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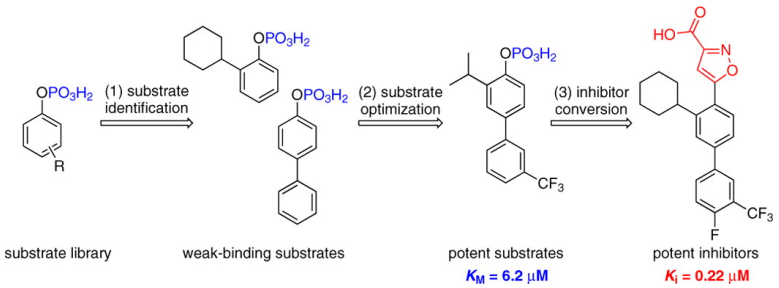

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Fragment-Based Substrate Activity Screening Method for the Identification of Potent Inhibitors of the *Mycobacterium tuberculosis* Phosphatase PtpB

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The pharmaceutical industry has invested considerable resources in the production and high throughput screening (HTS) of large compound libraries to identify drug-like molecules that interact with clinically relevant biomolecular targets. However, for intractable targets, such as protein tyrosine phosphatases (PTPs), this approach has had only limited success.[1] Alternative fragment-based approaches have therefore been developed wherein collections of low-molecular-weight molecules are screened to identify modest affinity binders that are subsequently optimized.[2]

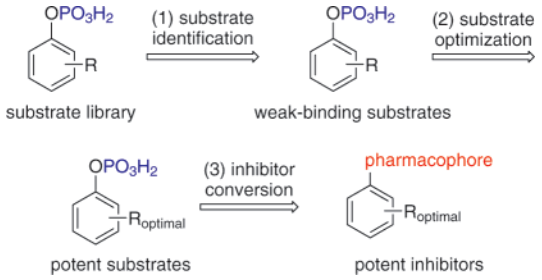
The PtpB protein tyrosine phosphatase encoded by *Mycobacterium tuberculosis* (*M. tb.*) is a promising target for new tuberculosis (TB) drugs.[3] *M. tb.* is one of the three deadliest international pathogens, yet the last new TB drug was introduced in the 1960s. Inhibitors against new targets are needed to shorten the 6 month course of the current standard treatment, cure multi-drug-resistant infections, and target persistent bacteria.[4] PtpB is a secreted virulence factor that functions within human macrophages.[5] Because a waxy coat impenetrable to many antibiotics surrounds the *M. tb.* bacilli, localization outside the bacterial cell makes PtpB an especially accessible therapeutic target. Here, we have used a new fragment-based approach termed substrate activity screening (SAS) to identify selective and low-molecular-weight PtpB inhibitors with submicromolar inhibitory activity.[6]

Our SAS method for identification of phosphatase inhibitors consists of three steps (Scheme 1): (1) a library of *O*-aryl phosphates with diverse, low molecular weight *O*-aryl groups is screened to identify phosphatase substrates using a simple spectrophotometric-based assay; (2) the identified *O*-aryl phosphate substrates are optimized by rapid analogue synthesis and evaluation; and (3) the optimized substrates are converted to inhibitors by direct replacement of the phosphate with known phosphate isosteres.

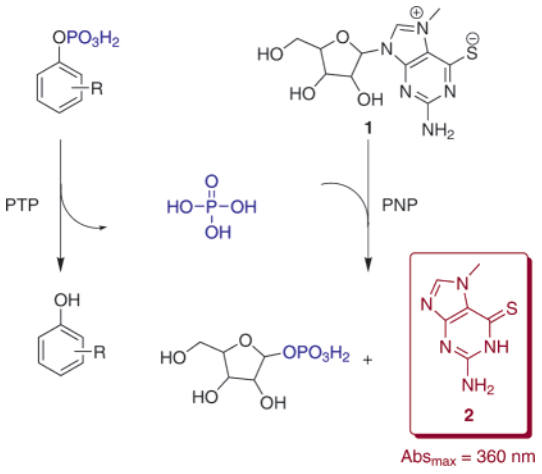
Step 1: A library of 140 *O*-aryl phosphates was prepared by solution-phase parallel synthesis from a diverse set of 140 phenol starting materials. The phenols were selected using hierarchical 2D extended connectivity analysis from thousands of commercially available phenols with molecular weights below 300 Da.[7] Each of the phenols was converted into the corresponding *O*-aryl phosphates using phosphorus oxychloride followed by aqueous workup.[8] All *O*-aryl phosphate library members were purified by preparative-scale reversed-phase HPLC and assayed for purity using LCMS and NMR spectroscopy.

