High Throughput Screen Identifies Small Molecule Inhibitors Specific for Mycobacterium tuberculosis Phosphoserine Phosphatase*

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Background: Phosphoserine phosphatase (PSP) is an essential enzyme involved in l-serine biosynthesis.

Results: High throughput screen was performed to identify specific PSP inhibitors with activity against intracellular bacteria.

Conclusion: Validation of PSP as a drug target would lead to identification of scaffolds with a novel mechanism.

Significance: This is the first report demonstrating selective inhibition of M. tuberculosis PSP enzyme.

The emergence of drug-resistant strains of Mycobacterium tuberculosis makes identification and validation of newer drug targets a global priority. Phosphoserine phosphatase (PSP), a key essential metabolic enzyme involved in conversion of O-phospho-L-serine to L-serine, was characterized in this study. The M. tuberculosis genome harbors all enzymes involved in L-serine biosynthesis including two PSP homologs: Rv0505c (SerB1) and Rv3042c (SerB2). In the present study, we have biochemically characterized SerB2 enzyme and developed malachite green-based high throughput assay system to identify SerB2 inhibitors. We have identified 10 compounds that were structurally different from known PSP inhibitors, and few of these scaffolds were highly specific in their ability to inhibit SerB2 enzyme, were noncytotoxic against mammalian cell lines, and inhibited M. tuberculosis growth in vitro. Surface plasmon resonance experiments demonstrated the relative binding for these inhibitors. The two best hits identified in our screen, chlorobiciocin and rosaniline, were bactericidal in vitro, and inhibited M. tuberculosis growth in vitro, and killed intracellular bacteria in a dose-dependent manner. We have also identified amino acid residues critical for these SerB2-small molecule interactions. This is the first study where we validate that M. tuberculosis SerB2 is a druggable and suitable target to pursue for further high throughput assay system screening.

The abbreviations used are: TB, tuberculosis; PSP, phosphoserine phosphatase; HTS, high throughput screening; PSAT, phosphoserine aminotransferase; HAD, haloacid dehalogenase; HPSP, human PSP; IPTG, isopropyl β-D-galactopyranoside; SPR, surface plasma resonance; RU, resonance units; RPMI, Roswell Park Memorial Institute; MBP, maltose-binding protein; r.m.s.d., root mean square deviation.

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High Throughput Screen Identifies PSP Specific Inhibitor

TABLE 1

List of bacterial strains and plasmids used in the present study

| Strains          | Description                        | Reference            |
|------------------|------------------------------------|----------------------|
| \( H_3Rv \)      | Virulent strain of \( M. \) tuberculosis | ATCC 27294           |
| \( M. \) bovis BCG Danish | Vaccine strain against tuberculosis | A kind gift from Prof. Anil K. Tyagi |

| Plasmids           | Description                                      | Reference           |
|--------------------|--------------------------------------------------|---------------------|
| pET28b             | T7 based expression system used to generate NH2-terminus His_\( \_ \)-terminal proteins | Novagen             |
| pET-28h-serB1      | pET28b carrying serB1                           | This work           |
| pET-28h-serB2      | pET28b carrying serB2                           | This work           |
| pMAL-c2x           | E. coli tac based expression system used to generate NH2-terminal MBP-tagged proteins | New England Biolabs |
| pMAL-c2x-serB1     | pMAL-c2x carrying serB1                         | This work           |
| pMAL-c2x-serB2     | pMAL-c2x carrying serB2                         | This work           |
| pMAL-c2x-D341G serB2 | pMAL-c2x carrying D341G serB2                 | This work           |
| pMAL-c2x-D185G serB2 | pMAL-c2x carrying D185G serB2                | This work           |
| pET28b-S273A serB2 | pET28b carrying S273A serB2                    | This work           |
| pET28b-S188A serB2 | pET28b carrying S188A serB2                    | This work           |
| pET28b-V186Q serB2 | pET28b carrying V186Q serB2                    | This work           |
| pMAL-c2x-K318E serB2 | pMAL-c2x carrying K318E serB2                | This work           |
| pMAL-c2x-K361A serB2 | pMAL-c2x carrying K361A serB2                | This work           |
| pMAL-c2x-R365A serB2 | pMAL-c2x carrying R365A serB2                | This work           |
| pMAL-c2x-E197A serB2 | pMAL-c2x carrying E197A serB2                | This work           |
| pMAL-c2x-D187A serB2 | pMAL-c2x carrying D187A serB2                | This work           |
| pMAL-c2x-E214A serB2 | pMAL-c2x carrying E214A serB2                | This work           |
| pMAL-c2x-V191A-serB2 | pMAL-c2x carrying V191A serB2                | This work           |
| pGEX-4T1           | E. coli tac based expression system used to generate GST fusion proteins | GE Healthcare |
| pGEX-4T1-serB1     | pGEX-4T1 carrying serB1                       | This work           |
| pET-DUET-psP_cat   | pET-Duet1 vector carrying His_\( \_ \)tagged PsP cytosolic domain (PsP_cat) | A kind gift from Dr. Y. Singh |

