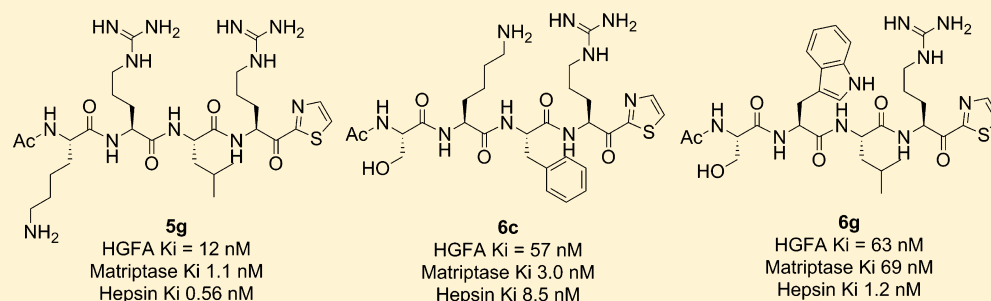# Inhibitors of HGFA, Matriptase, and Hepsin Serine Proteases: A Nonkinase Strategy to Block Cell Signaling in Cancer

Zhenfu Han,[†] Peter K. W. Harris,[†] Darin E. Jones,[†,§] Ryan Chugani,[†] Tommy Kim,[†] Manjula Agarwal,[†] Wei Shen,[‡] Scott A. Wildman,[†] and James W. Janetka*,[†]

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S *Supporting Information*

5g
HGFA Ki = 12 nM
Matriptase Ki 1.1 nM
Hepsin Ki 0.56 nM

6c
HGFA Ki = 57 nM
Matriptase Ki 3.0 nM
Hepsin Ki 8.5 nM

6g
HGFA Ki = 63 nM
Matriptase Ki 69 nM
Hepsin Ki 1.2 nM

ABSTRACT: Hepatocyte growth factor activators (HGFA), matriptase, and hepsin are S1 family trypsin-like serine proteases. These proteases proteolytically cleave the single-chain zymogen precursors, pro-HGF (hepatocyte growth factor), and pro-MSP (macrophage stimulating protein) into active heterodimeric forms. HGF and MSP are activating ligands for the oncogenic receptor tyrosine kinases (RTKs), c-MET and RON, respectively. We have discovered the first substrate-based ketothiazole inhibitors of HGFA, matriptase and hepsin. The compounds were synthesized using a combination of solution and solid-phase peptide synthesis (SPPS). Compounds were tested for protease inhibition using a kinetic enzyme assay employing fluorogenic peptide substrates. Highlighted HGFA inhibitors are Ac-KRLR-kt (**5g**), Ac-SKFR-kt (**6c**), and Ac-SWLR-kt (**6g**) with K_is = 12, 57, and 63 nM, respectively. We demonstrated that inhibitors block the conversion of native pro-HGF and pro-MSP by HGFA with equivalent potency. Finally, we show that inhibition causes a dose-dependent decrease of c-MET signaling in MDA-MB-231 breast cancer cells. This preliminary investigation provides evidence that HGFA is a promising therapeutic target in breast cancer and other tumor types driven by c-MET and RON.

KEYWORDS: *serine protease, peptidomimetic, breast cancer, HGFA, matriptase, hepsin, HGF, MSP, c-MET, RON, growth factor, kinase, cell signaling, ketothiazole, solid-phase peptide synthesis, inhibitor*

Increased activation and signaling of the oncogenic receptor tyrosine kinases (RTKs) c-MET and RON[1] triggers many downstream phenotypic changes necessary for tumor metastasis[2−4] including cell migration, invasion, proliferation, differentiation, survival, and angiogenesis. Targeting c-MET and RON kinase cell signaling pathways with RTK inhibitors[5] is a well-developed strategy for treating metastatic cancer. In fact, coexpression of c-MET and RON has been recently identified in several tumor types including bladder, ovarian, and node-negative breast cancer.[6−15] Crosstalk between c-MET and RON[16] has been shown in some cases to result from the formation of c-MET/RON heterodimers,[17] which is a mechanism tumors can utilize to promote a metastatic phenotype. Many potent kinase inhibitors of c-MET and some for RON have been developed, but most reported inhibitors are multitargeted and lack sufficient selectivity. With the exception of antibodies to c-MET[18] and HGF,[19,20] only limited studies have explored nonkinase extracellular targets upstream of c-MET or RON kinase receptor activation. One such target is hepatocyte

growth factor activator (HGFA) that has highly upregulated function in a number of different tumor types both in patient-derived cell lines[21] and in patient tissue samples most widely studied so far in breast cancer.

