Activity-based high-throughput profiling of metalloprotease inhibitors using small molecule microarrays†

Jun Wang,[a] Mahesh Uttamchandani,[b] Li Ping Sun[a] and Shao Q. Yao*[abc]

Received (in Cambridge, MA, USA) 27th October 2005, Accepted 1st December 2005
First published as an Advance Article on the web 4th January 2006
DOI: 10.1039/b515278j

We herein describe a high-throughput small molecule microarray (SMM) method that enables quick and cost-effective identification of potent inhibitors of metalloproteases in an activity-dependent manner, thereby offering a rapid means for inhibitor discovery and profiling.

Microarray-based technologies have received much attention due to their enormous potential in high-throughput screening.[1] Of the various platforms available, small molecule microarray (SMM) emerges as an important tool for rapid screening of large chemical libraries.[2] This method generally involves immobilization of chemicals in addressable grids on a glass slide, followed by screening with a fluorescently tagged protein. The critical limitation of SMM lies not so much in hit identification as in hit validation. This is because most SMM screening methods rely on non-covalent ligand–protein interactions, which invariably introduce false positives as a result of inconsequential affinity between the ligand and non-targeted regions of the protein. Without time-consuming validation, it remains unconfirmed whether any of the initial "hits" detected are relevant to the desired biological context.

Enzymes play a key role in virtually every biological process. They have long been considered valuable drug targets for potential treatments of major human diseases. Matrix metalloproteases (MMPs) for example, are a family of zinc-containing proteases which have been implicated in diseases such as arthritis, Alzheimer's disease and cancer. There is thus considerable interest in developing highly potent and selective inhibitors that target different human MMPs.

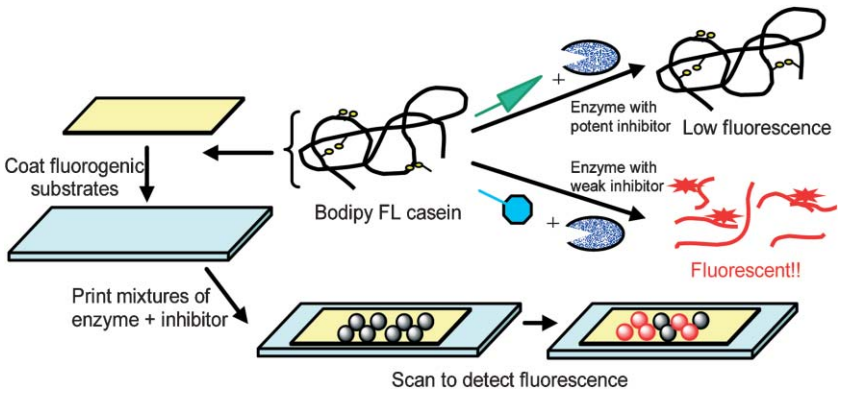
We aim to develop an SMM technology suitable for high-throughput identification of potential inhibitors on the basis of their ability to directly inhibit the catalytic activity of an enzyme, thus doing away with tedious hit validation processes. Herein, we explore one such platform which enables simultaneous evaluation/comparison of hundreds (or thousands) of small molecule inhibitors against an enzyme in an activity-based manner (Scheme 1). By demonstrating its utility in profiling potent hydroxamate-containing inhibitors (for both their potency and specificity) against metalloproteases, we find the approach to be well-suited for high-throughput discovery of potential MMP inhibitors. Previously, SMM had only been used successfully for activity-based profiling of substrate specificity, rather than inhibition, of proteases.[3] Our present work thus adds "inhibitor fingerprinting" to the expanding repertoire of SMM applications.

Our SMM strategy works by precoating a glass slide with a fluorogenic enzyme substrate (bodipy FL casein in our case[4]), followed by programmed spotting of mixtures of the target enzyme and an inhibitor, in individual nanodroplets, to predefined locations on the surface. Upon incubation and detection by fluorescence, relative potency of all spotted inhibitors is immediately revealed and simultaneously compared (Scheme 1). This strategy thus takes advantage of the parallel and miniaturized aspect of microarray, together with quantitative fluorescence readouts attainable from an enzyme-sensitive surface in response to a protease/inhibitor mixture (in a dose-dependent manner). Tagging of the enzyme with a fluorophore is not necessary, thus allowing it to be evaluated in its native form and in real time. Diamond *et al.* recently developed an SMM system by printing chemical libraries in glycerol droplets followed by introduction of an enzyme onto the slide with aerosol spray.[5] The strategy, however, has limited applications due to the need of glycerol droplets (which inhibit enzymatic reactions and prevent long-term slide storage) and complicated aerosol set-ups. Our approach needs only a conventional arrayer for spotting and standard bioconjugation chemistry for surface derivatization, making it adaptable by most researchers for high-throughput inhibitor screening.

