

Development of a Periplasmic FRET Screening Method for Protease Inhibitory Antibodies

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ABSTRACT: Proteases play critical roles in numerous physiological processes and thus represent one of the largest families of potential pharmaceutical targets. Previous failure of broad-spectrum small molecule inhibitors toward tumorigenic metalloproteinases in clinical trials emphasizes that selectivity is the key for a successful protease-inhibition therapy. With exquisite specificity, antibody-based inhibitors are emerging as promising therapeutics. However, the majority of current antibody selection technologies are based on binding and not on inhibition. Here, we report the development of a function-based inhibitory antibody screening method, which combines a simple periplasmic preparation and an ultra sensitive FRET assay, both processes are amenable to high-throughput applications. Using this method, inhibitory antibodies can be rapidly distinguished from non-inhibitory clones with satisfactory Z-factors. Coupled with ELISA, this method also provides a fast semi-quantitative estimation of IC_{50} values without antibody purification. We expect this technology to greatly facilitate the generation of highly selective biologic inhibitors, targeting many proteases that are important to medical research and therapeutic development.

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KEYWORDS: inhibitory antibody; matrix metalloproteinase; protease; fluorescence resonance energy transfer; high-throughput screening

Introduction

As extremely important signaling molecules, proteases precisely control a wide variety of physiological processes. It is not surprising that many diseases are associated with misregulation of protease expression or altered substrate proteolysis (Cudic and Fields, 2009; Drag and Salvesen, 2010;

Overall and Blobel, 2007). For instance, mounting evidence has implicated that proteolytic activities, such as extracellular matrix (ECM) degradation and regulation of cell signaling are virtually involved in all aspects of cancer progression, including tissue remodeling, angiogenesis, cancer cell migration and metastasis (Gialeli et al., 2011; Kessenbrock et al., 2010; Mason and Joyce, 2011). Therefore, matrix metalloproteinases (MMPs) and other extracellular proteinases (e.g., ADAM, ADAMTS, MT-SP families) have been recognized as important subclasses of regulatory enzymes for cancer research and attractive therapeutic targets for cancer treatments (Christopher and Oded, 2006; Cudic and Fields, 2009; Duffy et al., 2011; Umland, 2006). In addition, for many infectious diseases, such as malaria and HIV, protease activities are absolutely required for their life-cycle and invasion to host (Greenbaum et al., 2002; Wensing et al., 2010). One apparent pharmaceutical mechanism is to specifically block the abnormal or pathogenic proteolysis processes. To date, most therapeutic protease inhibitors in clinic or under development are peptides or their chemical compound mimics, originally derived from the protease substrates. Considering that ~2% of human genome encodes proteases (Overall and Blobel, 2007), specificity is highly desired for therapeutic protease inhibitors. The crucial importance of selectivity is highlighted by the extensive studies on inhibition of matrix metalloproteinases (MMPs) using zinc-chelating compounds (e.g., hydroxamates) as a strategy for treating cancer (Lee et al., 2004). Although pre-clinical results were encouraging, these small molecule inhibitors for broad-spectrum MMPs failed in clinical trials due to severe side effects, such as musculoskeletal pain and inflammation caused by poor selectivity (Turk, 2006; Zucker and Cao, 2009). It is now known that MMP families exhibit more complicated and paradoxical roles at different stages of cancer progression (Kessenbrock et al., 2010; Overall and Kleinfeld, 2006). In fact, some MMPs possess cancer-promoting activities whereas others have tumor-inhibiting functions. For example, MMP-8 favors host defense instead of stimulating tumor proliferation, suggesting its protective role in cancer processes (Decock et al., 2011). In addition, metalloproteinases exert different roles at different steps of cancer progression. For example, pro- and anti-tumorigenic

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effects of MMP-9 at different microenvironments (Egeblad and Werb, 2002). However, the high homology among catalytic domains of MMPs presents a great challenge in distinguishing them using small compound inhibitors (Cuniasse et al., 2005).

In these respects, inhibitory antibodies are emerging as attractive therapeutic agents, which can selectively block cancer-promoting proteases rather than cancer-suppressing proteases (Sela-Passwell et al., 2011). The advantages of antibody-based inhibitors include: (i) high affinity and high specificity due to the large antigen-antibody interaction areas, provided by multiple complementarity-determining regions (CDRs); (ii) extended half-life and the well-known mechanisms of antibody action; (iii) low immunogenicity and low toxicity; and (iv) the fact that a large number of proteases are potentially targetable by antibodies, since ~50% of human proteases are extracellular or cell surface anchored (Drag and Salvesen, 2010). Toward the development of antibody-based inhibitors for therapeutic applications, several highly selective mAbs targeting a small fraction of human proteases have been identified and have shown promising pharmaceutical potentials (Devy et al., 2009; Farady et al., 2007; Schneider et al., 2012; Sela-Passwell et al., 2012; Tape et al., 2011). Considering there are 23, 35, 19, and 18 members in MMP, ADAM, ADAMTS, and MT-SP families, respectively, and more than 270 human extracellular proteases have been identified, more inhibitory mAbs targeting disease-related proteinases are remained to be developed. Pharmacological blocking of cancer by the development of the next generation of potent and selective metalloproteinase inhibitors has been proposed as one of the major tasks of future cancer research (Overall and Kleinfeld, 2006; Zucker and Cao, 2009). In

addition, discovery of highly selective protease inhibitors will greatly facilitate our understanding on the protease degradome and associated pathogenic mechanisms (Dean and Overall, 2007).

