A rapid oxime linker-based library approach to identification of bivalent inhibitors of the Yersinia pestis protein-tyrosine phosphatase, YopH

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A bivalent tethered approach toward YopH inhibitor development is presented that joins aldehydes with mixtures of bis-aminooxy-containing linkers using oxime coupling. The methodology is characterized by its facility and ease of use and its ability to rapidly identify low micromolar affinity inhibitors. The generality of the approach may potentially make it amenable to the development of bivalent inhibitors directed against other phosphatases.

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Abstract

Protein-tyrosine phosphatases (PTPs) serve as important modulators of normal tyrosine kinase signaling and facilitators of pathological processes making the field of PTP inhibitor development an established area of research. The highly active PTP, YopH, is required for the virulence of Yersinia pestis, the causative agent of plague. Several groups have sought to pharmacologically attenuate YopH function through competitive inhibition. In theory, 'tethering', a 'fragment-based' approach that joins two or more structures via simple linkers, could be particularly amenable for the development of PTP inhibitors, because the binding of substrate involves both the recognition of phosphotyrosyl (pTyr) residues in well-formed catalytic clefts and interaction with secondary features of the enzyme outside the catalytic cleft. Indeed, tethered bidentate inhibitors containing at least one phenylphosphate mimetic have been previously reported for PTPs, including YopH. Nevertheless, we were interested in examining tethered bidentate constructs as potential YopH inhibitors, with a major focus on developing a protocol that could allow the rapid generation and screening of mixtures of inhibitors without purification of synthetic products. Previous expedited assemblies of multi-dentate PTP inhibitor libraries have used Huisgen [3+2] azide-alkyne cycloaddition 'click' chemistry. However, we were attracted to an alternate methodology that relies on the tethering of aldehyde-containing fragments by oxime bond formation with bis-aminooxy-containing linkers. The approach is made especially attractive by the fact that commercially available aldehyde building blocks can be used without modification.

Mixtures of bivalent linked constructs were formed using a phenylphosphate-mimicking aldehyde 'A' (5-formyl-2-hydroxybenzoic acid), whose function was to interact within the pTyr-binding pocket. Fragment A was joined by means of a homologous series of tethering components 'L' to a library of aromatic aldehydes 'B', whose function was to interact outside the catalytic site (Fig. 1). The components connecting A and B were derived from linear polymethylene chains having bis-aminooxy groups at each terminus. The synthetic protocol for assembling the bivalent tethered constructs employed oxime coupling reactions. The syntheses were conducted in DMSO using AcOH as catalyst and reactant ratios of (A:B:L:AcOH) = (1:1:1:2) to generate >90% tethered products. Since the proximities and orientations of secondary binding sites outside the catalytic cleft were not known, the structural composition of the B-fraction and the length of the tethering segment were optimized in parallel. To expedite this process, a two-stage protocol was employed that involved first constructing and then deconvoluting bivalent tethered fragment libraries. In the first stage of library development, mixtures of linker components...
were used to join 20 aldehydes B (a–t, Fig. 2) to the phenylphosphate mimic A. Two separate mixtures of linkers were used. These were formed by combining equal molar quantities of 'H2N–O–(CH2)ₙ–O–NH2', where \( n = 2–6 \) (L₂–₆) or \( n = 7–10 \) (L₇–₁₀). This allowed the simultaneous evaluation of multiple linker lengths. The predominant reaction products (1) resulting from these reactions would be expected to be of the forms A–L–A; A–L–B and B–L–B, as well as smaller quantities of partially reacted mono-adducts (Scheme 1).

Without purification, the crude mixtures of oxime products (1) synthesized as described above were screened directly in an in vitro YopH assay at 100 \( \mu \)M and 10 \( \mu \)M concentrations.²⁴ Uniformly poor inhibition was observed using the linker mixtures L₂–₆. Higher potencies were obtained using L₇–₁₀ linker mixtures, with the greatest inhibitory effects being observed for aldehydes a, e, j, k, l, m, and q (Fig. 3A). It should be noted that since aldehyde a is identical to fragment A, symmetrical constructs result when a is used as the B component. Each of these latter aldehydes was then reacted with the four individual linkers comprising the original L₂–₁₀ mixture and the reaction products were evaluated against YopH at 10 \( \mu \)M concentration (Fig. 3B). The greatest inhibitory potency was obtained from aldehyde q (4-benzyloxybenzaldehyde) using linker L₁₀. Longer chain lengths were not examined. Purification of this mixture yielded pure 2 (Fig. 4), which was shown to exhibit an IC₅₀ value of 2.4 \( \mu \)M.

In silico docking of 2 onto the catalytic cleft of YopH started with our earlier X-ray crystal structure of YopH in complex with the peptide Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-amide (PDB 1QZ0),²⁵,²⁶ where

Figure 1. Graphical representation of bivalent linked fragment protocol.