HGFA is a member of the S1 trypsin-like serine protease family exemplified by thrombin and the coagulation proteases but most structurally similar to Factor XIIa.[22] Characteristic of other coagulation cascade proteases, HGFA circulates in plasma as an inactive form and pro-HGFA at relatively high levels (40 nM). pro-HGFA is produced by the liver and is activated by other serine proteases in the plasma including thrombin[23] and kallikrein-1 related (KLK) peptidases.[24] Its biological function is a proteolytic process and activates the c-MET and RON tyrosine kinase ligands, HGF (hepatocyte growth factor), and MSP (macrophage stimulating protein), respectively, primarily

in injured tissues.[25] HGF and MSP are members of the plasminogen family of proteins that are secreted as inactive single-chain zymogens, pro-HGF, and pro-MSP. The latter are the only two known HGFA substrates and are enzymatically hydrolyzed at the Arg494-Val495 and the Arg483-Val484 peptide bonds, respectively, by either activated HGFA[26] or the cell-surface serine proteases matriptase[27] and hepsin.[28] Subsequent to enzymatic hydrolysis, the α-chain N-terminal fragment and β-chain C-terminal fragments of the processed growth factor spontaneously form a two-chain disulfide bridged heterodimer capable of binding to the extracellular domain of its respective receptor tyrosine kinase and causing its activation. The processing of HGF and MSP is thus critical for cell signaling through c-MET and RON.

Matriptase and hepsin are type II transmembrane serine proteases (TTSPs)[29,30] present on endothelial cells. They are also members of the S1 family of serine peptidases and are upregulated and aberrantly expressed in invasive tumors. The proteolytic activity of all three proteases, matriptase, hepsin, and HGFA, is regulated by the polypeptide inhibitors HAI-1 and HAI-2, which inhibit all three proteases at low nanomolar concentration. Imbalance of normal HGFA, HAI-1, and HAI-2 expression has been demonstrated to lead to invasive phenotypes in breast and other types of cancer.[31] Only limited studies have been pursued evaluating the individual roles that HGFA, matriptase, and hepsin play in cancer development or progression, and their importance in different types of cancer[32] is not yet understood. Inhibitors of HGFA, matriptase, or hepsin act in the tumor pericellular microenvironment to block cell signaling by preventing activation of both c-MET and RON receptor tyrosine kinases. Thus, potent and selective inhibitors of HGFA are powerful chemical tools for studying the role and regulation of cell signaling by serine proteases and are potential therapeutics for the treatment and prevention of metastatic cancer driven by c-MET and RON cell signaling.

Potent benzamidine-based inhibitors of matriptase[33] and weak inhibitors of hepsin identified through HTS[34] have been reported. There are several reports of inhibitory antibodies against HGFA,[35] matriptase, and hepsin, but there are no synthetic peptide-based or small molecule inhibitors of HGFA or hepsin. In this preliminary communication, we report on the design and synthesis of the first small molecular weight synthetic inhibitors of HGFA and hepsin. These inhibitors cause a dose-dependent decrease in the catalytic processing of pro-HGF and pro-MSP by HGFA and phosphorylation of c-MET in MDA-MB-231 invasive breast cancer cells.