However, from a technical point of view, one obstacle for the discovery of protease inhibitory antibodies is that most current antibody selection assays select antibodies based on binding and not based on inhibition. Inhibitory clones with weak affinities, which can be improved through well-established affinity maturation approaches afterwards, might be lost during the initial cycles of repeated competitive binding steps. Consequently, the chance that none of the selected binders exhibits inhibitory function is very high. For example, in the discovery of inhibitory scFv antibodies targeting the fibroblast activation protein (FAP), 384 clones were tested after phage panning, with 40 affinity clones being identified, but only one clone (E3) exhibited inhibitory function toward FAP (Zhang et al., 2012). To overcome this problem, a genetic selection method has been developed to directly isolate scFv antibodies neutralizing the HCV serine protease NS3 (Gal-Tanamy et al., 2005). This genetic selection relies on co-expression of three proteins simultaneously in cytoplasm: a clone from the an antibody library, a protease target, and its substrate enzyme. However, such a requirement is usually difficult to be achieved for most human proteases. Here we report the development of a novel function-based high-throughput screening method to facilitate the identification of inhibitory antibodies by performing a FRET assay directly in the periplasmic fraction preparation (Fig. 1). Inhibitory FRET assays were focused because of the following advantages: (i) ultra high sensitivity, for example, as low as 10 pM MMP can be detected; (ii)

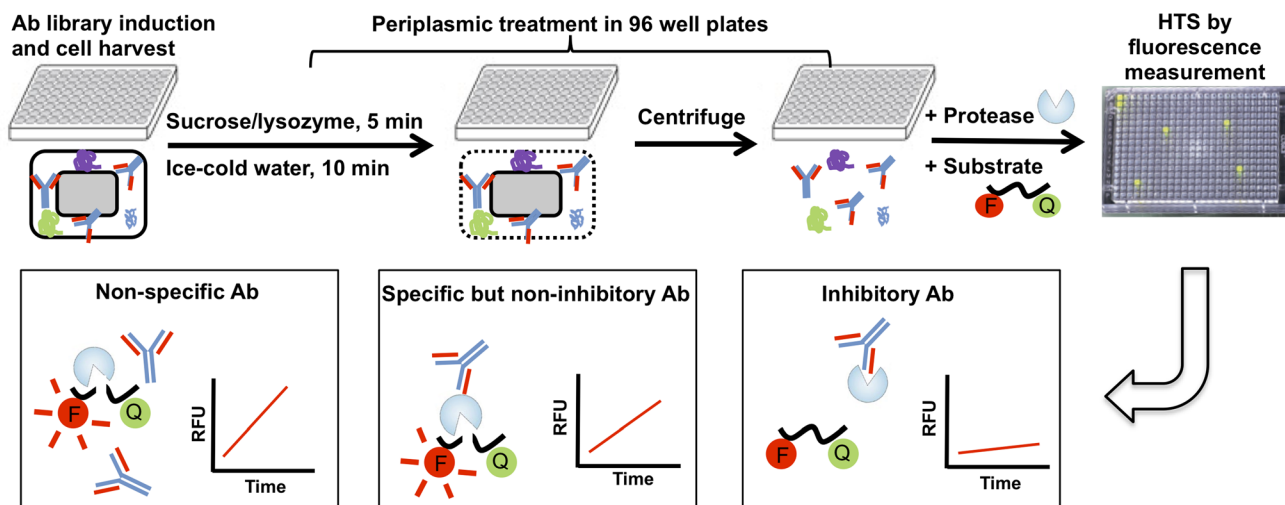


Figure 1. Procedure and expected results of periplasmic FRET assays. Antibody-expression cells were collected and treated with lysozyme and osmotic shock. Periplasmic fractions were clarified by centrifugation. Protease and fluorogenic substrate peptide were added to periplasmic preparations to perform FRET assays. Three scenarios are expected to be observed: non-specific antibodies, specific but non-inhibitory antibodies, and inhibitory antibodies. Only the antibody with inhibition function can suppress the FRET signal and thus distinguish from non-specific or non-inhibitory antibody clones.

availability of broad-range of protease specific peptide substrates; (iii) fast reaction rates; and (iv) amenability to high-throughput screening setups. Although widely applied for screening of small molecule inhibitors, FRET assays have not been adopted for inhibitory antibody selection. In this study, development of a periplasmic FRET assay is built on the facts that (i) antibodies or their fragments must be secreted to the periplasmic space for proper folding and disulfide bond formation and (ii) periplasmic space of *E. coli* contains only 4–8% of the total cell proteins with 7 out of the 25 known cellular proteases (French et al., 1996). Therefore, the possibility that the substrate peptide is cleaved by endopeptidase will be relatively low. We hypothesize that (i) the concentration of secreted antibody fragments in periplasmic space is sufficient for inhibitory FRET assays and (ii) native proteins present in periplasmic space do not interfere with FRET substrates. Combining a simple osmotic/enzymatic treatment step with an ultra-sensitive FRET assay that only requires picomolar ranges of protease, we expect that our method will greatly expedite the generation of highly selective antibodies inhibiting proteases that are important for cancer progression or other diseases.

