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A Strategy to Discover Inhibitors of *Bacillus subtilis* Surfactin-type Phosphopantetheinyl Transferase

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Abstract

Surfactin-type phosphopantetheinyl transferases (Sfp-PPTases) are responsible for modifying type I polyketide and nonribosomal peptide synthases of prokaryotes and have been implicated in the activation of a variety of pathogen-associated virulence factors. As such, inhibitors of this enzyme class represent enticing leads for antibiotic development and can serve as tools in studies of bacterial metabolism. Currently, no small molecule inhibitors of Sfp-PPTase are known, highlighting the need for efficient methods for PPTase inhibitor identification and development. Herein, we present the design and implementation of a robust and miniaturized high-throughput kinetic assay for inhibitors of Sfp-PPTase using the substrate combination of rhodamine-labeled coenzyme A and Black Hole Quencher-2 labeled consensus acceptor peptide YbbR. Upon PPTase-catalyzed transfer of the rhodamine-labeled phosphopantetheinyl arm onto the acceptor peptide, the fluorescent donor and quencher are covalently joined and the fluorescence signal is reduced. This assay was miniaturized to a low 4 μ L volume in 1,536-well format and was used to screen the Library of Pharmacologically Active Compounds (LOPAC¹²⁸⁰). Top inhibitors identified by the screen were further characterized in secondary assays, including protein phosphopantetheinylation detected by gel electrophoresis. The present assay enables the screening of large compound libraries against Sfp-PPTase in a robust and automated fashion and is applicable to designing assays for related transferase enzymes.

INTRODUCTION

A key biosynthetic step in the formation of fatty acids, non-ribosomal peptides, and polyketides is the coupling of the respective monomeric precursor units to the carrier domains of synthases. The linker between a conserved serine residue of the carrier protein and the nascent polymer is a phosphopantetheinyl group, derived from coenzyme A (CoA). Phosphopantetheinyl moieties are attached as post-translational modifications via the catalytic action of phosphopantetheinyl transferases (PPTases). Within bacteria, PPTases are grouped into two classes, the AcpS-type and Sfp-type PPTases (AcpS-PPTase and Sfp-PPTase, respectively), based upon primary structure,¹ and are responsible for modifying carrier protein domains in all primary and secondary metabolic pathways. The AcpS-type is

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COMPETING INTERESTS STATEMENT

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named for *holo*-acyl carrier protein synthase and is typically associated with fatty acid biosynthesis,² Sfp-type PPTases, corresponding to the activator of surfactin production in *Bacillus subtilis*, are generally responsible for modifying type I polyketide and nonribosomal peptide synthases of prokaryotes.³

The essential nature of PPTases to primary metabolism (i.e. fatty acid, teichoic acid, and lipid A biosynthesis) makes them enticing targets for antibiotic development.⁴⁻⁸ Additionally, Sfp-type PPTases are responsible for activating biosynthetic pathways that manufacture a variety of pathogen-associated virulence factors. Among these compounds are toxins such as mycolactone from *Mycobacterium ulcerans*, siderophores such as vibriobactin from *Vibrio cholerae* or mycobactin from *Mycobacterium tuberculosis*, as well as the mycolic acids that form the waxy cell wall of *Mycobacteria*; indeed, the biosyntheses of these compounds are now considered attractive targets for drug design.⁹⁻¹¹ Given the obligatory nature of this modification to these pathways, we have been interested in studying the effects that chemical modulation of this target will have on the virulence and viability of these pathogens.

While it has been suggested that the druggability of PPTases has been investigated,⁴⁻⁸ there are only limited reports of inhibitor development against AcpS PPTase.⁴⁻⁶ Furthermore, there is a lack of a simple, robust assay for PPTase that could be applied in an automated manner to the screening of large compound libraries.¹² Existing assays have largely utilized radiolabeled substrates and involve cumbersome treatments and separation steps. A pair of reports on inhibitor development for AcpS-PPTase^{5, 6} presented a time-resolved fluorescence resonance energy-transfer (TR-FRET) based assay for the enzyme; however, no follow-up studies have since been disclosed. The TR-FRET method involved the use of expensive reagents and required multiple liquid handling and incubation steps, making it difficult to automate and scale up. Further, that format only generated end-point data, making it vulnerable to noise and false positives.¹³

To this end, we have recently described work to develop a FRET-based homogenous screen for PPTase activity that drew upon two recent developments in the field.¹⁴ Our prior work¹⁵ has established that fluorescently modified-CoA (mCoA) is readily utilized as a substrate by Sfp-PPTase, converting the *apo*-acceptor substrate to the thiol blocked or *crypto*-product. Separately, Yin *et al*¹⁶ reported the identification of a consensus peptide motif (YbbR, sequence DSLEFIASKLA) which was shown to serve as a carrier protein surrogate for Sfp, accepting the phosphopantetheinyl arm transfer upon its second-position serine residue (Fig. 1A). Further, because YbbR was isolated as a collection of N-terminal extensions to the serine-containing motif, it was expected that attachment of detection labels to that end would be tolerated by the PPTase. Thus, we developed a first-generation assay using fluorescein-5-isothiocyanate modified-YbbR (FITC-YbbR) **1a** and rhodamine-mCoA **2b** substrates and observed a decrease in fluorescence intensity of the fluorescein donor emission when **1a** and **2b** are conjoined into the product **4ab**.¹⁴ These initial studies demonstrated that this fluorescein/rhodamine assay performed satisfactorily in 96-well format, and possessed the capacity to characterize an enzyme inhibitor.

With this method, we have determined that none of the currently disclosed chemical matter is active against Sfp,¹⁷ thus necessitating a large-scale inhibitor screening campaign. To this end, we undertook a high throughput screening feasibility study, taking advantage of our access to HTS and chemical probe development technology being made available to the public by the Molecular Libraries and Initiative of the NIH Roadmap¹⁸. We detail the evaluation of this fluorescein/rhodamine assay in highly miniaturized settings, where it functioned with significantly reduced performance in the 1,536-well format tested. To overcome this, we redesigned the assay to incorporate a non-emitting dark quencher to the

peptide substrate. This modified assay was miniaturized to a low 4 μ L volume and was used to identify and characterize Sfp-PPTase inhibitors from the LOPAC¹²⁸⁰ collection of known bioactives.

