Supplementary Data For:

Inhibitors of HGFA, matriptase and hepsin serine proteases: A non-kinase strategy to block cell signaling in cancer

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General synthesis, purification, and analytical chemistry procedures. Starting materials, reagents, and solvents were purchased from commercial vendors unless otherwise noted. ^{1}H NMR spectra were measured on a Varian 400 MHz NMR instrument. The chemical shifts were reported as ppm relative to TMS using residual solvent peak as the reference unless otherwise noted. The following abbreviations were used to express the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High-performance liquid chromatography (HPLC) was carried out on GILSON GX-281 using Waters C18 5μM, 4.6*50mm and Waters Prep C18 5μM, 19*150mm reverse phase columns, eluted with a gradient system of 5:95 to 95:5 acetonitrile:water with a buffer consisting of 0.05% TFA. Purity assessment and mass spectra (MS) data were obtained using a Hewlett-Packard HPLC/MSD using electrospray ionization (ESI) for detection. All reactions were monitored by thin layer chromatography (TLC) carried out on Merck silica gel plates (0.25 mm thick, 60F254), visualized by using UV (254 nm) or dyes such as ninhydrin, $KMnO_4$, *p*-anisaldehyde or CAM (ceric ammonium molybdate). Silica gel chromatography was carried out on a Teledyne ISCO CombiFlash purification system using pre-packed silica gel columns. All compounds used for biological assays are greater than 95% purity based on NMR and HPLC by UV absorbance at 210 nm and 254 nm wavelengths.

Procedure for the preparation of tetrapeptide ketothiazoles:

Tripeptide Synthesis: Tripeptide intermediates were synthesized on a 0.1 mmol scale using a CEM Liberty Microwave Peptide Synthesizer and Fmoc-amino acid-preloaded 2-Cl-trityl resins. Standard peptide coupling conditions were employed utilizing 5 equiv. of Fmoc-amino acid/4.5 equiv. HBTU/10 equiv. ^{i}Pr$_2$Net in DMF and the mixture was heated at 75 ^{o}C for 5 min by microwave. Piperidine/DMF (20% v/v) was employed for the deprotection of the Fmoc protecting group using the microwave for 5 min.

Acetyl capping of the tripeptides: The tripeptide resin was suspended in 15 mL of 0.5 M Ac_2O/DMF and 1 M ^{i}Pr$_2$NEt/DMF. The mixture was shaken at RT for 1 h. The resin was filtered and washed with DMF (10 mL x 4) followed by CH_2Cl_2 (10 mL x 4).

Cleavage of tripeptide resin: Ac-capped tripeptide resin was suspended and shaken in 15 ml of 25% v/v HFIP/ CH_2Cl_2 for 1 h. The mixture was filtered. The filtrate was concentrated then dried in vacuo, giving rise to crude Ac-capped tripeptide product.

Synthesis of tetrapeptide ketothiazoles:

Ac-KQFR ketothiazole (5c). Under nitrogen atmosphere, at 0 ^{o}C anhydrous DMF (5 mL) was added into the RB flask containing Ac-capped KQF tripeptide (0.081 g, 0.1 mmol) and HATU (0.042 g, 0.11 mmol). After stirring for 10 min, arginine ketothiazole (0.042 g, 0.1 mmol), then *N*,*N*-diisopropylethylamine (0.065 g, 0.5 mmol) were added. The mixture was stirred overnight while being warmed to RT naturally. The majority of DMF was removed and to the resulting residue was added 20 mL of water. The precipitate that formed was filtered and dried. To this precipitate was added 5 mL of TFA/thioanisole/water (95/2.5/2.5(v/v/v)). The mixture was stirred at RT for 4 h and then was added to 40 mL of cold ether. The precipitated crude product was collected by centrifugation, followed by carefully decanting out the ether solvent. The crude product was purified by HPLC (C18, 15*150 mm column; eluent: acetonitrile/water (0.1% TFA)) to give Ac-KQFR ketothiazole (0.032 g) in 46% yield. At the same time the isomer KQFR* ketothiazole (0.020 g) was also collected. ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 1.35 (m, 2 H) 1.45 - 1.77 (m, 7 H) 1.78 - 2.04 (m, 6 H) 2.06 - 2.29 (m, 2 H) 2.92 (t, *J*=7.63 Hz, 2 H) 3.00 (m, 2 H) 3.13 (m, 2 H) 4.12 - 4.20 (m, 1 H) 4.21 - 4.29 (m, 1 H) 4.59 (t, *J*=7.63 Hz, 1 H) 5.30 - 5.45 (m, 1 H) 7.17 (m, 5 H) 8.04 (br. s., 1 H) 8.07 (br. s., 1 H). MS (ESI): found: $[M + H]^{+}$, 687.5.

Ac-SQLR ketothiazole (5f) (yield: 42%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.85 (d, *J*=5.87 Hz, 3 H) 0.90 (d, *J*=5.87 Hz, 3 H) 1.48 - 1.76 (m, 5 H) 1.77 - 1.88 (m, 1 H) 1.89 - 2.02 (m, 1 H) 2.02 - 2.20 (m, 5 H) 2.26 - 2.47 (m, 2 H) 3.11 - 3.32 (m, 2 H) 3.84 (tt, *J*=11.44, 5.77 Hz, 2 H) 4.25 - 4.45 (m, 3 H) 5.47 (dd, *J*=9.39, 4.30 Hz, 1 H) 8.06 (d, *J*=3.13 Hz, 1 H) 8.11 (d, *J*=3.13 Hz, 1 H). MS (ESI): found: $[M + H]^{+}$, 612.5.