Materials and Methods

Cloning, Expression, and Refolding of MMP-14 Catalytic Domain

DNA fragment encoding the catalytic domain of human MMP-14 with its hinge region (Tyr112-Ile318, chMMP-14) was assembled from synthetic oligonucleotides and amplified by PCR (Hoover and Lubkowski, 2002). The obtained chMMP-14 gene was cloned into *NdeI/XhoI* sites of pET32b (Novagen, Madison, WI). Transformed *E. coli* BL21 (DE3) cells were grown in LB media supplemented with 100 µg/mL ampicillin at 37°C to reach an OD₆₀₀ of 0.6–1.0, then 1 mM isopropyl-β-D-thiogactopyranoside (IPTG) was added to induce chMMP-14 expression and inclusion body formation. After induction at 37°C for 6 h, the cells were harvested by centrifugation and resuspended in 1/10 culture volume of 50 mM Tris-HCl (pH 8.0). Lysozyme and Triton X-100 were added to resuspended cells with final concentrations of 100 µg/mL and 0.1%, respectively. After incubation at room temperature for 15 min, the cell samples were lysed by sonication and centrifuged by 10,000g for 25 min at 4°C. The pellet was solubilized in 6 M urea supplemented with 50 mM Tris-HCl (pH 8.0) and 30 mM 2-mercaptoethanol, then loaded onto a Ni²⁺-NTA affinity column (Qiagen, Valencia, CA) that was equilibrated with the solubilization buffer. After sufficient washing steps, chMMP-14 was eluted by 200 mM imidazole. To refold chMMP-14, the eluent was diluted to 50–100 µg/mL in the starting buffer containing 50 mM Tris-HCl (pH 8.0), 6 M Urea and 150 mM 2-mercaptoethanol, and dialyzed twice against large volume of the refolding buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 0.5 mM ZnCl₂, and 0.05% Brij35. The purity and concentration of refolded chMMP-14 were quantified by

silver stain (Bio-Rad Laboratories, Hercules, CA). Typically, 250 mL culture yielded ~5 mg purified chMMP-14, and ~35% refolding efficiency was usually achieved. Activities of refolded chMMP-14 were measured using a fluorogenic substrate peptide carrying a pair of fluorophore-donor and quencher-acceptor at its termini with a protease recognition site in the middle. Peptide XV (QXLTM 520-γ-Abu-Pro-Gln-Gly-Leu-Dab(5-FAM)-Ala-Lys-H₂; AnaSpec, Inc., San Jose, CA) or a MMP-14 specific peptide SensoLyte 520 (Kang et al., 2011) was used in FRET assays. The specific activity of produced chMMP-14 was calculated and compared with that of MMP-14 purchased from AnaSpec, Inc.

Production of DX-2400 and a Panel of Negative Control scFvs

The VH and VL sequences of DX-2400, a highly selective mAb inhibiting MMP-14, were obtained from Muruganandam et al. (2009). The gene encoding scFv format of DX2400, with a linker (GGGGSGGGSGGGGS) between VL and VH, was assembled, amplified, and cloned into *SfiI* sites of pMoPac16 vector (Hayhurst et al., 2003), which carries a pLac promoter, a pelB signal peptide and a C-terminal FLAG tag. pMoPac-DX2400 was transformed into *E. coli* strain Jude-1 and expression of DX-2400 scFv was induced in 1 L TB medium supplemented with 35 µg/mL chloramphenicol and 0.1 mM of IPTG at 20°C for 20 h. The cells were harvested and treated with osmotic shocks to recover periplasmic fraction as described in Goldman et al. (2003). In brief, the pelleted cells were suspended in 60 mL of 0.75 M sucrose in 0.1 M Tris (pH 7.5) followed by adding 1.6 mL of 50 mg/mL lysozyme and 120 mL of 1 mM EDTA and kept on ice for 10 min. 8 mL of 0.5 M MgCl₂ was then added for efficient release of periplasmic proteins. After incubation for 10 min on ice, the periplasmic fraction was clarified by centrifugation at 10,000g for 30 min. The DX-2400 scFv present in periplasmic preparation was purified by affinity chromatography using anti-FLAG resin according to manufacture procedure (Sigma-Aldrich, St. Louis, MO). Similarly, genes encoding scFvs of antibodies b12 (anti-HIV gp120), 2G12 (anti-HIV gp120), and M18 (anti-PA) were sub-cloned into *SfiI* sites of pMoPac16 vector, and each scFv was purified with anti-FLAG resin after expression. The homogeneity of the purified antibody fragments was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and antibody concentrations were measured with NanoDrop 2000 (Thermo Scientific, Waltham, MA).

Periplasmic FRET Assays

Antibody-expressing *E. coli* clones were grown in 96 well round bottom plates containing 250 µL of TB medium supplemented with chloramphenicol at 37°C until OD₆₀₀ reached 0.6–1.0. scFv expression was induced by 0.1 mM IPTG at 30°C for 10 h. Cells were centrifuged and the pellets were resuspended in 25 µL periplasmic buffer (200 mM Tris-HCl, pH 7.5; 20% sucrose; 30 U/µL lysozyme) by shaking at

2,000 rpm for 5 min using a microplate mixer (USA Scientific, Inc., Ocala, FL). The samples were then treated by osmotic shock with 25 μ L ice-cold ddH₂O followed by incubation on ice for 10 min. Plates were then centrifuged at 4,000 rpm for 15 min and clarified supernatants were transferred into 96 black assay plates (Corning, Inc., Corning, NY). In FRET assays, 500 pM refolded chMMP-14 and periplasmic antibody fraction were added to 96-well plates and incubated at RT for 30 min. 0.5 μ M peptide substrate XV was then added to start the reaction. Fluorescent signals (RFU) with excitation at 490 nm and emission at 520 nm were monitored continuously with 1 min intervals using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, San Diego, CA). Similar periplasmic FRET assays were performed with MMP-12 and MMP-13 (AnaSpec, Inc.) to validate that this periplasmic FRET assay can identify highly selective inhibitors.

