Activity-based fingerprinting and inhibitor discovery of cysteine proteases in a microarray†

Mahesh Uttamchandani,[a] Kai Liu,[a] Resmi C. Panicker[b] and Shao Q. Yao*[abc]

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A panel of 20 peptide vinyl sulfone probes has been synthesized and used to generate activity-based fingerprinting profiles of cysteine proteases in both gel- and microarray-based formats; the inhibitor fingerprints of representative small molecule inhibitors targeted against 4 cysteine proteases were also obtained, in high-throughput, using the same protein microarray platform.

Proteases are enzymes that catalyze the cleavage of amide bonds in peptides and proteins. They play an essential role in a variety of biological processes. Consequently, many proteases have been found to be critically involved in a wide range of human diseases, such as cancer, AIDS and Alzheimer's disease, making them the primary targets of potential therapeutics.[1] The major function of a protease lies in its ability to selectively discern and cleave only its target substrate sequence(s) in the presence of many unrelated proteins or/and peptides. Therefore, deciphering the substrate specificity, or the so-called "fingerprint", of a protease not only offers invaluable information for unravelling its physiological roles, but also facilitates the discovery of potent and selective inhibitors as potential drugs.[2]

Traditionally, enzyme fingerprinting experiments have been conducted using standard analytical techniques (*e.g.* microplate-based enzyme assays) with a whole spectrum of substrates and/or their analogs on a target enzyme, thus creating quantitative and reproducible profiles directly related to the enzyme's activity.[2] A variety of enzymes, including cytochrome P450, protein kinases and hydrolytic enzymes, have been successfully studied in this way.[3] More recently, the focus has been shifted to the development of novel methods allowing rapid fingerprinting of enzymes, on the basis of their activity, directly from a complex proteome or/and in miniaturized formats.[4,5] Potential high-throughput screening of enzyme inhibitors may also be realized from these efforts.[4,5a–c,g] A protein microarray provides a miniaturized platform on which thousands of proteins, including enzymes, can be assayed simultaneously on standard microscope glass slides.[6] We previously showed in a preliminary finding that activity-based probes (ABPs), originally developed by Cravatt *et al.* to profile enzymes from a crude proteome in a gel-based experiment,[7] could be used to detect enzymatic activity, as well as inhibition, in a protein microarray.[5a] This concept has recently been verified by others.[5c] Herein we report, for the first time, the extension of this strategy into the domain of high-throughput fingerprinting and inhibitor discovery of cysteine proteases immobilized on a protein microarray (Fig. 1).

Cysteine proteases were chosen as our target enzymes due to our ongoing interest in developing effective agents to combat numerous human diseases, *e.g.* SARS, in which key enzymes have been identified as cysteine proteases. Vinyl sulfone-containing small peptides were used as activity-based probes in our fingerprinting experiments (Fig. 1b), as they were previously shown to be highly specific towards cysteine proteases, caused by the covalent reaction between the active-site cysteine residues (present in all cysteine proteases) in the enzyme and the vinyl sulfone (a Michael acceptor) in the probes.[8a] In addition, a solid-phase strategy which allows efficient synthesis of peptide vinyl sulfones bearing different P and P′ substitutions is available.[8b]

Each peptide vinyl sulfone used in this study contains three units – a recognition unit consisting of any of 20 different amino acids (18 natural, 2 unnatural) mimicking the P_1 position of a protease substrate followed by a common Leu-Leu dipeptide linker, a vinyl

