Engineering a potent matriptase inhibitor from the natural hepatocyte growth factor activator inhibitor type-1 (HAI-1) protein

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Running Title: Engineering a potent matriptase inhibitor

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Abstract:
Dysregulated matriptase activity has been established as a key contributor to cancer progression through its activation of growth factors including the hepatocyte growth factor (HGF). Despite its critical role and prevalence in many human cancers, limitations to developing an effective matriptase inhibitor include weak binding affinity, poor selectivity, and short circulating half-life. We applied rational and combinatorial approaches to engineer a potent inhibitor based on the hepatocyte growth factor activator inhibitor type 1 (HAI-1), a natural matriptase inhibitor. The first Kunitz domain (KD1) of HAI-1 has been well established as a minimal matriptase binding and inhibition domain, while the second Kunitz domain (KD2) is inactive and involved in negative regulation. Here, we replaced the inactive KD2 domain of HAI-1 with an engineered chimeric variant of KD2/KD1 domains, and fused the resulting construct to an antibody Fc domain to increase valency and circulating serum half-life. The final protein variant contains 4 stoichiometric binding sites that we showed were needed to effectively inhibit matriptase with a Ki \(=70 \pm 5 \text{ pM}\), an increase of 120-fold compared to the natural HAI-1 inhibitor, to our knowledge making it one of the most potent matriptase inhibitors identified to date. Furthermore, the engineered inhibitor demonstrates a protease selectivity profile similar to wild-type KD1 but distinct from HAI-1. It also inhibits activation of the natural pro-HGF substrate and matriptase expressed on cancer cells with at least an order of magnitude greater efficacy than KD1.

High expression and dysregulated activity of the type II, membrane-anchored serine protease matriptase in the local tumor environment has been shown to correlate with poor patient prognosis in many human cancers including breast, colorectal, pancreatic, cervical, and prostate cancers (1–10). This dysregulation is partly driven by the high proteolytic processing and turnover of pro-hepatocyte growth factor (Pro-HGF) (9, 11) to a form of HGF that activates its cognate receptor, c-Met (12) (Fig. 1A). Matriptase is also known to activate other proteases and growth factors including urokinase plasminogen activator (uPA) (11), pro-macrophage stimulating protein (Pro-MSP) (13), and platelet derived growth factor-D (PDGF-D) (14), all of which play key roles in cancer growth and metastasis. Furthermore, matriptase has been identified as a critical driver of other diseases, including iron overload disease (15) and osteoarthritis (16), and has been shown to activate the human airway influenza virus (H1N1) (17) and human immunodeficiency virus (HIV) (18). Although the correlation of matriptase overexpression,
dysregulation, and disease progression is well-established, effective matriptase inhibitors are lacking, highlighting an important clinical need.

Matriptase naturally functions in developmental pathways as well as in tissue regeneration (19–23). The activity of matriptase is regulated in healthy tissue by the serine protease inhibitor hepatocyte growth factor activator inhibitor type-1 (HAI-1) (Fig. 1A). HAI-1 is primarily expressed on the surface of epithelial cells and naturally blocks the substrate-activating properties of matriptase (24–26), as well as other structurally related proteases such as the hepatocyte growth factor activator (HGFA) (9, 27), hepsin (28, 29), and kallakrein-4/5 (9, 30). The balance between substrate activation and protease inhibition is critical to the metastatic potential of tumor cells (Fig. 1B). As such, the ratio of HAI-1 expression to matriptase expression correlates with cancer aggression and patient prognosis, and has been established as a key biomarker (2, 5, 7, 10, 31, 32).

Inhibition of matriptase-driven cancer progression has been proposed as an attractive strategy for cancer therapy. In one study, induced cell surface expression of HAI-1 within the tumor environment of an orthotopic adenopancreatic cancer model resulted in reduced tumor size and eliminated metastatic nodule formation (2). In another study, the addition of soluble HAI-1 was shown to significantly lower pro-HGF activation and reduce breast cancer cell invasion in vitro (33), highlighting recombinant HAI-1 as a therapeutic approach. However, the therapeutic utility of HAI-1 is ultimately limited by its nanomolar inhibition constant to matriptase. In contrast, the protease inhibitor first Kunitz (KD1) sub-domain of HAI-1 (Fig. 1A) (26, 34) has been shown to inhibit matriptase activity with significantly greater potency than full-length HAI-1 (35). However, the small molecular weight of the KD1 domain (approximately 6 kDa vs 58 kDa for HAI-1) confers a short circulating half-life of 20 minutes, which greatly limits its therapeutic efficacy. While chemical conjugation of KD1 to polyethylene glycol (PEG) showed significant extension in serum half-life (35), this approach does not further improve the inhibition constant beyond wild-type KD1.

Alternative approaches to develop matriptase inhibitors include synthetic small molecules (36, 37), peptides (38), monoclonal antibodies (39), and constrained peptide scaffolds (40). While each strategy generated molecules that bound to and inhibited matriptase activity, none address all of the reported therapeutic limitations. An effective therapeutic candidate must bind matriptase with high affinity to effectively outcompete pro-HGF substrate activation, as well as possess a long serum half-life to mitigate the need for frequent dosing. To overcome these critical barriers, we used rational and combinatorial approaches to engineer a potent matriptase inhibitor based on a modified variant of the natural HAI-1 protein. In this work, the inactive second Kunitz (KD2) domain of HAI-1 was replaced with a chimeric variant of KD2/KD1 domains. This modified HAI-1 protein was then fused to an antibody Fc domain, resulting in a final construct with 4 putative sites that bound additively to matriptase with pM affinity. This engineered protein significantly inhibited pro-HGF activation and matriptase expressed on the surface of lung, breast, and prostate cancer cells.