Z Factor Determination

DX-2400 scFv as the positive control and other scFvs clones (b12, 2G12, M18) as the negative controls were cultured in alternate rows of three microplates to observe well to well and plate to plate variations. Periplasmic fractions were prepared as described above, and FRET signals were measured after 2 h of incubation with 500 pM chMMP-14 and 0.5 μ M peptide substrate XV. The means and standard deviation of RFU changes for DX-2400 and negative controls were calculated, and the Z' factor was determined according to the equation below, where σ is the standard deviation, μ is the mean, and P and N stands for the positive and negative control, respectively (Zhang et al., 1999).

$$Z\text{factor} = 1 - \frac{3(\sigma_P + \sigma_N)}{|\mu_P - \mu_N|}$$

Validation Using a Spiked scFv Antibody Library

DX-2400 and the three negative controls (b12, 2G12, M18) were separately cultured at 37°C then equal counts of each clone were mixed after OD₆₀₀ normalization. The spiked mixture was serially diluted and plated on the LB/Chl⁺ agar plates. Two hundred eighty-eight colonies were randomly picked and inoculated into three 96 well plates. After overnight culture, 2 μ L of each clone were inoculated into new microplates containing TB/Chl⁺ media with 0.6% glucose. When most of wells reached OD₆₀₀ of 0.6–1.0, measured by a microplate reader (BioTek), the protein expression was induced with fresh TB/Chl⁺ media containing 0.1 mM IPTG at 30°C for 10 h. After centrifuging down the cells, periplasmic fractions were prepared and FRET assays were performed as described above. The inhibition percentage was calculated from the initial and final RFU values. Dozens of clones with high (>90%), medium (40–90%), and low (<40%) inhibition percentages were randomly picked and their plasmids were isolated for tests by restriction

digestion, using a unique *Bam*HI site located in the linker region of DX-2400 scFv, and by DNA sequencing.

Semi-Quantitative IC₅₀ Estimation Without Antibody Purification

Concentrations of DX-2400 scFv present in the periplasmic fraction were estimated by ELISA using purified DX-2400 scFv with known concentration as a standard. DX-2400 scFv periplasmic fraction was serially diluted and inhibition assays were performed. Initial velocities were measured and used to calculate inhibition percentages. The semi-quantitative IC₅₀ curves were generated by plotting DX-2400 scFv concentrations estimated by ELISA against inhibition percentages measured by FRET assays. Similar experiments and calculations were performed for purified DX-2400 scFv to validate the processes.

Results

Development of a Periplasmic Inhibition FRET Assay

The mAb DX-2400, a specific inhibitor of MMP-14 (Devv et al., 2009), was employed in this study to facilitate the development of the assay. DX-2400 was formatted to a single chain Fv (scFv) and cloned into a periplasmic expression vector. scFvs of M18 (anti-anthrax toxin PA), b12 (anti-HIV-1 gp120), and 2g12 (anti-HIV-1 gp120) were also produced as controls. The catalytic domain of MMP-14 with its hinge region (chMMP-14) was cloned, expressed, and successfully refolded from inclusion body. The produced MMP-14 exhibited expected enzymatic activities toward fluorogenic peptide substrates with a specific activity comparable (~90%) to that of commercially available MMP-14. At beginning, we attempted to perform the FRET inhibition assays directly using culture supernatants containing M13 bacteriophage particles released from *E. coli* cells infected with DX-2400 phagemids. However, no significant changes of fluorescent signal were observed, most likely because the concentrations of phage particles in the preparation were lower than the detection limit of FRET assays (~10 nM of DX-2400 scFv). We also tested the feasibility of using soluble preparations of antibody-expressing cells for inhibitory assays. Unfortunately, not only the positive control, DX-2400 scFv, but also all the negative controls including host cells Jude-1 exhibited the same fluorescent signal, presumably because the FRET peptide substrate was non-specifically cleaved by certain native endoproteases of *E. coli* in the cytoplasmic space.

Inspired by above results and the fact that for proper folding antibodies or their fragments need to be secreted to periplasmic space, which has much less homogenous proteins compared to cytoplasmic fraction, we focused on development of a periplasmic FRET assay. As shown in Figure 1, individual antibody library clones in microplates were undertaken enzymatic and osmotic treatments to release antibodies, then periplasmic fractions were clarified