Electrophilic ketones,[36] alpha-ketoamides, and aldehydes are typical warheads used as mechanism-based inhibitors of serine proteases, which engage the catalytic active-site serine by covalent but reversible attachment. The warhead is attached at the P1 position of a peptide substrate and P1−P1′ is the site of amide bond hydrolysis indicating the P1 N-terminal amino and the P1′ C-terminal amino acid of the substrate. The covalent attachment closely mimics the tetrahedral intermediate inherent to the enzyme mechanism and is stabilized by the classical oxyanion hole in the active site of serine proteases. When HGFA was discovered and classified as a serine protease in 1992,[37] it was reported that Leupeptin showed only a weak 40% inhibition of HGFA at 20 μM, and TLCK, an irreversible inhibitor, showed only 28% inhibition of HGFA at 2 mM. Furthermore, benzamidine, ordinarily an inhibitor of serine proteases at 1 mM, only showed 14% inhibition at a concentration 10-fold higher. These data suggest HGFA is unique relative to other S1

proteases and might have a different inhibitor profile and substrate specificity. Therefore, we followed a systematic approach in our design of inhibitors based on the sequences of the only known HGFA substrates, pro-HGF and pro-MSP.

In order to rationally design mechanism-based inhibitors of HGFA, we synthesized tetrapeptide ketothiazole (KT) analogues based on the N-terminal portion (P4−P1) of the pro-HGF and pro-MSP substrate cleavage sites (Figure 1). Shown in
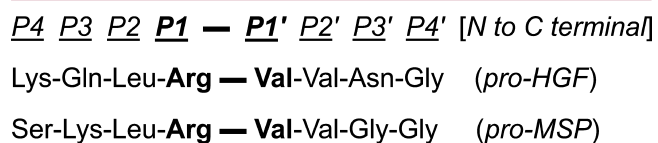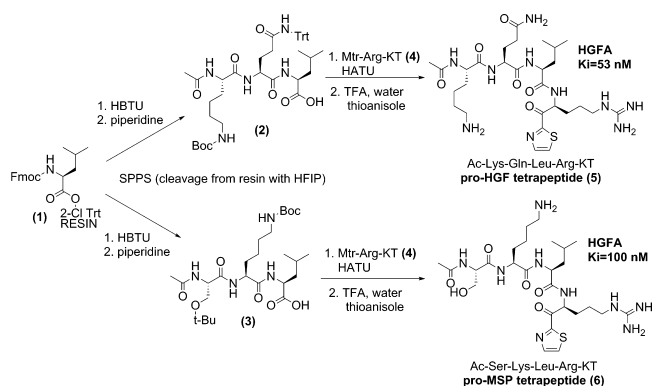
<u>P4</u> <u>P3</u> <u>P2</u> **<u>P1</u>** — **<u>P1'</u>** <u>P2'</u> <u>P3'</u> <u>P4'</u> [*N to C terminal*]

Lys-Gln-Leu-**Arg** — **Val**-Val-Asn-Gly   (*pro-HGF*)

Ser-Lys-Leu-**Arg** — **Val**-Val-Gly-Gly   (*pro-MSP*)

Figure 1. $P_4–P_4'$ polypeptide sequences of pro-HGF and pro-MSP highlighting cleavage site.

Scheme 1. Synthesis of Pro-HGF and Pro-MSP P_4−P_1 Tetrapeptide Ketothiazoles

Scheme 1, protected tripeptide acids were constructed using solid phase peptide synthesis from 2-Cl trityl resin (**1**). Reaction of **2** or **3** tripeptide acids with Mtr-protected Arg-KT (**4**) (Scheme 2)[36,38] and HATU in solution gave Ac-Lys(Boc)-

Scheme 2. Synthesis of Protected Arginine Ketothiazole
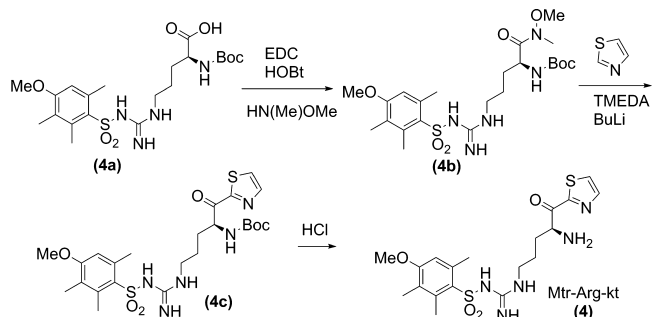
Gln(Trt)-Leu-Arg(Mtr)-kt and Ac-Ser(tBu)-Lys(Boc)-Leu-Arg-(Mtr)-kt. Deprotection with TFA/H2O/thioanisole and purification by reversed phase HPLC gave the pro-HGF and pro-MSP substrate mimetics, Ac-Lys-Gln-Leu-Arg-kt (**5**) and Ac-Ser-Lys-Leu-Arg-kt (**6**), respectively, in good overall yield. We tested previously reported inhibitors[27] and our new ketothiazoles, **5** and **6**, in an HGFA enzymatic assay using the fluorogenic substrate Boc-QLR-AMC with a recombinant form of the HGFA serine protease domain (Supporting Information). The pro-HGF and