**Results**

**Engineering the Kunitz domain 2 (KD2) of HAI-1 to bind matriptase**

We used HAI-1 as a starting scaffold for protein engineering to leverage its intrinsic ability to bind and inhibit matriptase. HAI-1 is comprised of an N-terminal domain (41), an internal domain (42), a first Kunitz domain (KD1), a low-density lipoprotein (LDL) like domain, a second Kunitz domain (KD2), a transmembrane domain, and an intracellular domain (Fig. 1A). KD1 has been well established as the minimal matriptase binding domain within HAI-1. KD2 has been shown to negatively regulate HAI-1 binding affinity and confer protease specificity (26, 34). In this work, removal of the KD2 domain resulted in a 10-fold improvement in HAI-1 inhibition of matriptase activity. In addition, matriptase inhibition was proposed to be driven by a 4 amino acid primary binding interface (Arg-Cys-Arg-Gly) found in KD1, but absent in KD2 (Fig. 1 and Fig. S1A). To create an improved matriptase inhibitor, we targeted the inactive KD2 domain of HAI-1 to convert it into a matriptase binding module, effectively doubling the binding sites within the HAI-1 protein.
Yeast surface display is a well-established protein engineering technology that has been used for characterizing and screening protein–based inhibitors (40, 43–45) (Fig. S1B). We found that KD1 and KD2 were well expressed on the surface of yeast as Aga2p mating protein fusions (Fig. S1C). Additionally, we showed that KD1 bound to soluble matriptase with an affinity (K\textsubscript{d}) of 13 ± 2 pM, while KD2 exhibited no detectable binding (Fig. 2C), a trend in agreement with previous results. KD2 has been suggested to retain the secondary matriptase binding site conserved from KD1 (26). In an attempt to supplement this binding site, we first introduced the primary matriptase binding site residues (Arg-Cys-Arg: KD2-graft 1, or Arg-Cys-Arg-Gly: KD2-graft 2) from KD1 into KD2 (Fig. S1A). Yeast-displayed versions of these constructs revealed high surface expression but a lack of matriptase binding similar to wild-type KD2 (Fig. S1D). These results indicated further engineering was required to effectively convert KD2 into a matriptase binding domain.

To further explore additional mutation space beyond the grafted primary binding motif, we applied error-prone polymerase chain reaction (epPCR) (46) to randomly introduce mutations throughout the KD2-graft 2 gene. The mutated DNA was transformed into \textit{S. cerevisiae} yeast cells, resulting in ~5x10\textsuperscript{7} transformants which were induced to express a library of yeast-surfaced displayed KD2 variants, averaging 2 amino acid mutations per gene. The library was screened using fluorescence-activated cell sorting (FACS) to isolate yeast clones that expressed KD2 variants and also bound to matriptase (Fig. 2A). Yeast cells that were collected were grown in culture and induced for KD2 expression for additional screening. Following two rounds of library screening, no detectable matriptase binding was observed. In an effort to increase the mutational load, DNA was recovered from the pooled yeast and subject to epPCR to introduce additional genetic diversity, with an average mutagenic frequency of 2 to 5 amino acids per gene. A new library of ~1x10\textsuperscript{7} yeast transformants was created and screened by FACS to identify KD2 variants that bound to matriptase. Parallel sorting and analysis for non-specific binding to secondary antibodies was also performed to reduce occurrence of false positive binding variants. From these efforts, a yeast population emerged that demonstrated significantly improved binding to matriptase compared with wild-type KD2 (Fig. 2A).

Characterization of sorted library variants reveal a KD2/KD1 chimera

DNA from the final sorted yeast population was isolated and sequenced to identify amino acid mutations that could confer increased matriptase binding compared to wild-type KD1 and KD2 (Fig. 2B and Table S1). Surprisingly, we identified a chimeric variant that essentially was a fusion of the N-terminus of KD2 and C-terminus of KD1 (clone 33, named KD2/1). The generation of KD2/1 was likely due to the presence of the wild-type KD1 gene within the library construction and transformation steps, allowing recombination of genetic regions of KD1 and KD2 to generate clone 33.

Select yeast-displayed variants were individually tested for binding to matriptase; however, only the KD2/1 chimera and wild-type KD1 showed any detectable binding signal (Fig. S2). It is likely that additional rounds of screening under more stringent conditions would have resulted in isolation of KD2/KD1 as a clonal yeast population. An equilibrium binding assay showed that yeast-displayed KD2/1 binds to matriptase with an affinity of K\textsubscript{d}= 220 ± 30 pM (Fig. 2C), which was 20-fold weaker than KD1. Notably, inhibitors that demonstrate protease binding do not always demonstrate protease inhibition of the target active site (47, 48). Thus, proteins were tested for functional matriptase inhibition by incubating an increasing number of yeast cells displaying KD2/1 or wild-type KD1 or KD2 with soluble matriptase and substrate. Fluorescent matriptase substrate activation was quantified over time and revealed that both yeast-displayed KD1 and KD2/1 domains significantly inhibited matriptase activity in a cell number dependent manner, while the yeast-displayed KD2 domain did not inhibit matriptase, even with up to 10\textsuperscript{5} yeast cells (Fig. 2D).

Development of a soluble matriptase inhibitor
After validating that yeast-tethered KD2/1 could bind to and inhibit matriptase, we next created a soluble, recombinant matriptase inhibitor. We first replaced the wild-type KD2 domain of full-length HAI-1 with the sequence of the engineered KD2/1 chimera. The construct was fused to the crystallizable fragment (Fc) domain of an antibody, which is an established protein engineering strategy that confers therapeutic properties including circulating half-life extension, immune system recruitment, and elevated binding affinity through avidity (49, 50). This protein was termed KD1-KD2/1-Fc (Fig. 3). In addition, given the favorable binding properties of yeast-displayed KD1 (Fig. 2C) an alternative HAI-1 design was created where the wild-type KD2 domain was replaced with a second wild-type KD1 domain, termed KD1x2-Fc. Wild-type KD1 monomer, full-length HAI-1 monomer, and a HAI-1 Fc fusion (HAI-Fc) were also produced as controls. The function of the KD1 domain can be diminished by introducing an R260A point mutation, which is known to ablate matriptase binding (34). Thus, we used this point mutation to create constructs containing a non-inhibitory KD1 domain to allow us to parse the importance of each Kunitz domain on matriptase inhibition (Fig. 3; HAI-R260A-Fc; KD1-R260A-KD2/1-Fc). Each protein construct was expressed in a transient mammalian cell expression system and underwent two-step purification. The resulting size exclusion chromatograms demonstrate expression and purification of all proteins (Fig. S3A-B), with the exception of KD1x2-Fc (Fig. S3C-D). These findings suggest that the 6 N-terminal amino acids from wild-type KD2, found in the KD2/1 chimera (Table S1), were critical for effective protein folding/ expression in the context of full-length HAI-1.