by centrifugation. Previous studies suggested that EDTA was required to efficiently prepare periplasmic fractions (Birdsell and Cota-Robles, 1967). However, as a metal-chelating reagent, EDTA is not compatible with assays containing MMPs or many other metalloproteinases. We therefore tested the results of periplasmic treatments with or without the addition of EDTA for these four antibody clones. Western blot results (Fig. 2A) showed that when EDTA was omitted, scFv fragments were detected in periplasmic fraction preparations with amounts sometimes as much as or even higher than those of EDTA treatments, demonstrating that periplasmic treatments can be performed properly without EDTA. Using ELISA with purified DX-2400 scFv as a standard, the concentrations of scFvs in periplasmic preparations were estimated to be 300–400 nM, which was in a good agreement with the values reported by Kazemier et al. (1996), 100–800 nM. Knowing that the detection limit of FRET assays for DX-2400 scFv was ~ 10 nM, the concentrations of periplasmic scFvs were at least 30 times higher than the limit so sufficient for inhibition assays. To establish a procedure suitable for high-throughput screening, conditions of periplasmic treatment using microplates and an automatic microplate mixer were optimized, including dilution factors, shaking speed and time, etc. Using this automatic microplate protocol, scFv fragments were successfully released to periplasmic preparations (Fig. 2B). These results suggested that in ~ 40 min, periplasmic fractions of a large number of antibody library clones can be efficiently prepared in parallel, making it possible for high-throughput applications.

In the following inhibition assay steps, the protease of interest and associated fluorogenic peptide substrate were added to periplasmic preparations and fluorescent signals were monitored. Three scenarios are expected (Fig. 1): (i) observation of non-specific antibodies that do not bind to target protease, which freely cleaves the peptide substrate and generates fluorescent signals; (ii) observation of specific but non-inhibitory antibodies that bind to protease at distanced epitopes, hence do not interfere with catalytic function of protease or fluorescent signal generation; and (iii) observation of inhibitory antibodies that bind to desired epitopes and block the catalytic function, resulting in no peptide cleavage

and fluorescent signal suppression. As a proof of concept, periplasmic fractions of DX-2400 scFv and control scFvs were subjected to FRET assays using refolded MMP-14 and fluorogenic peptide substrate XV. Fluorescent signals were monitored every 30 min and the results are shown in Figure 3A. For the periplasmic fraction prepared from host strain Jude-1, fluorescent signals continually increased, suggesting that the homogenous contents in periplasmic space did not interfere with the FRET assay. Similar fluorescence profiles were observed for periplasmic preparations of b12, 2G12, and M18 scFvs, an expected phenomenon because these antibodies were non-relevant to MMPs. Most significantly, the fluorescent signal of DX-2400 was dramatically suppressed due to its specific inhibitory function toward MMP-14; that is, after 30 min of reaction, the signal difference between DX-2400 and other control samples was more than 50-fold. Taking into account the simple preparation, high sensitivity, and short processing time, this method showed a promising potential for selection of scFv inhibitors in a high-throughput manner.

Rapid Identification of Inhibitory Clones

We further analyzed periplasmic FRET assays by studying the reaction kinetics. According to the results presented in Figure 3B, the FRET assay with DX-2400 showed a very slow initial velocity of 3.2 RFU/min, presumably due to auto-cleavage of the fluorogenic peptide. In clear contrast, other assays with scFvs exhibited initial velocities of 70–110/RFU/min, which were 20- to 35-fold faster than that of DX-2400. This difference in velocity can be observed as early as 5 min of reactions, suggesting a very rapid detection. Periplasmic preparations of these scFv clones were also characterized by ELISA using refolded chMMP-14 as the coating antigen. As expected, DX-2400 scFv demonstrated a strong binding signal, whereas 2G12 scFv, M18 scFv, and host cell Jude-1 exhibited basal ELISA signals. Considering labor, process time, and amount of antigen used for ELISA (5–10 μ g per 96-well plate), the periplasmic FRET assay can clearly identify the inhibitory clone from non-inhibitory clones using much less antigen (~ 0.05 μ g per 96-well plate), in an easy and rapid manner.

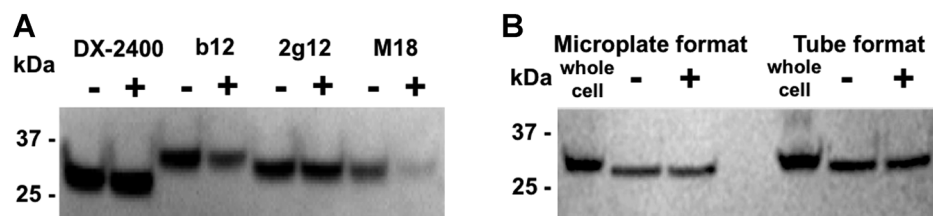


Figure 2. Release of periplasmic scFvs. **A:** Periplasmic fractions of four scFvs were prepared with (+) or without (–) adding of EDTA and released scFvs were detected by anti-FLAG-HRP; **(B)** Comparison of DX-2400 scFv periplasmic fraction preparations using microtube or microplate formats.