pro-MSP tetrapeptides **5** and **6** showed K_is of 53 and 81 nM, respectively. We demonstrated that inhibitor **5** was competitive with the substrate at the active-site and that the inhibition is reversible (Supporting Information), consistent with other reported ketone-based serine protease inhibitors.[38]

We confirmed in our assay that irreversible inhibitor TLCK showed no inhibition up to 100 μM and that Nafamostat had a K_i consistent with the single-point assay reported in 1992.[39] In order to understand substrate binding pocket specificity and develop structure–activity relationships (SAR), we rationally designed two focused libraries of peptides, one directed on changes to the pro-HGF peptide and the other on pro-MSP.

Using the X-ray structure of Ac-KQLR-CMK (PDB code: 2WUC)[35] and a computational model of the pro-HGF peptide, Lys-Gln-Leu-Arg-Val-Val-Asn-Gly, spanning the S4–S4′ pockets bound to the active site of HGFA (PDB code: 1YCO),[40] we noted the S4 and S3 pockets are large and not well occupied by the peptide side chains Lys and Gln. In the corresponding pro-MSP peptide the P4 position is a Ser and the P3 a Lys (Figure 2).
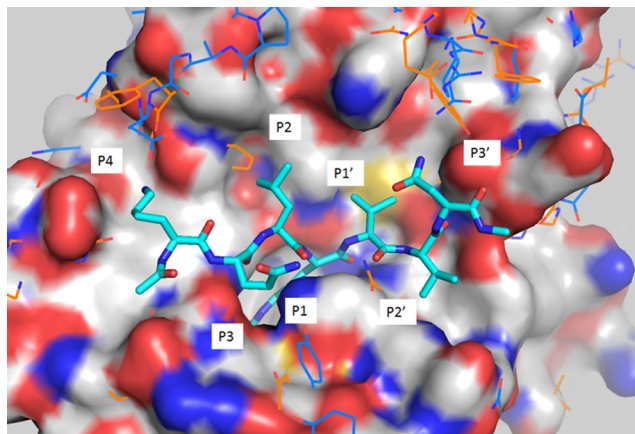

Figure 2. Model of pro-HGF P_4–$P_4{}'$ peptide (cyan) bound to HGFA (PDB code: 1YCO; white),[40] matriptase (PDB code: 2GV7; orange),[33] and hepsin (PDB code: 1Z8G; blue).[41]

Our data from the P4–P1 ketothiazoles **5** and **6** suggest that the Ser in P4 of the pro-MSP peptide decreases substrate binding relative to pro-HGF. Therefore, we hypothesized that larger side chains in P4 and P3 should lead to enhancements in potency due to increased hydrophobic interactions with these pockets of the enzyme. We also noticed that conserved residue Trp618 (Trp215 in chymotrypsin numbering) is forming part of the S4 pocket so Trp was one of the P4 residues chosen for our initial set of compounds. We found that replacement of Lys with Trp in P4 of Ac-KQLR-kt (**5**) had no effect on potency (**5b**), while replacement of Ser of Ac-SKLR-kt (**6**) in P4 with Trp leads to a 2-fold increase in activity (**6b**) (Table 1). However, changing the Lys of **5** to Ser (**5f**) decreased activity 3-fold. Also forming part of the S4 pocket is Asp576, and we surmised that replacement of Lys with Arg would not only fill the pocket better but would also lead to improved interaction with the side chain of Asp576. This substitution with Arg resulted in a 4-fold enhancement of potency seen with Ac-RKLR-kt (**6h**). This small set of compounds demonstrates that the S4 pocket of HGFA does prefer larger side chains but does not have a preference for a basic residue at P4 like has been demonstrated for matriptase[42] and hepsin.[41] Although the S3 site appears large, it is also more solvent exposed, and forming part of this pocket is a salt bridge between Glu545 and Arg624. The P3 amino acid in pro-MSP is

