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The Mechanism of Inhibition of Antibody-Based Inhibitors of Membrane-Type Serine Protease 1 (MT-SP1)

Christopher J. Farady¹, Jeonghoon Sun^{2,#}, Molly R. Darragh¹, Susan M. Miller¹, and Charles S. Craik^{1,2}

¹ Graduate Group in Biophysics, University of California, San Francisco

² Department of Pharmaceutical Chemistry, University of California, San Francisco. 600 16th St. Genentech Hall, San Francisco, CA. 94143

Summary

The mechanisms of inhibition of two novel scFv antibody inhibitors of the serine protease MT-SP1/matriptase reveal the basis of their potency and specificity. Kinetic experiments characterize the inhibitors as extremely potent inhibitors with K_I 's in the low picomolar range that compete with substrate binding in the S1 site. Alanine scanning of the loops surrounding the protease active site provide a rationale for inhibitor specificity. Each antibody binds to a number of residues flanking the active site, forming a unique three-dimensional binding epitope. Interestingly, one inhibitor binds in the active site cleft in a substrate-like manner, can be processed by MT-SP1 at low pH, and is a standard mechanism inhibitor of the protease. The mechanisms of inhibition provide a rationale for the effectiveness of these inhibitors, and suggest that the development of specific antibody-based inhibitors against individual members of closely related enzyme families is feasible, and an effective way to develop tools to tease apart complex biological processes.

Keywords

antibody; standard mechanism protease inhibitor; specificity; serine protease; HuCAL

Introduction

Of the 22 families of naturally-occurring protein-based protease inhibitors known to inhibit the S1 clan of serine proteases, 18 use an identical mechanism of inhibition¹. Standard mechanism (also known as Canonical, or Laskowski mechanism) inhibitors all insert a reactive loop into the active site of the protease, which binds in an extended β -sheet in a substrate-like manner². While some of these inhibitors have developed secondary mechanisms³, the primary mechanism of inhibition is extremely well conserved; so much so that crystal structures of unrelated inhibitors overlay perfectly in the protease active site⁴. As evidenced by this remarkable example of convergent evolution, the standard mechanism is an efficient, robust way to inhibit serine proteases. However, this robustness often comes at the expense of specificity. With the exception of a small number of parasitic anti-thrombin inhibitors that also bind to protease exosites⁵, the majority of standard mechanism protease inhibitors have

Correspondence to: Charles S. Craik.

[#]Current Address, Amgen Inc. Thousand Oaks, CA. 91320.

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relatively broad specificity. Bovine pancreatic trypsin inhibitor (BPTI) efficiently inhibits almost all trypsin-fold serine proteases with P1-Arg specificity, but can also inhibit chymotrypsin (P1-Phe specificity) with a K_I of 10 nM⁶.

Much effort has been spent on the development of specific protease inhibitors, for their use both as biological tools and potential therapeutic agents. As attempts to make specific small molecules have proven to be difficult⁷, researchers have often attempted to gain specificity using peptide or protein-based scaffolds. Constrained peptide phage display libraries have yielded extremely potent exosite inhibitors of factor VIIa (FVIIa)^{8, 9} and standard mechanism inhibitors of both chymotrypsin¹⁰ and urokinase-type plasminogen activator (uPA)¹¹ with moderate potency and specificity. An alternate approach has been to improve the specificity of naturally occurring protease inhibitors^{12; 13, 14}. For example, maturation of Alzheimer's amyloid β -protein precursor inhibitor (APPI) Kunitz domain, a canonical serine protease inhibitor, via competitive phage display improved its specificity for FVIIa by increasing its K_I against a panel of some related serine proteases by 2–5 orders of magnitude¹³. A third approach has been to mature specific protease inhibitors on other natural protein scaffolds, such as ankyrin repeats¹⁵ or antibodies. To this point, the characterized protease antibody inhibitors have been monoclonal antibodies raised from hybridomas, and have tended towards two types of inhibitors; inhibitors that interfere with multimerization (and thus activation) of the protease^{16, 17, 18}, and inhibitors that bind to loops and protein-protein interaction sites^{19, 20, 21; 22} and occlude substrate binding, instead of interfering with the catalytic machinery of the enzyme, and ensuring complete inhibition²³.

Previously, we reported the development of single chain variable fragment (scFv) antibody inhibitors of the serine protease membrane-type serine protease 1 (MT-SP1)²⁴. MT-SP1 (aka matriptase) was discovered and cloned in a search for serine proteases expressed in PC-3 cells, a prostate cancer cell line²⁵, and was independently determined to be a highly expressed protease in breast cancer tissue²⁶. Work from a number of groups has since shown that MT-SP1 may be a key upstream factor involved in the ECM remodeling and in signal transduction cascades involved in cell transformation²⁷. Ablation of MT-SP1 activity has been shown to decrease the invasiveness of both ovarian and prostate tumor cells, and modest orthotopic overexpression of MT-SP1 in mouse epidermal tissue led to spontaneous squamous cell carcinomas²⁸, further cementing MT-SP1's role in cancer, and suggesting the enzyme is causally involved in malignant transformation.

Here we have characterized the mechanism of inhibition of the two most potent scFv inhibitors of MT-SP1, E2 and S4. The inhibitors were selected from a fully synthetic human combinatorial antibody library in the scFv format (HuCAL, MorphoSys AG). HuCAL-scFv contains consensus frameworks with diversified light and heavy chain CDR3 regions reflecting the natural human amino acid composition²⁹. A combination of mutagenesis experiments, steady state kinetics, and stopped-flow kinetics reveal that, while the inhibitors gain specificity by making a number of critical interactions with surface loops on the protease, they can be standard mechanism inhibitors, which insert a reactive loop in a substrate like manner into the active site of the protease. This work suggests that the antibody scaffold can be used to create extremely specific standard mechanism protease inhibitors. Furthermore, the design of inhibitors that utilize macromolecular recognition factors (variable loops, protein-protein interaction sites) can help to differentiate highly homologous proteases, and can thus impart specificity upon the inhibitors.

