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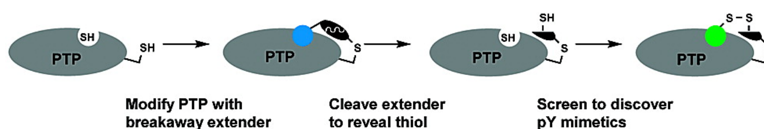

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Discovery of a New Phosphotyrosine Mimetic for PTP1B Using Breakaway Tethering

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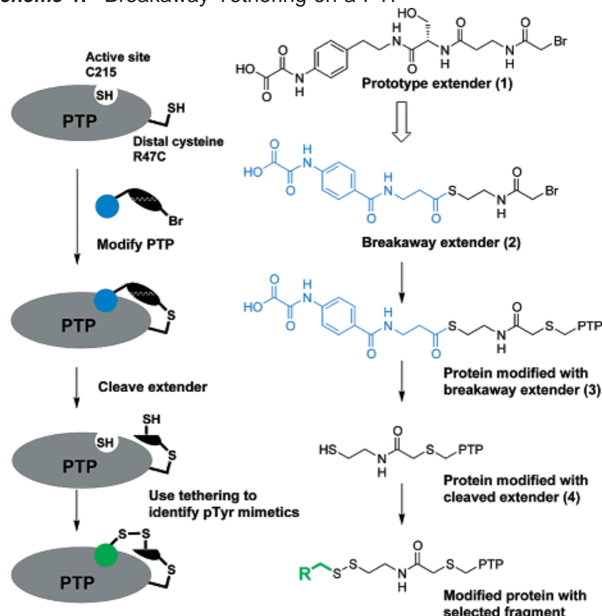
Protein tyrosine phosphatases (PTPs) represent an important but challenging target class for drug discovery.[1] One of the chief difficulties is that these enzymes are highly specific for the doubly charged phosphotyrosine (pY) residue. Several strategies have been used to find pY mimetics,[2] but the lack of selective drugs in clinical development highlights the need for new strategies. We recently demonstrated the use of covalent tethering to identify small molecule fragments that bind to a specific site on a protein.[3] Here we describe an expansion of this technology, "breakaway tethering", and use it to discover a pY mimetic that binds to the anti-diabetic target PTP1B in a new mode and belongs to a different molecular class than known pY mimetics.

Standard covalent tethering uses a cysteine residue to capture disulfide-containing fragments that bind to a nearby site on the protein; mass spectrometry is used to identify the fragments. Because PTP active sites are deep and highly conserved, introducing cysteine mutations within the active site itself is not desirable. Breakaway tethering circumvents this issue by introducing a cysteine residue *outside* the active site and alkylating it with an "extender" that reaches toward the active site, positioning a thiol to interrogate the pY binding region (Scheme 1). For PTP1B, we selected a position (Arg 47) distal to the active site but proximal to the pY-1 residue of substrate peptide.[4] We mutated Arg 47 to Cys (R47C) and labeled it with an extender that was designed using cocrystal structures of PTP1B with substrate peptides.[5] We protected the sensitive active site cysteine from alkylation by conjugating a pY mimetic (oxalic acid) to the extender thiol using a cleavable thioester linkage.

Scheme 1. Breakaway Tethering on a PTP[a]



[a] The breakaway extender protects the catalytic cysteine by sterically blocking it with a pY mimetic and also provides a spacer to probe the active site.

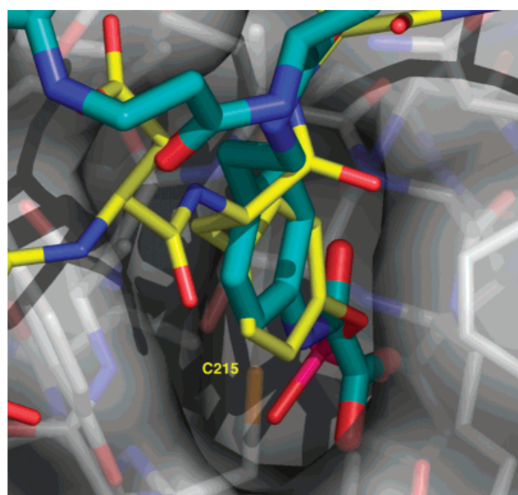
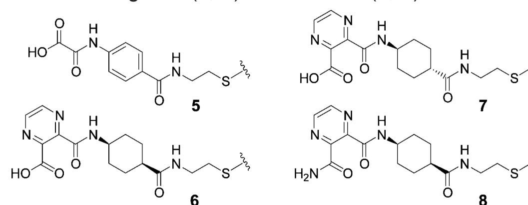


Figure 1. X-ray crystal structure of the prototype extender (**1**) (blue) covalently bound to PTP1B-R47C, superimposed on a peptide substrate containing a pY residue (yellow).[5] The active site cysteine (C215) is labeled. The figure was made using PyMol.[6]

Chart 1. Tethering Hits (**5**, **6**) and Nonhits (**7**, **8**)



The design was validated using a prototype extender (**1**). The prototype specifically and irreversibly labeled PTP1B at R47C and the cocrystal structure confirmed that the oxalic acid moiety occupied the active site (Figure 1). Having demonstrated that we could selectively modify PTP1B-R47C with an extender containing a pY mimetic, we next synthesized the "breakaway" extender (**2**) in which the oxalic acid group could be cleaved after protein modification. The breakaway extender rapidly and irreversibly modified PTP1B-R47C to give **3**. Cleavage of the thioester with hydroxylamine exposed the thiol to form **4** for tether screening (Figure S1, Supporting Information).