**KD1-KD2/1-Fc is a potent and selective inhibitor of matriptase**

We next evaluated the purified proteins for their ability to inhibit matriptase activity. We first tested each protein construct using an in vitro kinetic inhibition assay. Dose response plots were generated for each inhibitor (Fig. S4A) and inhibition constant (Ki) values were determined using equation (1) and equation (2) as previously reported (36, 38, 40). Table 1 lists the resulting Ki value for each inhibitor construct and the number of functional Kunitz domains present. As expected, HAI-R260A-Fc has no detectable inhibition of matriptase due to the ablating R260A mutation that disrupts wild-type KD1 function, and also confirms that the KD2 domain or Fc domain does not participate in matriptase inhibition. In contrast, the KD1-R260A-KD2/1-Fc protein exhibited a Ki of 550 ± 50 pM, indicating that the KD2/1 domain is a functional inhibitor of matriptase when incorporated into the HAI-1 Fc fusion protein. The HAI-1 monomer has a moderate Ki of 9.1 ± 1 nM, which improves slightly by 2-fold as a bivalent HAI-1 Fc fusion (Ki = 4.2 ± 0.5 nM). Additionally, the wild-type KD1 monomer has a Ki of 310 ± 20 pM, which is in agreement to previous Ki measurements for matriptase (34, 42), and is a 30-fold more potent inhibitor relative to the full length HAI-1 monomer. This improvement further demonstrates the negative regulation of KD2 in the context of the full length HAI-1 inhibitor.

Finally, KD1-KD2/1-Fc had the lowest Ki of 70 ± 5 pM, making it the most effective inhibitor in our panel. More specifically, KD1-KD2/1-Fc demonstrates a relative improvement in Ki of 4-fold compared with the wild-type KD1 monomer, a 60-fold improvement compared with HAI-Fc, and a 120-fold improvement compared with the HAI-1 monomer. The increased potency is due to the replacement of the KD2 domain with the matriptase binding KD2/1 chimera, as well as the homodimeric nature of the Fc fusion construct. These combined engineering efforts expand the number of matriptase binding sites from one domain in wild-type HAI-1 to four domains within the final KD1-KD2/1-Fc construct. Further experiments also determined that KD1-KD2/1-Fc follows a competitive inhibition modality for matriptase (Fig. S5) similar to that of wild-type KD1 monomer (26).

We next tested the selectivity of KD1-KD2/1-Fc against a panel of naturally soluble or cell anchored serine proteases, including trypsin 3 (51), urokinase (11), kallikrein 4 (30), and hepsin (28, 29); each of these proteases have a range of native affinities to wild-type HAI-1. We found that none of the proteins tested could inhibit trypsin 3 or urokinase activity (Fig. S4B-D and Table 2). In contrast, KD1-KD2/1-Fc and wild-type KD1
monomer inhibit kallikrein 4 similarly, at 8.0 ± 2 nM and 9.3 ± 2 nM respectively; while the wild-type HAI-1 monomer more weakly inhibits kallikrein 4 with Ki values above 100 nM. Additionally, KD1-KD2/1-Fc and wild-type KD1 monomer inhibit hepsin with Ki values of 1.5 ± 0.4 nM and 5.4 ± 2 nM respectively; while the wild-type HAI-1 monomer more weakly inhibits hepsin with a Ki value of 72 ± 30 nM. Overall, the relative selectivity of KD1-KD2/1-Fc for matriptase remains at >1,000-fold over trypsin and urokinase, 110-fold over kallikrein 4, and 20-fold over hepsin.

KD1-KD2/1-Fc inhibits matriptase-mediated activation of pro-HGF

We next tested the ability of KD1-KD2/1-Fc to inhibit matriptase cleavage and activation of human Pro-HGF (huPro-HGF), the natural substrate target that contributes to cancer metastasis (Fig. 1B, S6A-C). These results indicate that KD1-KD2/1-Fc inhibits matriptase mediated cleavage of huPro-HGF in a dose dependent manner, and qualitatively appears to be a more potent inhibitor than wild-type KD1 monomer. Reaction products from the huPro-HGF activation experiment were then incubated with Madin-Darby Canine Kidney (MDCK) cells to measure HGF-mediated cell migration via c-Met receptor binding and activation (11, 37, 52) (Fig. S6D). The addition of the KD1-KD2/1-Fc treated sample reduces MDCK migration in a dose dependent manner, compared to uninhibited control samples, further supporting that KD1-KD2/1-Fc potently inhibits activation of huPro-HGF by matriptase. Additionally, KD1-KD2/1-Fc qualitatively prevents cell scattering at lower concentrations than wild-type KD1 monomer.

KD1-KD2/1-Fc inhibits matriptase expressed on cancer cells

After demonstrating potent and selective inhibition of the in vitro form of matriptase, we next tested the ability of KD1-KD2/1-Fc to inhibit matriptase expressed on human cancer cell lines. We first confirmed expression and functional activity of matriptase on the surface of three human cancer cell lines, MDA-MB-231 (breast), A549 (lung), and PC3 (prostate), using a matriptase specific antibody and a commercial matriptase substrate. Positive matriptase expression levels correlating with matriptase functional activity were identified for each cell line tested (Fig. S7A-B). KD1-KD2/1-Fc was then tested and compared with wild-type KD1 and HAI-1 monomer proteins for inhibition of fluorescent matriptase substrate activation. Dose response curves of matriptase inhibition were then generated and fit to quantify IC_{50} values for each inhibitor tested (Fig. 4A). The results demonstrate that KD1-KD2/1-Fc inhibits matriptase up to 10-fold and 80-fold better compared to wild-type KD1 and HAI-1 inhibitors, respectively. KD1-KD2/1-Fc was also confirmed to bind to the surface of cancer cell lines, further confirming specific interactions with cell associated matriptase (Fig. S7C).