Results

We previously described the maturation and initial characterization of a number of scFv inhibitors of MT-SP1²⁴. The scFv's bound tightly to the catalytic domain of MT-SP1, and

showed a high degree of specificity, as they showed no appreciable inhibition of a panel of closely related serine proteases including factor Xa, thrombin, kallikrein, tissue plasminogen activator (tPA), and urokinase plasminogen activator (uPA) at inhibitor concentrations of 1 μ M. Here we characterize the mechanism of inhibition of the two most potent of this novel class of serine protease inhibitors, E2 and S4.

Steady State Kinetics

Previous experiments showed that E2 and S4 had K_D 's of 160 pM and 500 pM (as determined by surface plasmon resonance), and were potent inhibitors of MT-SP1. In the current study, a number of steady-state kinetic experiments were performed to understand the mechanism of inhibition of these inhibitors. The results of these experiments are summarized in Table 1. Double reciprocal plots revealed that both E2 and S4 are competitive inhibitors of MT-SP1 with respect to Spectrazyme-tPA, a small molecule para-nitroanilide (pNA) substrate of P1 arginine serine proteases. To further characterize the tight-binding nature of these inhibitors, accurate K_I values were determined. E2 and S4 are extremely tight-binding competitive inhibitors of MT-SP1 with K_I values of 8.0 ± 1.3 pM and 140 ± 6.0 pM, respectively.

To verify that the mode of inhibition is similar in the context of a macromolecular substrate, a discontinuous assay to measure the activation of uPA was developed. The K_M of uPA as a substrate for MT-SP1 was determined to be 1.7 ± 0.2 μ M, and the k_{cat} of MT-SP1 activation of sc-uPA was 0.89 ± 0.09 s⁻¹. Double reciprocal plots showed that the inhibitors were indeed competitive with respect to macromolecular substrates as well. From this data, approximate K_I 's of 12 pM for E2 and 160 pM for S4 could be extrapolated (Table 1). Since the substrate (uPA) concentration could not be increased above the K_M , the errors associated with the K_I values are large; nonetheless, they confirm that the inhibitors inhibit MT-SP1 equally well, regardless of the size of the protease substrate.

Pre-steady State Kinetics

A closer examination of the progress curves of the steady-state reactions when enzyme was added to a mixture of substrate and inhibitor revealed different binding mechanisms for E2 and S4 (Figure 1). The progress curves for S4 are linear, suggesting the binding of scFv to enzyme comes to equilibrium rapidly. Conversely, the progress curves for E2 inhibition are curved, suggesting slow-binding inhibition³⁰. To define the binding mechanisms of these scFv's, stopped-flow experiments were performed to evaluate the onset of inhibition during turnover at higher enzyme concentrations. Stopped-flow experiments measured the appearance of pNA, and were carried out as described in the materials and methods section.

The stopped flow traces from the S4 inhibitor experiments were fit by nonlinear regression to the rate equations for reversible, tight binding inhibition to obtain observed rate constants (k_{obs}) for the onset of inhibition (Equation 5)³¹. Plots of k_{obs} vs. [S4] are linear with positive y-intercepts (Figure 2a), consistent with a one-step reversible mechanism for binding of the inhibitor. The y-intercepts of the plots give an average off rate of $k_{-1} = 1.7 \times 10^{-2}$ s⁻¹, and a secondary plot of the slopes versus substrate concentration (Figure 2a, inset) defined the on rate as $k_1 = 1.2 \times 10^8$ M⁻¹s⁻¹. The K_I calculated from $k_{-1}/k_1 = 147$ pM, which is in very good agreement with the steady-state experimental K_I of 140 pM. From these data, it can be concluded that S4 binds and inhibits MT-SP1 with an extremely fast on-rate, and has a one-step binding mechanism as shown in Scheme 1.

The stopped flow traces from the E2 inhibitor experiments (Figure 2b) revealed a more complicated binding mechanism. In this case, the progress curves are fit well to a sum of two exponentials (Equation 7), indicating the presence of at least two steps in the binding process, which leads to the onset of inhibition. At minimum, a double exponential decay is consistent

with a two-step binding mechanism. This occurs when the first step in the binding process is more rapid than the second, and as a result, the first observed rate constant (k_{obs1}) shows a linear dependence on inhibitor concentration³². If k_{obs1} shows a hyperbolic dependence on inhibitor concentration, the mechanism of inhibition involves more than two steps. Unfortunately, due to the extremely tight nature of the enzyme-inhibitor interaction, the inhibitor concentration could not be increased to a high enough concentration to distinguish between a linear or hyperbolic dependence of k_{obs1} on inhibitor concentration. But, due to the presence of two exponential decays, an absolute minimal mechanism of E2 inhibition has two steps, and E2 can be classified as a slow, tight-binding inhibitor³⁰.

P-Aminobenzamidine Competition Assay

P-aminobenzamidine (pAB) has been reported as a weak competitive inhibitor of P1-arginine specific serine proteases³³, and can be used as a fluorescent probe to monitor substrate or inhibitor binding in the S1 site. The hydrophobic nature of the S1 site causes pAB to fluoresce with a maximum emission around 360 nm when bound to the enzyme, while pAB in aqueous solution has both a lower intensity and longer wavelength of emission at 376 nm. PAB has been used as a probe to monitor binding of inhibitors in the S1 site of inhibitors; competitive inhibitors displace pAB from the protease active site and reduce emission at 360 nm^{33, 34}, while non-competitive inhibitors do not³⁵. PAB has a K_I of 28.8 μ M for MT-SP1 (data not shown), and 1 μ M MT-SP1 incubated with 270 μ M PAB (to saturate the enzyme) shows a characteristic emission peak at 361 nm when excited at 325 nm (Figure 3). When one equivalent of either E2 or S4 are added to the pre-incubated MT-SP1/pAB, the fluorescence is sharply decreased (Figure 3). This suggests that both inhibitors bind at or near the S1 site, and most likely insert an arginine or lysine side chain in the pocket.