Ac-SRLR ketothiazole (6e) (yield: 26%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.85 (d, *J*=5.87 Hz, 3 H) 0.91 (d, *J*=5.87 Hz, 3 H) 1.52 - 1.77 (m, 8 H) 1.79 - 1.89 (m, 2 H) 1.99 - 2.18 (m, 4 H) 3.09 - 3.34 (m, 4 H) 3.83 (tt, *J*=11.35, 5.67 Hz, 2 H) 4.29 - 4.45 (m, 3 H) 5.46 (dd, *J*=9.20, 4.11 Hz, 1 H) 8.06 (d, *J*=3.13 Hz, 1 H) 8.12 (d, *J*=3.13 Hz, 1 H). MS (ESI): found: $[M + H]^{+}$, 640.5.

Ac-TKLR ketothiazole (6f) (yield: 59%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.85 (d, *J*=5.87 Hz, 3 H) 0.91 (d, *J*=5.48 Hz, 3 H) 1.12 - 1.24 (m, 3 H) 1.32 - 1.90 (m, 12 H) 1.99 - 2.18 (m, 4 H) 2.98 (t, *J*=7.43 Hz, 2 H) 3.15 - 3.30 (m, 2 H) 4.07 - 4.19 (m, 1 H) 4.24 (d, *J*=5.48 Hz, 1 H) 4.34 (td, *J*=8.90, 5.67 Hz, 2 H) 5.45 (dd, *J*=9.19, 4.50 Hz, 1 H) 8.07 (d, *J*=3.13 Hz, 1 H) 8.12 (d, *J*=3.13 Hz, 1 H). MS (ESI): found: $[M + H]^{+}$, 626.5.

Ac-KFLR ketothiazole (5d) (yield: 43%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.85 (d, *J*=6.26 Hz, 3 H) 0.90 (d, *J*=6.26 Hz, 3 H) 1.13 - 1.38 (m, 2 H) 1.40 - 1.77 (m, 9 H) 1.78 - 1.91 (m, 1 H) 1.99 (s, 3 H) 2.04 - 2.17 (m, 1 H) 2.82 - 3.05 (m, 3 H) 3.09 - 3.32 (m, 3 H) 4.08 - 4.21 (m, 1 H) 4.37 (dd, *J*=8.80, 6.06 Hz, 1 H) 4.65 (dd, *J*=8.61, 6.26 Hz, 1 H) 5.44 (dd, *J*=9.19, 4.50 Hz, 1 H) 7.15 - 7.43 (m, 5 H) 8.08 (d, *J*=3.13 Hz, 1 H) 8.13 (d, *J*=3.13 Hz, 1 H). MS (ESI): found: $[M + H]^{+}$, 672.5.

Ac-NKLR ketothiazole (6d) (yield: 39%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.85 (d, *J*=5.48 Hz, 3 H) 0.91 (d, *J*=5.87 Hz, 3 H) 1.32 - 1.48 (m, 2 H) 1.48 - 1.91 (m, 10 H) 1.93

- 2.17 (m, 4 H) 2.62 - 2.84 (m, 2 H) 2.98 (t, *J*=7.63 Hz, 2 H) 3.14 - 3.30 (m, 2 H) 4.20 - 4.42 (m, 2 H) 4.63 (t, *J*=7.04 Hz, 1 H) 5.45 (dd, *J*=9.39, 4.30 Hz, 1 H) 8.06 (d, *J*=3.13 Hz, 1 H) 8.11 (d, *J*=3.13 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 639.5.

Ac-RQLR ketothiazole (5e) (yield: 13%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.85 (d, *J*=5.87 Hz, 3 H) 0.91 (d, *J*=5.87 Hz, 3 H) 1.45 - 1.89 (m, 10 H) 1.90 - 2.19 (m, 6 H) 2.35 (td, *J*=7.43, 3.91 Hz, 2 H) 3.14 - 3.28 (m, 4 H) 4.25 (dd, *J*=8.02, 6.06 Hz, 1 H) 4.30 - 4.43 (m, 2 H) 5.47 (dd, *J*=9.39, 4.30 Hz, 1 H) 8.07 (d, *J*=3.13 Hz, 1 H) 8.12 (d, *J*=3.13 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 681.5.

Ac-WQLR ketothiazole (5b) (yield: 13%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.81 (d, *J*=5.87 Hz, 3 H) 0.88 (d, *J*=5.48 Hz, 3 H) 1.37 - 1.69 (m, 6 H) 1.70 - 1.91 (m, 3 H) 1.91 - 2.10 (m, 5 H) 2.98 - 3.35 (m, 4 H) 3.92 - 4.06 (m, 1 H) 4.11 - 4.23 (m, 1 H) 4.48 (t, *J*=6.65 Hz, 1 H) 5.39 (m, 1 H) 7.09 (t, *J*=7.24 Hz, 1 H) 7.14 - 7.26 (m, 2 H) 7.43 (d, *J*=8.22 Hz, 1 H) 7.52 (d, *J*=7.43 Hz, 1 H) 8.00 (d, *J*=2.74 Hz, 1 H) 8.05 (d, *J*=2.74 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 711.5.

Ac-KHLR ketothiazole (5a) (yield: 52%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.82 (d, *J*=4.30 Hz, 3 H) 0.87 (d, *J*=4.70 Hz, 3 H) 1.22 - 1.44 (m, 2 H) 1.44 - 1.73 (m, 9 H) 1.74 - 1.87 (m, 1 H) 1.96 (s, 3 H) 2.06 (m, 1 H) 2.83 - 2.99 (m, 2 H) 3.02 - 3.36 (m, 4 H) 4.06 - 4.22 (m, 1 H) 4.34 (m, 1 H) 4.58 - 4.72 (m, 1 H) 5.43 (dd, *J*=8.61, 4.30 Hz, 1 H) 7.23 (s, 1 H) 8.03 (d, *J*=2.35 Hz, 1 H) 8.08 (d, *J*=2.35 Hz, 1 H) 8.58 (s, 1 H). MS (ESI): found: $[M + H]^+$, 662.5.

