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Microarray-guided discovery of two-photon (2P) small molecule probes for live-cell imaging of cysteinyl cathepsin activities†

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A microarray immobilized with 105 aldehyde-containing small molecules was screened against mammalian cell lysates over-expressing cathepsin L to identify two potent inhibitors, which were subsequently converted into cell-permeable probes capable of live-cell imaging of endogenous cysteinyl cathepsin activities by two-photon fluorescence microscopy.

Proteases are critically involved in a multitude of vital biological processes, ranging from cellular signaling to tissue homeostasis.[1] Cathepsins, of which eleven are cysteine proteases, reside mostly in endolysosomal vesicles and hydrolyze peptides and proteins destined for these compartments.[2,3] The over-expression of these enzymes has been implicated in a number of pathological conditions. Consequently, the development of cell-permeable, small molecule probes capable imaging endogenous protease activities has gained considerable interest in recent years.[4] Of the various enzyme-detecting, small molecule probes available, those amenable to two-photon fluorescence microscopy (TPFM) provide additional key advantages over the usual one-photon imaging techniques, as they can afford superior imaging results with increased penetration depth, lower tissue autofluorescence and reduced photodamage/photobleaching.[5] Therefore TPFM is especially amenable for imaging of thick tissues. In the context of live-cell bioimaging of cysteinyl cathepsins using small molecule probes, there are several reported examples.[6] Their design principle is typically rational and involves taking a well-known substrate/inhibitor scaffold of a cysteinyl cathespin and introducing a suitable FRET (Förster resonance energy transfer) donor/acceptor pair or fluorogenic molecule. Two-photon (2P) small molecule probes for live-cell imaging of endogenous cysteinyl cathespins, however, are not presently available.[5,7] We sought to develop an alternative approach for high-throughput discovery of 2P small-molecule imaging probes against cysteinyl cathepsins using small molecule microarrays (SMMs).

SMMs are miniaturized assemblies of which compounds are spatially addressed in high-density grids, enabling simultaneous interrogation with suitable proteins and other biological targets. The platform offers a cheap and convenient method for rapid screening of thousands of compounds and has been successfully used in ligand/inhibitor identification, substrate fingerprinting and the profiling of cellular events.[8] More recently, it has also been used for high-throughput discovery of activity-based probes.[9] Herein, a microarray immobilized with 105 different aldehyde-containing small molecules were fabricated, and screened against lysates of mammalian cells over-expressing green fluorescent protein (GFP)-labeled cathepsin L (a key endolysosomal cysteinyl cathepsin which is known to be over-expressed in many tumor cells and tissues[2]). Two potent inhibitors were identified and subsequently converted into the corresponding cell-permeable, 2P small molecule probes, which were then used to image endogenous cysteinyl cathepsin activities in live HepG2 cancer cells (Fig. 1). The general structure of our small molecule inhibitors is shown in Fig. 1B, in which an aldehyde warhead (WH) was strategically chosen because 1) it is a well-known reversible but tight-binding inhibitor motif of cysteine proteases, 2) could be readily synthesized using solid-phase chemistry, and 3) later facilitate the inhibitor-to-probe conversion (*e.g.* by linking a quencher molecule *via* an amide bond; see Fig. 1A). From previous literatures,[3] it is known that cysteinyl cathespins prefer hydrophobic/aromatic P_2 and P_3 residues, while variations at the P_1 position are well-tolerated. Consequently in our small molecule library, a biotin-containing immobilization handle, Phe/Leu/Val residues and 35 different commercially available acid building blocks (aliphatic and aromatic) were installed at P_1, P_2 and P_3 positions, respectively. Both P_2 and P_3 building blocks were so chosen that the resulting compounds would possess reasonable cell permeability. We anticipated that, after SMM-guided identification of "hits", the biotin moiety in the inhibitor moiety may be conveniently replaced with a suitable fluorophore without compromising the probe/cathepsin recognition.