**Note:** The table lists bacterial strains and plasmids used in the study, along with their descriptions and references. The table is used to identify novel SerB2 specific inhibitors. These identified new scaffolds were (i) structurally different from known PSP inhibitors, (ii) selective in their ability to inhibit SerB2 enzyme in comparison with human PSP (HPSP) enzyme, and (iii) inhibited \( M. \) tuberculosis growth in vitro in a dose-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Strains, and Growth Conditions**—Most of the chemicals used in the present study unless mentioned were purchased from Sigma-Aldrich. Various strains and plasmids used in the study are shown in Table 1. *Escherichia coli* strains XL-1 Blue and BL-21 (DE3, plysS) were used for cloning and expression studies, respectively. *M. tuberculosis H$_3$Rv* and *Mycobacterium bovis* BCG strains were used for growth inhibition and macrophage infection studies. Various *E. coli* and mycobacterial strains were cultured in LB and Middlebrook medium, respectively, as per manufacturer’s standard protocols. The antibiotics were used in the following concentrations: ampicillin (50 \( \mu \)g/ml), kanamycin (25 \( \mu \)g/ml), tetracycline (10 \( \mu \)g/ml), and chloramphenicol (34 \( \mu \)g/ml).

**Multiple Sequence Alignment and Phylogenetic Analysis of PSP Homologs**—Protein sequences of PSP homologs from various organisms were retrieved from the National Center for Biotechnology Information protein database. Multiple sequence alignment analysis was performed using the Clustal Omega (version 1.2.0) alignment tool and edited using GeneDoc (27). The evolutionary history was inferred using the neighbor joining method (28, 29).

**Construction and Validation of SerB1 and SerB2 Homology Models**—The best templates for homology modeling of SerB1 and SerB2 proteins were identified using Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) analysis in the Protein Data Bank. The homology models for SerB1 and SerB2 were built using Discovery Studios 2.5 (Accelrys). The built models were further refined with repetitive loop modeling.
and energy minimization studies. The refined homology models were finally validated with PROCHECK, Verify_3D, and Errat programs (30–32).

Expression and Purification of PSP Proteins—For expression studies, both serB1 and serB2 were PCR-amplified and cloned into either pET28b or pMALc2x or pGEX4T-1. Various active site point mutants of SerB2 enzyme were generated by two-step PCR using gene specific primers having the desired mutations. E. coli BL-21 (ADE3, plysS) transformed with either wild type or mutant constructs were grown in LB medium at 37°C. Protein expression was induced at the presence of varying concentration of either L-serine or O-phospho-L-serine. For identification of catalytically important residues, enzyme assays were performed using affinity chromatography using nickel-nitrilotriacetic acid or amylose resin, respectively, as per manufacturer’s recommendations. Purified proteins were visualized on 10% SDS-PAGE by Coomassie Brilliant Blue staining. The purified fractions were dialyzed, concentrated using Amicon Ultra-15 centrifugal units (Millipore), and stored as aliquots in enzyme storage buffer (50 mM Tris, pH 7.4, 100 mM NaCl, and 10% glycerol). Protein concentration was estimated using Coomassie Plus protein assay reagent (Thermo Fisher).