Ac-SKLR ketothiazole (6) (yield: 57%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.81 (d, *J*=4.70 Hz, 3 H) 0.87 (d, *J*=4.30 Hz, 3 H) 1.26 - 1.91 (m, 12 H) 1.93 - 2.16 (m, 4 H) 2.94 (t, *J*=7.43 Hz, 2 H) 3.18 (m, 2 H) 3.79 (t, *J*=6.46 Hz, 2 H) 4.18 - 4.46 (m, 3 H) 5.43 (dd, *J*=9.00, 3.91 Hz, 1 H) 8.03 (d, *J*=2.74 Hz, 1 H) 8.08 (d, *J*=2.74 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 612.5.

Ac-SHLR ketothiazole (6a) (yield: 32%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.81 (d, *J*=5.09 Hz, 3 H) 0.86 (d, *J*=5.09 Hz, 3 H) 1.41 - 1.60 (m, 3 H) 1.60 - 1.72 (m, 2 H) 1.73 - 1.88 (m, 1 H) 1.90 - 2.15 (m, 4 H) 2.99 - 3.37 (m, 4 H) 3.63 - 3.83 (m, 2 H) 4.20 - 4.41 (m, 2 H) 4.68 (dd, *J*=8.61, 5.48 Hz, 1 H) 5.44 (dd, *J*=8.61, 3.13 Hz, 1 H) 7.24 (s, 1 H) 8.03 (d, *J*=2.35 Hz, 1 H) 8.08 (d, *J*=2.35 Hz, 1 H) 8.57 (s, 1 H). MS (ESI): found: $[M + H]^+$, 621.5.

Ac-SKFR ketothiazole (6c) (yield: 46%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 1.25 (m, 2 H) 1.42 - 1.81 (m, 7 H) 1.86 - 2.11 (m, 4 H) 2.80 - 3.06 (m, 4 H) 3.13 (m, 2 H) 3.64 - 3.86 (m, 2 H) 4.17 - 4.28 (m, 1 H) 4.32 (t, *J*=5.67 Hz, 1 H) 4.56 (t, *J*=7.83 Hz, 1 H) 5.38 (dd, *J*=8.61, 4.30 Hz, 1 H) 7.00 - 7.36 (m, 5 H) 8.04 (d, *J*=2.74 Hz, 1 H) 8.07 (d, *J*=2.74 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 646.5.

Ac-WKLR ketothiazole (6b) (yield: 38%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) ppm 0.86 (d, *J*=5.48 Hz, 3 H) 0.93 (d, *J*=5.48 Hz, 3 H) 1.09 - 1.20 (m, 2 H) 1.39 - 1.71 (m, 9 H) 1.73 - 1.88 (m, 1 H) 1.98 (s, 3 H) 2.01 - 2.14 (m, 1 H) 2.80 - 2.94 (m, 2 H) 3.10 - 3.27 (m, 4 H) 4.04 - 4.13 (m, 1 H) 4.15 - 4.24 (m, 1 H) 4.45 - 4.59 (m, 1 H) 5.42 (dd, *J*=9.00, 3.91 Hz, 1 H) 7.08 - 7.17 (m, 1 H) 7.18 - 7.28 (m, 2 H) 7.47 (d, *J*=7.83 Hz, 1 H) 7.57 (d, *J*=7.83 Hz, 1 H) 7.93 (dd,

J=13.50, 6.85 Hz, 1 H) 8.05 (d, *J*=2.74 Hz, 1 H) 8.09 (d, *J*=2.74 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 711.8.

Ac-KQLR ketothiazole (5) (yield: 32%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) □ ppm 0.80 (d, *J*=5.09 Hz, 3 H) 0.86 (d, *J*=5.09 Hz, 3 H) 1.26 - 1.84 (m, 12 H) 1.84 - 2.13 (m, 6 H) 2.29 (m, 2 H) 2.93 (t, *J*=7.43 Hz, 2 H) 3.17 (m, 2 H) 4.18 (t, *J*=7.04 Hz, 1 H) 4.23 - 4.37 (m, 2 H) 5.35 - 5.51 (m, 1 H) 8.01 (d, *J*=2.74 Hz, 1 H) 8.07 (d, *J*=2.74 Hz, 1 H). MS (ESI): found: $[M + H]^+$, 653.5.

Ac-KRLR-ketothiazole (5g) (yield: 44%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) □ ppm 0.77 - 0.91 (m, 6 H) 1.26 - 1.45 (m, 3 H) 1.46 - 1.58 (m, 5 H) 1.58 - 1.70 (m, 7 H) 1.70 - 1.85 (m, 4 H) 1.97 (s, 3 H) 1.99 - 2.11 (m, 2 H) 2.93 (t, J=7.63 Hz, 2 H) 3.15 (dt, J=13.79, 6.99 Hz, 4 H) 4.17 (dd, J=8.22, 5.87 Hz, 1 H) 4.24 - 4.30 (m, 1 H) 4.30 - 4.37 (m, 1 H) 5.41 (dd, J=9.20, 4.11 Hz, 1 H) 8.02 (d, J=2.74 Hz, 1 H) 8.07 (d, J=3.13 Hz, 1 H) MS (ESI): found: $[M + H]^+$, 681.7.