MATERIALS AND METHODS

Chemicals and consumables

4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid sodium salt (HEPES-Na) and $MgCl_2$ were purchased from GIBCO (Carlsbad, CA) and Quality Biological Inc. (Gaithersburg, MD), respectively. 7.5% bovine serum albumin (BSA) solution, Nonidet P-40 (NP-40), Tween-20, glycerol, coenzyme A (CoA), adenosine 3', 5'-diphosphate sodium salt (PAP), and 2'-Deoxy-N6-methyladenosine 3', 5'-bisphosphate (MRS 2179) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO, certified ACS grade) was obtained from Thermo-Fisher Scientific (Pittsburg, PA). Medium binding black solid-bottom 384- and 1,536-well plates (assay plates), and 1,536-well polypropylene plates (compound plates) were purchased from Greiner Bio One (Monroe, NC), 384-well polypropylene V-bottom plates (compound storage) were from Matrix/Thermo Scientific (Hudson, NH), black flat-bottom Costar 96-well plates (assay plates) were from Thermo-Fisher Scientific, and skirted V-bottom 96-well polypropylene plates (compound storage) were from Genesee Scientific (San Diego, CA). An analog of the YbbR peptide [H-DALEFIASKLA-OH] containing an S2A point mutation to make it a non-processable version of the original sequence was synthesized and HPLC purified by the Tufts University Core Facility (Medford, MA).

Instrumentation and equipment

Compound management and library plate compression to 1,536-well format was performed with a Cybi-well fixed tip automated pipettor (CyBio, Jena, Germany). Compound transfers for screening used a Kalypsys Workstation equipped with a 1,536 pin tool (Kalypsys Systems, San Diego, CA) and reagent dispenses were performed with a Flying Reagent Dispenser (FRD, Beckman Coulter, Fullerton, CA). Detection was accomplished with a ViewLux high-throughput CCD imager (Perkin Elmer, Waltham, MA).

Liquid handling for the FRET-quench and protein phosphatetheinylation assays was performed with manual multichannel pipettors, and microplate detection accomplished with an HTS-7000 plus plate reader (Perkin Elmer). Gel electrophoresis was performed on a Multiphor-II horizontal electrophoresis unit (GE Healthcare, Piscataway, NJ) powered by a Fisher brand FB-600 power supply (Thermo-Fisher Scientific). Fluorescence gels were imaged with a Typhoon Trio laser-based scanner (GE Healthcare).

Enzymes and Fluorogenic Substrates

Sfp-PPTase was produced and purified as previously described.³ Stock solutions of the enzyme (20 mg/mL, 765 μ M) were stored at $-80^{\circ}C$ in 50 mM HEPES-Na, 300 mM NaCl, pH 8.0, and 30% glycerol. Fluorescein-5-isothiocyanate (FITC) coupled to the 11 residue consensus peptide motif (FITC-YbbR) **1a** was purchased from GL Biochem (Shanghai, China) and supplied as a yellow powder of >95% purity.

Figure 1B depicts BHQ-2-YbbR **1c**, which was prepared from 6-aminocaproate-terminated YbbR that was assembled by standard Fmoc-based solid phase peptide synthesis methods using an automated synthesizer (Applied Biosystems Pioneers, Foster City, CA) with 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) activation.^{19, 20} After removal of the resin from the synthesizer, BHQ-2-carboxylic acid (BHQ-2000, Biosearch Technologies Inc, Navato, CA) was activated with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) and allowed

to couple to the peptide overnight at room temperature. In the morning the peptide was cleaved from the solid support in a cocktail containing 4% H₂O, 2% Triisopropylsilane in trifluoroacetic acid, and purified by HPLC.

Rhodamine-mCoA **2b** was prepared as previously described.¹⁴ Briefly, 1.1 equivalents of rhodamine maleimide (Invitrogen Corporation, Carlsbad, CA) was reacted with 1 equivalent of CoA in 10 mM NaH₂PO₄, pH 7.4 and followed to completion by HPLC. After completion, the reaction mixture was extracted 3 times with an equal volume of dichloromethane. The aqueous phase was placed under vacuum for two hours, and then stored in ~1 mM solutions in 10 mM NaH₂PO₄, at -20°C.

Miniaturized Fluorescein/rhodamine FRET-Quench Assay

The buffer consisted of 50 mM HEPES-Na, 10 mM MgCl₂, 0.1% BSA and 0.01% Tween-20. The substrate was a mixture of 5 μM FITC-YbbR **1a** and 10 μM rhodamine-mCoA **2b**. The assay was initiated by dispensing 3 μL of enzyme in buffer into a 1,536-well assay plates. The assay plate was then covered and incubated at room temperature for 10 minutes, followed by a 1 μL addition of substrate mixture in buffer to start the reaction, for a final assay volume of 4 μL. The plate was then centrifuged at 1,000 rpm for 15 seconds and kinetic fluorescence data were collected every minute for 30 minutes on a ViewLux high-throughput CCD imager with FITC [E_x/E_m = 480(20)/530(20) nm] optics.

Rhodamine/BHQ-2 Dark Quench Assay

The buffer consisted of 50 mM HEPES-Na, 10 mM MgCl₂, 0.1% BSA and 0.01% NP-40. The substrate was a mixture of 5 μM rhodamine-mCoA **2b** and 12.5 μM Black Hole Quencher-2 YbbR (BHQ-2-YbbR) **1c**. The assay was conducted as described above with the fluorescence signal collected using standard BODIPY [E_x/E_m = 525(25)/598(25) nm] optics.

Model Inhibitor Studies: Substrate and Product Mimetics

A 50 mM initial stock solution of the YbbR peptide analog [H-DALEFIASKLA-OH] in DMSO was prepared and diluted (1:2.5, 8-points) down to 81.9 μM in a 384-well polypropylene V-bottom plate. The assay was performed in a 384-well plate by pipetting 27 μL of enzyme solution into a 384-well assay plate, followed by 4 μL of each dilution (n = 3). The plate was covered and incubated at room temperature for 10 min, followed by a 9 μL addition of substrate mixture (5 μM rhodamine-mCoA **2b** and 12.5 μM BHQ-2-YbbR **1c**) to start the reaction. The plate was then centrifuged at 1,000 rpm for 15 seconds, and an initial fluorescence read was collected on a ViewLux reader. The assay plate was removed from the reader, covered, incubated for 30 minutes at room temperature, and a second fluorescence read was performed.

