Cinnamic aldehyde derived probes for the active site labelling of pathogenesis associated enzymes†

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Michael acceptor based natural product derived probes are selective and sensitive chemical tools for the identification and characterization of pathologically relevant enzymes in MRSA.

With the emergence of multiresistant bacterial strains, infectious diseases once again pose a major threat to public health.[1] One fundamental challenge is the identification and functional characterization of resistance free enzyme targets that are essential for bacterial viability or pathogenesis and which are not addressed by currently described antibiotics. We recently introduced two strategies for the identification and functional characterization of bacterial enzymes based on bioactive β-lactam[2] and β-lactone[3,4] compound libraries. β-Lactam and β-lactone scaffolds represent electrophilic entities that are prone to react with nucleophilic enzyme active sites forming a covalent bond. The β-lactam and β-lactone probe libraries were successfully applied in activity based protein profiling (ABPP) experiments (pioneered by Cravatt, Bogyo and co-workers)[5,6] to identify enzymes that play crucial roles in resistance and virulence. Inspired by the success of this strategy we here explore cinnamic aldehyde (CA) as an electrophilic scaffold for bacterial target identification. Cinnamic aldehyde is the key flavour compound in cinnamon and an important FDA approved food additive that contains an α,β-unsaturated carbonyl Michael pharmacophore.[7] Previous studies with α,β-unsaturated carbonyl Michael acceptor systems revealed that these compounds exhibit a preference for the modification of cysteine active site residues.[8,9] The high reactivity of many Michael acceptor systems compromises their application for selective biological studies. Peptide scaffolds that are attached to CA, however, have been reported to be selective inhibitors of protein tyrosine phosphatases (PTPs).[10] Unmodified CA displays potent anti-cancer activities, suggesting a promising biological potential of this compound in the binding and inhibition of pharmacologically relevant enzyme classes.[11]

Following our goal to identify novel therapeutic targets in pathogenic bacteria we synthesized a CA peptide probe library to monitor the activity and function of cysteine active site containing enzymes that play crucial roles in bacterial pathogenesis, such as PTPs and cysteine proteases.[12] The general probe design of our library consisted of the CA core scaffold that was appended by a short alkyne handle as a benign tag for visualization and enrichment of labelled proteins (Fig. 1A). The modification of the alkyne tag *via* the 1,3-dipolar Huisgen cycloaddition (click chemistry, CC) allows us to introduce the bulky reporter group (*e.g.* rhodamine) after enzyme binding and cell preparation (Fig. 1B).[13] Labelled enzymes can be run on SDS gels, visualized by fluorescent scanning and subsequently identified by mass spectrometry.

Our general synthetic procedure is shown in Fig. S1, ESI†. All amino acid positions in the CA tripeptides were varied by charged, aromatic or hydrophobic residues in order to increase the chance of specific probe–protein interactions. The synthesis of the peptide scaffold was carried out on a Rink-amide resin and followed standard Fmoc solid phase chemistry procedures. The N-terminal end of the peptides was coupled with the free acid of the reactive CA binding group in the last step of synthesis. In addition to CA we also attached the corresponding cinnamic acid (CAC) moiety. In total the library consists of three CAC (please refer for structures and compositions to Fig. S1, ESI†) and 11 CA peptide probes (Fig. 1A).

Previous studies of tripeptide CA and CAC scaffolds revealed potent inhibition of PTP1B, with K_i values ranging