Table 1. HGFA, Matriptase and Hepsin Inhibitory Activity of Pro-HGF and Pro-MSP Substrate-Based Inhibitors

structure	HGFA K_i (nM)	matriptase K_i (nM)	hepsin K_i (nM)
nafamostat	25	0.02	0.53
leupeptin (Ac-LLR-H)	188	696	61
Ac-KQLR-kt (pro-HGF; 5)	**53**	**0.92**	**0.22**
Ac−KHLR-kt (**5a**)	96	22	0.41
Ac-WQLR-kt (**5b**)	65	32	0.21
Ac-KQFR-kt (**5c**)	58	0.69	0.58
Ac−KFLR-kt (**5d**)	80	15	2.1
Ac-RQLR-kt (**5e**)	60	0.32	0.28
Ac-SQLR-kt (**5f**)	182	9.2	0.34
Ac−KRLR-kt (**5g**)	12	1.1	0.57
Ac-WRLR-kt (**5h**)	21	5.5	0.21
Ac-SKLR-kt (pro-MSP; 6)	**81**	**58**	**1.2**
Ac−SHLR-kt (**6a**)	332	104	0.60
Ac-WKLR-kt (**6b**)	56	8.6	0.55
Ac-SKFR-kt (**6c**)	57	3.0	8.5
Ac-NKLR-kt (**6d**)	79	12	1.4
Ac-SRLR-kt (**6e**)	24	5.8	0.68
Ac-TKLR-kt (**6f**)	103	8.7	0.61
Ac−SWLR-kt (**6g**)	63	69	1.2
Ac-RKLR-kt (**6h**)	17	0.83	0.47

Lys suggesting a possible interaction with the side chain of Glu545. We proposed that a His or Arg side chain might be able to interact in a similar fashion but also more effectively interfere with the Arg624. We found that replacement of P3 with His resulted in similar inhibition of HGFA for the pro-HGF analogue (**5a**) but a 4-fold decrease in activity for the pro-MSP analogue (**6a**). However, the corresponding Arg replacements did give enhanced activity as predicted and exemplified by **5g** (K_i = 12 nM) and **6e** (K_i = 29 nM). Compound **5d** with Phe on the P3 position also increased HGFA inhibition with a K_i = 41 nM. We also found substitution of the P2 Leu with Phe in the pro-HGF peptide (**5c**) or the pro-MSP peptide (**6c**) was tolerated with no change in activity. Modifying the P2 position has potential for increasing selectivity against matriptase, which prefers small hydrophobic side chains[41] since the S2 pocket is attenuated compared to HGFA. SAR from this study demonstrates that HGFA prefers Arg or Trp in the S4 and S3 pockets, and cooperativity between these two pockets suggests that the best potency results when one of the side chains is basic such as Ac-WRLR-kt (**5h**, K_i = 21 nM) and Ac-WKLR-kt (**6b**, K_i = 56 nM). However, good activity is still achieved in the absence of a basic side chain when either the P4 or the P3 residue is Trp as in Ac-WQLR-kt (**5b**, K_i = 65 nM) and Ac-SWLR-kt (**6g**, K_i = 63 nM).