50 mM stock solutions of CoA and PAP **3** in DMSO were prepared and diluted (1:3, 8-points) down to 68.6 μM in a 384-well polypropylene V-bottom plate. Similarly, a 10 mM stock solution of MRS 2179 in DMSO was prepared and diluted (1:2, 16-points) down to 305 nM. Using a CyBi-well, the compound dilution series were transferred in duplicates to a 1,536-well compound plate. CoA, PAP, and MRS 2179 were assayed in 1,536-well format as described in the qHTS protocol section.

Compound Library

The Library of Pharmacologically Active Compounds (LOPAC¹²⁸⁰, Sigma-Aldrich) of 1,280 known bioactives were received as 10 mM DMSO solutions and formatted as 1,536-well compound plates of 6 concentrations (1:5 dilution) at 5 μL per well. Additional details on the preparation of the compound library for quantitative high-throughput screening (qHTS) have been previously described.²¹

qHTS Protocol and Data Analysis

An FRD was used to dispense reagents into the assay plates. 3 μ L of reagents, consisting of enzyme (in columns 1 and 2 as the catalyzed control, 5 to 48 for compounds) and enzyme buffer (in columns 3 and 4 as the uncatalyzed control) were dispensed into 1,536-well assay plates. Compounds (23 nL) were transferred via Kalypsys pintool equipped with 1,536-pin array.¹² The plate was incubated for 10 min at room temperature, followed by a 1 μ L addition of substrate to start the reaction, for a final assay volume of 4 μ L. The plate was then centrifuged at 1,000 rpm for 15 seconds, and kinetic fluorescence data were collected on a ViewLux reader. The plate was then covered, incubated for 30 minutes, and a second fluorescence read was performed. Library plates were screened starting from the lowest and proceeding to the highest concentration with pin-tool wash steps interleaved. The data were then analyzed as previously described.^{12, 21, 22} For activity calculations, delta values were computed as the difference in fluorescence intensity between last and first time points. Percent activity was derived from the catalyzed, or neutral control, and the uncatalyzed, or 100% inhibited, control values, respectively, using in-house software (<http://ncgc.nih.gov/pub/openhts/>).

Re-test of Screening Actives

Candidate inhibitors selected for confirmatory testing were re-sourced as 10 mM initial stock solutions in DMSO or as powder from Sigma-Aldrich. The samples were then serially diluted (1:2, 22-points) row-wise down to 4.77 nM in a 384-well polypropylene V-bottom plate (leaving columns 1 and 2 empty for controls). Using a CyBi-well, the solutions were then transferred to columns 5 to 48 ($n = 2$ per dilution point) of a 1,536-well compound plate. The assay protocol for confirmation was identical to that described in the qHTS protocol section, with the first four columns of the 1,536-well compound plate being reserved for placement of controls. Pin-transfer of 23 nL of compound solution into 4 μ L of assay mixture resulted in final compound concentrations between 57.2 μ M and 27.3 pM.

FITC-Rhodamine and Gel Assays

These experiments were performed in the San Diego laboratory with inhibitor compounds that were resourced as solids from Sigma-Aldrich, dissolved as 10 mM stock solutions in DMSO, and further formulated as 1:3 dilution series over 12 points in full skirted 96-well polypropylene PCR plates, with top and bottom concentrations of 10 mM and 56.4 nM, respectively. These stocks were sealed with adhesive foil and stored at room temperature over CaCO₃ dessicant until use.

Compound activities were evaluated with the FITC-Quench assay as follows: 2.5 μ L of compound solution or DMSO were transferred to black half-well polystyrene plates (Costar), followed by 37.5 μ L of a 1.33X enzyme solution (16.62 nM Sfp-PPTase in a buffer containing 13.3 mM MgCl₂, 66.6 mM HEPES-Na, 1.33 mg/mL BSA), and the plates incubated at room temperature for 15 minutes. Reactions were initiated by the addition of 10 μ L of 5X substrate solution (50 μ M Rhodamine-mCoA **2b** and 25 μ M FITC-YbbR **1a** in 10 mM Tris-HCl pH 7.4); the plate was centrifuged for 1 minute at 3,500 RPM in a Marathon 8K microplate centrifuge (Thermo-Fisher Scientific), and the reactions continuously monitored (15 reads, 2 minute cycle time) in a HTS7000 plus microplate reader with standard fluorescein filters [$E_x/E_m = 485(20)/535(30)$]. Data were analyzed by subtracting the RFU value at read 10 from the value at read 1, and converted to % activity using a slope generated from the inhibited and uninhibited controls present each plate. These data were plotted and fit with GraphPad Prism version 5.00 (Graphpad, Inc, La Jolla, CA) using the four-parameter dose response equation with the ordinary (least squares) setting, with error bars and error values representing one standard deviation.

Gel-based assay

Compounds were evaluated by monitoring of fluorescence transfer to whole carrier protein substrates via polyacrylamide gel electrophoresis.¹⁵ Actinorhodin ACP was cloned from genomic DNA of *Streptomyces coelicolor* A(3)2 by initial amplification using Pfu DNA polymerase with primers 5'AATGGCAACCCTGCTGACCACCGACGATCTG (ActACP_F) and 5'TCATGCCGCCTCGGCAGTGCGCCGTTGATC (ActACP_R) into pET28b and expressed as a hexahistidine-tagged product using standard protocols. We elected to use the acyl carrier protein of the actinorhodin biosynthetic pathway (ActACP) as a protein substrate given its small size, high recombinant yield when expressed in *E. coli*, and facile applicability to native polyacrylamide gel electrophoretic techniques. The final protein preparation was concentrated to 10 mg/mL, as determined using the Bradford assay with a BSA/lysozyme mixture as a standard, diluted with an equal volume of 50% glycerol, and stored in 1 mL aliquots at -80°C . The top 8 compound concentrations from the stock plates generated during evaluation with the FITC-Rhodamine assay were used in the present protein phosphopantetheinylation experiment. 1 μL of compounds or DMSO were transferred to unskirted polypropylene 96 well PCR plates, followed by the addition of a 2X enzyme solution (30 nM Sfp-PPTase in a buffer containing 50mM HEPES-Na, 10 mM MgCl_2 , 2 mg/mL BSA, pH 7.6); the plates were then incubated at room temperature for 15 minutes. Reactions were initiated by addition of 9 μL of a 2.1X reagent solution (20 μM Act-ACP, 20 μM Rhodamine-mCoA **2b**, 50 mM KOAc, pH 8.0) After 30 minutes incubation, the reaction was terminated by the addition of a 2X quench/load solution containing 20 mM EDTA, 20 mM Tris, 192 mM glycine, 4 M Urea, pH 8.6 (pH unadjusted).