The 140-member *O*-aryl phosphate library was then screened against PtpB using a fast and sensitive continuous spectrophotometric coupled assay method based upon the quantitation of inorganic phosphate (P_i) released into solution (Scheme 2).[9] Purine nucleoside phosphorylase (PNP) catalyzes the conversion of P_i and nucleoside **1** to ribose 1-phosphate and **2** (Scheme 2). Substrate turnover results in a spectrophotometric shift in maximum absorbance from 330 nm for substrate **1** to 360 nm for product **2**, and thus this assay can be used to continuously monitor the kinetics of P_i released by phosphatase-catalyzed hydrolysis of *O*-aryl phosphate substrates. This coupled assay was adapted for screening in 96-well plates and spectrophotometric plate readers to enable high throughput screening of the *O*-aryl phosphate library.

Scheme 1. Identification of Phosphatase Inhibitors by Substrate Activity Screening



Scheme 2. Spectrophotometric Method for Detection of Phosphatase Substrates



Importantly, our substrate-based assay eliminates all false positives commonly found in HTS assays, including false positives due to irreversible inactivation of the protein target, protein precipitation, and aggregation of putative inhibitors. Because inhibition of PNP by a putative phosphatase substrate would lead to false negatives in our assay, as a control, a subset of substrates was evaluated for inhibition of PNP and none were found to inhibit PNP at all concentrations examined.

Step 2: Screening the initial library of 140 *O*-aryl phosphates yielded several promising fragment substrates with greatly improved K_M values relative to the standard assay substrate *p*-nitrophenyl phosphate **9** (Figure 1 contains a subset of the initial fragment results). Of note, the biphenyl scaffold **5** is regarded as a "privileged

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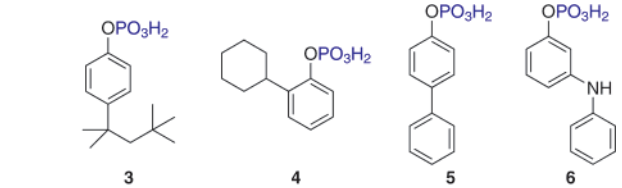


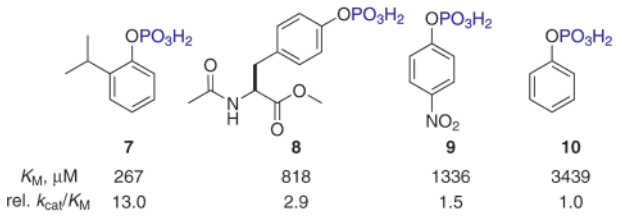


Figure 1. Selected initial substrate hits obtained against PtpB.

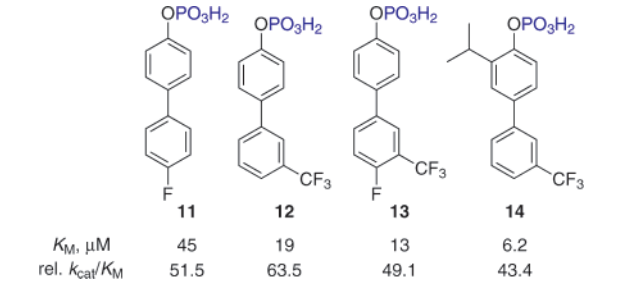


Figure 2. Selected hits from focused library of biphenyl phosphates.

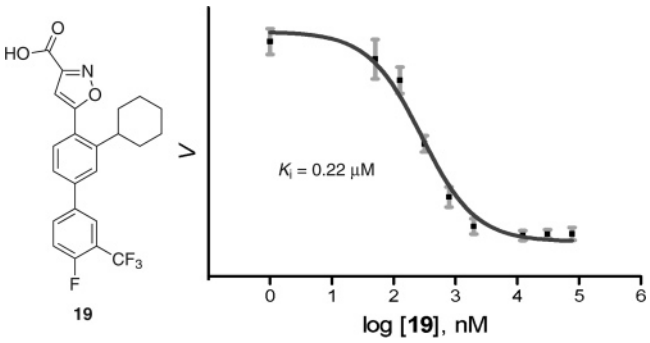


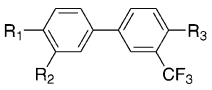
Figure 3. Plot of averaged initial rate versus log [**19**] used to determine the averaged K_i for inhibitor **19**.

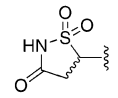
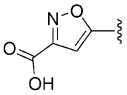
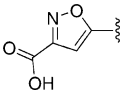
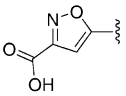
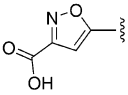
structure" with drug-like properties, and therefore it was the focus of further optimization.[10]

To explore the substrate activity relationships (SAR) of the biphenyl fragment **5**, a small, focused library (45 members) of biphenyl phosphates was synthesized and screened against PtpB. Determination of the K_M values provided clear SAR as represented by the select substrates shown in Figure 2. The *m*-trifluoromethyl-substituted biphenyl phosphate **12** showed a >4-fold decrease in K_M relative to substrate **5**. Likewise, the *p*-fluoro-substituted derivative **11** conferred a ~2-fold decrease in K_M. Importantly, merging these two substituents provided substrate **13** with >6-fold decreased Michaelis constant. Finally, the 2-isopropyl substituent from the initial fragment library (**7**, Figure 1) was merged with the disubstituted biphenyl hit **13** to provide substrate **14** with >13-fold improved apparent affinity.

