Small molecule microarray-facilitated screening of affinity-based probes (A*f*BPs) for γ-secretase†

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A small molecule microarray (SMM) platform is developed herein, which enables high-throughput discovery of affinity-based probes (A*f*BPs) against γ-secretase.

Amyloid β-protein (Aβ), a central pathogenic feature of Alzheimer's disease (AD), is generated through proteolysis of amyloid precursor protein (APP) by membrane-associated aspartic proteases including β- and γ-secretases.[1] β-secretase has been well characterized; the study on γ-secretase, however, has been much more challenging, because the enzyme is a multimeric membrane protein complex, comprising presenilin (PS), nicastrin, anterior pharynx defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2). The proteolytically active form of PS is made up of two subunits, termed the N-terminal fragment (NTF) and C-terminal fragment (CTF). Despite recent advances, much remains to be understood about what the γ-secretase complex interacts with and how it carries out the proteolytic process. Due to well-known difficulties associated with biochemical studies of membrane proteins, much work on γ-secretase has so far relied on the development of the so-called affinity-based probes (A*f*BPs), which are typically generated from known inhibitors of γ-secretase by attaching a photo-crosslinking group (*i.e.* benzophenone (BP) or diazirine) and a fluorescent dye (*i.e.* tetraethylrhodamine (TER)) to the inhibitors.[2] For example, A*f*BPs based on L685 458, a potent hydroxylethylene-based γ-secretase inhibitor, were first developed by Li *et al.*[2a] Recently, probes based on DAPT and arylsulfonamide derivatives have also been reported.[2b–d] The key advantage of all these probes is their ability to selectively bind the active subunits of γ-secretase complex (*i.e.* PS subunits) from the whole cell extract and, upon UV-photolysis and in gel-analysis, accurately report the active state of γ-secretase without the need of isolating the enzyme complex. As a result, these probes have provided invaluable tools for the studies of Alzheimer's disease, both in its pathology and potential therapeutics. Similar strategies are widely used for the development of A*f*BPs against other classes of enzymes in the field of activity-based protein profiling (ABPP) pioneered by Evans and Cravatt[3]

To convert an inhibitor into an effective AfBP, the following two steps are taken: (1) identification of a suitable inhibitor (or an inhibitor scaffold) against the target enzyme (or the class of enzymes). This typically limits the AfBP strategy only to proteins for which inhibitors are known *a priori*.[2] (2) Introduction of a BP–fluorophore linker into the molecule, which in many cases may abolish its target-binding ability and, as a result, leads to inactive probes.[2b] Consequently, a much larger number of potential probes (than the final probes obtained) are usually synthesized chemically and tested biologically. Clearly, there is an urgent need to develop new ways for rapid screening and identification of A*f*BPs against different protein targets, including both well-known and less-characterized ones. Herein, we report the first small molecule microarray (SMM)-facilitated approach for high-throughput identification of A*f*BPs, in which γ-secretase was used as our model target.

Small molecule microarrays (SMMs) are becoming increasingly important for the large-scale determination of protein–ligand interactions, high-throughput screening of enzyme substrates/inhibitors and the development of biosensors and biomarkers for disease analysis.[4] In our studies, we realized one of the key steps in most current SMM technologies requires the site-specific immobilization of small molecules onto the glass slide through the introduction of a suitable chemical "tag" (and the connecting linker) into the small molecules (*e.g.* biotin in Fig. 1).[4b] Upon SMM screening, the "hits" identified were in fact the "tagged" small molecules. We reasoned that the direct conversion of biotin into a linker containing both BP and a dye should retain the full protein-binding ability of the "hits", thus making them suitable A*f*BPs against the target protein. So by combining concepts in microarray technologies and activity-based protein profiling, new A*f*xBPs may be rapidly identified.