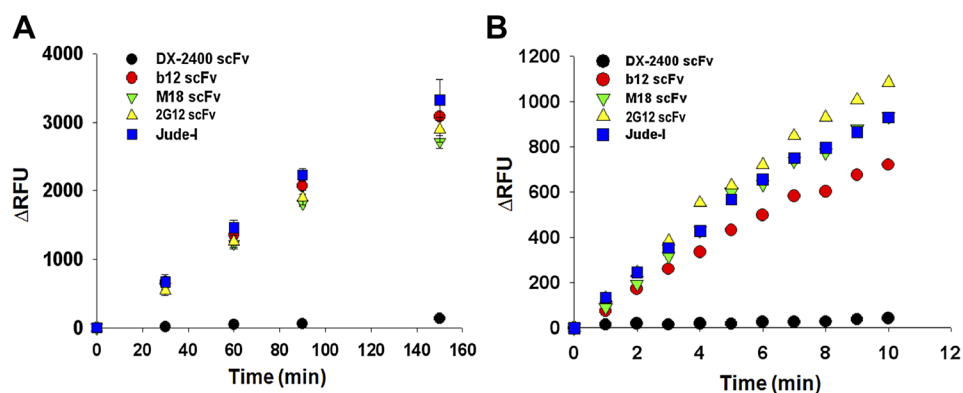


Figure 3. Identification of inhibitory antibodies. **A:** Result of periplasmic FRET inhibition assays. Fluorescent signals were detected using end-point mode every 30 min. **B:** Kinetic measurements for the first 10 min of reaction with continuous shaking. DX-2400 scFv (black circle, slope = 3.2 RFU/min, $r^2 = 0.85$); b12 scFv (red circle, slope = 73.6 RFU/min, $r^2 = 0.99$); M18 scFv (green triangle, slope = 96.2 RFU/min, $r^2 = 0.98$); 2G12 scFv (yellow triangle, slope = 109.7 RFU/min, $r^2 = 0.99$); Jude-1 (blue rectangle, slope = 93.1 RFU/min, $r^2 = 0.99$).

Inhibition Specificity Tests and Semi-Quantitative IC₅₀ Measurement

Because high selectivity is a critical criterion for protease inhibition therapies, we tested the feasibility of using periplasmic FRET assays to study inhibition specificity. In addition to MMP-14, MMP-12 (a macrophage metalloelastase), and MMP-13 (a collagenase) were subjected to FRET assays using periplasmic preparation containing DX-2400 scFv, a MMP-14 specific inhibitor (Devy et al., 2009). Peptide substrate XV was chosen because it can be cleaved by these three MMPs, though MMP-12 has a lower activity on XV than MMP-13 and MMP-14 (communications with AnaSpec, Inc.). As the results of Figure 4 show, MMP-14 activity was completely inhibited by DX-2400 scFv-containing periplasmic preparation, but MMP-12 and MMP-13 were not affected. These data demonstrate that specificity tests can be performed by periplasmic FRET assays, which are rapid, straightforward and do not require antibody purification.

To discover antibody-based inhibitors, it is important to estimate IC₅₀ of a large number of lead candidates at early development stages. However, protein purification often presents a bottleneck for high-throughput screening. Here, we extended periplasmic FRET assays for a rapid and semi-quantitative IC₅₀ estimation without antibody purification (Fig. 5). In Step 1, serial dilutions of periplasmic fraction were subjected to FRET assays, and the initial RFU velocities were measured and converted to inhibition percentages. In Step 2, the same periplasmic preparations were used to estimate antibody concentrations by ELISA with purified antibody fragments as a standard. Finally in Step 3, the correlation between antibody concentrations and inhibition percentages was plotted to generate an IC₅₀ curve. Using this method, periplasmic DX-2400 scFv exhibited an apparent IC₅₀ of 2 nM, which is ~5-fold lower than that of purified DX-2400 scFv (10 nM), indicating that this method is semi-quantitative. By removing the bioprocess bottleneck, that is, antibody purification, this rapid IC₅₀ estimation is

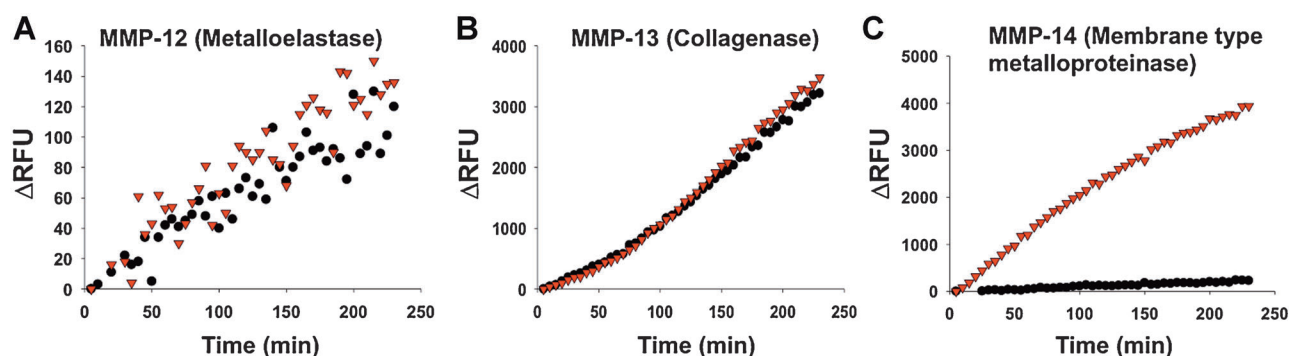


Figure 4. Inhibitor specificity measured by periplasmic FRET. MMP-12 (A), MMP-13 (B), and MMP-14 (C) were incubated with periplasmic fractions containing DX-2400 scFv (black circle) and host cell periplasmic fractions, that is, without DX-2400 scFv (red triangle). FRET assays were performed in present of their universal substrate peptide XV.