Thermolysin and collagenase, two well-characterized metalloproteases, were chosen in our study, as they exhibit similarity to many vertebrate metallopeptidases, in particular to those of the MMP family.[6] Earlier reports showed that both enzymes, like most MMPs, show substrate specificity at P′ sites, with a strong preference of hydrophobic residues at their P_1' position.[6b] Little is known, however, about their specificities at P_2' and P_3' positions.[6c] We therefore synthesized a 400-member small molecule library with the scaffold HONH-Suc(2-*i*Bu)–P_2'–P_3'–Gly–Gly–Lys(biotin)–$CONH_2$, as shown in Fig. 1. Each inhibitor in the library comprises a succinic hydroxamate "warhead" (a highly potent zinc-binding group against metalloproteases), in which the P_1' residue was maintained as an isobutyl group throughout. The design was based on the structures of Marimastat, Batimastat, and GM6001, three broad-spectrum potent hydroxamate inhibitors of MMPs (See Supporting Information†). With variations across P_2' and P_3' positions in the library, we aimed to profile both the potency and selectivity of individual members against different metalloproteases, in particular MMPs. A flexible linker and biotin

[a]*Department of Chemistry, National University of Singapore, 3 Science Drive 3, 117543, Singapore. E-mail: chmyaosq@nus.edu.sg; Fax: (+65) 67791691; Tel: (+65) 68741683*
[b]*Department of Biological Sciences, National University of Singapore, 117543, Singapore*
[c]*NUS MedChem Program of the Office of Life Sciences, National University of Singapore, 117543, Singapore*

† Electronic supplementary information (ESI) available: Procedures for the synthesis and characterization of inhibitors, microarray and microplate experiments, as well as kinetic experiments and computer modeling. See DOI: 10.1039/b515278j



Scheme 1 Nanodroplet SMM strategy for high-throughput profiling of potential MMP inhibitors.

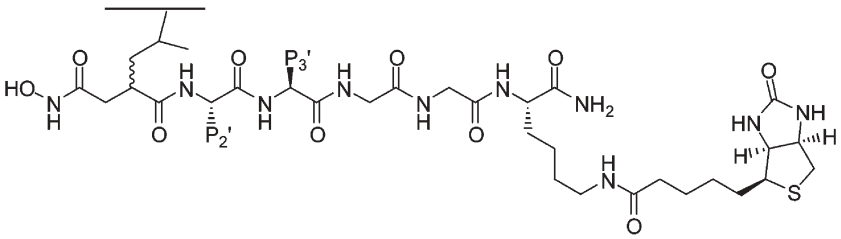


Fig. 1 Structure of 400-member hydroxamate inhibitors. Diversity was generated at P2′ and P3′ positions with 20 natural amino acids.

were incorporated into each inhibitor for future proteomic applications. Synthesis of the library is described in the Supporting Information.† Briefly, the monosubstituted succinyl hydroxymate was incorporated into the 400-member library synthesis using solid-phase peptide chemistry and IRORI™ split-and-pool sorting technology. The resulting 400 hydroxamates were cleaved individually from the solid support and purified by precipitation. The final products were prepared in DMSO stocks (to a uniform concentration†), and used for subsequent SMM screening. The quality of the final products was confirmed by LC-MS, indicating most inhibitors (>90%) were of correct molecular mass and sufficient purity (>80% purity).