The samples were resolved on a non-denaturing 20% polyacrylamide gel containing 20 mM Tris and 192 mM glycine using the Multiphor-II horizontal electrophoresis unit. The gels were imaged with a Typhoon Trio laser scanner with standard tetramethylrhodamine (TAMRA) settings using the 532 nm green laser for excitation and 580(30) emission filter with a 50 μm pixel size. The inhibition effect was scored visually, in a manner similar to that used by Dexheimer et al.²³, by comparing band intensities with control reactions that contained 20, 15, 10, 5 and 0 nM Sfp-PPTase (100, 75, 50, 25 and 0% activity, respectively) as a reference.

RESULTS

Fluorescein/Rhodamine FRET-Quench Assay

Initially, we evaluated the existing FRET-quench system¹⁴ (Fig. 1A) for signal strength and viability in a miniaturized setting. The assay was miniaturized from 96- to 1,536-well format and tested according to the protocol described above utilizing FITC optics for fluorescence detection. 3 μL of enzyme solution, or buffer serving as a no-enzyme control, were dispensed. The reaction was initiated by the addition of 1 μL substrate. The time-course data revealed a significant baseline drift of the uncatalyzed control samples (substrate and buffer only, open circles Figure 2A). The signal from the catalyzed reaction decreased ~51% during the 30 minute reaction monitoring. However, the signal from the uncatalyzed control wells decreased also by a significant value of ~24%, ultimately resulting in a low signal-to-background ratio and a poor Z' factor²⁴ of 0.27 (Fig. 2A). Attempts to stabilize this drift with increased concentrations of substrate or by dispensing the two substrates separately were unsuccessful (data not shown). This effect was also observed in 384-well format but was less dramatic and could be partially mitigated by running the reaction in low-binding plates (such an option was not available for the system-specific 1,536-well plates). Therefore we concluded that one or both substrates undergo irreversible binding and

aggregation to the plate walls, an effect exacerbated by the high surface-to-volume ratio of 1,536-well plates.

Rhodamine/BHQ-2 Dark Quench Assay Design

To address the baseline drift and to increase the available signal window, we converted the initial donor-acceptor system to a pure fluorescence-quenching format by replacing the former fluorescein donor label with a dark, non-emitting quencher molecule, BHQ-2^{25, 26} (Fig. 1B). This configuration was designed to spectrally match the emission of rhodamine, which in the new format serves as a fluorescence emission donor. Linking BHQ-2 to the YbbR peptide allowed us to maintain the rhodamine-mCoA **2b** component of the reagent set and continue to report enzyme activity as a decrease in signal (hereinafter referred to as Δ RFU) relative to rhodamine quenching upon product formation. Further, we anticipated that by shifting detection to a longer wavelength, the assay would be less sensitive to autofluorescence from compound library members, a prevalent occurrence in the blue region of the light spectrum.²⁷

Assay implementation

The new reagent, BHQ-2-YbbR **1c** was assembled by conventional solid phase peptide synthesis and its identity confirmed by mass spectral analysis. With the new reagent in hand, the uncatalyzed reaction was screened for baseline drift in the presence of different detergents before assembling the complete dark quench assay. Substrate concentrations of 5 μ M rhodamine-mCoA **2b** and 12.5 μ M BHQ-2-YbbR **1c** were chosen based on previously estimated limits of inner filter effect, solubility of BHQ-2, and the K_m ratio of the two substrates.¹⁴ The substrate mixture, prepared as a 4X stock, was dispensed (1 μ L) into plates containing 3 μ L buffer. The baseline drift, expressed as the difference in fluorescence signal between reaction initiation and 30 minutes later, was monitored in the presence of 0.01% NP40, 0.01% Tween-20, and 0.01% Brij-35, respectively (Fig. 3). Tween-20 (Δ RFU = 263 ± 30) and Brij-35 (Δ RFU = 239 ± 19) conditions both exhibited a twofold larger drift as compared to NP40 (Δ RFU = 100 ± 14). Thus, the signal was best stabilized by the addition of 0.01% NP40 to the buffer. The use of detergent in the assay buffer carried an additional benefit in suppressing the interference from promiscuous inhibitors acting by colloidal aggregation.²⁸

The above conditions were evaluated in a direct comparison run versus the fluorescein-rhodamine pair in 1,536-well format, with an Sfp-PPTase concentration of 50 nM. Only a minimal baseline drift of the uncatalyzed reaction control was observed with the new reagent set (Fig. 2B). The catalyzed reaction signal (black circles) decreased ~62% during the 30 minute reaction monitoring, versus only ~6% observed for the uncatalyzed (open circles) control, leading to a robust Z' factor of 0.76. This represented a dramatic improvement in assay performance when compared with the fluorescein-rhodamine system in 1,536-well format (Fig. 2A). Additionally, we note that only 50 nM enzyme was required to achieve ~62% signal change in the catalyzed reaction when using the Dark Quench reagent set, while double that concentration of PPTase was needed in the fluorescein-rhodamine assay to observe a smaller ~51% change. Utilization of NP40 instead of the originally used Tween-20 in the fluorescein-rhodamine FRET-quench assay did relieve some of the uncatalyzed reaction drift (similar to the trend observed with the rhodamine-BHQ2 Dark Quench pair in Fig. 3, data not shown) but the use of this detergent did not lead to a significant improvement in the signal increase (catalyzed rate) or signal-to-background ratio of that original reaction. Thus, aside from the issue of baseline stability, the improved donor-quencher format still yielded a superior signal-to-background.