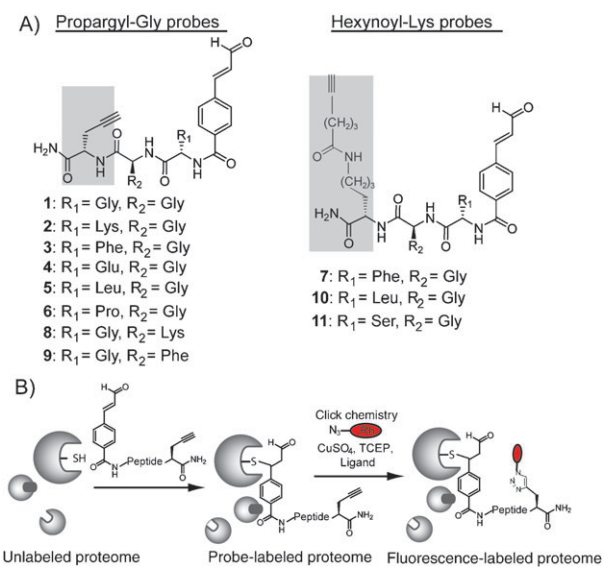

Fig. 1 Structures and applications of CA peptide probes. (A) Structures of all CA probes. (B) Proteomes are first treated with the CA probes and subsequently appended with a fluorescent dye *via* CC.

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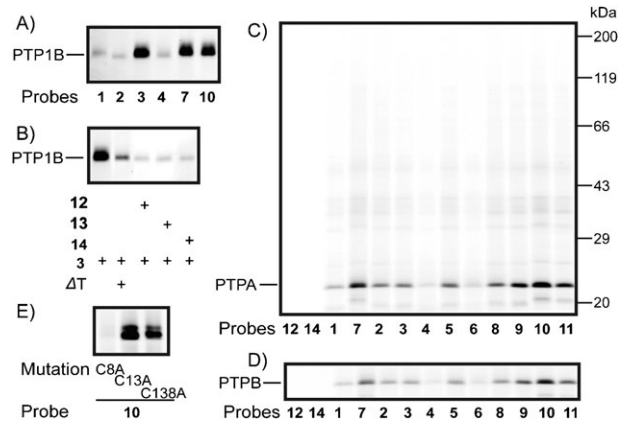

Fig. 2 Labelling of protein tyrosine phosphatases. (A) Labelling of recombinant human PTP1B with 50 μM of individual probes. (B) Heat control (ΔT) and active site competition by a pre-incubation of the enzyme with a 40-fold excess of CAC probes **12**, **13** and **14** and subsequent labelling with 50 μM **3**. (C) Labelling of recombinant MRSA PTPA in *E. coli* proteome with the probe library (50 μM). (D) Labelling of MRSA PTPB with the probe library (50 μM). (E) Labelling of PTPA mutants with 50 μM **10**.

from 5.4 μM to 79 nM, respectively.[14,15] PTP1B is an important drug target for the treatment of both type 2 diabetes and obesity.[16] To test whether the concept of utilizing tripeptide CA probes is effective in labelling this PTP we incubated the commercially available recombinant enzyme with a selection from our probe library.

In fact, several probes exhibited strong labelling intensities (50 μM probe concentration) of the recombinant enzyme (250 ng) with **3** being one of the best (Fig. 2A). These results indicate that PTP1B prefers aromatic (**3** and **7**) or hydrophobic (**10**) residues in its active site. Heat denaturation of PTP1B prior to probe labelling resulted in a strongly reduced fluorescent signal which shows that the binding of probes to the enzyme occurred specifically only with an intact and properly folded enzyme (Fig. 2B). Since CAC tripeptide scaffolds, with a free acid moiety, have been reported to be potent PTP1B inhibitors we tested labelling of the recombinant enzyme with probe **12** which closely mimics a published Gly-Glu-Glu-CAC inhibitor.[14] No labelling could be observed indicating that α,β unsaturated acids may not be reactive enough to covalently attach to the enzyme active site. To test whether the CAC probes bind to the PTP1B active site we pre-incubated the enzyme with a 40-fold excess of **12**, **13** and **14** and subsequently added 50 μM of the reactive CA probe **3** (Fig. 2B). In fact, labelling was efficiently reduced, emphasizing that the CAC based inhibitor exerts its activity *via* reversible binding and competes for the same active site as the CA probe.