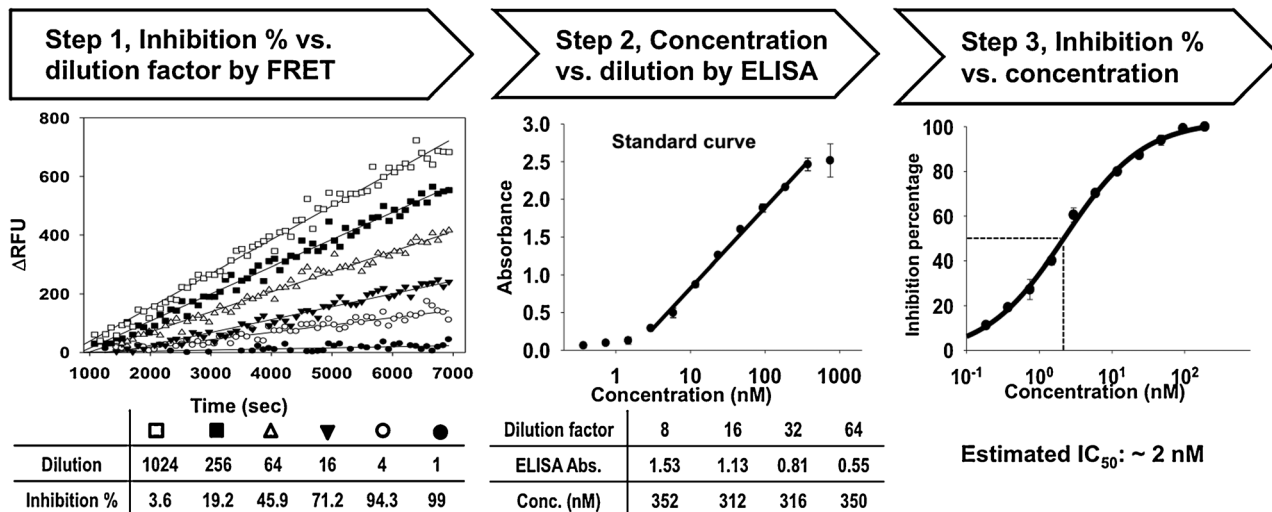


Figure 5. Rapid semi-quantitative IC_{50} measurement without antibody purification. Step 1, establishing the correlation between inhibition percentage and dilution factor by performing periplasmic FRET assays with serial dilutions of periplasmic preparation; Step 2, measuring antibody concentration in periplasmic preparation by ELISA using purified antibodies as a standard; Step 3, estimating IC_{50} by combining the results of Steps 1 and 2.

therefore amenable to access massive scFv candidates in parallel.

Satisfactory Z factors and Isolation of Inhibitory Ab From a Spiked Library

To evaluate the variation associated with individual measurements and the dynamic range of the assay system, repeats of periplasmic FRET assays were performed for DX-2400 scFv (as the positive control) and other scFvs and Jude-1 (as negative controls). As results shown in Figure 6, clear detection windows were observed for each pair of positive and negative controls. The Z' factors were determined to be in the range of 0.64–0.69, confirming that it is a stable and excellent assay system with a high statistical confidence suitable for HTS of inhibitory scFvs.

An antibody library, composed of equal counts of DX-2400, b12, 2G12, and M18 was constructed. Random clones from this spiked library were picked to culture in 96-well microplates (Fig. 7A). After antibody induction, periplasmic FRET assays were performed and initial and final RFU (after 2 h of reaction) were measured to calculate inhibition percentages. Out of 288 tested clones, ~30% exhibited strong inhibition (>90%), whereas the majority of the remaining clones demonstrated no or weak inhibition (<40%) with a clear detection gap between 40% and 90% of inhibition (Fig. 7B). Six inhibitory clones (>90%) and 10 non-inhibitory clones (<40%) were chosen for further analysis (squared in Fig. 7B). Restriction enzyme digestion and plasmid DNA sequencing confirmed that all the six clones exhibiting >90% inhibition were DX-2400, and all the 10 non-inhibitory clones (<40%) were 2G12 (five clones), b12 (one clone) or M18 (four clones). Notably, there were

three isolated cultures exhibiting 60–80% inhibition (circled in Fig. 7B). Plasmid DNA analysis proved that these three suspicious cultures contained multiple plasmids. One culture was the mixture of M18 and DX-2400 and two cultures were the mixtures of 2G12 and DX-2400.

Discussion

As a membrane associated protease, MMP-14 has been recognized as one of the most crucial MMPs in both development and invasion of tumors. High MMP-14 expression is associated with early death of breast cancer patients and is correlated with lymph node metastases,

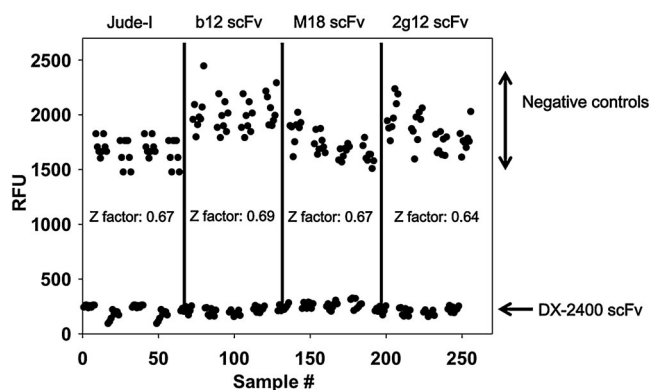


Figure 6. Z' factor plot of periplasmic FRET assays. Positive control was DX-2400 scFv and negative controls were host cell (Jude-1) and non-relevant scFvs (b12, 2G12, and M18).

