressor activity and epigenetic inactivation of hepatocyte growth

factor activator inhibitor type 2/SPINT2 in papillary and clear cell renal cell carcinoma. *Cancer Res.,* **2005**, *65*(11), 4598-4606.

[33] Antalis, T.M.; Bugge, T.H.; Wu, Q. Membrane-anchored serine proteases in health and disease. *Prog. Mol. Biol. Transl. Sci.,* **2011**, *99*, 1-50.

[34] Tsuji, A.; Torres-Rosado, A.; Arai, T.; Le Beau, M.M.; Lemons, R.S.; Chou, S.H.; Kurachi, K. Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. *J. Biol. Chem.,* **1991**, *266*(25), 16948-16953.

[35] Adib, T.R.; Henderson, S.; Perrett, C.; Hewitt, D.; Bourmpoulia, D.; Ledermann, J.; Boshoff, C. Predicting biomarkers for ovarian cancer using gene-expression microarrays. *Br. J. Cancer,* **2004**, *90*(3), 686-692.

[36] Zacharski, L.R.; Ornstein, D.L.; Memoli, V.A.; Rousseau, S.M.; Kisiel, W. Expression of the factor VII activating protease, hepsin, in situ in renal cell carcinoma. *Thromb. Haemost.,* **1998**, *79*(4), 876-877.

[37] Cao, J.G.; Peng, S.P.; Sun, L.; Li, H.; Wang, L.; Deng, H.W. Vascular basement membrane-derived multifunctional peptide, a novel inhibitor of angiogenesis and tumor growth. *Acta Bioch. Bioph. Sin.,* **2006**, *38*(7), 514-521.

[38] Netzel-Arnett, S.; Hooper, J.D.; Szabo, R.; Madison, E.L.; Quigley, J.P.; Bugge, T.H.; Antalis, T.M. Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. *Cancer Metast. Rev.,* **2003**, *22*(2-3), 237-258.

[39] Dhanasekaran, S.M.; Barrette, T.R.; Ghosh, D.; Shah, R.; Varambally, S.; Kurachi, K.; Pienta, K.J.; Rubin, M.A.; Chinnaiyan, A.M. Delineation of prognostic biomarkers in prostate cancer. *Nature,* **2001**, *412*(6849), 822-826.

[40] Wu, Q.; Parry, G. Hepsin and prostate cancer. *Front. Biosci.,* **2007**, *12*, 5052-5059.

[41] Bugge, T.H.; Antalis, T.M.; Wu, Q. Type II transmembrane serine proteases. *J. Biol. Chem.,* **2009**, *284*(35), 23177-23181.

[42] Stamey, T.A.; Warrington, J.A.; Caldwell, M.C.; Chen, Z.; Fan, Z.; Mahadevappa, M.; McNeal, J.E.; Nolley, R.; Zhang, Z. Molecular genetic profiling of Gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. *J. Urol.,* **2001**, *166*(6), 2171-2177.

[43] Stephan, C.; Yousef, G.M.; Scorilas, A.; Jung, K.; Jung, M.; Kristiansen, G.; Hauptmann, S.; Kishi, T.; Nakamura, T.; Loening, S.A.; Diamandis, E.P. Hepsin is highly over expressed in and a new candidate for a prognostic indicator in prostate cancer. *J. Urol.,* **2004**, *171*(1), 187-191.

[44] Klezovitch, O.; Chevillet, J.; Mirosevich, J.; Roberts, R.L.; Matusik, R.J.; Vasioukhin, V. Hepsin promotes prostate cancer progression and metastasis. *Cancer Cell,* **2004**, *6*(2), 185-195.

[45] Tanimoto, H.; Yan, Y.; Clarke, J.; Korourian, S.; Shigemasa, K.; Parmley, T.H.; Parham, G.P.; O'Brien, T.J. Hepsin, a cell surface serine protease identified in hepatoma cells, is overexpressed in ovarian cancer. *Cancer Res.,* **1997**, *57*(14), 2884-2887.

[46] Betsunoh, H.; Mukai, S.; Akiyama, Y.; Fukushima, T.; Minamiguchi, N.; Hasui, Y.; Osada, Y.; Kataoka, H. Clinical relevance of hepsin and hepatocyte growth factor activator inhibitor type 2 expression in renal cell carcinoma. *Cancer Sci.,* **2007**, *98*(4), 491-498.