With these initial experiments that validated the general utility of our probes in PTP labelling we next tested the binding and inhibition of the probe library against two PTPs from the pathogenic bacteria methicillin resistant *Staphylococcus aureus* (MRSA). The precise role of these PTPs remains elusive so far but it is assumed that they are involved in virulence mechanisms.[17,18] We cloned and overexpressed PTPA and PTPB from MRSA recombinantly in *Escherichia coli* and added 50 μM of all probes (Fig. 2C and D). Interestingly, several probes exhibited strong labelling intensities of both enzymes in an activity dependent manner as visualized by the corresponding heat control (Fig. S2, ESI†). Peptide sequences of probes which performed best in PTPA labelling contained no charged residues, which indicates that this enzyme prefers small hydrophobic (Gly) or aromatic (Phe) residues in its active site. A similar labelling profile was obtained for PTPB, which reflects a close relationship between these two enzymes (Fig. 2D).[18] In contrast, our three CAC probes in which the aldehyde moiety is replaced by a free carboxylic acid did not show any enzyme labelling, indicating that the α,β unsaturated acid is not electrophilic enough to acylate the enzyme active site.

Many covalent PTP inhibitors attach to a conserved active site cysteine residue which functions as the catalytic nucleophile that attacks the tyrosine phosphate to form a transient phosphocysteinyl intermediate.[19] The cinnamic aldehyde probes most likely attach *via* this cysteine residue and inhibit enzyme activity. To test this hypothesis and to figure out which of the three cysteines in uncharacterized MRSA PTPA is relevant for catalysis we individually mutated all three cysteines (C8, C13 and C138) to alanine and checked the labelling efficiency of the corresponding mutants with probe **10** (Fig. 2E). It turned out that C13A and C138A were strongly labelled, however, C8A revealed no labelling intensity, indicating that this residue functions as the catalytically active site.

We next evaluated if PTP active site labelling also results in inhibition of catalytic activity. PTPA activity was measured by the catalysed dephosphorylation reaction of *p*-nitrophenylphosphate that resulted in the generation of *p*-nitrophenol as a chromogenic product that can be measured at 410 nm on a UV-Vis spectrometer. Several CA probes revealed inhibition of substrate turnover, with IC_{50} values ranging from 5 μM for probe **10** to 271 μM for probe **4** (Fig. 3A, Fig. S3, ESI†). Interestingly, probe **10** which had already revealed the most intense labelling also exhibited the lowest IC_{50} value of all tested compounds, while CA probe **4**, which weakly labelled PTPA, also weakly inhibited its activity. However, the hydrophobic nature of **10** caused solubility problems above 100 μM in the assay buffer and restricted the evaluation of full PTP inhibition. As mentioned previously, CAC based probes were not capable of PTPA labelling due to their low reactivity.

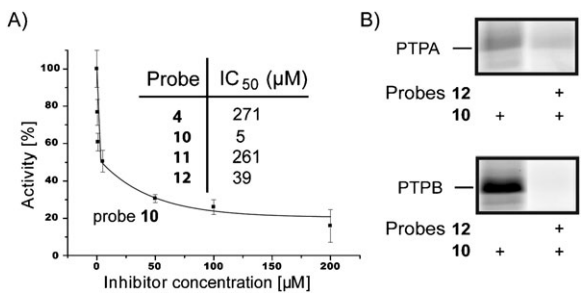

Fig. 3 Inhibition of PTP activity. (A) IC_{50} values of PTPA inhibition with CA and CAC compounds. (B) Active site competition of PTPA and PTPB by a pre-incubation of the enzyme with a 100-fold excess of CAC probe **12** and subsequent labelling with 50 μM probe **10**.

However, we were able to obtain good inhibition of PTPA with an IC_{50} value of 39 μM (**12**), indicating that cinnamic acid based tripeptides are promising reversible inhibitors for bacterial PTPs. In addition, pre-incubation of PTPA and PTPB with a 100-fold excess of CAC compound **12** and subsequent incubation with 10 μM CA probe **10** resulted in strongly reduced labelling for PTPB and a significant reduction for PTPA, providing further confirmation that both probes compete for the same active site (Fig. 3B).