Ac-WRLR-ketothiazole (5h) (yield: 24%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) □ ppm 0.82 (d, J=5.87 Hz, 3 H) 0.89 (d, J=5.87 Hz, 3 H) 1.10 - 1.32 (m, 3 H) 1.34 - 1.45 (m, 2 H) 1.48 (d, J=6.65 Hz, 3 H) 1.53 - 1.68 (m, 3 H) 1.68 - 1.83 (m, 1 H) 1.95 (s, 3 H) 2.02 (d, J=10.56 Hz, 1 H) 2.97 (t, J=5.87 Hz, 2 H) 3.12 (t, J=6.85 Hz, 2 H) 3.15 - 3.22 (m, 2 H) 4.02 (dd, J=8.02, 5.67 Hz, 1 H) 4.15 (t, J=7.63 Hz, 1 H) 4.47 (t, J=7.04 Hz, 1 H) 5.37 (dd, J=9.00, 4.30 Hz, 1 H) 7.02 - 7.12 (m, 1 H) 7.17 (t, J=7.63 Hz, 1 H) 7.21 (s, 1 H) 7.42 (d, J=8.22 Hz, 1 H) 7.51 (d, J=7.83 Hz, 1 H) 8.00 (d, J=3.13 Hz, 1 H) 8.05 (d, J=3.13 Hz, 1 H)MS (ESI): found: $[M + H]^+$, 739.7.

Ac-SWLR-ketothiazole (6g) (yield: 20%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) □ ppm 0.74 (d, J=6.65 Hz, 3 H) 0.71 (d, J=6.26 Hz, 3 H) 0.99 - 1.15 (m, 2 H) 1.35 (t, J=7.04 Hz, 2 H) 1.51 - 1.64 (m, 3 H) 1.72 (s, 3 H) 1.76 (br. s., 1 H) 2.00 (dd, J=13.69, 4.70 Hz, 2 H) 3.00 - 3.18 (m, 3 H) 3.25 (d, J=5.48 Hz, 2 H) 3.60 - 3.81 (m, 3 H) 4.11 - 4.26 (m, 2 H) 4.62 (t, J=5.87 Hz, 1 H) 5.33 (dd, J=9.59, 4.11 Hz, 1 H) 7.08 - 7.15 (m, 1 H) 7.15 (s, 1 H) 7.17 - 7.23 (m, 1 H) 7.43 (d, J=8.22 Hz, 1 H) 7.57 (d, J=8.22 Hz, 1 H) 7.63 (br. s., 1 H) 7.77 (br. s., 1 H) 8.00 (d, J=3.13 Hz, 1 H) 8.06 (d, J=2.74 Hz, 1 H) MS (ESI): found: $[M + H]^+$, 670.5.

Ac-RKLR-ketothiazole (6h) (yield: 31%) ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) □ ppm 0.76 - 0.91 (m, 6 H) 1.24 - 1.47 (m, 3 H) 1.47 - 1.56 (m, 3 H) 1.56 - 1.70 (m, 7 H) 1.70 - 1.86 (m, 4 H) 1.97 (s, 3 H) 2.00 - 2.12 (m, 1 H) 2.93 (t, J=7.63 Hz, 2 H) 3.06 - 3.24 (m, 4 H) 4.19 (t, J=7.04 Hz, 1 H) 4.27 (dd, J=8.80, 6.06 Hz, 1 H) 4.33 (d, J=9.00 Hz, 1 H) 5.41 (dd, J=9.00, 4.70 Hz, 1 H) 8.03 (d, J=3.13 Hz, 1 H) 8.08 (d, J=3.13 Hz, 1 H)MS (ESI): found: $[M + H]^+$, 681.7.

Synthesis of Boc-QLR-AMC fluorogenic substrate:

Boc-R(NO_2)-AMC. Under nitrogen atmosphere, pyridine (60 mL) was added into the RB flask containing Boc-R(NO_2)-OH (4.653 g, 14.6 mmol) and 7-amino-4-methylcoumarin (3.829 g, 21.9 mmol). Diisopropylcarbodiimide (2.023 g, 16.0 mmol) was added and the mixture was stirred overnight. The mixture was filtered. The filtrate was concentrated then dried in vacuo. The resultant residue was purified by silica gel chromatography with dichloromethane/methanol combinations as eluent giving rise to Boc-R(NO_2)-AMC (2.964 g) in 43% yield. MS (ESI): found $[M + H]^+$, 477.4.

HCl.H_2N-R(NO_2)-AMC. 4 N HCl in dioxane (25 mL) was added into the RB flask containing Boc-R(NO_2)-AMC (2.964 g, 6.2 mmol) and the mixture stirred for two hours. The dioxane was removed in vacuo and to the resultant residue methanol was added then concentrated in vacuo three times, giving rise to the title compound in quantitative yield. MS (ESI): found $[M + H]^+$, 377.3.

Boc-QL-OH. Under nitrogen atmosphere, anhydrous DMF (10 mL) was added into the RB flask containing Boc-Q-OH (0.500 g, 2.0 mmol), H-L-OMe.HCl (0.406 g, 2.2 mmol), EDCI.HCl (0.467 g, 2.4 mmol), and HOBt (0.466 g, 3.1 mmol). *N*,*N*-diisopropylethylamine (0.787 g, 6.1 mmol) was added and the mixture was stirred overnight. The majority of DMF was removed in vacuo and to the resulting residue was added 20 mL of water. The precipitate was isolated by filtration then purified by silica gel chromatography with dichloromethane/methanol combinations as eluent to give Boc-QL-OMe (0.711 g) in 95% yield. MS (ESI): found $[M + Na]^+$, 396.4. Methanol/water (1:1 v/v, 10 mL) was added into the RB flask containing the Boc-QL-OMe (0.711 g, mmol) and LiOH (0.068 g, 2.8 mmol). The reaction was stirred overnight. The mixture was concentrated in vacuo and to the resulting residue was added 30 mL of water. 0.5 M HCl was added dropwise until pH=4.5 was reached, then the mixture was extracted three times with ethyl acetate. The ethyl acetate layers were collected, dried with Na_2SO_4, then concentrated in vacuo to give rise to Boc-QL-OH (0.603 g) in 49% yield. MS (ESI): found $[M + Na]^+$, 373.4.