MT-SP1/Inhibitor Digest

The reactive site of many standard mechanism serine protease inhibitors have been determined by incubating protease and inhibitor at low pH, where the inhibitor can be cleaved in a substrate-like manner, causing a processing of the inhibitor into two fragments, with the cleavage occurring between the P1 and P1' residue^{36, 37}. When MT-SP1 and E2 are incubated at pH 6.0 for an extended period of time (>120 hrs), E2 is processed into two bands (Figure 4). This processing is not seen at pH 8.0, or without MT-SP1 at pH 6.0. MT-SP1 shows no proteolytic activity below pH 6.0, thus making pH 6.0 the lowest pH at which processing can occur. ESI mass spectrometry verifies that the processing event takes place between R131 and R132 in E2. The N-terminal fragment has a mass of 12,013 Da (expected MW, 12,014 Da) and the C-terminal fragment has a mass of 15,624 Da (expected MW, 15,627 Da). This places the reactive loop in the CDR3 of the heavy chain of E2, which would be expected from the HuCAL library from which these scFv's were matured, as the scaffold had large, diverse CDR3's²⁹. No S4 processing was observed upon incubation with MT-SP1 for extended periods of time at low pH, suggesting a different, non-canonical mechanism of inhibition for the S4 scFv.

Inhibitor Point Mutants

To verify the mechanism of inhibition of the scFv inhibitors, point mutants of the arginine residue in the CDR3 loops of the inhibitors were constructed. It would be expected that mutations to residues that bind in the S1 site of the protease would have the greatest effect on binding. The mutational data is summarized in Table 2. E2 R131A and R132A had K_I values of 78 nM and 454 pM, respectively, as opposed to a K_I of 12.3 pM for the wild-type E2. The mutation of R131 to an alanine has a 6500-fold effect on protease inhibition as would be expected from a residue that binds in the S1 site. This is consistent with the data from the inhibitor digest at low pH. The mutation of R132 caused a 38-fold increase in K_I , suggesting that the P1' arginine also makes significant contacts with the protease. The CDR3 loop of S4

also has a double arginine motif, R128 and R129. Both arginines were mutated to alanine and had significant effects on protease inhibition: S4 R128A had and K_I of 2.8 μM , while the R129 alanine mutant had and K_I of 3.9 nM, a 4×10^4 -fold and 56-fold difference, respectively.

MT-SP1 Point Mutations

To footprint the binding site of the inhibitors, site-directed mutagenesis was used to alanine scan the surface of the protease domain³⁸. Based on the crystal structure of MT-SP1³⁹, thirty point mutants were identified as potential partners in macromolecular interactions (Table 3). The majority of these residues were located on the loops flanking the protease active site. Proteolytic activity against Spec-tPA was used to assure that the point mutations did not drastically affect MT-SP1 structure or function. The differences between the mutant and wild-type protease k_{cat}/K_M values were less than two fold in most cases, suggesting that the mutations had minimal effect on protease structure. MT-SP1 T98A was a 6-fold less efficient enzyme than the wild-type, which could be attributed primarily to a lower k_{cat} . MT-SP1 D217A had a 3-fold decrease in protease specific activity, which was due to an increased K_M of 210 μM . The F99A, Q192A, and W215A substitutions in MT-SP1 all resulted in inactive enzymes. The inactive variants eluted from a gel filtration column at same size as the zymogen protease, suggesting they are inactive because they could not autoactivate (data not shown).

The K_I values for E2 and S4 were determined against the MT-SP1 point mutants. As a positive control, the fold-specific serine protease inhibitor BPTI was also screened against the protease point mutants, since the mechanism of inhibition is known⁴⁰ and a co-crystal of BPTI and MT-SP1 has been solved³⁹. As would be expected from a fold-specific protease inhibitor, most point mutants had little effect on BPTI inhibition. The I41A substitution moderately improved BPTI binding to MT-SP1, F94A, F97A, and E169A moderately decreased BPTI inhibition (corresponding to <1 kcal/mol binding energy), and D60bA and D217A mutations had a more significant affect on BPTI inhibition (Figure 5b). Analysis of the crystal structure suggest that the increased K_I of MT-SP1 D60bA could be due to D60b hydrogen bonding with R20 of BPTI, and forming an intramolecular H-bond with R60c, which packs against BPTI. A deletion of the H-bonding ability of this side chain would account for the moderate increase (12.6-fold) in K_I . The structure does not readily explain the 43-fold increase in the K_I of BPTI for MT-SP1 D217A, but it is possible that the mutation affects the structure of the 220's loop, which would also account for the increased K_M of Spec-tPA for D217A.

Based on the alanine scanning data, S4 makes contacts of moderate strength with a number of residues on the six surface loops surrounding the active site (Figure 5a, 5d). Interactions with the side chains of I41, D60b, T98, and Q175 account for modest binding energy (pink residues, Figure 5d); alanine mutations of these residues increased the K_I 's of S4 3–6 fold, corresponding to a decrease in free energy of binding of 0.5–1 kcal/mol. S4 makes stronger interactions with the side chains of F94, D96, H143, Y146, and D217 (red residues, Figure 5d), decreasing the free energy of binding by 1.5–2.0 kcal/mol. Interestingly, a mutation of R60c to alanine decreased the K_I of S4 6-fold. This suggests that the protease/scFv interaction has not been completely optimized, and could be improved. Taken together, the mutational data suggest that S4 makes a number of moderate contacts with the loops flanking the active site, making the strongest interactions with the 140's and 90's loops, and thereby bridging the active site.

E2 also makes interactions with a number of loops surrounding the MT-SP1 active site (Figure 5c), including the base of the 60's loop, the 90's loop, the 170's loop, the 220's loop, and the 140's loop. In contrast to S4, though, which makes a number of interactions of moderate strength, E2 gains much of its binding energy from interactions with two residues, D96 and F97. Mutations of each of these residues to alanine increased the K_I of E2 to >1 μM , corresponding to a decrease in free energy of binding of >7.5 kcal/mol. The Q175A variant, on the loop adjacent to the 90's loop, also has a significant effect on E2 inhibition, increasing

the K_I of E2 by 69-fold (3.0 kcal/mol). The 90's loop and 170's loop flank the extended binding sites of MT-SP1,^{39; 41}, and F97 helps form the S4 pocket, suggesting E2 binds in the extended binding pockets of MT-SP1. Though E2 makes minor interactions with Y146, Q221a, and K224, the majority of the binding energy of E2 for MT-SP1 comes from interactions with the 90's loop, and minor interactions with residues flanking the 90's loop (I60, Q175). This defines the 90's loop as a 'hot-spot' for E2 binding; and as the 90's loop sequence is unique to MT-SP1, it helps explain E2's specificity for MT-SP1.