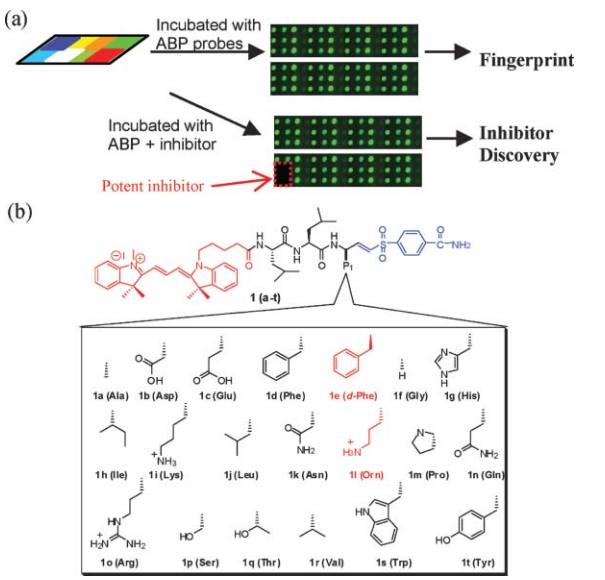

Fig. 1 (a) Schematic showing activity-based enzyme fingerprinting (top) and inhibitor discovery (bottom) in a protein microarray. (b) Structures of 20 probes used in the study, with variations at the P_1 position (box). Highlighted in red are unnatural amino acids.

[a]*Department of Biological Sciences, National University of Singapore, 4 Science Drive 4, 117543, Singapore. E-mail: chmyaosq@nus.edu.sg; Fax: +65 6779 1691; Tel: +65 6516 2925*
[b]*Department of Chemistry, National University of Singapore, 117543, Singapore*
[c]*NUS MedChem Program of the Office of the Life Sciences, National University of Singapore, 117543, Singapore*
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sulfone moiety serving as the warhead for covalent reaction with the enzyme (*via* its active-site Cys residue), and a fluorescent reporter unit, Cy_3 (Fig. 1b).

It is well known that for many cysteine proteases, *e.g.* caspases, the most critical component in a protease substrate is the P_1 residue.[9] We envisaged that, by varying the P_1 residue in our probes with a variety of diverse functional groups and generating a panel of cysteine protease-specific, activity-based probes, one might use them to obtain quick (but not necessarily detailed) substrate fingerprint profiles of these enzymes not only in a gel-based proteomics experiment, but more importantly, in high throughput, in a protein microarray format (Fig. 1a, top scheme). Of equal importance, when a putative enzyme inhibitor is introduced during the microarray screening, one may generate an "inhibitor fingerprint" against all immobilized proteins, thus allowing potential high-throughput discovery of inhibitors through simultaneous assessment of both their potency and specificity (Fig. 1a, bottom scheme).

Chemical synthesis of the 20 probes was performed as previously described (Scheme S1 in ESI).[8] The final products, **1 (a–t)**, were purified by preparative RP-HPLC and unambiguously characterized (Table S1 in ESI). It should be noted that with this versatile solid-phase method, a variety of vinyl sulfone probes containing variable P and/or P′ positions may be readily synthesized in future.[8b]

Both activity-based fingerprinting and inhibitor fingerprinting require quantitative assessments of the enzymatic activity of multiple enzymes (and their inhibition) in a protein microarray, and unfortunately slide-to-slide variation is one of the major problems facing all microarray-based work.[6] In order to address this issue, as well as to increase throughput, we adopted the subarray methods developed by Miyake *et al.* (with some necessary adjustments)[5c] and performed all enzymatic experiments in a single protein microarray (Fig. 1a). Briefly, 48 subarrays of proteins were spotted on the same glass slide, with each subarray containing all tested proteins (with or without control proteins and dye) individually spotted, in duplicate (or triplicate), in a preprogrammed manner. In this way, each subarray could comprise ~100 different spots of up to 44 different proteins (see Fig. 2a for a representative subarray image), allowing potentially up to 5000 enzymatic assays (*e.g.* 48 × 100 = 4800) to be simultaneously carried out in a glass slide with different probe–inhibitor combinations. To ameliorate the aforementioned method which required a Teflon-coated surface to generate subarrays and is thus prohibitively expensive,[5c] we attempted to optimize grid spacing and sample application protocols. We found that different samples could be readily applied to each of the 48 subarrays on the same chip, without cross contamination, by careful design of subarray placement and controlled application of the reaction mix during screening.