The previous experiments validated the utility of CA probes to label and inhibit recombinant bacterial phosphatases. Based on these results we wanted to test if the probes were capable of labelling enzymes in whole bacterial proteome lysates. For an initial evaluation we chose to analyse the labelling pattern of our library in a proteome of the antibiotic resistant MRSA strain. Enzymes which are labelled in MRSA proteomes could be involved in pathogenesis and resistance and may represent valuable targets for therapeutic interventions.

We therefore used a selection of our best probes to profile the MRSA membrane proteome. Although we were not able to detect PTPA and PTPB which may be less abundant or inactive under the test conditions, one new protein target was selectively labelled (Fig. 4A). Subsequent identification by LC-MS analysis revealed the identity of this protein as a virulence associated Staphylococcal secretory antigen (SsaA2). To validate the MS result, we recombinantly expressed SsaA2 and subsequently confirmed its labelling by the corresponding probes (Fig. 4B). SsaA2 is an uncharacterized protein that has been previously reported to be involved in *Staphylococcus epidermidis* sepsis and infective endocarditis.[20] The protein contains a cysteine protease sub-domain that is supposed to process the protein into its mature form. Most likely SsaA2 is labelled by the attachment of the active site cysteine residue onto the CA tripeptide probe. To support this notion, we pre-incubated the proteomic mixture with 5 mM *N*-ethylmaleimide (NEM) to alkylate all free cysteine residues. Subsequent labelling with probe **10** revealed no residual labelling, indicating that a reactive cysteine was blocked and was no longer susceptible to CA probe alkylation (Fig. 4B). In addition, we identified the reactive cysteine by an active site mutation of Cys171 to alanine. As expected, the mutant enzyme revealed no probe labelling (Fig. 4C). Probes **7** and **10** exhibited the best labelling intensities of SsaA2 while probe **1** showed no labelling, indicating that the enzyme does not prefer two adjacent Gly residues next to the CA group. This information could be useful to gain insight into the native substrate preferences of this uncharacterized virulence associated protein. To estimate the strength of probe–protein interactions, we compared the reactivity profile of **10** with SsaA2 across a concentration range from 100 to 0.2 μM (Fig. 4D). The protein can still be detected at concentrations as low as 1 μM, which indicates an affinity suitable for monitoring the function and activity of this putatively important enzyme in future studies.

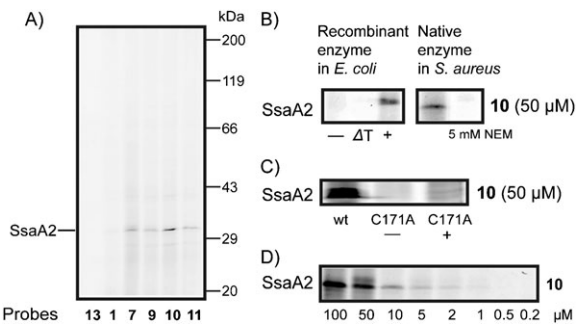

Fig. 4 Labelling of the MRSA proteome. (A) Labelling of the MRSA membrane proteome with a selection of CA and CAC probes (50 μM). SsaA2 = Staphylococcal secretory antigen. (B) Labelling of recombinant SsaA2 (– before induction, + after induction, Δ*T* heat control). Blocking of free cysteine residues in native SsaA2 with NEM. (C) SsaA2 active site cysteine mutation (– before induction, + after induction). (D) Concentration dependence of SsaA2 labelling.

In conclusion, we utilized cinnamic aldehyde as a prototype Michael acceptor system for the activity based labelling of pathogenesis associated enzymes including phosphatases as well as a secretory antigen from MRSA. Based on our library approach we were able to identify substrate preferences of individual uncharacterized enzymes which may help to guide the design of specific inhibitors. These findings illustrate that our Michael acceptor based natural product derived probes are useful, selective and sensitive chemical tools for the identification and characterization of physiologically and pathologically relevant enzymes in complex proteomes.

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