Boc-QLR(NO_2)-AMC. Under nitrogen atmosphere, anhydrous DMF (10 mL) was added into the RB flask containing Boc-QL-OH (0.603 g, 1.7 mmol), HCl.H_2N-R(NO_2)-AMC (0.406 g, 2.2 mmol), EDCI.HCl (0.322 g, 1.7 mmol), and HOBt (0.257 g, 1.7 mmol). *N*,*N*-diisopropylethylamine (0.904 g, 7.0 mmol) was added and the mixture was stirred overnight. The majority of DMF was removed in vacuo and to the resulting residue was added 20 mL of water. The mixture was extracted three times with ethyl acetate. The ethyl acetate layers were collected, dried with Na_2SO_4, then concentrated in vacuo. The resultant residue was purified by silica gel chromatography with dichloromethane/methanol combinations as eluent giving rise to Boc-QLR(NO_2)-AMC (0.250 g) in 20% yield. MS (ESI): found $[M + H]^+$, 718.5.

Boc-QLR-AMC. Into the solution of Boc-QLR(NO_2)-AMC (0.250 g, 0.35 mmol) in MeOH (15 mL) was added Pd/C(10%) (0.111 g) followed by several drops of acetic acid. The mixture was stirred under H_2 atmosphere for 21 h. Additional Pd/C(10%) (0.184 g) was added with a few drops of acetic acid. The mixture was stirred for 24 h, then filtered. The filtrate was concentrated. 1/5 of the resulting residue was purified by HPLC (C18, 15*150 mm column; eluent: acetonitrile/water (0.05% TFA)) to give the title compound (0.037 g) in 78% yield. ^{1}H NMR (400 MHz, METHANOL-d_4) □ ppm 0.92 (d, *J*=6.30 Hz, 3 H) 0.98 (d, *J*=6.26 Hz, 3 H) 1.45 (s, 9 H) 1.54 - 2.14 (m, 9 H) 2.25 - 2.41 (m, 2 H) 2.49 (s, 3 H) 3.17 - 3.29 (m, 2 H) 4.00 - 4.14 (m, 1 H) 4.34 - 4.47 (m, 1 H) 4.49 - 4.61 (m, 1 H) 6.28 (s, 1 H) 7.46 - 7.60 (m, 1 H) 7.71 - 7.80 (m, 1 H) 7.80 - 7.89 (m, 1 H). MS (ESI): found: $[M + H]^+$, 673.6.

Expression and purification of N-terminal His-tag HGFA serine protease domain:

Using 5’ primer gtccaactgtcaccggatctc, 3’ primer ggcggcaagctttcaggagggagccacaagccgcc, and standard PCR protocols, the nucleotide sequence encoding amino acids 373-655 of HGFA was

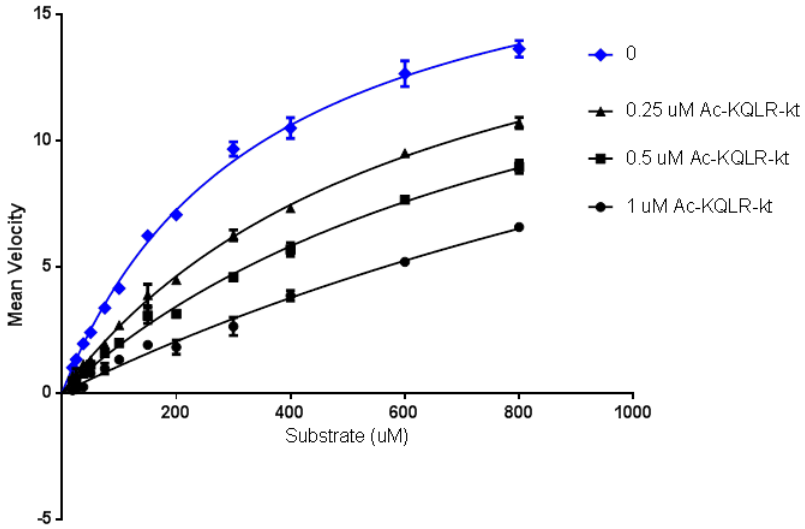
synthesized. This PCR product was cloned into the SfoI-HindIII sites of a modified pFastBac HT baculovirus expression vector (Addgene, Cambridge, MA). This vector contains a six amino His tag followed by a seven amino spacer and a seven amino acid TEV cleavage site placed immediately downstream of the Honey Bee melittin signal peptide. Using a modified Bac to Bac Expression System (Life Technologies, Carlsbad, CA), recombinant HGFA bacmids were obtained by transforming DH10Bac *Escherichia coli* cells. To obtain HGFA containing baculovirus, purified bacmids were transfected into Sf9 insect cells. After 5 days in culture at 27 ºC, media was harvested from transfected Sf9 cells. This media was used to prepare baculovirus infected insect cells (BIICs). These BIIICs were used to infect High 5 insect cells. Four days post infection, media was harvested and recombinant protein prepared as follows. Media was chilled to 4 ºC and spun 4000xg for 20 minutes (all subsequent steps were performed at 4 ºC unless noted). Clarified media was passed first through a Whatman GF/B 1 um (# 1821-047, GE Healthcare Life Sciences, Piscataway, NJ) and then a 0.22 um PES membrane (#99955, TPP Techno Plastic Products AG, Trasadingen, Switzerland) and then concentrated using a Pall Centramate tangential flow system and Centramate T-series Cassette (#OS010T12, Pall Corporation, Port Washington, NY). Concentrated media was then buffer exchanged in two steps, five volumes of 50 mM Na-phosphate, 500 mM NaCl, pH 6.2, followed by five volumes of 50 mM Na-phosphate, 500 mM NaCl, pH 7.5. The concentrated and buffered exchanged insect cell media was again filtered as above and made 25 mM imidazole (#I202, Sigma-Aldrich, St. Louis, MO) and was mixed with nickel agarose beads (# H-321-25, Gold Biotechnology, Inc., St. Louis MO). After mixing this slurry for 12 hours, nickel agarose beads were allowed to settle by gravity and then loaded into a column. Beads were washed with buffer (25 mM Na-phosphate, 500 mM NaCl, 25 mM imidazole, pH 8) and the bound protein eluted using (25 mM Na-phosphate, 500 mM NaCl, 250 mM imidazole, pH 8). Using a Amicon Ultra-4 Centrifugal filters (#UFC801008, Merck Millipore, Ltd., Tullagreen, Ireland), peak protein fractions were concentrated and run over a Superdex-200 10/300 GL column (GE Healthcare Life Sciences, Piscataway, NJ) in 10 mM Tris, 200 mM NaCl, 0.2 mM EDTA, pH 8. HGFA containing fractions were pooled, concentrated, made 50% glycerol, and stored at minus 20 ºC. Protein was quantitated using a modified Lowry protein assay (#500-0006, Bio-Rad Laboratories, Hercules, CA).