We next performed a cancer cell invasion assay to further test the ability of KD1-KD2/1-Fc to inhibit cell expressed matriptase activation of huPro-HGF. Invasion assays are often used to measure the phenotypic behavior of cancer cells in response to growth factor stimulation and protease inhibition involving the matriptase-HAI-1 / Pro-HGF-Met pathway (Fig. 1B) (33, 53). To stimulate cancer invasion, we first transfected HEK cells to overexpress soluble huPro-HGF (Fig. S6A-B). The HEK-huPro-HGF cell line was used to construct a co-culture assay to model cancer cell invasion in response to paracrine growth factor stimulation. A significant increase in invasion of both breast (MDA-MB-231) and lung (A549) cancer cells was observed upon incubation with HEK-huPro-HGF cells (Fig. 4B), compared with controls of cancer cells alone or with untransfected HEK cells. Furthermore, when KD1-KD2/1-Fc was added to the reaction containing HEK-huPro-HGF cells, the invaded cell number decreased significantly compared to conditions without inhibitor. Collectively, these results indicate that KD1-KD2/1-Fc can potently bind to and inhibit soluble and cell expressed matriptase, and block huPro-HGF activation to impede cell migration and invasion.

Discussion

Matriptase represents a critical protease that when unregulated participates in the aggressive advancement of many human maladies. In the context of cancer, loss of the natural matriptase inhibitor HAI-1 has been found to result in more
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aggressive cancer progression and poor patient prognosis. Previous attempts to develop potent and selective matriptase inhibitors centering around wild-type HAI-1 or KD1 have limitations due to weak matriptase binding affinity or short circulating half-life (34, 35). Previous reports (26, 34) and our current study have also supported that the KD2 domain imposes negative regulation on the inhibitory capacity of the KD1 domain (up to 30-fold) within the context of wild-type HAI-1. To overcome these limitations, we applied rational and combinatorial protein engineering strategies in an attempt to convert the KD2 domain of HAI-1 into a matriptase binding domain.

Despite the sequence similarities between KD1 and KD2, we did not identify KD2 variants that could bind to matriptase. Homology modeling has previously been used to study key differences between KD1 and KD2 domains (26). With the exception of Leu 284, all secondary binding regions were proposed to be conserved between the two domains. Thus, the main difference between KD1 and KD2 appears to be the primary matriptase binding site (Arg-Cys-Arg-Gly) found in KD1, but absent in KD2. We found that grafting this dominant matriptase binding sequence from KD1 into KD2 was not sufficient to convert it into a binding domain. These results indicate that structural features beyond the primary binding site additionally influence matriptase binding to KD1 and are absent in KD2. Moreover, error-prone PCR was not sufficient to introduce favorable mutations into this modified KD2 domain to confer matriptase binding. One possible explanation is that the relatively small sequence space explored in our screens compared to theoretical library size limited our outcome, especially if multiple cooperative mutations are needed. Alternatively, a recent study indicated the importance of concerted inter-domain interactions within HAI-1 for overall folding and function (42). In particular, the internal domain of HAI-1 was shown to interact with KD1, but also improves its availability for reaction with protease (i.e. stimulates its inhibitory activity), a finding also observed in earlier work (34). Furthermore, these findings may explain why KD2 mutants screened in isolation did not reveal matriptase binding attributes, and why the six KD2 residues that precede the KD1 sequence were critical in the context of the KD2/KD1 chimera for folding and functional activity.

Our protein engineering efforts serendipitously identified a chimeric domain comprised of KD2 and KD1 regions that demonstrated subnanomolar matriptase binding and potent matriptase inhibition compared to the wild-type KD2 domain. Further creation of a bivalent KD1-KD2/1-Fc fusion results in a protein construct that contains four matriptase binding domains: two wild-type KD1 domains and two engineered KD2/KD1 (KD2/1) chimeric domains, accentuating its potency 120-fold relative to wild-type HAI-1 (Table 1). This dramatic improvement can be attributed to replacement of the sterically regulating wild-type KD2 domain with the engineered KD2/1 matriptase-binding chimera. The KD2/1 chimeric domain was critical to this work, as a protein variant created by replacing the KD2 domain with another wild-type KD1 domain (KD1x2-Fc) was unable to be recombinantly expressed in mammalian cell culture. Interestingly, this loss of recombinant expression was also observed in a previous study in which disruption of the internal domain and KD1 domain interface of HAI-1 resulted in a 10-fold loss in expression level (42).

Notably, the Ki for KD1-KD2/1-Fc improves 4-fold relative to wild-type KD1 monomer, suggesting stoichiometric binding of one matriptase molecule to one functionally inhibiting domain of KD1-KD2/1-Fc. This relationship is also observed in the 2-fold relative difference in Ki between wild-type HAI-1 monomer compared with HAI-Fc. Stoichiometric 1:1 wild-type HAI-1 and matriptase complexes have been previously observed to occur naturally (54). This model of inhibition assumes that all four KD1-KD2/1-Fc functional domains are equally accessible for simultaneous matriptase inhibition. Results obtained with the KD1-R260A-KD2/1-Fc inhibitor support this possibility, in which the KD2/1 domains functionally inhibit matriptase within the context of the Fc fusion construct with a Ki= 550 ± 50 pM. It is important to note that although non-inhibitor depleting conditions were used to quantify the Ki value for all inhibitors tested, Ki values may be an overestimate, as the concentration of matriptase used (50 pM) was at

| Table 1 |
the lowest limit for assay detection and may also not be 100% active under the assay conditions. Further testing using solid phase or solution based assay formats could help confirm the Ki for this very tight matriptase inhibitor, assuming improved detection limits. However, the Ki of wild-type KD1 monomer we report (310 ± 20 pM) closely aligns with prior literature values for rat (328 ± 181 pM) and human (380 ± 70 pM) matriptase, which use similar assay methods and thus strongly validates our results (34, 42).

KD1-KD2/1-Fc additionally retains the highest selectivity to matriptase amongst a panel of serine proteases tested (Table 2). Interestingly, while KD1-KD2/1-Fc and wild-type KD1 inhibit kallikrein 4 with low nanomolar Ki values, wild-type HAI-1 monomer only weakly inhibits kallikrein 4 with a Ki > 100 nM. This result further demonstrates the regulatory role that KD2 plays on the KD1 domain within the context of full-length HAI-1. This regulation is also observed for hepsin, where KD1-KD2/1-Fc and wild-type KD1 monomer inhibit hepsin more effectively compared to the wild-type HAI-1 monomer. Like matriptase, the relative fold difference in Ki values for hepsin between KD1-KD2/1-Fc and wild-type KD1 monomer is also 4-fold, which further supports the stoichiometric inhibition hypothesis stated above. Hepsin is reported to share a similar role as matriptase in cancer progression, and thus dual targeting of hepsin and matriptase by KD1-KD2/1-Fc may serve as an attractive therapeutic feature (28, 29, 35).