Figure 2. Structures of aldehydes used in synthesis of linked bivalent constructs. [Note that aldehyde ‘a’ is equivalent to phenylphosphate mimic ‘A’.]
‘F2Pmp’ represents the non-hydrolyzable pTyr mimetic, phosphomethylphenylalanine (Fig. 5A).27,28 Comparing two of the best docking poses of 2 with the binding orientation of the parent F2Pmp-containing peptide indicates that the positioning of the linker oxime methylene situated proximal to the catalytic cleft occurs in a hydrophobic region identical to that occupied by the Leu side chain of the peptide (Fig. 5B). Additionally, the overall alignments of the methylene linkers were highly uniform for both docked poses of 2.

Differences in binding were observed mainly in the placement of the terminal 4-benzyloxy group. Therefore, components of inhibitors derived from fragment B, should not be assumed to bind uniquely in defined, specific pockets. Rather, the overall inhibitory potency of bivalent linked constructs may represent the combined effects of interacting in multiple orientations/pockets. Additionally, as shown in Figure 5, binding of 2 with the YopH protein involves hydrophobic interactions extending over a considerable distance. Disruption of these hydrophobic interactions by means of surfactants could potentially reduce the binding affinity. Indeed, addition of 0.01% of Triton X-100 to the binding assay did shift the binding curve to the right. Such ‘detergent effects’ have been previously interpreted to potentially indicate inhibition by ‘promiscuous’ mechanisms.29–31 However, given the extended hydrophobic interactions between the long alkyl linker segment of 2 and the 4-benzyloxy group, the observed binding behavior is more consistent with the idea of multiple binding orientations/pockets rather than ‘promiscuous’ binding.

Figure 3. Inhibition of pNPP hydrolysis in an in vitro YopH activity assay. (A) 100 μM of unpurified mixtures resulting from reaction of the indicated aldehydes (Fig. 2) with a mixture of linkers, L7–10. (B) 10 μM of unpurified mixtures resulting from reaction of the indicated aldehydes with linkers of defined length, L7–L10.

Figure 4. Structure of bivalent construct 2.
protein surface, surfactant effects may reflect disruption of critical protein-ligand interactions. Finally, the selectivities of the bivalent linked constructs for YopH versus other PTPs were not evaluated.

The primary intent of the work was to develop a quick and facile approach to the preparation of bivalent tethered inhibitors that could be executed without purification of reaction products. For a series of YopH-directed inhibitors, this was accomplished by generation and de-convolution of mixtures of linker segments using oxime chemistries. The methodology presented is characterized by its facility and ease of use and its ability to rapidly identify low micromolar affinity inhibitors. The generality of the approach may make it applicable to the development of bivalent inhibitors directed against other phosphatases.

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References and notes


Inhibitor concentrations of 10 μM and 100 μM were tested for inhibition of YopH hydrolysis of pNPP. The inhibitors were reconstituted in either in dry DMSO or in the pNPP assay buffer (25 mM Hepes buffer (pH 7.0–7.6), 50 mM NaCl, 2.5 mM EDTA, 10 mM DTT), preparing 1000-fold stock solutions for each of the two concentrations. A pre-reaction mix was prepared by combining 63 μl of the pNPP buffer, 5 μl of BSA stock solution at 5 mg/ml, 2 μl of the appropriate 100-fold stock for the test inhibitor, and 10 μl of the purified catalytic domain of YopH, prepared as indicated in Ref. 26 (corresponding to 0.25 μg of the enzyme), and was allowed to incubate at 37 °C for 15 min. The reaction was then initiated by adding 120 μl of the pNPP solution (1.5 mg/ml of pNPP (Novabiochem)) in pNPP buffer (25 mM Hepes buffer (pH 7.0–7.6)) to the reaction mixture, for a total reaction volume of 200 μl, with the final concentration of pNPP being 4.15 mM. The reaction mixture was incubated (8 min, 37 °C) with agitation on an Eppendorf shaker, and the enzymatic reaction was stopped by adding 20 μl of 12% H₃PO₄ in H₂O (V/V). Absorbance measurements at 405 nm were performed in triplicates and the average value for each was used for quantifying the extent of reaction. Control reactions for each set of tested compounds included a negative control tube lacking the enzyme to measure background, and an uninhibited positive control tube lacking inhibitor. For the negative control tube, 10 μl of pNPP buffer was added in place of YopH, and for the positive control tube 2 μl of DMSO or pNPP buffer was used in place of inhibitor. For measuring the inhibition, Triton X-100 was added to the pre-reaction mix at a final concentration of 0.01%, replacing 2 μl of the pNPP buffer with 2 μl of 1% Triton X-100. IC₅₀ values were determined by testing a range of inhibitor concentrations that blanketed the 50% inhibition level at the specified reaction endpoint. The IC₅₀ values were calculated by non-linear regression analysis of the dose-response curves.

Modeling was performed using ICM Chemist Pro software by Molsoft LLC. (http://www.molsoft.com).