Chromogenic Proteolytic Assay of HGFA Inhibitors:

Inhibitors (0-50 µM final concentration in reaction) were diluted in DMSO (2% DMSO final concentration in reaction) and then mixed with recombinant HGFA (12.5 nM final concentration in reaction) in TNC buffer (25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100, pH 8). After incubating for thirty minutes at 25^{o} C, chromogenic substrate, Pefachrome FVIIa, (#093-01, Enzyme Research Laboratories, South Bend, IN)) was added to a final concentration of 250 µM in a final reaction volume of 40 microliters. Changes in absorbance at 405 nm were measured over time in a Biotek Synergy 2 plate reader (Winnoski, VT). Using Gen 5 2.00 software program (Biotek, Winnoski, VT), a four parameter curve fit was used to determine the inhibitor IC_{50}s from a plot of the mean reaction velocity versus the inhibitor concentration. The IC_{50} values represent the average of three separate experimental determinations.

HGFA Competition Assay of inhibitor Ac-KQLR-kt (5) and substrate:

To determine whether the ketothiazoles are behaving as competitive inhibitors, different amounts of Ac-KQLR-kt (**5**) (0, 0.25, 0.5, and 1.0 µM) were mixed with Pefachrome serially diluted in TNC buffer. HGFA was added to 12.5 nM and changes in absorbance at 405 nm were measured over time in a Biotek Synergy 2 plate reader. From the plots of the mean reaction velocity versus substrate concentration and the Michaelis-Menten enzyme kinetics equation within GraphPad Prism version 6.04 for Windows, the reaction V_{max} and subsequent K_m for each concentration of inhibitor was determined.



	1 uM Ac-KQLR-kt	0.5 uM Ac-KQLR-kt	0.25 uM Ac-KQLR-kt	0
Vmax	23.93	19.17	19.27	19.79
Km	2137	917.7	632.9	345.7

	1 uM Ac-KQLR-kt	0.5 uM Ac-KQLR-kt	0.25 uM Ac-KQLR-kt	0
R square	0.9862	0.9944	0.9973	0.9962

Fluorescent Inhibitor Assays:

Inhibitors (0-10 µM final concentration in reaction) were diluted in DMSO (2% DMSO final concentration in reaction) and then mixed with either recombinant HGFA serine protease domain, Matriptase (Charles Craik, UCSF), or activated[1] Hepsin (#4776-SE-010, R&D Systems, Minneapolis, Minnesota) in black 384 well plates (Corning # 3575. Corning, NY). The final assay concentration for HGFA, Matriptase, and Hepsin were 10 nM, 0.2 nM, and 0.3 nM, respectively in TNC buffer (25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100, pH 8). After thirty minutes incubation[2] at room temperature, Boc-QLR-AMC substrate (synthesis described above) was added to the HGFA assays and Boc-QAR-AMC substrate (#ES014, R&D Systems, Minneapolis, Minnesota) was added to the Matriptase and Hepsin assays. The final substrate concentrations were at the K_m for the respective enzymes; 35 µM, 100 µM, and 150 µM. Changes in fluorescence (excitation at 380 nm and emission at 460 nm) were measured at room temperature over time in a Biotek Synergy 2 plate reader (Winnoski, VT). From a plot of the mean reaction velocity versus the inhibitor concentration, a non-linear four parameter curve fit was performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, San

Diego, CA, www.graphpad.com) to determine inhibitor IC_{50}s. K_i values were calculated using the Cheng and Prusoff equation ($K_i = IC_{50}/(1+[S]/K_m)$ and the IC_{50} values that were determined from the average of three separate experimental determinations.

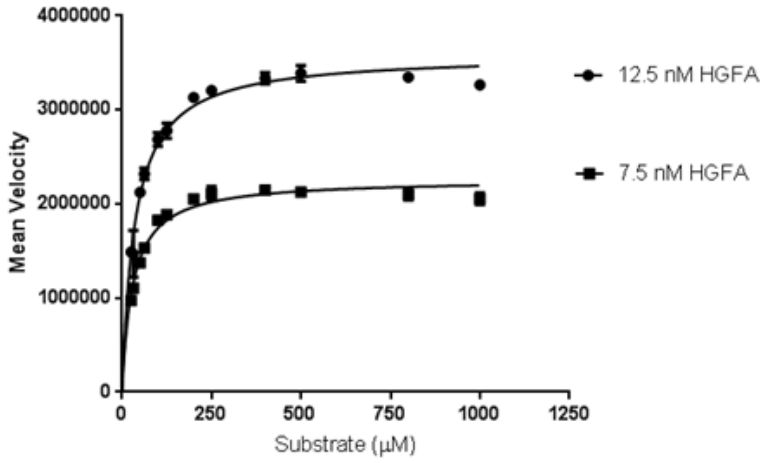
[1]Hepsin Activation: Based upon the manufacturer's recommendations, recombinant Hepsin (#4776-SE-010, R&D Systems, Minneapolis, Minnesota) was diluted 5.5 fold in TNC buffer (25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100, pH 8) and incubated at 37°C. After twenty-four hours, the Hepsin was diluted in glycerol to 50%. This stock Hepsin (1.2 µM) was stored in a -20°C freezer and diluted in TNC buffer for use in assays.