Cell based activity assays further demonstrate the superior potency of KD1-KD2/1-Fc in inhibiting matriptase activity both soluble and cell associated form. The huPro-HGF activation assay qualitatively confirmed that KD1-KD2/1-Fc inhibits matriptase activation of the pro-domain form of HGF, with reduced MDCK cell scattering at lower inhibitor concentrations than wild-type KD1 monomer (Fig. S6). KD1-KD2/1-Fc efficacy is further observed by a 10-fold greater inhibition of matriptase activity on cancer cells relative to wild-type KD1 monomer (Fig. 4A). The greater magnitude of improvement in cancer cell matriptase inhibition (10-fold) compared to soluble matriptase (4-fold) is possibly due to avidity effects of the KD1-KD2/1-Fc construct on cell anchored matriptase. KD1-KD2/1-Fc also significantly reduces lung and breast cancer cell invasion in vitro (Fig. 4B). The extent of reduced invasion aligns with previous inhibition results using this standard invasion model (33, 53). Notably, the invasion assay also included media containing 2% fetal bovine serum to maintain HEK cell viability, identified to contain significant levels of active proteases capable of cleaving commercial matriptase substrate. This high background of protease activity, combined with the heterogeneity of other serum proteins and constitutively overexpressed huPro-HGF, likely contributes to preventing a greater reduction in cancer cell invasion observed with KD1-KD2/1-Fc. Further testing using optimized media conditions might result in a greater extent of matriptase inhibition in cancer cells upon treatment with KD1-KD2/1-Fc. The KD1-KD2/1-Fc construct has a Ki for matriptase of 70 ± 5 pM, which is amongst the tightest Ki values measured for protein based matriptase inhibitors. In addition to wild-type KD1 (Ki = 310 pM ± 20 pM) (42, 53), previous efforts have generated matriptase inhibitors based on constrained peptides (Ki = 830 ± 140 pM (40) and Ki = 290 ± 54 pM) (38) and antibodies (Ki = 720 pM) (39). Peptide and KD1-based inhibitors, as well as synthetic small molecule inhibitors (36, 37), have short circulating half-lives and restricted molecular surface area for binding matriptase with high affinity. The engineered KD1-KD2/1-Fc protein fulfills several attractive design criteria. First, use of the native HAI-1 as a starting point for therapeutic development leverages the affinity and specificity of the natural inhibitor. Second, replacing the KD2 domain with an active matriptase-binding domain is expected to be minimally perturbing to native HAI-1. Third, fusion of the engineered construct to an Fc domain creates a bivalent protein, in this case, resulting in 4 matriptase binding sites that improves protease inhibition. Fourth, fusion to an Fc domain is expected to increase serum half-life through increased molecular weight and FcRn-mediated recycling (50) requiring less frequent therapeutic dosing, and allowing manufacturing processes that are similar to antibodies. Matriptase imaging experiments have also suggested that cell anchored HAI-1 can serve as a natural reservoir for secreted
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proteases, effectively increasing their local concentration and activity at the leading edge of cancer invasion (55, 56). High affinity inhibitor binding is therefore critical to effectively outcompete the interaction of proteases to native cell surface HAI-1. The engineered matriptase binding protein described here thus has the potential to function as a HAI-1 “decoy” in cancer and other disorders where matriptase underlies disease pathophysiology.

Experimental Procedures

Media and reagents. Yeast media: (YPD media) 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract; (SD-CAA media) 20 g/L dextrose, 6.7 g/L yeast nitrogen base lacking amino acids, 5.4 g/L Na2HP04, 8.6 g/L NaH2PO4 · H2O, and 5 g/L Bacto casamino acids; (SG-CAA media) same as SD-CAA, but dextrose is substituted for 20 g/L galactose; (Low pH, SD-CAA media) same as to SD-CAA, but phosphate components were substituted with 13.7 g/L sodium citrate dehydrate and 8.4 g/L citric acid anhydrous, and pH was adjusted to 4.5. BMMY, BMGY media, and RDB plates for P. pastoris strain GS115 were prepared as described (52). Mammalian cells and media: (Complete growth media) Dulbecco’s modified Eagle medium (Fisher Scientific) containing 10% fetal bovine serum (FBS)(Fisher Scientific). Cell lines used include: Human Embryonic Kidney (HEK) cells, PC3 (prostate cancer), MDA-MB-231 (breast cancer), and A549 (lung cancer) – (ATCC).

Yeast cell surface binding assays and library screening conditions. Induced EBY100 yeast cells were counted (OD600 of 1 = 10⁷ cells/ml), 1x10⁸ cells/sample were washed with 1 mL matriptase assay buffer, and then mixed with soluble matriptase (final concentration 0 to 1 nM, serial dilution) and matriptase assay buffer (final concentration 200 µL to 100 mL). Ligand depleting conditions were addressed using methods previously described, and estimated by assuming each yeast displayed 50,000 copies of inhibitor per cell (57, 58). Reactions were incubated for 48 hr at room temperature to reach equilibrium. Samples were then incubated with a 1:250 dilution of anti-HA, mouse primary antibody (Fisher Scientific)) for final 30 min at room temperature. Following washing with matriptase assay buffer, samples were labeled with secondary antibodies to measure yeast expression (1:100 dilution of anti-mouse phycoerythrin PE (Invitrogen)) and matriptase binding (1:100 dilution of anti-His fluorescein isothiocyanate FITC (Bethyl)) in 50 uL of matriptase assay buffer. Samples were incubated at 4°C for 15 min, washed, and maintained on ice until loading onto a flow cytometer for analysis (BD Accuri) or library sorting (BD Aria II). Data analysis and three parameter curve fits performed with GraphPad Prism software, version 6. Sorted cells were recovered in SD-CAA liquid media and incubated at 30°C overnight or until reaching an optical density (O.D.) of 4-8. Yeast surface protein expression was then induced by culturing cells in SG-CAA media at 20°C overnight. The initial library (approximately 5x10⁷ yeast) was screened twice in the presence of 10 nM matriptase, then isolated yeast were lysed for DNA extraction (Zymoprep; Fisher Scientific). This DNA was subjected to additional mutagenesis (see above) before retransformation into yeast. This library was screened seven times under equilibrium sorting conditions with 10 nM matriptase. Parallel screening for binding against secondary reagents alone was used to reduce false positives. The final pool of isolated yeast was lysed for DNA extraction (Zymoprep; Fisher Scientific), and transformed into DH10B electrocompetent E. coli cells for plasmid amplification and sequencing (Sequetech, MCLAB).