We went on to optimize the array-based enzyme-labeling reactions under different pH conditions and varied labeling time. Our results indicated that a 15-minute labeling reaction under slightly acidic conditions (pH = 5) gave the highest detection signal with minimal background (Fig. S1 and S2 in ESI). These conditions were therefore adopted in all our subsequent experiments, unless otherwise indicated. We next evaluated the uniformity of labeling reactions across the 48 subarrays on the same slide (Fig. S3 in ESI); upon treatment with the same labeling

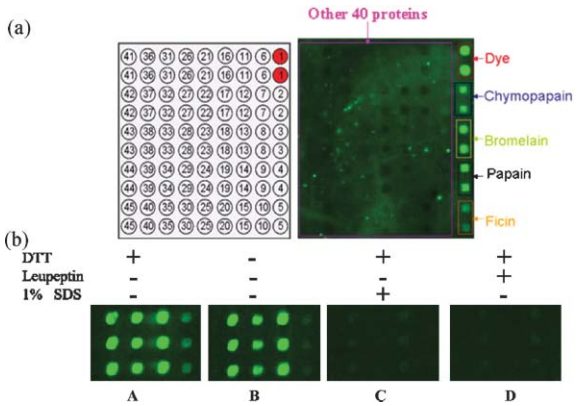

Fig. 2 (a) Activity-based screening of cysteine proteases in a protein submicroarray. A total of 90 spots were in this subarray, with duplicated spots of Cy3 dye (**1**), 4 different cysteine proteases (**2**: chymopapain; **3**: papain; **4**: bromelain; **5**: ficin), and 40 other non-cysteine protease enzymes (**6–45**; see ESI for full list). The array was screened with a master mix of 20 probes **1 (a–t)**. (b) The four cysteine proteases labeled by probe **1a** with different additives (*i.e.* DTT, leupeptin and 1% SDS). Each cysteine protease in a subarray (left to right: chymopapain, bromelain, papain and ficin respectively) was spotted in triplicate (top to bottom).

mixture, followed by standard post-reaction processing, the fluorescence-scanned images obtained for all 48 subarrays showed a high degree of similarity, as exemplified by spots representing the same enzymes having < 10% standard deviation across all subarrays. This thus validated our subarray approach in generating highly accurate and quantitative data for subsequent fingerprinting experiments.

To demonstrate that our probes and strategy could be used as a general miniaturized tool for high-throughput detection/screening of cysteine proteases immobilized on a protein microarray, a subarray containing four known cysteine proteases and 40 other non-cysteine protease enzymes (*e.g.* other classes of proteases, phosphatases, lipases and others; see Fig. 2a and legend) was prepared and screened against a "master mix", which contains an equal amount of each of the 20 vinyl sulfone probes, **1 (a–t)**. The "master mix" serves as a "universal" chemical which allows quick detection of most cysteine protease activity in a single screening. Indeed, as shown in Fig. 2a, only the 4 cysteine proteases, but not any other enzymes, were detected on the microarray. As shown in Fig. 2b, the labeling signals increased as a result of the addition of reducing agent DTT (*e.g.* image **A** *vs.* **B**), an activator of cysteine protease activity, and no labeling signal was detected when the array was treated with either 1% SDS (which denatures proteins) or 0.1 mM leupeptin (a general cysteine protease inhibitor) (*e.g.* images **C** and **D** *vs.* **B**). All these lines of evidence indicate that profiles obtained using a microarray-based screening strategy are indeed activity-dependent.

Next the 4 cysteine proteases (*i.e.* chymopapain, bromelain, papain and ficin) were labeled with each of the 20 vinyl sulfone probes, **1 (a–t)**, in both gel-based experiments (top panel in Fig. 3a) and on protein microarrays (bottom panel in Fig. 3a). The throughput and scalability of microarray- over gel-based fingerprinting immediately became evident. A single glass slide with 48 subarrays was sufficient to accommodate all 4 proteases (or more if desired) with all 20 probes simultaneously. In contrast,