[2]K_is calculated for selected inhibitors without pre-incubation are consistently ~1.5 fold higher than those with incubation.

K_m Determinations:

In black 384 well plates (Corning #3575), 12.5 nM HGFA was mixed with various amounts of Boc-QLR-AMC and 1 nM Matriptase and 0.3 nM Hepsin were mixed with various amounts of Boc-QAR-AMC. Changes in fluorescence (excitation at 380nm and emission at 460nn) were measured at room temperature over time in a Biotek Synergy 2 plate reader (Winnoski, VT). Using From plots of the mean reaction velocity versus substrate concentration and the Michaelis-Menten enzyme kinetics equation within GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com), the reaction V_{max} and subsequent K_m for each of the substrates were determined.

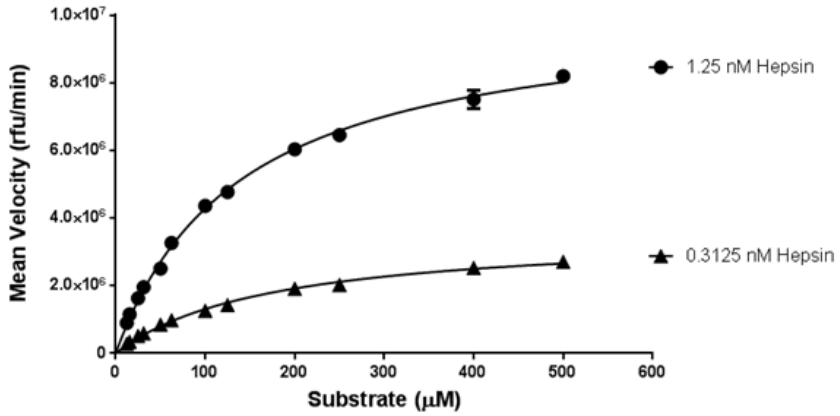
K_m determination for Boc-QLR-AMC and HGFA:



	12.5 nM HGFA	7.5 nM HGFA
Km	36.06	29.88

	12.5 nM HGFA	7.5 nM HGFA
R square	0.9716	0.9610

K_m determination for Boc-QAR-AMC and hepsin:

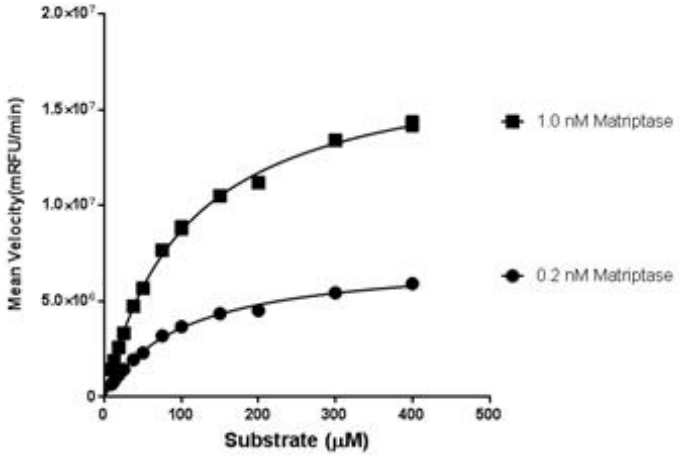


	1.25 nM Hepsin	0.3125 nM Hepsin
Km	139.7	173.0

	1.25 nM Hepsin	0.3125 nM Hepsin
Vmax	133264	61821

	1.25 nM Hepsin	0.3125 nM Hepsin
R square	0.9965	0.9952

K_m determination for Boc-QAR-AMC and matriptase:



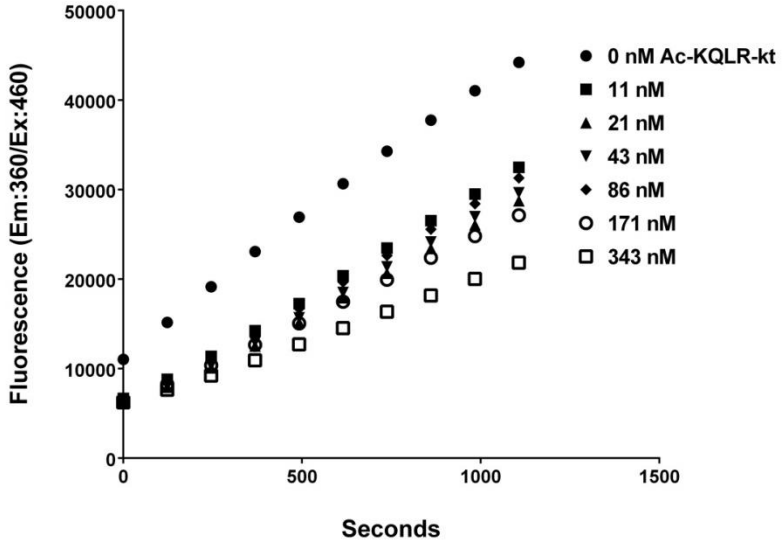
	0.2 nM Matriptase	1.0 nM Matriptase
Vmax	7.256e+006	1.793e+007
Km	101.6	106.4