Protease inhibition assay. First, 0.05 nM matriptase (R&D Systems) was added to soluble inhibitor (final concentrations ranging from 0-50nM) containing 100 µL final matriptase assay buffer volume. Soluble matriptase substrate (1 µM; Boc-QAR-AMC) (R&D Systems) was added to initiate the reaction. Matriptase inhibition assays were carried out on yeast surface displayed proteins using the same matriptase and substrate conditions, except yeast were first counted (ranging from 10 to 10⁵ yeast cells/sample) and incubated with matriptase for at least 1 hour prior to addition of the substrate in 100 µL final matriptase assay buffer volume. Additional protease inhibition assays were carried out using 0.5 nM of urokinase, trypsin 3, kallikrein 4, and
hpsin, with 1 μM of each enzyme specific substrate, Z-GGR-AMC, Mca-RPKPVE-Nval-WRK(Dnp)-NH2, Boc-VPR-AMC, and Boc-QRR-AMC, respectively. All enzymes and substrates purchased from R&D Systems were assumed 100% active; buffers and assay conditions were prepared as each R&D Systems protocol describes with 100 μL final volume reactions. Soluble inhibitor (final concentrations ranging from 0 to 150 nM, excluding inhibitor depleting conditions) was added initially to each enzyme containing its respective assay buffer, then the reaction was initiated at least 30 min later by addition of substrate and measuring fluorescent output over time at 380nm/460nm (matriptase, urokinase, kallikrein 4, and hpsin) and 320nm/405nm (trypsin 3). Protease activation of fluorescent substrate (relative fluorescent units (RFU)/s) was measured for at least 30 min using 96-well clear black bottom plate (Fisher Scientific) and a kinetic microplate reader (Synergy H4, Biotek), corrected for background, and then converted to initial relative velocity, \( v/v_o \). Relative velocities were plotted against inhibitor concentration, and apparent inhibition constants, \( K_i^{app} \), values were determined by fitting each curve to the Morrison binding equation (1) as previously described (38, 40, 59) using GraphPad Prism version 6 software. Inhibition constants, \( K_i \), were then calculated using equation (2).

\[
\frac{v}{v_o} = 1 - \frac{\left( [E] + [I] + K_i^{app} \right)^2 - \left( [E] + [I] \right)^2}{2[E]} \\
(2) \quad K_i = \frac{K_i^{app}}{1 + \frac{[I]}{K_M}}
\]

**KD2 variant cloning and library construction.**

The natural KD2 domain is comprised of amino acids Cys375 to Cys425 (Genbank Accession ID: AY358969.1). KD2 variant constructs Arg-Cys-Arg (graft 1), or Arg-Cys-Arg-Gly (graft 2) were created using PCR and gene products were cloned into the pTMY yeast display vector previously described (52) using NheI and MluI restriction sites. Plasmids were transformed into the yeast strain EBY100 using electroporation, expanded in SD-CAA media at 30°C, and then induced for surface expression using SG-CAA media at 20°C (45). All yeast displayed proteins were cloned and prepared in this manner. Yeast expression levels were measured using an anti-HA epitope tag antibody (1:250 dilution of anti-HA, mouse primary antibody (Fisher Scientific)), and an anti-HAI-1 antibody (1:100 dilution of rabbit anti-HAI-1 primary antibody (Fisher Scientific)), followed by analysis using flow cytometry (BD Accuri). The KD2 graft 2 library was constructed using error-prone PCR as previously reported (46). In short, variable concentrations of Mn\(^{2+}\) (0.075 mM or 0.15 mM) were added to reactions to alter the mutational frequency (final average 2 amino acid mutations/gene), while elevated ratios of dCTP and dTTP nucleotides were added in order to account for mutational bias. Error-prone PCR was carried out using a low fidelity Taq polymerase and primers: Forward Primer 5’-3’: gtatcttctgtcttgac and Reverse Primer 5’-3’: tgcagttcctggaaag. Final PCR products were amplified under high fidelity conditions (without Mn\(^{2+}\), equal dNTP ratios, and Phusion polymerase) and then transformed, along with digested pTMY plasmid, into EBY100 competent cells as previously described (60). Cell samples were collected post transformation, serially diluted, and plated on SD-CAA plates to quantify a transformation efficiency of 5x10\(^7\) cells. Cells were then expanded in SD-CAA media at 30°C and 5x10\(^8\) cells were induced for surface expression in SG-CAA media at 20°C in preparation for library sorting. Additional rounds of mutagenesis followed the same method as above (46), but the concentration of Mn\(^{2+}\) was increased to 0.3 mM or 0.4 mM to increase the mutational frequency to 2-5 amino acid mutations/gene. It also appears the wild-type KD1 gene (Cys250 to Val303; Genbank Accession ID: AY358969.1) was present during PCR and transformation, allowing recombination of genetic regions of KD1 and KD2 to generate clone 33. Final PCR products were then amplified as before and transformed into EBY100 with an estimated transformation efficiency of 1x10\(^7\) cells. Yeast cells were expanded and induced for expression prior to sorting as described above.