To apply our strategy to the screening of A*f*BPs against γ-secretase, we developed a solid-phase method for

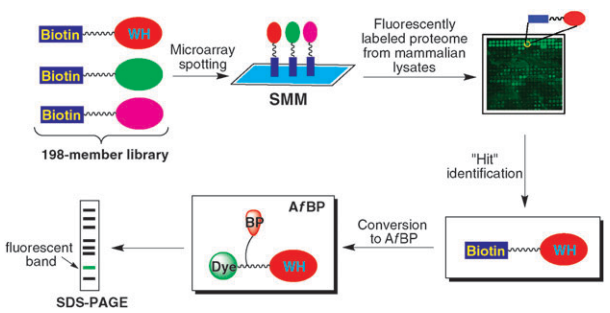

Fig. 1 Overall strategy of the small molecule microarray (SMM)-facilitated platform for high-throughput identification of A*f*BPs.

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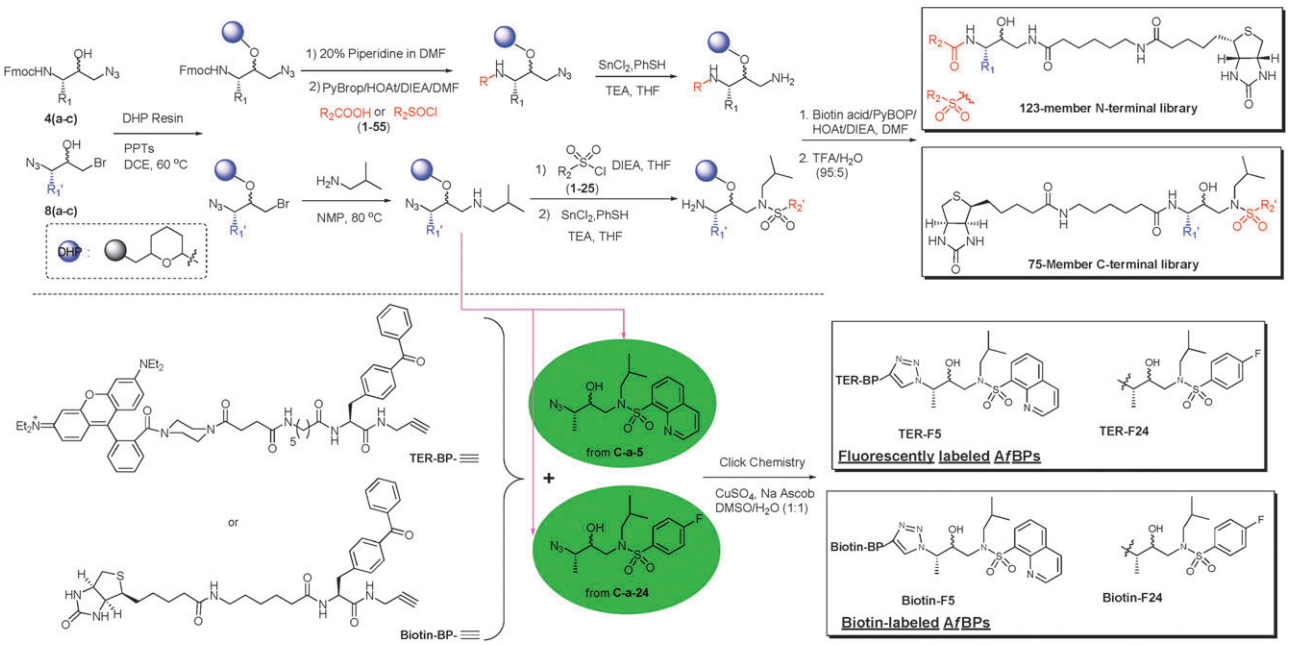

Scheme 1 Synthetic strategy of the N- and C-terminal libraries and click assembly for A*f*BPs.