Discussion

We have described the mechanism by which two novel scFv antibodies inhibit the cancer-associated serine protease MT-SP1/matriptase. The S4 antibody has a fast association rate with MT-SP1 ($1.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ as measured by stopped-flow kinetics) and binds very tightly to the protease, making numerous contacts with the loops surrounding the active site of MT-SP1. The fast on-rate is likely influenced by electrostatic steering, which can increase k_{on} 's by more than 10^4 over the basal diffusion controlled association rate⁴². Mutational data supports this hypothesis, as nearly all the residues S4 makes significant contacts with are polar or charged. The inhibitor competes with pAB for the S1 site, and the R128A variant of S4 nearly abolishes protease inhibition. Despite these data, S4 cannot be considered a standard mechanism inhibitor of MT-SP1 without further structural characterization. Standard mechanism inhibitors have a characteristic two-step binding mechanism; an initial binding step, followed by a tightening of the enzyme-inhibitor complex, and as such have an association rate approximately two orders of magnitude slower than S4. Furthermore, S4 is not processed by MT-SP1 at low pH, meaning the substrate-like binding cannot be assumed.

E2, on the other hand, displays all the characteristics of a standard mechanism serine protease inhibitor. While a crystal structure would help to definitively determine the mechanism of inhibition, the data here are consistent with E2 being a standard-mechanism inhibitor. The enzyme-inhibitor complex slowly reaches equilibrium, E2 binds in a substrate-like manner, and inserts an arginine in the S1 site of MT-SP1 (R131), which is important, but not absolutely critical to inhibition. Furthermore, E2 shows a slight degree of fold specificity; it inhibits the mouse homolog of MT-SP1, epithin, with a K_I of 40nM²⁴ and can inhibit trypsin, a digestive protease with extremely broad specificity, with an IC_{50} of 45 μM (data not shown). In contrast to most standard mechanism serine protease inhibitors, though, E2 is highly specific for a single serine protease, MT-SP1. E2 gains much of its specificity through interactions with the 90's loop of MT-SP1, and makes significant interactions with residues D96 and F97 of the protease. Perhaps not surprisingly, known protease inhibitors that do exhibit a high degree of specificity, such as anticoagulant protease inhibitors from ticks and leeches, often employ a similar mechanism of inhibition; they combine the robustness of competitive, active site inhibition with protein extensions that bind to recognition sites on target enzymes^{5, 43}.

To our knowledge, these scFvs are the first documented case of mechanistic protease inhibitors on an antibody scaffold that bind in the active site. A number of monoclonal antibody protease inhibitors have been reported in the literature^{17; 18; 19; 21; 22; 44}, but despite diverse mechanisms, all have the same underlying mode of action; they bind to a small, linear peptide sequence and prevent either a protein-protein or enzyme-substrate interaction. While often sufficient for inhibition, these mAb's can have curious inhibitory profiles in which they cannot inhibit the hydrolysis of small-molecule substrates, or have different levels of inhibition against different substrates^{19, 21}. Because they are selected *in vitro* against the active form of the enzyme, antibodies developed by phage display have the inherent advantage of recognizing three-dimensional epitopes and the topography of the enzyme active site. With this comes the opportunity for tighter binding due to greater buried surface areas and minimal entropic penalties upon binding, and more complete inhibition through insertion of residues into the

protease active site. E2 and S4 have clearly used these advantages; they have fast on-rates, very low K_D 's, bind in the active site groove, and make contacts with a number of loops flanking the active site.

The HuCAL-scFv library²⁹ contains consensus framework sequences for all frequently occurring V_H and V_L subfamilies with a germline sequence for the CDR1 and CDR2 in each subfamily. Both the heavy and light chain CDR3 regions were diversified according to the natural amino acid composition and cover the natural length variation of the V_H and V_L CDR3 regions. In retrospect, this proves to be an ideal scaffold for serine protease inhibition; it allows for a large, rigidified reactive loop to be inserted into the protease active site while the rest of the antibody stabilizes the CDR3 of the heavy chain and makes additional contacts with the protease. While only the most potent scFv inhibitors of MT-SP1 were characterized, all inhibitors had heavy chain CDR3 loops of at least 17 residues, suggesting that large heavy chain CDR3's were critical to MT-SP1 inhibition.

The explosion in antibody research over the past 15 years has revolutionized biotechnology. Antibodies have been developed into extremely useful drugs and imaging devices, and have become critical tools for nearly all biological research. Here, scFv fragments have shown the ability to specifically inhibit a single member of a family of closely related enzymes. While these molecules will be useful in helping dissect the complex biology of MT-SP1,⁴⁵ the mechanisms through which they work once again reveals the innate binding flexibility of antibodies, and the power of protein engineering. That these inhibitors have developed the robust inhibition mechanism of standard mechanism serine protease inhibitors, suggests that we can develop antibodies to mimic any protein-protein interaction, and precisely modulate nearly any biological process.

Materials and Methods

Protein Expression, Purification, and Mutagenesis

MT-SP1 and MT-SP1 mutants were expressed in *E. coli* and purified from inclusion bodies as previously described²⁵. Antibodies were selected from the HuCAL scFv library (MorphoSys AG, Martinsreid, Germany)²⁹. Expression and purification of inhibitory scFv antibodies was also previously described²⁴. Point mutants were made using the Stratagene Quickchange kit (Stratagene, La Jolla, CA). One or two base changes were sufficient to create the point mutant in each case, and all sequences were verified by DNA sequencing.