These improvements in assay performance allowed us to optimize the dark quench system further in an effort to decrease enzyme concentration. We tested the reaction rate as a function of Sfp-PPTase concentration. Enzyme concentrations of 15, 50, and 100 nM were examined, with fluorescence data collection every 5 minutes over a 30 minute interval. Figure 4 shows the reaction progress of each enzyme concentration and the Z' factor calculated at each time-point. All enzyme concentrations tested exhibited robust assay performance, with Z' factor values at 30-minute reaction of 0.72, 0.84, and 0.74, respectively. Based on these results, an enzyme concentration of 15 nM was chosen for the 1,536-well screen. At this concentration, the 30 minute kinetic window was chosen to balance the need to run the reaction under initial-rates regime, at the lowest enzyme concentration possible and at minimal substrate conversion, and the desire for robust fluorescence intensity change of the catalyzed vs. uncatalyzed reactions, giving an acceptable Z' score. In the final screening mode, the 30-minute kinetic data were collected in a discontinuous manner¹² where, rather than measure fluorescence continuously for 30 minutes, we collected only two data points per well: the first read performed immediately after addition of the substrate reagent, and the second after 30 minutes of reaction. The net result from such implementation is that each plate spends a minimal amount of time inside the detector, thereby allowing a maximal screening throughput.

With the basic assay parameters established, enzyme and substrate reagent components were tested for stability. Fresh enzyme and substrate solutions were prepared at time zero and stored at 4°C. At selected time points (0, 0.5, 1, 2, 4, and 21 hours), the reagents were tested by running the enzymatic assay in 1,536-well plates (*vide supra*). Excellent reagent integrity was noted for at least 21 hours of storage (Fig. 5), indicating that an unattended overnight screening using the present combination of enzyme and substrate was feasible.

Model Inhibitors: Substrate and Product Mimetics

In the absence of known inhibitors of Sfp-PPTase, we validated the assay by using several molecules which represented either components of the native reaction or were analogues thereof. This collection included CoA itself, representing a competitive substrate versus rhodamine-mCoA; MRS 2179, a competitive inhibitor versus CoA; a YbbR analogue peptide [sequence H-DALEFIASKLA-OH], unprocessable due to mutation of the acceptor serine residue; and PAP 3, one of the reaction products. Evaluation of these compounds with the developed conditions is presented in Figure 6. The CoA substrate and its analogue MRS 2179 yielded the strongest inhibition (IC_{50} values of 4.2 μ M for CoA and 7.7 μ M for MRS 2179, respectively), while the effect of the PAP 3 product was weaker (IC_{50} value of 14.6 μ M). Unsurprisingly, the point-mutant version of YbbR was weakly recognized by the enzyme, yielding an IC_{50} value of 616 μ M. We note the IC_{50} value obtained for PAP 3 in the present assay is within the error of measurement to that observed in the fluorescein-rhodamine assay investigated earlier.¹⁴

qHTS of LOPAC¹²⁸⁰ Collection

Following this validation, the dark quench system was screened against a 6-point dilution series of the LOPAC¹²⁸⁰ compound library with final compound concentrations ranging from 18.3 nM to 57.2 μ M. At the end of the screen, data were analyzed, with top hits presented in Table 1. Importantly, the Z' factor remained nearly constant throughout the experiment, with an average value of 0.73, indicating a stable assay (Fig. 7A). In total, 163 samples exhibited inhibitory activity which tracked with sample concentration (Fig. 7B), with a small fraction, totaling 24 compounds, exhibiting complete concentration-response curves and 139 samples displaying incomplete responses (either partial curve without an upper asymptote or a single top-concentration activity point). The complete screening results are available in PubChem (bioassay identifier 1490).

Follow up screening and secondary assays

From these hits, nine compounds possessing complete concentration responses and lacking obvious highly reactive functionalities, and one partial-response compound (mitoxantrone **5**), were selected for follow-up testing in a set of secondary assays to validate the biochemical activity with Sfp-PPTase observed in the screen. This collection of experiments included a retest of the material in the donor-quencher screening assay on site at the screening center, followed by resourcing of the substances and evaluation in the FRET-quench screen at the San Diego laboratory. Afterward, an orthogonal enzymatic assay utilizing whole acyl carrier protein as a substrate and following the product formation by polyacrylamide gel electrophoretic separation was used as a secondary screen.¹⁵ As seen in Table 1, 6 out of the 9 full-response inhibitors maintained remarkably similar IC₅₀ values when tested across the different assay platforms and at different sites. From the three compounds which failed to confirm, mitoxantrone **5** was identified in the primary screen as being a partial-response weak active, and as such its failure to reproduce was not surprising. The variable assay results observed with BAY 11-7085 **6** and benserazide **7**, combined with the structural features of these molecules (vinyl nitrile and polyphenol, respectively), indicate that these hits may be unstable under the assay conditions and can thus be considered likely false positives or too reactive for further development.

DISCUSSION

As a first step toward identifying new inhibitor scaffolds of Sfp-PPTase, we attempted to miniaturize our recently described FRET-Quench assay using the FITC-YbbR **1a** and rhodamine mCoA **2b** acceptor (Fig. 1A).¹⁴ However, this method did not perform satisfactorily in 1,536-well format due to the overall weak signal difference and drift in the uncatalyzed control (Fig. 2A), the latter being attributed to the strong binding and aggregation of the FITC-YbbR **1a** reagent to the microtiter plates.