combinatorial synthesis of hydroxylethylene-based small molecule inhibitors (Scheme 1). Hydroxylethylenes are well-known scaffolds as transition state analogs of aspartic proteases, and have been included in the inhibitor design of numerous aspartic proteases including γ-secretase. In the 123-member N-terminal sublibrary, R_1 and R_2 positions, corresponding to the P_1 and P_2 residues, respectively, upon binding to the aspartic protease, were varied with aromatic/aliphatic moieties (R_1 = Phe, Leu, Ala; R_2 = aromatic/aliphatic acid (**1-55**) as shown in Fig. S4 in the ESI†). In the 75-member C-terminal sublibrary, R_1' and R_2' positions, corresponding to the P_1 and $P_{1/2}'$ positions, respectively, were similarly varied. In both sublibraries, a biotin tag (and a connecting linker) was introduced in each compound for subsequent immobilization onto SMM. Since most known aspartic proteases recognize the four key residues (P_2, P_1, P_1' and $P_{2/3}'$) flanking the scissile bond of the substrates, our 198-member combined library could potentially be used for screening of other aspartic proteases besides γ-secretase. As shown in Scheme 1, the synthetic strategy was so chosen because (1) it's solid-phase and amenable to large-scale synthesis of hydroxylethylene-containing compounds from readily available Fmoc-protected L-amino acids; (2) the diversity of the library can be easily introduced throughout the P_2–P_2' positions; (3) the key azido intermediates in the synthesis (shaded in Scheme 1) could be easily recovered and converted to the corresponding A*f*BPs using "click chemistry". Briefly, **4(a–c)**, synthesized from the corresponding commercial available L-amino acids following previous reported procedures with some modifications,[5] were first immobilized at the –OH group onto 3,4-dihydro-2H-pyran (DHP) resin under acid-catalyzed conditions. Subsequently, deprotection of the Fmoc group followed by standard acylation with different acid building blocks (**1–55** in Fig. S4†) and azide reduction, gave the corresponding amines. Finally, attachment of a biotin linker then TFA cleavage gave the 123-member N-terminal compounds. Similarly, **8(a–c)**, again from the corresponding three L-amino acids, were attached to DHP resin, followed by alkylation reaction with *i*-butylamine, acylation with commercially available sulfonyl chloride building blocks (**1–25** in Fig. S5†), reduction with $SnCl_2$/PhSH, attachment of the biotin linker then TFA cleavage, gave the 75-member C-terminal compounds. Finally, the resulting 198 compounds were analyzed by LC-MS, which confirmed most were of sufficient purity (>80%) and were subsequently used for direct immobilization onto avidin-functionalized slides to generate the corresponding small molecule microarrays.

To assess whether the SMM could be used to detect aspartic protease–small molecule interactions, we carried out our preliminary screening with the recombinantly purified protein, histo-aspartic protease (HAP), which is one of the aspartic proteases from the parasite *Plasmodium falciparum*.[6] HAP was fluorescently labeled with Cy3 before being applied onto the SMM. As negative controls, three inactive mutant proteins of HAP (E278K, H34A and S37A mutants), previously shown to have minimum enzymatic activity, were used. Subsequently, the slides were scanned using an ArrayWoRx microarray scanner installed with the relevant filters (Cy3: $\lambda_{ex/em}$ = 548/595 nm). Only HAP showed distinct binding profiles against the SMM, while none of the three active-site mutants showed any significant binding (see ESI†). This indicates that our SMM platform was able to sensitively report activity-based binding events of aspartic proteases towards their small molecule inhibitors. Previous SMM strategies have mainly relied on incubation with a purified protein of interest.[4] Recently, Koehler and coworkers have shown cell lysates expressing a target protein could be used directly for ligand identification in SMM as well.[7] In our case, since it is notoriously difficult to isolate γ-secretase, we decided to carry out our SMM screening using the fluorescently labeled membrane fraction of mammalian cell lysates. The γ-30 cell line, which is a γ-secretase overexpressing CHOK1 mammalian cell line,[8] was used to