Steady-State Kinetics

All reaction volumes were 120 μ L and were carried out in 50mM Tris, pH 8.8, 50 mM NaCl, 0.01% Tween-20 unless otherwise stated. All reactions were carried out in triplicate. Reactions were run in 96-well, medium binding, flat-bottomed plates (Corning), and cleavage of substrate was measured in a UVmax Microplate Reader (Molecular Devices Corporation, Palo Alto, CA.). MT-SP1 and mutant protease concentrations were determined by 4-methylumbelliferyl *p*-guanidinobenzoate (MUG-B) active-site titration⁴⁶ in a Fluormax-2 spectrofluorimeter. Kinetic parameters of MT-SP1 and mutant proteases were determined at 0.2 nM enzyme, with Spectrazyme-tPA (hexahydrotyrosyl-Gly-Arg-pNA, American Diagnostica, Greenwich, CT) concentrations varying from 1 μ M to 400 μ M. K_M and k_{cat} were determined using the Michaelis-Menten equation.

Tight-binding inhibitors require that the effective decrease in free enzyme be taken into account when determining K_I values³¹. This is accomplished by pre-incubating enzyme and inhibitor so that the system can reach equilibrium, adding substrate, and then measuring steady-state velocities at varying inhibitor concentrations and fitting the data to equation 1.

$$v_i/v_s = \frac{\left\{ [E_T - I_T - K_i^*] + [(I_T + K_i^* - E_T)^2 + (4K_i^* E_T)]^{1/2} \right\}}{2E_T} \quad (1)$$

K_i^* 's are then plotted against substrate concentration to extrapolate the K_i at zero substrate concentration (equation 2).

$$K_i^* = K_i(1 + [S]/K_m) \quad (2)$$

When measuring the effect mutations had on the strength of the interaction between the protease and inhibitor, IC_{50} values were used instead of K_i 's as determined above. Though less accurate than K_i 's⁴⁷, IC_{50} 's are easier to calculate when screening large numbers of inhibitor point mutants, and are sufficient to monitor relative changes in inhibition versus the wild type system. IC_{50} 's were determined by incubating inhibitor and 0.2 nM enzyme for at least 5 hours at room temperature to assure steady-state behavior of the system³. There was no appreciable decrease in protease activity during the incubation period. Steady-state velocities were then plotted against inhibitor concentration and fit to equation 3.

$$v = v_{\min} + \frac{(v_{\max} - v_{\min})}{(1 + 10^{([I] - IC_{50})})} \quad (3)$$

Relative K_i 's were then calculated from IC_{50} values according to equation 4.

$$K_i = \frac{IC_{50}}{(1 + [S]/K_m)} \quad (4)$$

Though nearly all protease mutants had a minimal (less than 2-fold) effect on substrate K_M , and the substrate concentration was held well above the K_M , this correction normalizes the IC_{50} with respect to the strength of the protease/substrate interaction. All graphs and equations were fit using Kaledagraph 3.6 (Synergy Software, Reading, PA).

Macromolecular Substrate Assay

In an assay analogous to that used to monitor FVIIa activation of FX⁸, we developed a coupled assay that monitors MT-SP1 activation of uPA. 50 pM MT-SP1 was incubated with varying concentrations (final concentrations, 12.5–400 nM) of single-chain uPA (American Diagnostica). At varying time points (0–150min), aliquots of the reaction were removed and quenched with 10 nM E2. There was no residual MT-SP1 activity after the quench, and E2 showed no inhibition of uPA at 10 nM concentrations. The amount of active uPA was measured by monitoring the activity for uPA against the paranitroanilide uPA substrate Spectrazyme-UK (American Diagnostica). The mode of inhibition was determined from double reciprocal plots, and kinetic parameters and inhibition constants were determined using the Michaelis-Menten equation. The K_M of Spec-UK for uPA was determined to be 42 μ M, and the k_{cat} of uPA turnover was 1.0 s^{-1} .

Stopped-Flow Kinetics

Stopped-flow experiments were conducted using a HiTech SF-61DX2 instrument (TgK Scientific Ltd., Bradford On Avon, U.K.). Data were collected in dual beam mode using photomultiplier detection of absorbance data at 405 nm. MT-SP1 (10 nM for E2 experiments, 1 nM for S4 experiments) was rapidly mixed with a solution of substrate (Spec-tPA, 200 μ M–800 μ M) and inhibitor (10–300 nM for S4, 100–340 nM for E2) and the appearance of pNA was monitored for 20 sec (for S4) or 150 sec (for E2). Concentrations of enzyme and

length of experiments were varied between the two systems to ensure robust signal and equilibration of the system.

The stopped flow traces from the S4 inhibitor experiments were fit by nonlinear regression to the rate equations for reversible, tight binding inhibition³¹ (equation 5).

$$P = v_s t + (v_i - v_s)(1 - e^{-k_{obs}t})/k_{obs} \quad (5)$$

The appearance of the product (P) is a function of the initial (v_i) and final (v_s) velocities, and an apparent first-order rate constant, k_{obs} for the onset of inhibition. Plots of k_{obs} versus inhibitor concentration were linear, and fit to equation 6, as would be expected when the inhibitory mechanism consists of one reversible binding step, as in scheme 1.

$$k_{obs} = k_{-1} + k_1[I]/(1 + [S]/K_M) \quad (6)$$

E2 stopped flow traces fit poorly to equation 5, but fit well to a mechanism with two observed rate constants (equation 7)³².

$$P = v_s t + (v_i - v_s)(1 - e^{-k_{obs1}t})/k_{obs1} + (v_i - v_s)(1 - e^{-k_{obs2}t})/k_{obs2} \quad (7)$$

P-Aminobenzamidine Fluorescence

Experiments were carried out in PBS on a Fluorolog 3 (Instruments SA Inc. Edison, NJ) fluorimeter. Emission spectra of MT-SP1/pAB were obtained by excitation at 325 nm using a 4 nm excitation and 2 nm emission bandpass and were scanned from 335–430nm. Spectra were corrected for emission due to free pAB and protease. Data corrections were performed with Datamax 2.20 software (Instruments SA).