To circumvent this shortcoming, we embarked to overhaul the assay and address the following aspects. First, we wished to convert the assay from a FRET-quench format to a simple donor-quencher output. Doing so would eliminate the optical crosstalk effect where the fluorescence acceptor is partially excited at the wavelength used to excite the donor and produces a small amount of tail-band emission, interfering with the donor signal being monitored. By implementing the simple donor-quencher format, the crosstalk effect is eliminated, as there is only one emitting species present in the assay. Second, we considered it advantageous to red-shift the reporter signal by utilizing a rhodamine fluorophore that excites/emits at longer wavelengths in order to avoid the fluorescein spectral region, where approximately 0.2% of a diverse compound collection has been shown to absorb or emit light.²⁷ Third, in realizing these reagent modifications, we aimed at placing the quencher onto the YbbR peptide to allow the use of the new substrates at concentrations which better reflect the K_m ratio of the native CoA and YbbR substrates. By keeping rhodamine attached to CoA and labeling the YbbR peptide with a non-emitting BHQ-2 (Fig. 1B), we were able to re-design the assay to meet all three criteria set above with only a single reagent modification.

In practice, this new assay system was greatly improved, with a stronger signal due to the more complete fluorescence quenching within the rhodamine-BHQ-2 product (Fig. 2B). This, in combination with the stability of the no-enzyme control, allowed a reduction of Sfp-PPTase level to a nanomolar concentration (Fig. 4), sensitizing the assay to inhibition and leading to significant savings of enzyme. The combination of having a low enzyme concentration and substrates used at concentrations close to their K_m ratio resulted in a more sensitive screening assay. The red-shifted nature of the assay detection combined with the micromolar concentration of rhodamine fluorophore was expected to reduce the number of

fluorescent compounds capable of interfering with the signal, thereby effectively lowering the false-positive burden on the screen. Furthermore, any compounds interfering in this manner can be easily identified because we perform the screen by monitoring reaction progress in kinetic mode. Given the limited number of sample handling steps, this is easily accomplished without limiting the throughput of the screen after complete robotic automation.

Before testing the present assay against a compound library, we assessed its ability to report on model inhibitors. The profiles observed for molecules native to the Sfp-PPTase system, such as unlabeled CoA (acting as a substrate competing with its labeled counterpart) and the reaction product PAP **3**, served as an initial proof of principle (Fig. 6). Further, we demonstrated that the enzyme is inhibited by a CoA-like molecule, MRS 2179. Lastly, as expected from changing the key serine residue to an alanine, the mutated YbbR peptide, exhibited only a minimal effect on the reaction. While we had anticipated that this peptide might serve as a representative inhibitor competitive with respect to YbbR, it should not be overlooked that deletion of a hydrogen bond donor/acceptor pair from such a minimalized motif may cause structural perturbations that significantly disrupt the peptide's binding capacity for Sfp-PPTase.

With the assay in hand, we proceeded to screen the LOPAC¹²⁸⁰ collection. The LOPAC¹²⁸⁰ represents a diverse makeup of bioactive compounds and approved drugs and is an excellent starting point to assess an assay's viability, identify preliminary inhibitor structures, and gauge the hit rate. To ensure a high quality screen, we performed the assay in concentration-response mode. In this format, the screen performed outstandingly with a stable signal window, and a high and consistent Z' factor. As anticipated from red-shifting the reporting fluorophore, and with its micromolar concentration, there was practically no fluorescent interference affecting the data analysis. The only library compounds which affected the initial fluorescence read by values greater than three standard deviations from the median were the known fluorescent drug idamycin^{27, 29} and the intensely blue-colored Reactive Blue 2.³⁰ The identification of strong inhibitors possessing complete concentration-response curves, as well as a number of partial-response weak inhibitors demonstrated the ability of the present assay to respond to inhibitors of varying potencies expected to be found in diverse compound collections.

It is not unusual for a primary screening assay to be sensitive to both genuine enzyme inhibitors and to effectors acting by format- or assay technology-dependent mechanisms irrelevant to the enzymology at hand. To further prioritize the screening hits detected herein, we subjected the top actives to two additional comparative tests. First, the re-sourced compounds were assayed in the initial fluorescein-rhodamine reagent format in 96-well plates, an example of an orthogonal confirmation assay.¹³ By maintaining the basic substrate composition but changing the reporter system, we wished to identify possible compounds which acted by nonspecifically disrupting the HTS reporters and confirm the activity of the newly resourced matter with a medium throughput technique. Further, we subjected the panel to analysis in an Sfp-PPTase enzymatic assay utilizing whole protein instead of the surrogate YbbR peptide. The excellent inter-assay concordance observed for the top inhibitors is indicative of the robustness of these different assay platforms and the similarity in their sensitivity, making them valuable as a panel to validate HTS actives. The concordance also reaffirms the general reliability of the new donor-quencher assay as a tool for early discovery of PPTase inhibitors.

The top active compounds found in the screen represented a diverse set. The most potent trial HTS active, the thiadiazole SCH-202676 **8** has been reported as a reversible allosteric agonist and antagonist of G-protein coupled receptors (GPCR).³¹ A recent mechanistic study

of this compound by Lewandowicz *et al.* highlighted the possibility that it may act on GPCRs via a thiol-modification pathway dependent on the presence of dithiothreitol in the test medium.³² Although additional studies into the mechanism of Sfp-PPTase inhibition by SCH-202676 **8** will be required, we note that our assay conditions do not contain reducing agents in any of the reagent formulations, and thereby would not be conducive to the mechanism advanced by Lewandowicz *et al.* This raises the possibility the SCH-202676 **8** may be the first true submicromolar inhibitor of Sfp-PPTase.

In addition to this compound, several low-micromolar hits deserve mention. 6-nitroso-1,2-benzopyrone (NOBP) **9** is a type of aromatic C-nitroso ligand capable of extracting zinc out of zinc-finger proteins such as HIV-1 nucleocapsid protein and poly(ADP-ribose) polymerase.³³ While it may be possible that in the present reaction NOBP **9** acts by binding the catalytic magnesium, this mechanism of action appears unlikely due to the thousand-fold excess of Mg²⁺ relative to the NOBP **9** concentration. Another hit, PD 404,182 **10**, was discovered as a phosphoenolpyruvate-competitive inhibitor of 3-deoxy-D-manno-octulosonic acid 8-phosphate (KDO) synthase and has been studied as a potential antibiotic against gram negative bacteria.³⁴ Its further development was hampered by its hydrophobic nature, the absence of crystal structure, and lack of *in vivo* activity.³⁵ Lastly, among the top actives were two molecules with extended ring systems: guanidinylnaltrindole di-trifluoroacetate (GNTI) **11** is a selective κ opioid receptor antagonist,³⁶ while sanguinarine **12** is a natural product with noted anti-proliferative and pro-apoptotic effects in some cancer cell lines.³⁷ Further characterization of the top hit compounds for *in vivo* activity is currently underway.