We next used the nanodroplet strategy to screen the 400 hydroxamates against thermolysin (Fig. 2) and collagenase.† To validate our results, separate experiments were performed in standard microplate format.† Advantages of the approach were immediately evident. First, the entire 400-member library (in duplicate) was readily accommodated on a single slide, effectively allowing >800 assays to be performed with merely 6 μl of bodipy FL casein (Fig. 2a). With few exceptions, results obtained from SMM and microplate formats were in good agreement, giving a relatively high Pearson correlation coefficient (r = 0.852†). Second, the relative potency of each inhibitor was immediately revealed by the fluorescence intensity generated from its corresponding nanodroplet (small boxes in Fig. 2a) with more potent inhibitors giving weaker fluorescence signals, thus avoiding tedious hit validation. This was unambiguously confirmed by enzyme kinetic experiments carried out in microplates on selected inhibitors.† Notably, the nanodroplet SMM strategy was able to discern slight differences in inhibitor potency. Finally, because the enzyme "inhibitor fingerprint" was generated in a single experiment under uniform conditions, the results could be used directly for further SAR analysis to address not only potency, but more importantly selectivity, of any given inhibitor (Fig. 2b): the dendrograms, before (left) and after (right) cluster analysis, show the relative potency of each inhibitor against thermolysin with regards to its P_2' and P_3' substitutions. Our results indicate that Cys, Glu and Asp were disfavored at both P_2' and P_3' positions. Potent thermolysin inhibitors appear to be those containing aromatic (*i.e.* Trp/Tyr), small (*i.e.* Ala), hydrophobic (*i.e.* Leu/Ile), basic (*i.e.* Lys/Arg) and polar (*i.e.* Gln/Asn) residues in a variety of P_2'/P_3' combinations, with considerable variations across rows and columns, indicating cooperativity from both P_2' and P_3' residues is critical to achieve maximum inhibition. Interestingly, screening results obtained with collagenase were distinctly different from those with thermolysin, with potent inhibitors comprising predominantly aromatic (*i.e.* Tyr/Trp/Phe) and hydrophobic residues (*i.e.* Leu/Ile) at the P_2' position, and Trp at the P_3' position. This underlines the potential of our platform in detecting subtle substrate preferences amongst different MMPs. One of the most potent inhibitors identified from our screen was HONH–Suc(2-*i*Bu)–Tyr–Lys–Gly–Gly–Lys(Biotin)–$CONH_2$ (IC_{50} = 9.9 nM; K_i = 2.4 nM), consisting of Tyr and Lys at its P_2' and P_3' sites, respectively, and was 10-fold more potent than GM6001. This finding, to our knowledge, provides the first direct evidence of P_2'/P_3' selectivity in thermolysin inhibitors. It is further supported by inspection of the active site structure of thermolysin, showing predominantly hydrophobic S_2' and solvent-accessible S_3' pockets.[7]

In conclusion, we have developed a nanodroplet SMM strategy for high-throughput profiling of inhibitors against metalloproteases, potentially extendable to other enzymes. It enables potent and highly selective inhibitors to be directly identified without the need of time-consuming hit validation. Our strategy thus provides a new tool in the ever expanding SMM technologies for the inhibitor fingerprinting of enzymes. Notwithstanding, a key issue