Inhibitor Digest

2 μ M E2 or S4 was incubated with 0.1 nM MT-SP1 for 120 hours at room temperature. Proteins were incubated in 100 mM NaCl, 100 mM MES, pH 6.0, or 100 mM NaCl, 50 mM TRIS, pH 8.0. Proteolysis was monitored by gel-shift on a 12% polyacrylamide gel with a 4.5% stacking gel, and stained with coomassie brilliant blue. ESI mass spectrometry was carried out on an LCT Premier mass spectrometer (Waters Corp. Milford, MA), and molecular masses were determined using MassLynx (Waters) deconvolution software.

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References

1. Rawlings ND, Morton FR, Barrett AJ. MEROPS: the peptidase database. *Nucleic Acids Res* 2006;34:D270–2. [PubMed: 16381862]
2. Laskowski M Jr, Kato I. Protein inhibitors of proteinases. *Annu Rev Biochem* 1980;49:593–626. [PubMed: 6996568]
3. Eggers CT, Wang SX, Fletterick RJ, Craik CS. The role of ecotin dimerization in protease inhibition. *J Mol Biol* 2001;308:975–91. [PubMed: 11352586]
4. Tyndall JD, Nall T, Fairlie DP. Proteases universally recognize beta strands in their active sites. *Chem Rev* 2005;105:973–99. [PubMed: 15755082]

5. Rydel TJ, Tulinsky A, Bode W, Huber R. Refined structure of the hirudin-thrombin complex. *J Mol Biol* 1991;221:583–601. [PubMed: 1920434]
6. Castro MJ, Anderson S. Alanine point-mutations in the reactive region of bovine pancreatic trypsin inhibitor: effects on the kinetics and thermodynamics of binding to beta-trypsin and alpha-chymotrypsin. *Biochemistry* 1996;35:11435–46. [PubMed: 8784199]
7. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002;295:2387–92. [PubMed: 11923519]
8. Dennis MS, Eigenbrot C, Skelton NJ, Ultsch MH, Santell L, Dwyer MA, O'Connell MP, Lazarus RA. Peptide exosite inhibitors of factor VIIa as anticoagulants. *Nature* 2000;404:465–70. [PubMed: 10761907]
9. Roberge M, Santell L, Dennis MS, Eigenbrot C, Dwyer MA, Lazarus RA. A novel exosite on coagulation factor VIIa and its molecular interactions with a new class of peptide inhibitors. *Biochemistry* 2001;40:9522–31. [PubMed: 11583151]
10. Krook M, Lindbladh C, Eriksen JA, Mosbach K. Selection of a cyclic nonapeptide inhibitor to alpha-chymotrypsin using a phage display peptide library. *Mol Divers* 1997;3:149–59. [PubMed: 9680646]
11. Hansen M, Wind T, Blouse GE, Christensen A, Petersen HH, Kjølgaard S, Mathiasen L, Holtet TL, Andreasen PA. A urokinase-type plasminogen activator-inhibiting cyclic peptide with an unusual P2 residue and an extended protease binding surface demonstrates new modalities for enzyme inhibition. *J Biol Chem* 2005;280:38424–37. [PubMed: 16141208]
12. Wang CI, Yang Q, Craik CS. Isolation of a high affinity inhibitor of urokinase-type plasminogen activator by phage display of ecotin. *J Biol Chem* 1995;270:12250–6. [PubMed: 7744876]
13. Dennis MS, Lazarus RA. Kunitz domain inhibitors of tissue factor-factor VIIa. II. Potent and specific inhibitors by competitive phage selection. *J Biol Chem* 1994;269:22137–44. [PubMed: 8071338]
14. Stoop AA, Craik CS. Engineering of a macromolecular scaffold to develop specific protease inhibitors. *Nat Biotechnol* 2003;21:1063–8. [PubMed: 12923547]
15. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grütter MG, Plückthun A. High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol* 2004;22:575–82. [PubMed: 15097997]
16. Rezacova P, Lescar J, Brynda J, Fabry M, Horejsi M, Sedlacek J, Bentley GA. Structural basis of HIV-1 and HIV-2 protease inhibition by a monoclonal antibody. *Structure* 2001;9:887–95. [PubMed: 11591344]
17. Puchi M, Quinones K, Concha C, Iribarren C, Bustos P, Morin V, Geneviere AM, Imschenetzky M. Microinjection of an antibody against the cysteine-protease involved in male chromatin remodeling blocks the development of sea urchin embryos at the initial cell cycle. *J Cell Biochem* 2006;98:335–42. [PubMed: 16408295]
18. Fukuoka Y, Schwartz LB. The B12 anti-tryptase monoclonal antibody disrupts the tetrameric structure of heparin-stabilized beta-tryptase to form monomers that are inactive at neutral pH and active at acidic pH. *J Immunol* 2006;176:3165–72. [PubMed: 16493076]
19. Petersen HH, Hansen M, Schousboe SL, Andreasen PA. Localization of epitopes for monoclonal antibodies to urokinase-type plasminogen activator: relationship between epitope localization and effects of antibodies on molecular interactions of the enzyme. *Eur J Biochem* 2001;268:4430–9. [PubMed: 11502203]
20. Matias-Roman S, Galvez BG, Genis L, Yanez-Mo M, de la Rosa G, Sanchez-Mateos P, Sanchez-Madrid F, Arroyo AG. Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. *Blood* 2005;105:3956–64. [PubMed: 15665118]
21. Xuan JA, Schneider D, Toy P, Lin R, Newton A, Zhu Y, Finster S, Vogel D, Mintzer B, Dinter H, Light D, Parry R, Polokoff M, Whitlow M, Wu Q, Parry G. Antibodies neutralizing hepsin protease activity do not impact cell growth but inhibit invasion of prostate and ovarian tumor cells in culture. *Cancer Res* 2006;66:3611–9. [PubMed: 16585186]
22. Obermajer N, Premzl A, Zavasnik Bergant T, Turk B, Kos J. Carboxypeptidase cathepsin X mediates beta2-integrin-dependent adhesion of differentiated U-937 cells. *Exp Cell Res* 2006;312:2515–27. [PubMed: 16774752]