[47] Tang, X.; Mahajan, S.S.; Nguyen, L.T.; Beliveau, F.; Leduc, R.; Simon, J.A.; Vasioukhin, V. Targeted inhibition of cell-surface serine protease Hepsin blocks prostate cancer bone metastasis. *Oncotarget,* **2014**, *5*(5), 1352-1362.

[48] Chen, Z.; Fan, Z.; McNeal, J.E.; Nolley, R.; Caldwell, M.C.; Mahadevappa, M.; Zhang, Z.; Warrington, J.A.; Stamey, T.A. Hepsin and maspin are inversely expressed in laser capture microdissectioned prostate cancer. *J. Urol.,* **2003**, *169*(4), 1316-1319.

[49] Li, W.; Wang, B.E.; Moran, P.; Lipari, T.; Ganesan, R.; Corpuz, R.; Ludlam, M.J.; Gogineni, A.; Koeppen, H.; Bunting, S.; Gao, W.Q.; Kirchhofer, D. Pegylated kunitz domain inhibitor suppresses hepsin-mediated invasive tumor growth and metastasis. *Cancer Res.,* **2009**, *69*(21), 8395-8402.

[50] Zacharski, L.R.; Ornstein, D.L. Heparin and cancer. *Thromb. Haemost.,* **1998**, *80*(1), 10-23.

[51] Ganesan, R.; Kolumam, G.A.; Lin, S.J.; Xie, M.H.; Santell, L.; Wu, T.D.; Lazarus, R.A.; Chaudhuri, A.; Kirchhofer, D. Proteolytic activation of pro-macrophage-stimulating protein by hepsin. *Mol. Cancer Res.,* **2011**, *9*(9), 1175-1186.

[52] Koschubs, T.; Dengl, S.; Durr, H.; Kaluza, K.; Georges, G.; Hartl, C.; Jennewein, S.; Lanzendorfer, M.; Auer, J.; Stern, A.; Huang, K.S.; Packman, K.; Gubler, U.; Kostrewa, D.; Ries, S.; Hansen, S.; Kohnert, U.; Cramer, P.; Mundigl, O. Allosteric antibody inhibition of human hepsin protease. *Biochem. J.,* **2012**, *442*(3), 483-494.

[53] Cheng, Y.; Prusoff, W.H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.,* **1973**, *22*(23), 3099-3108.

[54] Barre, O.; Dufour, A.; Eckhard, U.; Kappelhoff, R.; Beliveau, F.; Leduc, R.; Overall, C.M. Cleavage specificity analysis of six type II transmembrane serine proteases (TTSPs) using PICS with proteome-derived peptide libraries. *PloS One,* **2014**, *9*(9), e105984.

[55] Goswami, R.; Wohlfahrt, G.; Tormakangas, O.; Moilanen, A.; Lakshminarasimhan, A.; Nagaraj, J.; Arumugam, K.N.; Mukherjee, S.; Chacko, A.R.; Krishnamurthy, N.R.; Jaleel, M.; Palakurthy, R.K.; Samiulla, D.S.; Ramachandra, M. Structure-guided discovery of 2-aryl/pyridin-2-yl-1H-indole derivatives as potent and selective hepsin inhibitors. *Bioorg. Med. Chem. Lett.,* **2015**, *25*(22), 5309-5314.

[56] Goswami, R.; Mukherjee, S.; Wohlfahrt, G.; Ghadiyaram, C.; Nagaraj, J.; Chandra, B.R.; Sistla, R.K.; Satyam, L.K.; Samiulla, D.S.; Moilanen, A.; Subramanya, H.S.; Ramachandra, M. Discovery of Pyridyl Bis(oxy)dibenzimidamide Derivatives as Selective Matriptase Inhibitors. *ACS Med. Chem. Lett.,* **2013**, *4*(12), 1152-1157.