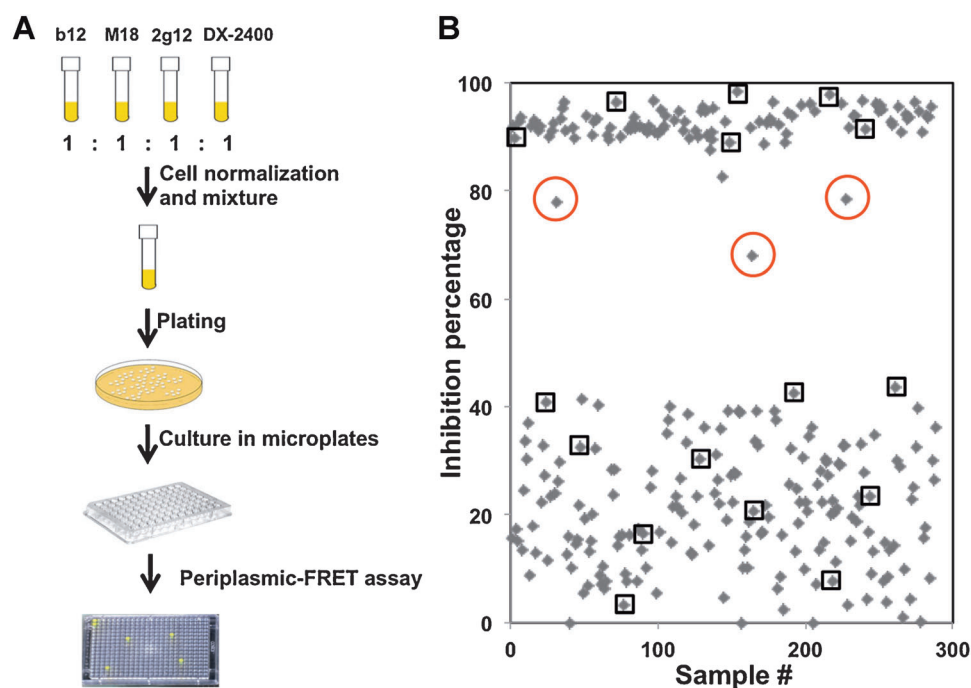


Figure 7. Identification of inhibitory antibody clones from a spiked library. **A:** Experiment procedure. **B:** Inhibition profiles of 288 clones. Six inhibitory clones (>90% of inhibition, squared), 10 non-inhibitory clones (<40% of inhibition, squared), and three suspicious clones (60–80%, of inhibition, red circled) were chosen for analysis by restriction enzyme digestion and DNA sequencing.

progression, invasion, large tumor size, and increasing tumor stage. All these evidences support that MMP-14 is a strong therapeutic target for cancer (Jiang et al., 2006). Towards the development of MMP-14 specific antibody-based inhibitors, there are two inhibition mechanisms to be considered: (i) competitive inhibition by binding to the catalytic cleft and (ii) allosteric inhibition by binding to exosites (Ganesan et al., 2010). DX-2400, a potent and specific inhibitory antibody toward MMP-14, was isolated using phage display through the subtraction panning on TIMP-2/MMP-14 complex (Devy and Dransfield, 2011). This epitope specific selection strategy, in principle, may exclude allosteric inhibitors targeting exosites of protease. In general, conventional antibody selection methods apply repeated rounds of binding against a certain antigen to enrich high affinity clones, and then the selected tight binders are characterized for inhibitory function. At least two issues associated with this conventional selection approach make screening of inhibitory antibodies difficult: (i) functional clones with weak affinities might be lost during repeated cycles of competitive binding selection and (ii) for functional tests, the selected high affinity binders need to be individually cloned, expressed and purified, which can be laborious and costly.

In distinct contrast to binding-based selection, the function-based screening method developed in this study makes selection of inhibitory antibodies facile and straightforward. After one round of phage panning to decrease the size the antibody libraries, periplasmic FRET assays will be

performed to directly select inhibitory clones. This selection method relies on protease activity and thus can identify both competitive and allosteric inhibitors. The sample preparation of this assay is simple and fast—the enzymatic and osmotic treatment followed by centrifugation can be finished in ~40 min using microplates. The following FRET reactions can be as short as 30 min to generation > 50-fold RFU difference between inhibitory and non-inhibitory clones. With ultra-high sensitivity, periplasmic FRET assays only need picomolar range of proteases, making this approach economically attractive. In addition to scFv, our results demonstrated that other antibody fragments formats, such as, Fab and Fab-geneIII fusion, were also amenable to periplasmic FRET assays. Z' factor evaluation and test with a spiked library suggested that this robust system could be applied in a high-throughput manner. Combined with ELISA results, semi-quantitative IC_{50} can also be estimated without antibody purification, providing a rapid assay to compare relative potency of lead candidates.

The present study represents a proof of concept for the establishment of a function-based inhibitory antibody screening method. Applying this method to discovery of antibody-based inhibitors from novel synthetic libraries is currently under investigation in our lab. As the pharmaceutical industry continues to expand its portfolio of protein therapeutic agents, new methods are continuously being sought to improve protein drug discovery and production, especially to develop time- and cost-effective HTS for

functional leads. In these regards, the periplasmic FRET assay demonstrated in this paper provides an excellent approach to directly select inhibitory clones as a promising alternative to conventional binding-based selection.

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