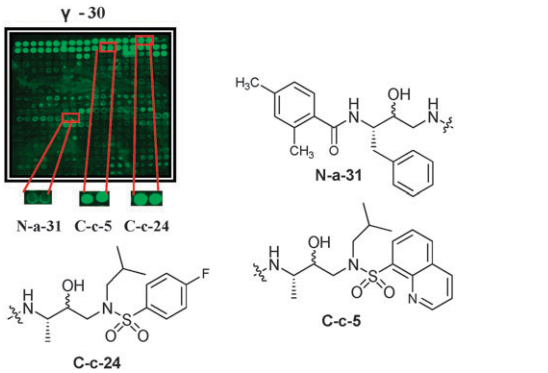

Fig. 2 SMM of the 198-member library screened against fluorescently labeled membrane fraction of γ-30 cell lysate, with (left) microarray image showing selected binders and non-binder and (right) their chemical structures. All compounds were printed in duplicate (horizontally). See ESI for spotting pattern.†

ensure a sufficient amount of active γ-secretase was present in the lysate. The cells were grown as previously described.[8] Subsequently, the membrane fraction was isolated, solubilized and fluorescently labeled with Cy3 before being applied onto the SMM. As shown in Fig. 2, highly reproducible and distinct binding profile of the 198-member library against the membrane fraction of γ-30 lysates could be obtained. Interestingly, none of the N-terminal sublibrary showed any significant fluorescent binding. Of the strong binders, most were identified as members of the C-terminal sublibrary containing an alanine residue at the P_1 position. **C-c-5** and **C-c-24** showed the strongest relative fluorescence binding, and therefore were chosen for further studies. **N-a-31**, a non-binder, was also chosen as a negative control.

To make the corresponding A*f*BPs of above three compounds, their azido intermediates (shaded) were taken, and "click" assembled with TER-BP alkyne (for in gel activity-based profiling) and Biotin-BP alkyne (for pull-down experiments), as shown in Scheme 1. Previously, the modularity of "click chemistry" had been successfully explored to facilitate the synthesis of other classes of activity-based probes.[9] To demonstrate the ability of our SMM-derived A*f*BPs for UV-initiated, activity-based profiling of γ-secretase in cellular lysates, we carried out in-gel fluorescence labeling and pull-down experiments. Detailed

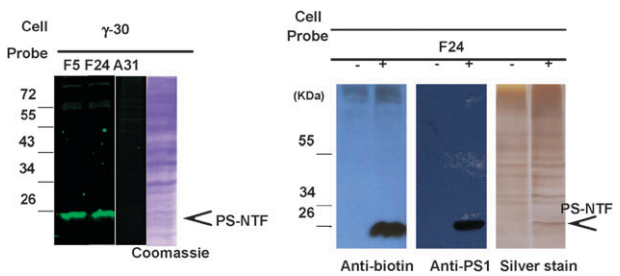

Fig. 3 (left) In-gel fluorescence profiling of γ-30 lysates using different A*f*BPs. The arrowed band was identified as PS-NTF. (right) After pull-down, western blotting showed the biotinylated protein in γ-30 cell lysate labeled by **Biotin-F24** as PS-NTF.

procedures were described in ESI. As shown in Fig. 3a, the two positive probes identified from SMM, **F5** and **F24** (corresponding to **C-c-5** and **C-c-24** in SMM, respectively) strongly labeled a 26-KDa protein in the total lysate of γ-30 mammalian cell line with high specificity. This band was subsequently confirmed as the N-terminal fragment of PS (PS-NTF) by both western blotting with anti-PS antibody and pull-down experiments (Fig. 3b). On the contrary, the total lysate of γ-30 treated with the negative control **A31** (corresponding to **N-a-31** in SMM) failed to give any noticeable labeled band. From our results, it is interesting to note only PS-NTF was labeled by our probes. Previous work on the γ-secretase complex had identified that both NTF and CTF are needed to form the catalytic core of PS.[1,2] A quick survey of other known A*f*BPs of γ-secretase revealed that most also labeled only PS-NTF.[2b,c] This thus unambiguously confirms our SMM-facilitated screening approach as a valuable tool for rapid discovery of A*f*BPs.

In conclusion, a small molecule microarray-facilitated screening strategy has been developed for high-throughput identification of affinity-based probes. This approach does not require known inhibitors of a protein target and minimizes risks involved in the loss of protein-binding property of these inhibitors due to linker introduction. We further demonstrated the utility of this method by successfully identifying highly specific γ-secretase probes from a generic hydroxylethylene small molecule library.

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