In conclusion, we have designed and implemented an improved homogeneous assay for monitoring the Sfp-PPTase activity in real time. The mix-and-read scheme yielded a robust performance in miniaturized 1,536-well format and was used in an HTS setting to discover what we believe are the first low- and sub-micromolar small molecule inhibitors of this enzyme. The simplicity and universality of principle should make this format applicable to designing screening assays for other PPTases and transferase enzymes that have been shown to accept fluorescent substrate analogues.

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REFERENCES

1. Lambalot RH, Gehring AM, Flugel RS, Zuber P, LaCelle M, Marahiel MA, Reid R, Khosla C, Walsh CT. *Chem Biol* 1996;3:923–936. [PubMed: 8939709]
2. Lambalot RH, Walsh CT. *J Biol Chem* 1995;270:24658–24661. [PubMed: 7559576]
3. Quadri LE, Weinreb PH, Lei M, Nakano MM, Zuber P, Walsh CT. *Biochemistry* 1998;37:1585–1595. [PubMed: 9484229]
4. Chu M, Mierzwa R, Xu L, Yang SW, He L, Patel M, Stafford J, Macinga D, Black T, Chan TM, Gullo V. *Bioorg Med Chem Lett* 2003;13:3827–3829. [PubMed: 14552789]
5. Gilbert AM, Kirisits M, Toy P, Nunn DS, Failli A, Dushin EG, Novikova E, Petersen PJ, Joseph-McCarthy D, McFadyen I, Fritz CC. *Bioorg Med Chem Lett* 2004;14:37–41. [PubMed: 14684293]
6. Joseph-McCarthy D, Parris K, Huang A, Failli A, Quagliato D, Dushin EG, Novikova E, Severina E, Tuckman M, Petersen PJ, Dean C, Fritz CC, Meshulam T, DeCenzo M, Dick L, McFadyen IJ, Somers WS, Lovering F, Gilbert AM. *J MedChem* 2005;48:7960–7969.
7. McAllister KA, Peery RB, Meier TI, Fischl AS, Zhao G. *J Biol Chem* 2000;275:30864–30872. [PubMed: 10903317]

8. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. *Nat Rev Drug Discov* 2007;6:29–40. [PubMed: 17159923]
9. Onwueme KC, Vos CJ, Zurita J, Ferreras JA, Quadri LE. *Progress Lipid Res* 2005;44:259–302.
10. Barry CE 3rd, Lee RE, Mdluli K, Sampson AE, Schroeder BG, Slayden RA, Yuan Y. *Progress Lipid Res* 1998;37:143–179.
11. Chalut C, Botella L, de Sousa-D'Auria C, Houssin C, Guilhot C. *Proc. Nat. Acad. Sci. USA* 2006;103:8511–8516. [PubMed: 16709676]
12. Michael S, Auld D, Klumpp C, Jadhav A, Zheng W, Thorne N, Austin C, Inglese J, Simeonov A. *Assay Drug Dev Technol* 2008;6:637–657. [PubMed: 19035846]
13. Inglese J, Johnson RL, Simeonov A, Xia M, Zheng W, Austin CP, Auld DS. *Nat Chem Biol* 2007;3:466–479. [PubMed: 17637779]
14. Foley TL, Burkart MD. *Anal Biochem*. 2009 in press, doi:10.1016/j.ab.2009.06.037.
15. La Clair JJ, Foley TL, Schegg TR, Regan CM, Burkart MD. *Chem Biol* 2004;11:195–201. [PubMed: 15123281]
16. Yin J, Straight PD, McLoughlin SM, Zhou Z, Lin AJ, Golan DE, Kelleher NL, Kolter R, Walsh CT. *Proc. Nat. Acad. Sci. USA* 2005;102:15815–15820. [PubMed: 16236721]
17. Foley, TL.; Young, BS.; Burkart, MD. The 233rd Natl Meeting of the Am Chem Soc; Chicago, IL. 2007;
18. Austin, CP.; Brady, LS.; Insel, TR.; Collins, FS. *Science*. Vol. 306. New York, N.Y: 2004. p. 1138–1139.
19. Wellings DA, Atherton E. *Meth Enzymol* 1997;289:44–67. [PubMed: 9353717]
20. Glover KJ, Martini PM, Vold RR, Komives EA. *Anal Biochem* 1999;272:270–274. [PubMed: 10415099]
21. Yasgar A, Shinn P, Michael S, Zheng W, Jadhav A, Auld D, Austin C, Inglese J, Simeonov A. *J Assoc Lab Automat* 2008;13:79–89.
22. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP. *Proc. Nat. Acad. Sci. USA* 2006;103:11473–11478. [PubMed: 16864780]
23. Dexheimer TS, Pommier Y. *Nat Prot* 2008;3:1736–1750.
24. Zhang JH, Chung TDY, Oldenburg KR. *J. Biomol. Screen* 1999;4:67–73. [PubMed: 10838414]
25. Simeonov A, Kulkarni A, Dorjsuren D, Jadhav A, Shen M, McNeill DR, Austin CP, Wilson DM III. *PLoS ONE* 2009;4:e5740. [PubMed: 19484131]
26. USA Pat, 7019129.
27. Simeonov A, Jadhav A, Thomas CJ, Wang YH, Huang RL, Southall NT, Shinn P, Smith J, Austin CP, Auld DS, Inglese J. *J Med Chem* 2008;51:2363–2371. [PubMed: 18363325]
28. Feng BY, Simeonov A, Jadhav A, Babaoglu K, Inglese J, Shoichet BK, Austin CP. *J. Med. Chem* 2007;50:2385–2390. [PubMed: 17447748]
29. Perez-Ruiz T, Martinez-Lozano C, Sanz A, Bravo E. *Electrophoresis* 2001;22:134–138. [PubMed: 11197162]
30. Marks BD, Qadir N, Eliason HC, Shekhani MS, Doering K, Vogel KW. *Assay Drug Dev Technol* 2005;3:613–622. [PubMed: 16438657]
31. Fawzi AB, Macdonald D, Benbow LL, Smith-Torhan A, Zhang H, Weig BC, Ho G, Tulshian D, Linder ME, Graziano MP. *Mol Pharmacol* 2001;59:30–37. [PubMed: 11125021]
32. Lewandowicz AM, Vepsäläinen J, Laitinen JT. *British J Pharmacol* 2006;147:422–429.
33. Rice WG, Schaeffer CA, Harten B, Villinger F, South TL, Summers MF, Henderson LE, Bess JW, Arthur LO, McDougal JS, Orloff SL, Mendeleyev J, Kun E. *Nature* 1993;361:473–475. [PubMed: 8429889]
34. Birck MR, Holler TP, Woodard RW. *J Am Chem Soc* 2000;122:9334–9335.
35. Sansom C. *Drug Disc Today* 2001;6:499–500.
36. Stevens WC, Jones RM, Subramanian G, Metzger TG, Ferguson DM, Portoghese PS. *J Med Chem* 2000;43:2759–2769. [PubMed: 10893314]
37. Ahmad N, Gupta S, Husain MM, Heiskanen KM, Mukhtar H. *Clin Cancer Res* 2000;6:1524–1528. [PubMed: 10778985]