The solid-phase synthesis of the 105-member aldehyde library was carried out as shown in Scheme 1.[8d] Briefly, Fmoc-Lys(Mtt) aldehyde was loaded onto a theonine-functionalized resin by acid-catalyzed oxazolidine formation. Subsequently, Mtt was removed with 1% TFA followed by biotin coupling. Next, solid-phase combinatorial library synthesis was carried out by using standard solid-phase peptide synthesis (SPPS) using Fmoc chemistry protocols. At the end, compounds were cleaved off the resin,

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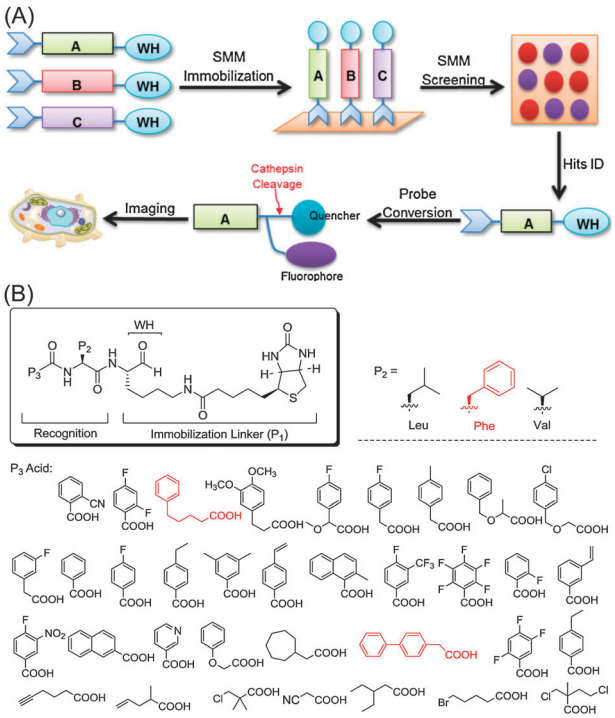
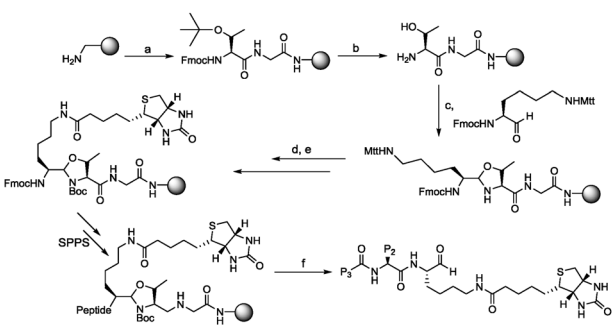


Fig. 1 (A) Overall strategy of the SMM-guided, high-throughput discovery of cell-permeable, 2P small molecular probes for imaging of endogenous cysteinyl cathepsins. (B) General structure of the 105-member small molecule library. Biotin is located at the P_1 position. P_2 consists of Phe/Leu/Val residues. P_3 consists of 35 different aromatic/aliphatic building blocks. Highlighted (in red) are the P_2/P_3 structures of the two hits identified from the SMM screening results.



Scheme 1 Reagents and conditions. (a) i: Fmoc-Gly-OH/HBTU/HOBt/DMF, 4 h; ii: 20% piperidine/DMF, 30 min; iii: Fmoc-Thr(OtBu)-OH/HBTU/DIEA/DMF, 4 h; (b) i: 20% piperidine/DMF, 30 min; ii: TFA/DCM (1 : 1) 1 h; iii: 10% DIEA/DCM. (c) 1% DIEA/MeOH, 2 h, 60 °C. (d) Boc_2O, 1% DIEA/DCM, 2 h. (e) i: 1% TFA/DCM, 30 min; ii: Biotin/HBTU/DIEA/DMF, 4 h. (f) i: TFA/TIS/EDT (95 : 5 : 0.1); ii: ACN/H_2O/TFA (60 : 40 : 0.1), 30 min, 60 °C.

precipitated, and immobilized directly onto avidin-functionalized glass slides to generate the corresponding SMM.