After establishing that the newly introduced thiol could form a disulfide bond with the known oxalate **5** (Chart 1), we performed a tether screen against 15 000 disulfide-containing fragments. Compound **6** was identified as one of the strongest hits and is chemically distinct from previously reported pY mimetics. It also

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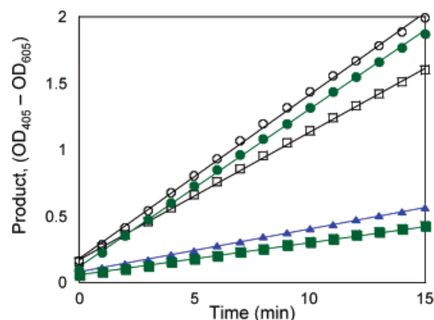

Figure 2. Activity of PTP1B-R47C (○), PTP1B-R47C with 50 μM compound **6** (●), extender-modified PTP1B-R47C (**4**) (□), extender-modified PTP1B-R47C (**4**) with 50 μM compound **6** (■), and uncleaved extender-modified PTP1B-R47C (**3**) (▲). The *y*-axis units are arbitrary.

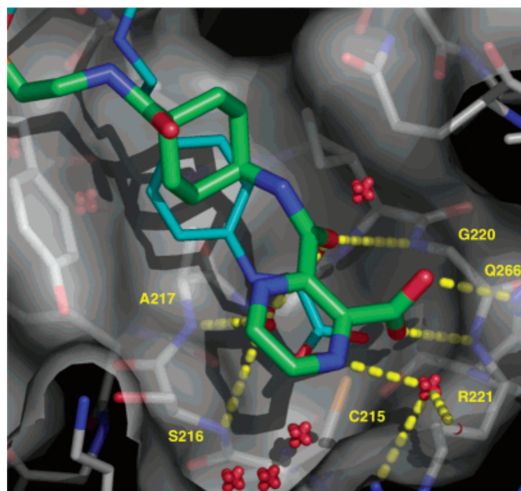

Figure 3. X-ray crystal structure of compound **6** (green) disulfide bonded to the breakaway extender, superimposed on the prototype extender (**1**, blue). Ordered water molecules are shown as red crosses, and hydrogen bonds are shown as yellow dashed lines. The figure was made using PyMol.[6]

exhibited sharp SAR: the closely related molecules **7** and **8** were not selected (Chart 1) in subsequent screens.

Enzymatic assays confirmed that PTP1B-R47C and extender-modified PTP1B-R47C (**4**) had comparable activities (Figure 2). However, 50 μM compound **6** only inhibited extender-modified PTP1B-R47C (**4**), and this inhibition was comparable to that of tethered oxalic acid (**3**). Inhibition correlated with the degree of protein modification and was reversible with reducing agents. The K_is of the free tethering hits **5** and **6** were determined using native PTP1B under reducing conditions. These experiments revealed that **6** had a K_i of 4.1 mM while the known pY mimetic (**5**) showed less than 20% inhibition at 5 mM, suggesting a K_i in excess of 10 mM. Furthermore, inhibition by **6** was competitive with substrate. The potency of **6** is comparable to that of pY itself (K_m = 4.9 mM) and other phenyl phosphate derivatives[2f,g] as well as phosphonates and oxalates that have served as starting points for potent inhibitor design.[2d,e]

To further characterize the mode of binding, we determined the X-ray crystal structure of compound **6** disulfide-bonded to extender-modified PTP1B-R47C (**4**) (Figures 3 and S2). The structure shows the fragment binding in the active site of PTP1B, with the carboxylic acid and pyrazine moieties making several hydrogen bonds to the active site. The cyclohexyl ring sits snugly in the hydrophobic pocket normally occupied by the phenyl ring of pY. In addition, the WPD loop of the protein is in the "open" conformation, whereas most PTP1B inhibitors bind with the loop in the closed conformation.[7] Significantly, the open conformations of PTP1B and the closely related TCPTP exhibit differences that could be exploited in designing and optimizing selective inhibitors.[8]

We have demonstrated that breakaway tethering can be used to discover new pY mimetics. The ability to start from a known substrate mimimizes perturbation of protein structure and increases the opportunity to probe the active site using tethering. Breakaway tethering expands tethering technology to probe active sites from afar, even when these sites contain catalytic cysteine residues, and could thus be applied to other target classes such as cysteine proteases, and potentially to any protein where modification within the active site itself is not desirable.

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Supporting Information Available: Experimental details (PDF). Structures have been deposited in the protein data bank (1NWE and 1NWL). This material is available free of charge via the Internet at http://pubs.acs.org.

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