Figure 1. Assay Principle

Panel A is the Fluorescein-rhodamine assay scheme. Apo-fluorescein-YbbR peptide **1a** is a fluorescently labeled acceptor peptide for Sfp phosphopantetheinyl transferase. Enzymatic modification of **1a** with the 4'-phosphopantetheinyl arm from rhodamine-mCoA **2b** generates the *crypto*-YbbR **4ab** FRET peptide and 3'-phosphoadenosine-5'-phosphate (PAP) **3** as products. Enzymatic activity is detected by observing the decrease of fluorescent emission from **1a** that is quenched in the FRET peptide **4ab**. Panel B is the Dark quench assay scheme. Rhodamine-mCoA **2b** and Black Hole Quencher-2 labeled acceptor peptide YbbR **1c** are used as substrates. Upon PPTase-catalyzed transfer of the rhodamine-labeled phosphopantetheinyl arm onto the acceptor peptide, the fluorescent donor and quencher are covalently joined in the reaction product and the fluorescence signal is reduced.

Figure 2. Assay Optimization

Panel A is the reaction progress of the uncatalyzed enzyme ($\circ$, $n = 64$) and catalyzed enzyme ($\bullet$, $n = 64$) at 100 nM using the FITC FRET Quench protocol in 1,536-well format (delta values were computed using the 30 minute and 1 minute time-point RFU). Panel B is the reaction progress of the uncatalyzed enzyme ($\circ$, $n = 64$) and catalyzed enzyme ($\bullet$, $n = 48$) at 50 nM using the Dark Quench protocol in 1,536-well format (delta values were computed using the 30 minute and 1 minute time-point RFU).

Figure 3. Effect of different detergents on the reaction progress of the uncatalyzed enzyme in the Dark Quench system

Fluorescent intensity signal trend of substrate mixture (5 μ M rhodamine-CoA **2b** and 12.5 μ M BHQ-2-YbbR **1c**) in the presence of 0.01% NP40 (■), Tween20 (○), or Brij35 (●) freshly dispensed in 1,536-well plates (n = 64/condition).

Figure 4. Sfp-PPTase Titration

Panel A is the reaction progress at 0 (□), 15 (▲), 50 (●), and 100 nM (■) of Sfp-PPTase (n = 64 wells concentration/time-point). Panel B is the Z'-factor 15 (■), 50 (□), and 100 nM (●) after the plate has been incubated for 5, 10, 15, 20, 25, and 30 minutes.

Figure 5. Reagent Stability

The left axis is the signal change of the of the uncatalyzed enzyme ($\circ$, $n = 64$) and catalyzed enzyme ($\bullet$, $n = 64$) at 50 nM using the Dark Quench protocol in 1,536-well format at 0, 0.5, 1, 2, 4, and 21 hours of reagent storage. The right axis shows the corresponding Z'-factor ($\diamond$).

Figure 6. Substrate and Product Mimetics

The percent inhibition activity of Coenzyme-A (unlabeled substrate, ●), MRS 2179 (substrate mimetic, ○), PAP (reaction product, ■), the unlabeled YbbR peptide (substrate mimic, ◆), and the YbbR analog peptide (substrate mimetic, ◇) containing a point mutation to make it a non-processable version of the original sequence.

Figure 7. LOPAC¹²⁸⁰ qHTS. Panel A is the screen performance represented by the reproducible high Z' factor as a function of screening plate number. Panel B is a compound activity plot of each LOPAC compound, with potential inhibitors positioned in the front (black solid dots ●), while the inactive samples are represented by open circles (○).

Table 1

HTS Actives

Values shown are IC₅₀'s in μ M for Screen (six-point LOPAC HTS utilizing the rhodamine-BHQ-2 dark quench system), Retest (22-point concentration response using the HTS assay), FITC (12-point concentration response using the fluorescein-rhodamine FRET quench assay), and Gel (eight-point concentration response using whole protein assay with SDS PAGE separation). NC = not calculated. ND = no data available.

HTS Active	Screen	Retest	FITC	Gel	HTS Active	Screen	Retest	FITC	Gel
	35	Inactive	38	Inactive		3.5	10	1.7	1.0

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HTS Active	Screen	Retest	FITC	Gel	HTS Active	Screen	Retest	FITC	Gel
 Bay 11-7085 6	2.2	9.8	19	Inactive	 sanguinarine Cl 12	1.3	3.4	4.9	3.1
 benserazide HCl 7	22	Inactive	NC	9.3	 guandryl-naltindole difluoroacetate 11	2.8	4.3	4.8	3.1

HTS Active	Screen	Retest	FITC	Gel	HTS Active	Screen	Retest	FITC	Gel

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6-nitroso-1,2-benzopyrone **9**

HTS Active	Screen	Retest	FTTC	Gel	HTS Active	Screen	Retest	FTTC	Gel
	2.2	6.0	2.8	3.1		4.0	6.6	ND	ND