We screened the SMM with mammalian cell lysates over-expressing GFP-fused cathepsin L. Direct application of total mammalian lysates, rather than recombinantly purified proteins, saved substantial time and costs by avoiding the tedious and often problematic protein purification processes. This method should also be amenable for future high-throughput screening of other mammalian proteins. As shown in Fig. 2, from the SMM results, two hits (**D02** and **D17**) were identified, both of which showed significantly stronger binding toward cathepsin L than any other immobilized ligands. Both **D02** and **D17** carried a Phe residue at the P_2 position, and a hydrophobic aromatic moiety at the P_3 position. To confirm that both compounds were indeed genuine hits, their IC_{50} values against recombinant cathepsin L were measured; with values of 14.5 ± 0.08 nM (for **D02**) and 20.8 ± 0.22 nM (for **D17**) under our assay conditions, both were shown to be potent inhibitors of the enzyme. Our results also corroborated well with other known cathepsin inhibitors, most of which also contain hydrophobic/aromatic P_2/P_3 residues. It should be noted that, although cathepsin L was used as the representative enzyme in our SMM screening, both **D02**/**D17** are likely potent inhibitors of other cysteinyl cathespins.

Subsequently, these two potent small molecule inhibitors were converted into imaging probes based on our earlier described design principle (Fig. 1A). As the P_1 residue in the inhibitors did not play a significant role in binding to cathepsin L, and the aldehyde WH sat where the scissile bond of a cysteinyl cathepsin substrate would have been located, we replaced the biotin moiety in **D02**/**D17** with a two-photon dye, **DL-1** (an 4,6-bis(4-hydroxystyryl)pyrimidine derivative[10]), and the aldehyde WH with Disperse Red 1 (**DR-1**; a fluorescence quencher) linked to the P_1 residue *via* an amide bond, giving **ZK-1** and **ZK-2**, respectively (Fig. 2). In the absence of a cysteinyl cathepsin, the intrinsic fluorescence of these two imaging probes would be mostly quenched due to the intramolecular FRET effect between **DL-1** and **DR-1**. Upon binding to active enzymes, however, the probes, with the newly introduced amide bond serving as the scissile bond, would be proteolytically processed (to remove **DR-1**) and emit strong fluorescence. Synthesis of the probes were shown in Fig. 2 (right), with the key step involving a highly efficient and modular assembly of the compounds using click chemistry.[11] The final probes were purified to homogeneity and fully characterized (see ESI†).

We next evaluated whether the proteolytic cleavage and fluorescence increase of **ZK-1** and **ZK-2** in the presence of cysteinyl cathepsins could be monitored spectroscopically. Mammalian lysates from HepG2 cells (a liver cancer cell line known to over-express endolysosomal cysteinyl cathepsins) were used as the enzyme source. As shown in Fig. 3A, a time-dependent proportional increase in fluorescence (max λ_{em} = 522 nm) was observed, evidently due to successful proteolytic cleavage of the probes and release of the quencher. Finally, we examined whether the probes could be used in live-cell imaging experiments to detect endogenous cysteinyl cathepsin activities (Fig. 3B); HepG2 cells treated with either **ZK-1** or **ZK-2** for 2 h showed exclusive fluorescence (green channel) in endolysosomal compartments which were readily stained by Lyso Tracker™ (red channel).[12] The same cells pre-treated with E-64 (a broad-spectrum cysteinyl cathepsin inhibitor) showed no detectable fluorescence in the probe channel, indicating these fluorescence signals were a direct result of endogenous cysteinyl cathepsin activities.