23. Maun HR, Eigenbrot C, Lazarus RA. Engineering exosite peptides for complete inhibition of factor VIIa using a protease switch with substrate phage. *J Biol Chem* 2003;278:21823–30. [PubMed: 12657647]
24. Sun J, Pons J, Craik CS. Potent and selective inhibition of membrane-type serine protease 1 by human single-chain antibodies. *Biochemistry* 2003;42:892–900. [PubMed: 12549907]
25. Takeuchi T, Shuman MA, Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci U S A* 1999;96:11054–61. [PubMed: 10500122]
26. Lin CY, Anders J, Johnson M, Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. *J Biol Chem* 1999;274:18237–42. [PubMed: 10373425]
27. Uhland K. Matriptase and its putative role in cancer. *Cell Mol Life Sci* 2006;63:2968–78. [PubMed: 17131055]
28. List K, Szabo R, Molinolo A, Sriuranpong V, Redeye V, Murdock T, Burke B, Nielsen BS, Gutkind JS, Bugge TH. Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev* 2005;19:1934–50. [PubMed: 16103220]
29. Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellenhofer G, Hoess A, Wolle J, Pluckthun A, Virnekas B. Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* 2000;296:57–86. [PubMed: 10656818]
30. Morrison JF, Walsh CT. The behavior and significance of slow-binding enzyme inhibitors. *Adv Enzymol Relat Areas Mol Biol* 1988;61:201–301. [PubMed: 3281418]
31. Williams JW, Morrison JF. The kinetics of reversible tight-binding inhibition. *Methods Enzymol* 1979;63:437–67. [PubMed: 502865]
32. Hiromi, K. Kinetics of fast enzyme reactions: theory and practice. Kodansha Ltd.; Wiley; Tokyo New York: 1979.
33. Evans SA, Olson ST, Shore JD. p-Aminobenzamidine as a fluorescent probe for the active site of serine proteases. *J Biol Chem* 1982;257:3014–7. [PubMed: 7037776]
34. Parry MA, Maraganore JM, Stone SR. Kinetic mechanism for the interaction of Hirulog with thrombin. *Biochemistry* 1994;33:14807–14. [PubMed: 7993908]
35. Fernandez AZ, Tablante A, Beguin S, Hemker HC, Apitz-Castro R. Draculin, the anticoagulant factor in vampire bat saliva, is a tight-binding, noncompetitive inhibitor of activated factor X. *Biochim Biophys Acta* 1999;1434:135–42. [PubMed: 10556567]
36. Ozawa K, Laskowski M Jr. The reactive site of trypsin inhibitors. *J Biol Chem* 1966;241:3955–61. [PubMed: 5950769]
37. McGrath ME, Hines WM, Sakanari JA, Fletterick RJ, Craik CS. The sequence and reactive site of ecotin. A general inhibitor of pancreatic serine proteases from *Escherichia coli*. *J Biol Chem* 1991;266:6620–5. [PubMed: 2007606]
38. Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* 1989;244:1081–5. [PubMed: 2471267]
39. Friedrich R, Fuentes-Prior P, Ong E, Coombs G, Hunter M, Oehler R, Pierson D, Gonzalez R, Huber R, Bode W, Madison EL. Catalytic domain structures of MT-SP1/matriptase, a matrix-degrading transmembrane serine proteinase. *J Biol Chem* 2002;277:2160–8. [PubMed: 11696548]
40. Luthy JA, Praissman M, Finkenstadt WR, Laskowski M Jr. Detailed mechanism of interaction of bovine -trypsin with soybean trypsin inhibitor (Kunitz). I. Stopped flow measurements. *J Biol Chem* 1973;248:1760–71. [PubMed: 4735140]
41. Sriprapundh, D., Craik, CS. (2006).
42. Schreiber G, Fersht AR. Rapid, electrostatically assisted association of proteins. *Nat Struct Biol* 1996;3:427–31. [PubMed: 8612072]
43. Rezaie AR. Kinetics of factor Xa inhibition by recombinant tick anticoagulant peptide: both active site and exosite interactions are required for a slow- and tight-binding inhibition mechanism. *Biochemistry* 2004;43:3368–75. [PubMed: 15035608]

44. Martin F, Volpari C, Steinkuhler C, Dimasi N, Brunetti M, Biasiol G, Altamura S, Cortese R, De Francesco R, Sollazzo M. Affinity selection of a camelized V(H) domain antibody inhibitor of hepatitis C virus NS3 protease. *Protein Eng* 1997;10:607–14. [PubMed: 9215580]
45. Bhatt AS, Welm A, Farady CJ, Vasquez M, Wilson K, Craik CS. Coordinate Expression and Functional Profiling Identify and Extracellular Proteolytic Signaling Pathway. *Proc Natl Acad Sci U S A*. 2007
46. Jameson GW, Roberts DV, Adams RW, Kyle WS, Elmore DT. Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration. *Biochem J* 1973;131:107–17. [PubMed: 4737291]
47. Chou T. Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms, and different types and mechanisms of inhibition. *Mol Pharmacol* 1974;10:235–47. [PubMed: 4212316]

Figure 1.

Progress curves of MT-SP1 inhibition by scFv inhibitors reveal multiple mechanisms of inhibition. The addition of enzyme (0.2 nM) to a mixture of substrate (300 μ M Spec-tPA) and inhibitor results in a decrease in proteolytic activity. S4 inhibition results in a linear progress curve, suggesting rapid-equilibrium inhibition, while the curved nature of the E2 progress curve suggests slow-binding inhibition.

Figure 2.