[57] Goswami, R.; Mukherjee, S.; Ghadiyaram, C.; Wohlfahrt, G.; Sistla, R.K.; Nagaraj, J.; Satyam, L.K.; Subbarao, K.; Palakurthy, R.K.; Gopinath, S.; Krishnamurthy, N.R.; Ikonen, T.; Moilanen, A.; Subramanya, H.S.; Kallio, P.; Ramachandra, M. Structure-guided discovery of 1,3,5 tri-substituted benzenes as potent and selective matriptase inhibitors exhibiting *in vivo* antitumor efficacy. *Bioorg. Med. Chem.,* **2014**, *22*(12), 3187-3203.

[58] Tidwell, R.R.; Geratz, J.D.; Dubovi, E.J. Aromatic amidines: comparison of their ability to block respiratory syncytial virus induced cell fusion and to inhibit plasmin, urokinase, thrombin, and trypsin. *J. Med. Chem.,* **1983**, *26*(2), 294-298.

[59] Somoza, J.R.; Ho, J.D.; Luong, C.; Ghate, M.; Sprengeler, P.A.; Mortara, K.; Shrader, W.D.; Sperandio, D.; Chan, H.; McGrath, M.E.; Katz, B.A. The structure of the extracellular region of human hepsin reveals a serine protease domain and a novel scavenger receptor cysteine-rich (SRCR) domain. *Structure,* **2003**, *11*(9), 1123-1131.

[60] Friedrich, R.; Fuentes-Prior, P.; Ong, E.; Coombs, G.; Hunter, M.; Oehler, R.; Pierson, D.; Gonzalez, R.; Huber, R.; Bode, W.; Madison, E.L. Catalytic domain structures of

MT-SP1/matriptase, a matrix-degrading transmembrane serine proteinase. *J. Biol. Chem.,* **2002**, *277*(3), 2160-2168.

[61] Maignan, S.; Guilloteau, J.P.; Pouzieux, S.; Choi-Sledeski, Y.M.; Becker, M.R.; Klein, S.I.; Ewing, W.R.; Pauls, H.W.; Spada, A.P.; Mikol, V. Crystal structures of human factor Xa complexed with potent inhibitors. *J. Med. Chem.,* **2000**, *43*(17), 3226-3232.

[62] Subedi, M.; Minn, I.; Chen, J.; Kim, Y.; Ok, K.; Jung, Y.W.; Pomper, M.G.; Byun, Y. Design, synthesis and biological evaluation of PSMA/hepsin-targeted heterobivalent ligands. *Eur. J. Med. Chem.,* **2016**, *118*, 208-218.

[63] Katz, B.A.; Luong, C.; Ho, J.D.; Somoza, J.R.; Gjerstad, E.; Tang, J.; Williams, S.R.; Verner, E.; Mackman, R.L.; Young, W.B.; Sprengeler, P.A.; Chan, H.; Mortara, K.; Janc, J.W.; McGrath, M.E. Dissecting and designing inhibitor selectivity determinants at the S1 site using an artificial Ala190 protease (Ala190 uPA). *J. Mol. Biol.,* **2004**, *344*(2), 527-547.

[64] Franco, F.M.; Jones, D.E.; Harris, P.K.; Han, Z.; Wildman, S.A.; Jarvis, C.M.; Janetka, J.W. Structure-based discovery of small molecule hepsin and HGFA protease inhibitors: Evaluation of potency and selectivity derived from distinct binding pockets. *Bioorg. Med. Chem.,* **2015**, *23*(10), 2328-2343.

[65] Steinmetzer, T.; Schweinitz, A.; Sturzebecher, A.; Donnecke, D.; Uhland, K.; Schuster, O.; Steinmetzer, P.; Muller, F.; Friedrich, R.; Than, M.E.; Bode, W.; Sturzebecher, J. Secondary amides of sulfonylated 3-amidinophenylalanine. New potent and selective inhibitors of matriptase. *J. Med. Chem.,* **2006**, *49*(14), 4116-4126.

[66] Ekici, O.D.; Paetzel, M.; Dalbey, R.E. Unconventional serine proteases: variations on the catalytic Ser/His/Asp triad configuration. *Protein Sci.,* **2008**, *17*(12), 2023-2037.

[67] Mori, S.; Itoh, Y.; Shinohata, R.; Sendo, T.; Oishi, R.; Nishibori, M. Nafamostat mesilate is an extremely potent inhibitor of human tryptase. *J. Pharmacol. Sci.,* **2003**, *92*(4), 420-423.