**Cancer cell binding assay.** 5x10\(^5\) cancer cells were resuspended in cold 1xPBS with 1 mg/mL bovine serum albumin (0.1% BPBS) (50 μL to 10mL final volume), containing soluble inhibitor (50 pM to 1 μM serial dilution) or a 1:100 dilution soluble human matriptase antibody (Fisher Scientific). Cell solutions were incubated at 4°C.
for at least 3 hours (for single point binding assays) or overnight (for binding curve assays). Cells were then washed with 1mL cold 0.01% BPBS, and protein binding was measured using 1:100 anti-mouse phycoerythrin (PE) (Invitrogen). Following incubation at 4°C for 15 min, cells were washed with 1mL cold 0.01% BPBS and analyzed using flow cytometry (BD-Accuri). Mean cell binding (Mean-RFU) was quantified from at least 10,000 cell events and corrected for controls incubated with antibodies alone. Values were then plotted against inhibitor concentration, normalized to saturating conditions, and Kd<sub>app</sub> values were calculated by fitting graphs to a four-parameter equation (GraphPad version 6).

Pro-HGF activation assay. A gradient of soluble inhibitors (0 to 5 nM, serial dilution) were incubated with 0.05 nM soluble matriptase in 10μL matriptase assay buffer for 30 min at room temperature. Soluble Pro-HGF (125 nM) was then added to the solutions and the reactions were incubated for an additional 2 hr at room temperature. Reaction products were then boiled for 10 min at 95°C in the presence of loading dye and reducing agent, and then loaded onto a 12% SDS-polyacrylamide gel (GenScript) and subjected to electrophoresis for protein fragment separation. Protein bands were then transferred to a nitrocellulose membrane, and probed with a primary anti-HGF α-chain antibody (Abcam), followed by an anti-rabbit horse radish peroxidase (HRP) antibody (Fisher Scientific). Protein presence was then detected and imaged using SuperSignal West Femto HRP substrate (Fisher Scientific).

MDCK cell migration/scatter assay. 3x10<sup>3</sup> Madin-Darby Canine Kidney (MDCK) cells were seeded in 96-well plates in 100 μL 2% serum-supplemented media. Plates were cultured for 24 hr in a humidified tissue culture incubator at 37°C, 5% CO<sub>2</sub> atmosphere. Following incubation, cells were washed twice with warm 1xPBS, and serum free media was added (for 100 μL final reaction volume). Soluble inhibitors (0 to 250 nM serial dilution) were then immediately added and samples were incubated at 37°C, 5% CO<sub>2</sub> for 1 h. 100 μM final matriptase substrate (Boc-QAR-AMC; R&D) concentration was then added to initiate the reaction and fluorescence at 380nm/460nm was measured once per hour for 5 hr using a microplate reader (Synergy H4, Biotek). The reactions were incubated at 37°C, 5% CO<sub>2</sub> between reads. The obtained matriptase activity rates, RFU/hr, were then normalized to conditions lacking inhibitor, and plotted against inhibitor concentration tested. Graphs were fitted using a three-parameter equation (GraphPad version 6) and IC<sub>50</sub> values were calculated.

Cancer cell invasion assay. 24-well 8μm pore inserts (Corning) were coated with 50 μg of Matrigel (Corning) following manufacturing protocols and inserted into a 24-well plate.1x10<sup>4</sup> cancer cells in 0.5 mL 2% serum-supplemented media were seeded onto the Matrigel coated inserts and 1x10<sup>5</sup> human embryonic kidney (HEK) cells in 0.75 mL 2% serum-supplemented media were then seeded onto the lower chamber; 100 nM final concentration of soluble inhibitor was added to the lower chamber immediately. The 24-well plates were cultured for 48 hr in a humidified tissue culture incubator at 37°C, 5% CO<sub>2</sub> atmosphere. Following incubation, non-invading cells were removed and invaded cells were stained.
using crystal violet then imaged at three random center fields of view at 10x magnification. Images were analyzed using Image J, adjusted for brightness-contrast and cell borders defined by watershed command, and cell numbers were quantified with threshold particle setting <500 pixels excluded to reduce artifacts.

**Recombinant protein production.** HAI-1 is comprised of amino acids Met1 to Glu449 (Genbank Accession ID: AY358969.1); KD1 is comprised of amino acids Cys250 to Val303 (Genbank Accession ID: AY358969.1), and Pro-HGF is comprised of amino acids Met1 to Ser728 (HGF Isoform 3, Genbank Accession ID: NP_001010932). DNA encoding the open reading frame of the HAI-1 monomer, Pro-HGF, and Fc-fusion constructs was cloned into the pCEP4 mammalian expression plasmid (Invitrogen). Genes were cloned into the pCEP4 vector using NotI and HindIII restriction sites, and included a C-terminal hexa-histidine tag (Pro-HGF, monomer inhibitors) or a mouse IgG2a Fc domain was genetically linked using NotI and XhoI restriction sites. pCEP4 vectors were amplified and transfected into adherent human embryonic kidney (HEK) cells using Lipofectamin 2000 (Fisher Scientific). Transfected HEK cells were selected using 400 μg/mL Hygromycin B (Fisher Scientific) and cultured in DMEM containing 10% FBS in a humidified incubator at 37°C, 5% CO₂. Selected HEK cells were then expanded in T-225 culture flasks (Fisher Scientific) until reaching 70% confluence and recombinant protein expression was initiated by the addition of 25mL serum-free DMEM and protein expression occurred for at least one week. The KD1 monomer was cloned into the pPIC9 yeast expression plasmid, transformed into the yeast strain P. pastoris, and expressed using reagents, media, and protocols exactly as previously described (52). Protein-containing supernatants from HEK cells and P. pastoris was purified by Ni-NTA metal chelating chromatography and eluted using 500mM imidazole (for monomer inhibitors and Pro-HGF containing a hexa-histidine-tag) or Protein A affinity chromatography eluted using pH 5 citrate elution buffer (for inhibitors containing a Fc fusion). Eluted protein samples were then buffer exchanged into 1xPBS or 1xPBS with 500mM NaCl (Pro-HGF only). Each protein was additionally purified by size exclusion chromatography (s75 10/300 GL, s200 Increase 10/300 GL; GE Healthcare). Purified protein was then characterized using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and concentrations were quantified by UV-Vis absorbance (280nm) and extinction coefficients: (HAI Fc fusion variants; 179810cm⁻¹M⁻¹, HAI monomer; 57100cm⁻¹M⁻¹, KD1 monomer; 11835 cm⁻¹M⁻¹, Pro-HGF; 149180cm⁻¹M⁻¹). Protein yields typically ranged from 1 to 22 mg/mL. Purified proteins were stored in 1xPBS (inhibitors) or 1xPBS with 500mM NaCl (Pro-HGF) at 4°C and tested within three weeks or flash frozen with 0.01% Tween 80 for long term storage at -80°C.