Stopped-flow experiments confirm disparate mechanisms of inhibitor binding to MT-SP1. (a) Linear plots of k_{obs} vs. S4 concentration confirm that S4 has a one-step binding mechanism, as illustrated in Scheme 1. Individual traces are carried out at different substrate concentrations. Black (x) experiments were run at 200 μ M Spec-tPA, green (o) experiments, 300 μ M Spec-tPA, blue ($\square$) experiments, 500 μ M Spec-tPA, and red ($\circ$) experiments at 800 μ M Spec-tPA. The y-intercepts of the observed rate constant plots (a) gave an average off rate of $k_{-1} = 1.7 \times 10^{-2} \text{ s}^{-1}$, and a secondary plot of the slopes versus substrate concentration (a, inset) defined the on rate as $k_1 = 1.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. (b) The raw stopped-flow trace monitoring E2 inhibition of MT-SP1 by measuring the appearance of pNA at 405 nm fits well to the double exponential Equation 7 with two observed rate constants. The inset shows the residuals of the non-linear

regression fit. Final concentrations for this trace were 240 nM E2, 10 nM MT-SP1, and 500 μ M Spec-tPA.

Figure 3.

Inhibitors displace pAB from the MT-SP1 active site. pAB (270 μ M) incubated with 1 μ M MT-SP1 emits a strong emission peak with a maximum at 361 nm when excited at 325 nm due to hydrophobic interactions between pAB and the P1 pocket of the protease. When one equivalent of either S4 (blue trace) or E2 (green trace) are added to 1 μ M MT-SP1 saturated with pAB, the fluorescence decreases, suggesting pAB is released into the aqueous environment where it is weakly fluorescent. Therefore, binding of both S4 and E2 are competitive with pAB binding, and both inhibitors bind in or near the P1 pocket in a manner that precludes binding of pAB.

Figure 4.

E2 is processed by MT-SP1 at pH 6.0. 2 mM E2 was incubated at pH 6.0 with (lane 3) and without (lane 1) 0.1 μ M MT-SP1 for 120 hours. Samples were run on a 12% polyacrylamide gel and stained with coomassie blue. At pH 6.0, E2 was processed into two products, with molecular masses determined to be 15,624 Da, and 12,013 Da by ESI mass spectrometry. These masses, when added together, account for the mass of the full-length inhibitor (27,219 Da) and the water molecule added to the products during the hydrolysis reaction. This processing does not take place when E2 and MT-SP1 are incubated at pH 8.0 (lane 2). The diagram below shows the site of the scissile bond in the middle of the heavy chain of E2.

Figure 5.

MT-SP1 alanine point mutants and their effect on protease inhibition by BPTI (*b*), E2 (*c*), and S4 (*d*). *5a* shows the 6 MT-SP1 surface loops surrounding the protease active site consisting of a binding cleft and the catalytic triad (sticks). The space-filling models of *b*, *c* and *d* are oriented in the same manner, with the catalytic triad in yellow. Point mutants that had minimal effect on protease inhibition are shaded in gray, mutations that had a 3–10-fold increase in inhibitor K_I are shaded pink, and point mutants that increased inhibitor K_I by >10-fold are shaded in red. Point mutants that decreased inhibitor K_I 's are shaded in green. The values of point-mutant/inhibitor K_I 's are given in Table 3. MT-SP1 point mutants have a minimal effect on BPTI inhibition, S4 interacts with moderate affinity to all 6 protease loops surrounding the active site, and E2 binds with high affinity to the 90's and 170's loop. Figure was prepared using PyMOL.

Scheme.

Table 1

Kinetic Parameters of scFv Inhibitors

	k_{on}^* ($10^6 M^{-1} s^{-1}$)	k_{off}^* ($10^{-3} s^{-1}$)	K_d^* (nM)	Mode of Inhibition	K_i (pM)	Macro molecular MOI	Macro molecular K_i (pM)
E2	2.1	0.38	0.16	Competitive	8.0 ± 1.3	Competitive	12
S4	11.5	5.8	0.51	Competitive	140 ± 6	Competitive	160

* Values Determined by SPR. Sun *et al.* 2003

Table 2Inhibitor Point Mutant K_I 's vs. MT-SP1

	K_I (nM)	Fold Difference
E2	0.012	
E2 R131A	78	6500
E2 R132A	0.454	38
S4	0.07	
S4 R128A	2800	4.0×10^4
S4 R129A	3.9	56

* All error values > 6%

Table 3

MT-SP1 Point Mutant/Inhibitor K_i Values

	BPTI			E2			S4		
	K_i (pM)	Fold Difference		K_i (pM)	Fold Difference		K_i (pM)	Fold Difference	
MT-SP1	49.7			12.3			70.4		
Q38A	20.7	0.42		6.1	0.5		73.6		1
I41A	12.4	0.25 (4-fold)		12.3	1		208		3
I60A	35.8	0.72		50.4	4.1		40.2		0.57
D60aA	37.2	0.75		25.1	2		125		1.8
D60bA	628	12.6		20.8	1.7		427		6.1
R60cA	134	2.7		11.4	0.93		11.7		0.17 (6-fold)
F60eA	24.1	0.48		11.4	0.93		102		1.4
R60fA	73.4	1.5		10.9	0.89		88.9		1.3
Y60gA	43.5	0.88		12.7	1		151		2.1
R87A	38.6	0.78		9.4	0.76		54.5		0.77
F94A	170	3.4		36.2	2.9		1036		15
N95A	83.6	1.7		45.4	3.7		108		1.5
D96A	150	3		>1uM	> 10^5		897		13
F97A	224	4.5		>1uM	> 10^5		154		2
T98A	76.4	1.5		83.2	6.7		239		3.4
H143A	48.5	1		14.2	1.2		1671		24
Q145A	83.4	1.7		15.4	1.3		116		1.6
Y146A	116	2.3		76.8	6.2		1405		20
T150A	57.8	1.2		20.1	1.6		94.6		1.3
L153A	116	2.3		21.7	1.8		116		1.6
E169A	163	3.3		23.1	1.9		199		2.8
Q174A	129	2.6		11.6	0.94		63.7		0.9
Q175A	39.7	0.8		851	69		246		3.5
D217A	2137	43		32	2.6		838		12
Q221aA	63.4	1.3		40.5	3.3		65.7		0.93
R222A	42.8	0.87		10.5	0.85		61.1		0.87
K224A	111	2.2		46.3	3.8		59.1		0.84

* K_i s calculated from IC₅₀ values, all errors > 6%