Having this data in hand, we wanted to determine the selectivity profile of the inhibitors relative to matriptase and hepsin. We developed a similar assay using Boc-QAR-AMC as the substrate. Shown in Table 1, the majority of compounds evaluated are more selective for matriptase and hepsin; most pronounced with hepsin where some inhibitors have K_is < 1 nM. There are several compounds that are equipotent for HGFA compared to matriptase but leupeptin is 3-fold selective for HGFA over matriptase and only 3-fold favoring hepsin. The pro-HGF peptide (**5**) is 200-fold more potent for hepsin and 50-fold for matriptase, while the pro-MSP peptide (**6**) is only slightly more potent for matriptase and 70-fold potent for hepsin versus HGFA. Ac-SWLR-kt (**6g**) is equipotent for both HGFA and

matriptase with a K_i = 66 nM but K_i = 1.2 nM for hepsin. Compound **6g**, Ac-SKFR-kt (**6c**), Ac-KFLR-kt (**5d**), and Ac-SRLR-kt (**6e**) are promising leads for obtaining HGFA selectivity over both hepsin and matriptase.

We employed a biochemical assay in order to demonstrate that inhibitors can block the proteolytic activation of the endogenous growth factors, pro-HGF and pro-MSP, by HGFA in a dose-dependent manner. In order to determine efficiency of our recombinant HGFA we performed a concentration response of HGFA using a fixed concentration of pro-HGF and pro-MSP. Since both the single chain inactive pro-HGF or pro-MSP and the active heterodimers have the same molecular weight, SDS gels were developed under reducing conditions. Lanes with pro-HGF (Figure 3) and pro-MSP (Figure 4) contain one band at 90
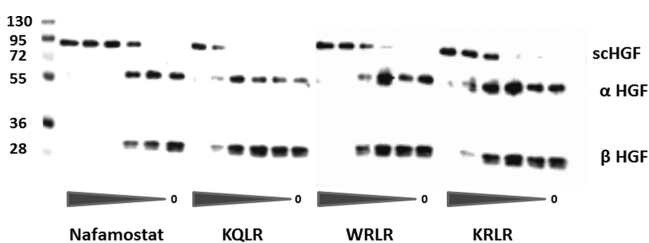

Figure 3. Inhibition of HGFA-mediated scHGF (pro-HGF) cleavage by inhibitors. Immunoblot of scHGF cleavage reactions: Pro-HGF (30 ng) was cleaved with 1 nM HGFA in the presence of inhibitors (5-fold dilutions from 12.5 μM).
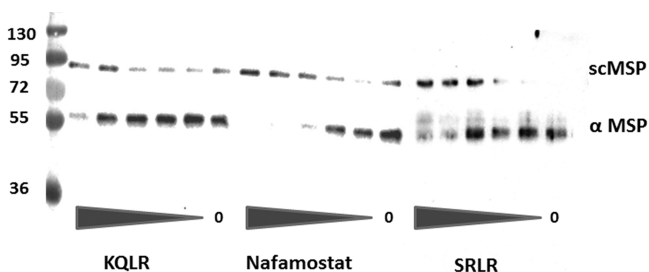

Figure 4. Inhibition of HGFA-mediated scMSP (pro-MSP) cleavage by inhibitors. Immunoblot of scMSP cleavage reactions: Pro-MSP (50 ng) was cleaved with 10 nM HGFA in the presence of inhibitors (5-fold dilutions from 12.5 μM).

and 75 kDa, respectively, whereas activated HGF and MSP appear as two bands as the 60 kDa α-chain and 30 kDa β-chain in HGF and at 50 and 25 kDa for MSP (note: MSP Ab only recognizes the α-chain). Shown in Figure 4, we found that nafamostat and the three inhibitors **5**, **5h**, and **5g** all showed a dose-dependent inhibition of pro-HGF activation with similar EC_{50} values in direct correlation with those found from the HGFA enzyme assay. These inhibitors in addition to **6e** also all show dose-dependent inhibition of pro-MSP proteolysis by HGFA as shown in Figure 4. While it is difficult to quantitate the level of MSP activation since the pro-MSP has some active two-chain MSP present, the EC_{50} values correlate with the level of potency seen in the enzyme assay. These results show the inhibitors inhibit the processing of both known protein substrates of HGFA.