	0.2 nM Matriptase	1.0 nM Matriptase
R square	0.9950	0.9969

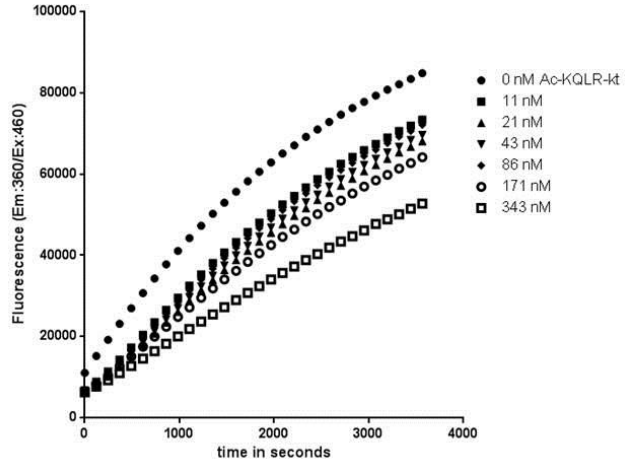
Dilution recovery experiments with Ac-KQLR-kt (5) and HGFA:

To demonstrate reversibility of HGFA ketothiazole inhibitors and to examine the dissociation of the enzyme-inhibitor complex, dilution recovery experiments were performed. High concentration HGFA (7.5 µM) were mixed with different concentrations of Ac-KQLR-kt (**5**), between 0-120 µM. After incubating for 20 minutes at room temperature, these reactions were diluted rapidly (1:350) in TNC buffer containing 250 µM Boc-QLR-AMC substrate (~7 times K_m). Activity was monitored by recording the change in fluorescence (excitation at 380 nm and emission at 460 nn) over time in a Biotek Synergy 2 plate reader. GraphPad Prism was used to plot the change in fluorescence over time.

**These results suggest that Ac-KQLR-kt (5) is a reversible inhibitor of HGFA as the enzyme activity is recovered slowly over time even at an inhibitor concentrations 7x K_i.*



7.5 uM HGFA protease incubated for 20 minutes in the presence of various amount of Inhibitor. Reactions then diluted 350-fold with 250 uM Boc-QLR-AMC and perform kinetic read in Syngergy 2, Ex 360 nm, Em 460 nm.



Biochemical Assay for Proteolysis of pro-HGF and pro-MSP by HGFA:

For pro-HGF proteolysis, inhibitors (0-12.5 µM final concentration in reaction) were diluted in DMSO (2% DMSO final concentration in reaction) and then mixed with recombinant HGFA (1.0 nM final concentration) in TNC buffer. After 30 minutes incubation at room temperature (25^{o} C), 25 ng of pro-HGF (R&D Systems, NKG011306A) was added. After 1 hour incubation at 37^{o} C, reactions were stopped by adding SDS gel loading buffer containing DTT (reducing) and then run on 12% PAGE. Proteins were transferred to Millipore Immobilon-P membranes (Billerica, MA) and then immunoblotted at 4^{o} C with anti-HGF antibody (R&D Systems, AF-294-NA) and HRP conjugated donkey anti-goat IgG (Santa Cruz, SC-2020) diluted 1:500 and 1:5000 in 5% milk/TBST (5% Carnation nonfat dried milk/10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0), respectively. Membranes were washed in TBST, immersed in Millipore Luminata Crescendo Western-HRP Substrate for 5 minutes, and then exposures made on a BioRad ChemiDoc MP Imaging System (Hercules, CA). For pro-MSP proteolysis assays, the procedures were similar, except that the HGFA concentration was 75 nM, pro-MSP (R&D Systems ZN081306A) concentration was 50 ng per reaction, and 1:500 dilution of anti-MSP antibody (R&D Systems, AF352) was used for MSP detection during immunoblotting.

c-MET Phosphorylation (Y1234/1235) in cells treated with HGFA processed pro-HGF and MSP:

MDA-MB-231 cells were maintained in RPMI medium (Sigma R8758, St. Louis, MO) containing 10% fetal bovine serum (Sigma F2442) and 1XPenicillin/Streptomycin (Pen/Strep) antibiotics (Thermo Fisher SV30010). For cMet phosphorylation measurements, MDA-MB-231 cells were switched to starve medium (RPMI medium containing 1 mM Sodium Pyruvate (Corning 25-000-ci), 10 mM HEPES (Corning 25-060-ci), 0.225% Glucose (Corning 25-037-ci), 1X PenStrep). After 18 hours, cells were switched to fresh starve media and then treated with HGFA Proteolysis reactions containing 1 nM HGFA, 50 ng pro-HGF and various amounts of inhibitors. After 15 minutes incubation, media was removed by aspiration and the remaining cells were washed twice with cold Dulbeccos Phosphate Buffered Saline (Life Technologies #14190-136). Next cells were scraped into in Lysis Buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Sodium Deoxcholate, pH 7.5) containing 1mM sodium fluoride, 1 mM sodium orthovanandate, 1X Sigma (P8340) Inhibitor Cocktail, and one Roche Complete Mini, EDTA-free Protease Inhibitor Cocktail tablet per 10 ml buffer and stored frozen. Lysate aliquots were mixed with reducing SDS gel loading buffer and run on 10% PAGE. Immunoblots were performed as described previously, except primary antibodies, anti-phospho cMet (Cell Signaling, CS3077) and anti-total cMet (Cell Signaling, CS3127) were used and diluted in 5% BSA/TBST. Secondary antibodies, HRP-anti rabbit antibody (CS7074) and HRP-anti mouse (CS7076) were used, respectively. All antibodies were used at a 1:1000 dilution. Images were obtained and quantitated using BioRad ChemiDoc MP Imaging System. cMet phosphorylation signals were normalized using the total cMet signals and % inhibition calculated using the ratio of inhibited versus uninhibited HGFA Proteolytic reactions. Results were plotted in GraphPad Prism and a four parameter curve fit was used to determine the inhibitor EC_{50}s.