Step 3: The final step of this method involves direct conversion of the optimal substrates to inhibitors by replacement of the phosphate functionality with a phosphate isostere. Introducing the phosphate isostere at this later stage greatly facilitates optimization efforts because phosphate substrates are inherently more straightforward to prepare than corresponding inhibitors containing phosphate mimetics.

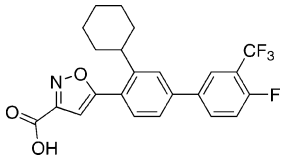
Table 1. Inhibitors of *M. tb.* PtpB



	R_1	R_2	R_3	K_i, μM[a]
15		-H	-H	8.80 ± 0.11
16		-H	-H	2.50 ± 0.26
17			-H	0.85 ± 0.09
18			-F	0.50 ± 0.02
19			-F	0.22 ± 0.03

[a] K_i values represent the average of at least six independent measurements and at least two enzyme concentrations.

Table 2. Selectivity of Inhibitor **19** against a Panel of PTPs



	PtpB	PtpA	VHR	TC-Ptp	CD45	LAR
K_i, μM	0.22 ± 0.03	>50	>50	>50	7.70 ± 0.31	21.4 ± 0.78
selectivity		>225	>225	>225	35	98

A variety of phosphate isosteres have been reported and could be introduced in place of the phosphate,[11] but many contain at least two acidic sites and lead to inhibitors with poor cellular permeability.[12] We therefore selected two promising monoacidic phosphate isosteres, isothiazolidinone[13] and isoxazole carboxylic acid,[14] which have effectively been used in PTP1B inhibitors with good cell permeability and activity.

A series of inhibitors was prepared by modified literature procedures (see Supporting Information), and K_i values against PtpB were determined using the standard inhibition assay for phosphatases with *para*-nitrophenyl phosphate (*p*NPP) as the chromogenic substrate.[15] The assay was performed with 0.025% Triton-X 100 detergent, and inhibition was found to be independent of both enzyme and detergent concentrations. Additionally, the K_i curves did not display any unusual steepness[16] (see Figure 3 for an initial rate plot for the most potent inhibitor). Together, these controls demonstrate the observed inhibition is specific and not due to compound aggregation or micelle formation, which are common assay artifacts.[16]

Compounds **15** and **16** allow direct comparison of the selected pharmacophores. Isothiazolidinone **15** has a K_i of 8.8 μM as a racemic mixture, while isoxazole carboxylic acid **16** has a K_i of 2.5 μM (Table 1). Due to the modest increase in potency, the

isoxazole carboxylic acid isostere was chosen for the synthesis of all additional inhibitor analogues.[17]

Addition of the isopropyl group at R_2, which was observed to be a favorable element for binding in substrate **7**, provided inhibitor **17** with a K_i of 850 nM. Inhibitor **18** with the fluoro group at R_3 was prepared based upon SAR obtained from the small, focused biphenyl substrate library (Figure 2) and showed further increased potency, with a K_i of 500 nM. Finally, replacement of the isopropyl group of **18** with a cyclohexyl group decreased the K_i to 220 nM, as predicted from substrate **4** (Figure 3).

It has been shown previously that, for reversible difluorophosphonic acid inhibitors of PTP1, inhibitor K_i values correlate to substrate K_M values but not to k_{cat}/K_M values.[18] Indeed, our inhibitors exhibit the same trend, with good correlation between substrate K_M values and inhibitor K_i values for the isoxazole carboxylic acid series of inhibitors. Moreover, no correlation was observed between k_{cat}/K_M values and inhibitor K_i values (Figures 1 and 2). On the basis of transition state theory, these results are consistent with the selected phosphate mimetics binding as substrate rather than transition state analogues.[19]

Isoxazole inhibitor **19** with a K_i of 220 nM represents the most potent inhibitor of PtpB known in the literature.[20] In addition, inhibitor **19** showed good selectivity against a panel of mycobacterial (PtpA) and human PTPs (Table 2). Structural characterization and biological evaluation of PtpB inhibitor **19** are currently in progress. The small size of this compound (MW = 433 Da) leaves room for additional elaborations to improve its properties.

In conclusion, we have developed a new substrate-based fragment approach termed SAS for the identification of novel PTP inhibitors. Application of this method to *M. tb*. PtpB resulted in the development of inhibitor **19**, the most potent PtpB inhibitor reported to date.[20] Additionally, the method should be generally applicable to many different phosphatases.[21]

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Supporting Information Available: Complete experimental details, spectral data for all compounds described, and full author list for refs 4 and 13. This material is available free of charge via the Internet at http://pubs.acs.org.

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