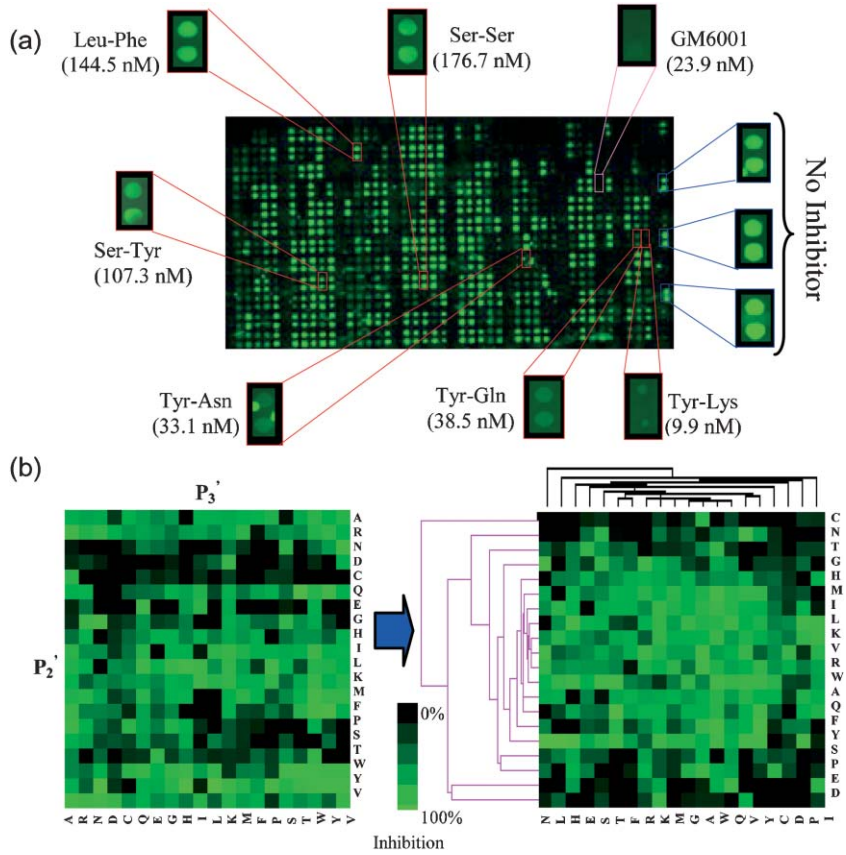


Fig. 2 (a) Microarray image of the 400-member library screened against thermolysin. Samples were spotted in duplicate. Spots of selected inhibitors (labeled by their P_2'–P_3' sequence) with IC_{50} (in brackets) were boxed. (b) Image in (a) shown as dendrogram before (left panel) or after Cluster Analysis (right panel) based on inhibition potency. See Supporting Information for details.†

remains to be addressed before the technique can be applied for routine high-throughput screening of enzyme inhibitors; with the current method, inhibitor/enzyme mixtures are individually prepared before spotting, and the microarray is processed immediately post-spotting. This inevitably limits the throughput of the screening, especially with multiple enzymes. We are currently investigating possible solutions to this and will report our findings in due course.

Funding support was provided by the National University of Singapore (NUS) and the Agency for Science, Technology and Research (A*STAR) of Singapore.

Notes and references

1 For recent reviews, see: (*a*) J. S. Merkel, G. A. Michaud, M. Salcius, B. Schweitzer and P. F. Predki, *Curr. Opin. Biotechnol.*, 2005, **16**, 447–452; (*b*) K. T. Tomizaki and H. Mihara, *ChemBioChem*, 2005, **6**, 782–799; (*c*) D. P. Walsh and Y. T. Chang, *Comb. Chem. High Throughput Screening*, 2004, **7**, 557–564; (*d*) I. Shin, S. Park and M. R. Lee, *Chem. Eur. J.*, 2005, **11**, 2984–2901; (*e*) M. Uttamchandani, D. P. Walsh, S. Q. Yao and Y. T. Chang, *Curr. Opin. Chem. Biol.*, 2005, **9**, 4–13.

2 F. G. Kuruvilla, A. F. Shamji, S. M. Sternson, P. J. Hergenrother and S. L. Schreiber, *Nature*, 2002, **416**, 653–657.

3 (*a*) C. M. Salisbury, D. J. Maly and J. A. Ellman, *J. Am. Chem. Soc.*, 2002, **124**, 14868–14870; (*b*) Q. Zhu, M. Uttamchandani, D. B. Li, M. L. Lesaicherre and S. Q. Yao, *Org. Lett.*, 2003, **5**, 1257–1260; (*c*) R. Srinivasan, X. Huang, S. L. Ng and S. Q. Yao, *ChemBioChem*, 2006, DOI: 10.1002/cbic.200500340.

4 We previously showed that, depending on the types of fluorogenic substrates precoated, the glass slide may be used to detect different enzymes including proteases and phosphatases: M. Uttamchandani, X. Huang, G. Y. J. Chen and S. Q. Yao, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 2135–2139.

5 D. N. Gosalia and S. L. Diamond, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 8721–8726.

6 (*a*) M. Whittaker, C. D. Floyd, P. Brown and A. J. H. Gearing, *Chem. Rev.*, 1999, **99**, 2735–2776; (*b*) M. Ilies, M. D. Baciu, A. Scozzafava, M. A. Ilies, M. T. Capriou and C. T. Supuran, *Bioorg. Med. Chem.*, 2003, **11**, 2227–2239; (*c*) E. W. S. Chan, S. Chattopadhaya, R. C. Panicker, X. Huang and S. Q. Yao, *J. Am. Chem. Soc.*, 2004, **126**, 14435–14446.

7 D. E. Tronrud, A. F. Moneingo and B. W. Matthews, *Eur. J. Biochem.*, 1986, **157**, 261–268.