In conclusion, by using a small molecule microarray as a high-throughput screening platform, we have successfully discovered two potent inhibitors of cathepsin L which were converted strategically into the corresponding two-photon

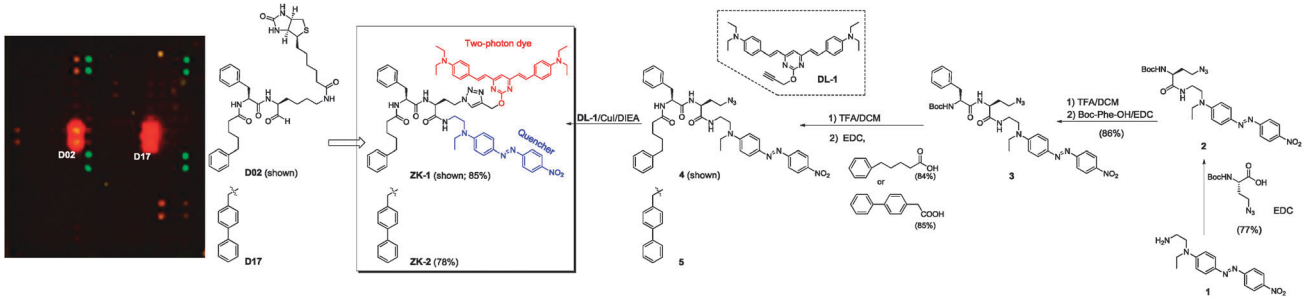


Fig. 2 (Left) SMM image screened against mammalian cell lysates over-expressing GFP-fused cathepsin L. All compounds on SMM were spotted in duplicate. The green spots were dye references. See spotting pattern and ID in ESI.† (Right) Structures of hits identified and the corresponding cell-permeable imaging probes (**ZK-1** and **ZK-2**; boxed) and their synthetic scheme. (Red) **DL-1**. (Blue) **DR-1**.

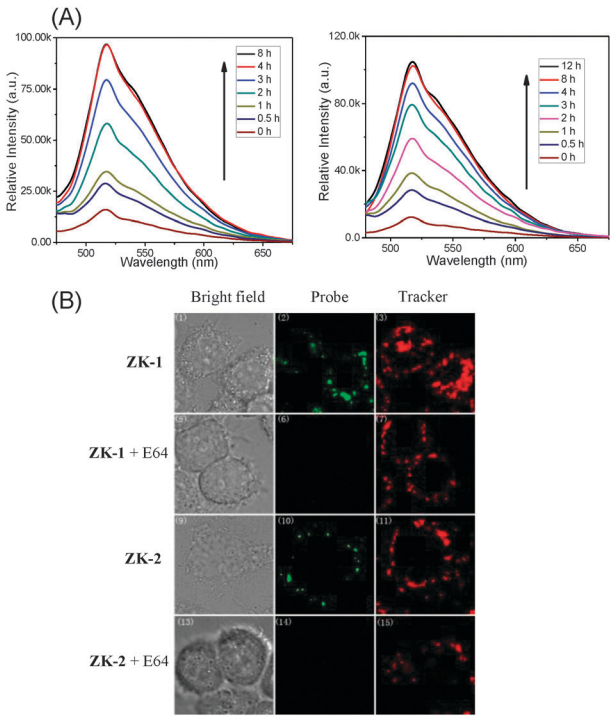


Fig. 3 (A) Time-dependent emission spectra (λ_{em} = 522 nm) of **ZK-1**/**-2** in HepG2 lysates (Hepes buffer, *p*H 5.5) at room temperature. (B) Two-photon confocal images of live HepG2 cells upon treatment with **ZK-1**/**-2** (2 μM) for 2 h. Probe channel (pseudo-colored in green): λ_{em} = 550 nm. Tracker channel (pseudo-colored in red): λ_{em} = 590 nm channel. Endolysosomal tracker (LysoTracker® Red DND-99; Invitrogen) was used. In panels where a general cysteine protease inhibitor (E64) was used, HepG2 cells were pre-incubated with 15 μM of E64 before probe treatment and imaging. All images were acquired under the same settings.

small molecule imaging probes, both of which were found to be cell-permeable and could detect endogenous cysteinyl cathepsin activies from cell lysates or live mammalian cells of HepG2 cancer cells. Future efforts will focus on using these 2P small molecule probes to directly image cysteinyl cathepsin activities in thick tissues.

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12 Fluorescent signals from **ZK-1**/**-2** were not expected to completely overlap with the tracker signals, as the former represented only endolysosomal cysteinyl cathepsin activities which might not be distributed throughout the entire organelles.