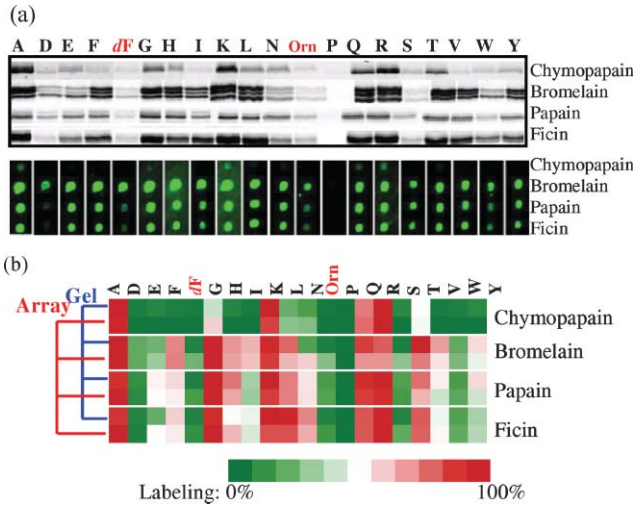

Fig. 3 (a) Activity-based fingerprinting of 4 cysteine proteins with each of 20 vinyl sulfone probes, **1 (a–t)**, in gel (top) and microarray (bottom) formats. The brightest features in the gel and microarray denote the positively labeled cysteine proteases. The probe used in each labeling reaction was identified by its P_1 residue as shown in Fig. 1 (on top of gels): A (Ala), D (Asp), E (Glu), F (Phe), *d*F (*d*-Phe), G (Gly), H (His), I (Ile), K (Lys), L (Leu), N (Asn), Orn, P (Pro), Q (Gln), R (Arg), S (Ser), T (Thr), V (Val), W (Trp), Y (Tyr). (b) A colored heat map of the results, generated using the Tree View software (http://rana.lbl.gov/EisenSoftware.htm).

8 separate SDS protein gels (10 lanes/gel, for $20 \times 4 = 80$ labeling reactions) were needed to replicate the results on the traditional gel-based format. The resulting fingerprints of each protein revealed characteristic and quantifiable signatures with each of the 20 probes (Fig. 3b). Bromelain has a broad-based substrate specificity and was therefore labeled strongly by many of the probes. Chymopapain and papain, two homologous cysteine proteases known to prefer basic residues, *e.g.* lysine and arginine, at the P_1 position, accordingly showed the strongest labeling by the two corresponding probes. The highly homologous fingerprints obtained on both gel and protein microarray platforms further underscore the merit of our microarray-based activity fingerprinting platform to accommodate many (different or related) proteins in future high-throughput characterization of cysteine proteases.

In our previous work,[5a] we showed that activity-based probes could be used to study enzyme inhibition in a protein microarray. This has been validated by Miyake *et al.*[5c] Herein, we show that, with the subarray approach described earlier, microarray screening can be readily adapted for the determination of accurate and quantitative inhibition data (*e.g.* IC_{50}), in a single experiment and on the same slide, against a whole panel of arrayed proteins. In this way, a quantitative "inhibitor fingerprint" of various inhibitor(s) may be obtained in high throughput, allowing each inhibitor to be assessed not only for its relative potency against a given enzyme, but also its selectivity against many other enzymes simultaneously. Three small molecules, leupeptin, as well as two synthesized vinyl sulfones (for structures see Table S2, ESI),[8b] were screened on the 48-subarray protein microarray. The fluorescence signal of each concentration point was quantified and the resulting data fitted to reveal the IC_{50} (see Fig. S4 and Table S3, ESI). Of particular interest is **VS16**, which in our microarray assay inhibited chymopapain at 1.3 μM, at a 30- to 70-fold greater selectivity than the other three cysteine proteases. We are currently investigating the basis of this high selectivity. Inhibition potencies were further reconfirmed using plate based assays (ESI). This thus highlights the potential of our microarray-based strategy for efficient and convenient high-throughput inhibitor screening.

In conclusion, we have successfully developed a microarray-based, high-throughput strategy for activity-based fingerprinting (and inhibitor fingerprinting) of cysteine proteases (and their potential inhibitors). The strategy relies on the miniaturization capability of protein microarray technology, allowing subarrays of many proteins to be assayed simultaneously, in a single slide. By applying different probe–inhibitor combinations under various reaction conditions highly accurate and quantitative data may be generated. The present panel of P_1 diversified vinyl sulfone probes should serve as a useful toolbox for fingerprinting most cysteine proteases. By adopting a similar solid-phase approach,[8b] other vinyl sulfone probes, with variations across both P and P′ positions, may be conveniently synthesized in future and used for detailed fingerprinting of cysteine proteases and their inhibitors. Our present approach thus provides a useful tool for future research in the emerging field of "Catalomics".[10]

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