Since we demonstrated robust and potent inhibition of pro-HGF processing by HGFA inhibitors, we next wanted to show that the inhibition would have effects on cell signaling through c-MET kinase. Therefore, we developed a phosphorylation assay using the invasive breast cancer cell line, MDA-MB-231. This cell

line has high expression of c-MET and pro-HGFA but not pro-HGF. We incubated HGFA with inhibitor, followed by the addition of pro-HGF in cell culture with the MBA-MB-231 cells. Shown in Figure 5, several inhibitors were effective at decreasing
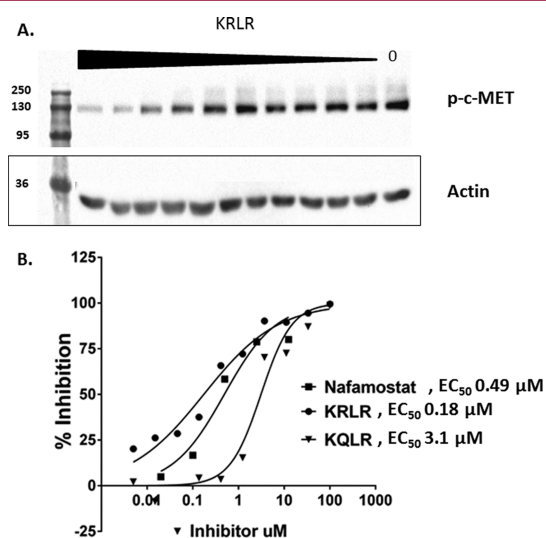

Figure 5. MDA-MB-231 c-MET phosphorylation of cells treated with pro-HGF/HGFA reactions (3-fold dilutions of inhibitors starting at 100 μM). (A) Immunoblot of pY1234/135 c-MET. (B) Percent inhibition of c-MET phosphorylation.

c-MET phosphorylation in a dose-dependent manner. Thus, inhibitors of HGFA show promise as nonkinase inhibitors of HGF-mediated c-MET kinase signaling in cancer. The most potent compound **5g** had an EC_{50} of 180 nM.

In conclusion, we have rationally designed the first peptidomimetic inhibitors of HGFA serine protease. We utilized the sequence and predicted binding conformations of the pro-HGF and pro-MSP protein substrates of HGFA to discover a series of potent mechanism-based inhibitors. We have developed an initial understanding of structure−activity relationships (SAR) for HGFA, matriptase, and hepsin by evaluating tetrapeptide ketothiazole libraries. The data from our inhibitors suggests that HGFA has an unusual inhibitor profile relative to other trypsin-like serine proteases. HGFA prefers one basic side chain in either the S3 or S4 subsites for maximal binding, but interestingly, substitution of Arg or Lys with Trp is also well-tolerated. We discovered that Ser or Trp in the P4 and Phe in the P2 position of the ketothiazole inhibitor help improve relative selectivity for HGFA over matriptase and hepsin. All inhibitors cause a dose-dependent decrease in pro-HGF and pro-MSP native substrate proteolysis and activation by HGFA. Furthermore, inhibitors causes decreased c-MET phosphorylation in MDA-MB-231 invasive breast cancer cells. This is the first demonstration that a small molecule inhibitor of HGFA can block c-MET phosphorylation and cancer cell signaling. The results from this study enable the rational design of more potent and selective inhibitors of HGFA, which can be used to study the role of these proteases in cancer and developed further as new therapeutics for metastatic cancer. We are currently pursuing structural biology data of HGFA-inhibitor binding through X-ray crystallography and are testing inhibitors for their effects on RON kinase phosphorylation and other phenotypic effects, which will be reported in due course.

ASSOCIATED CONTENT

S **Supporting Information**

Experimental details on the synthesis, purification, and characterization of Boc-QLR-AMC, **5**, **6**, **5a–5h**, and **6a–6h** are provided. Experimental details on the expression and purification of HGFA are included. Protocols and data analysis for the enzyme, biochemical, and phosphorylation assays are also supplied. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

HGF, hepatocyte growth factor; MSP, macrophage stimulating protein; HGFA, hepatocyte growth factor activator; kt, ketothiazole; AMC, 7-amino-4-methylcoumarin; TLCK, N-α-tosyl-L-lysine chloromethyl ketone; PNGB, 4-nitrophenyl 4-guanidinobenzoate; Cha, cyclohexylalanine; RTK, receptor tyrosine kinase; SPPS, solid-phase peptide synthesis

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