[68] Steinmetzer, T.; Donnecke, D.; Korsonewski, M.; Neuwirth, C.; Steinmetzer, P.; Schulze, A.; Saupe, S.M.; Schweinitz, A. Modification of the N-terminal sulfonyl residue in 3-amidinophenylalanine-based matriptase inhibitors. *Bioorg. Med. Chem. Lett.,* **2009**, *19*(1), 67-73.

[69] Venukadasula, P.K.; Owusu, B.Y.; Bansal, N.; Ross, L.J.; Hobrath, J.V.; Bao, D.; Truss, J.W.; Stackhouse, M.; Messick, T.E.; Klampfer, L.; Galemmo, R.A. Jr. Design and synthesis of nonpeptide inhibitors of hepatocyte growth factor activation. *ACS Med. Chem. Lett.,* **2016**, *7*(2), 177-181.

[70] Owusu, B.Y.; Bansal, N.; Venukadasula, P.K.; Ross, L.J.; Messick, T.E.; Goel, S.; Galemmo, R.A.; Klampfer, L. Inhibition of pro-HGF activation by SRI31215, a novel approach to block oncogenic HGF/MET signaling. *Oncotarget,* **2016**, *7*(20), 29492-29506.

[71] Galemmo, R.A. Jr.; Maduskuie, T.P.; Dominguez, C.; Rossi, K.A.; Knabb, R.M.; Wexler, R.R.; Stouten, P.F. The de novo design and synthesis of cyclic urea inhibitors of factor Xa: initial SAR studies. *Bioorg. Med. Chem. Lett.,* **1998**, *8*(19), 2705-2710.

[72] Galemmo, R.A. Jr.; Wells, B.L.; Rossi, K.A.; Alexander, R.S.; Dominguez, C.; Maduskuie, T.P.; Stouten, P.F.; Wright, M.R.; Aungst, B.J.; Wong, P.C.; Knabb, R.M.; Wexler, R.R. The de novo design and synthesis of cyclic urea inhibitors of factor Xa: optimization of the S4 ligand. *Bioorg. Med. Chem. Lett.,* **2000**, *10*(3), 301-304.

[73] Han, Z.; Harris, P.K.; Jones, D.E.; Chugani, R.; Kim, T.; Agarwal, M.; Shen, W.; Wildman, S.A.; Janetka, J.W. Inhibitors of HGFA, matriptase, and hepsin serine proteases: A nonkinase strategy to block cell signaling in cancer. *ACS Med. Chem. Lett.,* **2014**, *5*(11), 1219-1224.

[74] Han, Z.; Harris, P.K.; Karmakar, P.; Kim, T.; Owusu, B.Y.; Wildman, S.A.; Klampfer, L.; Janetka, J.W. Alpha-ketobenzothiazole serine protease inhibitors of aberrant HGF/c-MET and MSP/RON kinase pathway signaling in cancer. *ChemMedChem,* **2016**, *11*(6), 585-599.

[75] Bhatt, A.S.; Welm, A.; Farady, C.J.; Vasquez, M.; Wilson, K.; Craik, C.S. Coordinate expression and functional profiling identify an extracellular proteolytic signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.,* **2007**, *104*(14), 5771-5776.

[76] Kwon, H.; Kim, Y.; Park, K.; Choi, S.A.; Son, S.H.; Byun, Y. Structure-based design, synthesis, and biological evaluation of Leu-Arg dipeptide analogs as novel hepsin inhibitors. *Bioorg. Med. Chem. Lett.,* **2016**, *26*(2), 310-314.

[77] Chevillet, J.R.; Park, G.J.; Bedalov, A.; Simon, J.A.; Vasioukhin, V.I. Identification and characterization of small-molecule inhibitors of hepsin. *Mol. Cancer Ther.,* **2008**, *7*(10), 3343-3351.

[78] Kyrieleis, O.J.; Huber, R.; Ong, E.; Oehler, R.; Hunter, M.; Madison, E.L.; Jacob, U. Crystal structure of the catalytic domain of DESC1, a new member of the type II transmembrane serine proteinase family. *FEBS J.,* **2007**, *274*(8), 2148-2160.