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**Conflicts of Interest:**
The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions:**
ACM conceived and coordinated the study, performed and analyzed (or assisted others) the experiments in each figure, prepared artwork, and wrote the paper. DK helped perform and analyzed the experiments shown in Table 1 and Table 2, Fig4B, and SI Fig5 and helped produce and test constructs in Fig3. SAH helped design and optimize the experiments shown in Fig2A-C and helped produce and test constructs in.
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Fig3. RAPS helped design, perform, and analyze the experiments shown in Fig2D and Fig4A and SI Fig7. CHC helped design the experiments shown in Fig4B and SI Fig6. JRC provided the experimental resources and helped edit the manuscript draft and figures and data analysis. All authors reviewed the manuscript text and results, provided editing comments, and approved the final version of the manuscript.

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Tables:

| Inhibitor                  | Ki [pM]          | Functional Domains | KD1-KD2/1-Fc Ki Ratio |
|----------------------------|------------------|--------------------|-----------------------|
| KD1x2, Fc                  | Not Expressed    | 4                  | N/A                   |
| HAI-R260A, Fc              | >100,000         | 0                  | N/A                   |
| HAI-WT, monomer            | 9,100 ± 1,000    | 1                  | 120                   |
| HAI-WT, Fc                 | 4,200 ± 500      | 2                  | 60                    |
| KD1-R260A-KD2/1-Fc         | 550 ± 50         | 2                  | 7                     |
| KD1-WT, monomer            | 310 ± 20         | 1                  | 4                     |
| KD1-KD2/1-Fc               | 70 ± 5           | 4                  | –                     |

**Table 1:** Summary of the Ki values quantified from dose response plots (Fig. S4A) for each soluble inhibitor tested. Values were fit and calculated using equations 1 and 2 and reported as the mean and standard deviation of triplicate measurements.

| Protease   | Ki [pM] KD1-KD2/1-Fc | Ki [pM] KD1 WT | Ki [pM] HAI-1 WT | [Ki KD1 WT] / [Ki KD1-KD2/1-Fc] | [Ki HAI-1 WT] / [Ki KD1-KD2/1-Fc] |
|------------|----------------------|----------------|-----------------|---------------------------------|-----------------------------------|
| Matriptase | 70 ± 5               | 310 ± 20       | 9,100 ± 1,000   | 4                               | 120                               |
| Trypsin 3  | >100,000             | >100,000       | >100,000        | –                               | –                                 |
| Urokinase  | >100,000             | >100,000       | >100,000        | –                               | –                                 |
| Kallikrein | 8,000 ± 2,000        | 9,300 ± 2,000  | >100,000        | 1                               | 1                                 |
| Hepsin     | 1,500 ± 400          | 5,400 ± 2,000  | 72,000 ± 30,000 | 4                               | 48                                |

**Table 2:** KD1-KD2/1-Fc protease selectivity profile. Summary of the Ki values quantified from dose response plots (Fig. S4B) for each soluble protease and inhibitor tested. KD1 WT = wild-type KD1 monomer; HAI-1 WT = wild-type HAI-1 monomer. Values were fit and calculated using equations 1 and 2 and reported as the mean and standard deviation of triplicate measurements. Approximate fold selectivity values for wild-type KD1 monomer and HAI-1 monomer are reported relative to KD1-KD2/1-Fc.
Figures:

**Fig. 1.** (A) Schematic of the HAI-1 inhibitor, which naturally regulates matriptase activity and levels of Pro-HGF activation, thus preventing cancer progression in healthy tissue. (B) Biological representation of the tumor environment. Dysregulated matriptase cleaves Pro-HGF into activated HGF, which is competent to bind to and stimulate its cognate receptor, c-Met. Ligand-receptor dimerization then triggers intracellular signaling pathways that in turn stimulate cellular phenotypic responses, including cell growth, proliferation, and migration.
Fig. 2. (A) Library screening identifies a chimera of KD1 and KD2 that binds matriptase. Representative FACS plots are shown from separate yeast library sorting rounds, including sorting gates used to isolate phenotypically improved KD2 variants. (B) Sequence alignment of clone 33, which is a chimera of KD2/KD1, (termed KD2/1) with KD2 graft 2 in green, wild-type KD1 in orange, and shared sequence space of KD1 and KD2 in white. (C) Matriptase equilibrium binding of yeast-displayed KD1 wild-type (blue; $K_d = 13 \pm 2$ pM), KD2/1 chimera (red; $K_d = 220 \pm 30$ pM), and KD2 wild-type (green; no binding); reported as mean and standard deviation values. (D) Yeast-displayed KD domains that bind matriptase also inhibit its activity. Bar graphs indicate quantified matriptase activity for varying number of yeast cells. Color scheme is the same as in panel C. Significance quantified with pair wise $T$-test; $^*p<0.0001$, $^{**}p<0.0003$, $^{***}p<0.0004$, $^{****}p<0.0024$. 
Fig. 3. Schematic depiction of the HAI-1 based protease inhibitor panel engineered to contain varying functional domains. Engineered domains include KD1-R260A, which contains a point mutation that disrupts matriptase binding, and the chimeric domain KD2/1. Several constructs are genetically fused to the Fc region of an antibody domain to increase valency and molecular size.
**Fig. 4.** Ability of soluble inhibitors to inhibit matriptase activity on PC3 (prostate), MDA-MB-231 (breast), and A549 (lung) cancer cells. (A) Dose response of normalized matriptase inhibition. Mean IC\(_{50}\) values reported with log standard deviation. Wild-type HAI-1 monomer (blue); wild-type KD1 monomer (red); KD1-KD2/1-Fc (green). (B) Effects of KD1-KD2/1-Fc on cancer cell invasion in the presence of human embryonic kidney (HEK) cells or HEK cells transfected to overexpress pro-hepatocyte growth factor (HEK(+-ProHGF)). Cancer cells alone (blue bars) represent negative controls. Significance quantified with pair wise \(T\)-test; *\(p<0.008\), **\(p<0.05\), ***\(p<0.017\), ****\(p<0.054\).