Steady State Kinetics of SerB2 Enzyme—To determine kinetic parameters for SerB2 enzyme, assays were performed in 25 μl of reaction volume (100 mM Tris, pH 7.4, 5 mM MgCl2, 5 mM DTT) using varying concentration of O-phospho-L-serine (20–300 μM) and 1 μM His6-SerB2 enzyme at 37°C for 10 min. The formation of inorganic phosphate (P,) in enzyme reaction was monitored by measuring absorbance at 630 nm using Quanti-chrome phosphate assay kit (Bioassay Systems) as per manufacturer’s recommendations. Purified proteins were visualized on 10% SDS-PAGE by Coomassie Brilliant Blue staining. The purified fractions were dialyzed, concentrated using Amicon Ultra-15 centrifugal units (Millipore), and stored as aliquots in enzyme storage buffer (50 mM Tris, pH 7.4, 100 mM NaCl, and 10% glycerol). Protein concentration was estimated using Coomassie Plus protein assay reagent (Thermo Fisher).

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Far Ultraviolet Circular Dichroism Studies—CD spectra of various proteins were recorded on a JASCO-J-815 spectropolarimeter using a 1-mm-path length cuvette with a scan rate of 10 nm/min and averaged over five scans. The raw CD data were converted into molar ellipticity (ΦM) as follows,

$$\Phi_M = (100*\Phi_{obs})/[d*C]$$

(Eq. 1)

where Φobs is the observed ellipticity (in degrees), d is the path length (in centimeters), and C is the protein concentration (molar). The ΦM of MBP was calculated and subtracted from the ΦM of MBP-SerB2 fusion protein to obtain molar ellipticity of free SerB2. ΦM was converted to mean residue ellipticity (ΦMRE) as follows,

$$\Phi_{MRE} = \Phi_M/(n - 1)$$

(Eq. 2)

where n is the total number of amino acids in the protein.

Determinant of Specificity of Primary Hits—The hits identified in our HTS were also evaluated for their ability to inhibit mycobacterial Ser/Tyr phosphatase (PstP, Rv0018c), alkaline phosphatase (Bangalore Genei, Bangalore, India) and HPSP enzyme (Calbiochem) in vitro. PstP and alkaline phosphatase inhibition studies were performed in the presence of 100 μM of any phosphopeptide (K-R-pT-I-R-R; Millipore) and 200 μM p-nitrophenyl phosphate, respectively, as per standard protocols. HPSP inhibition studies were performed in conditions similar to those standardized for SerB2 protein.

Surface Plasmon Resonance Studies—Surface plasmon resonance (SPR) experiments were performed at 25°C using BIACORE T200 (GE Healthcare). SerB2 was diluted to a concentration of 400 μg/ml in 10 mM sodium acetate buffer, pH 4.0. The protein was immobilized on CMS sensor chip by the use of amine coupling chemistry as per standard protocols. The free surface was blocked with 1M ethanolamine-HCl (pH 8.5) and washed with 50 mM NaOH to remove free SerB2. The immobilization levels ranged from 8,000 to 10,000 resonance units (RU). For binding studies drugs at concentration of 1 mM in running buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 0.005% Tween 20, 2% DMSO) were injected at a flow rate of 30 μl/min for 2 min over the immobilized protein or a reference surface without protein. The surfaces were then washed with the running buffer and regenerated twice using 10 mM glycine, pH 2.5.
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Cytotoxicity of Primary Hits—THP-1 (human acute monocytic leukemia) or Vero (green monkey kidney epithelial cell line) were cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% FBS. These cell lines were procured from the National Cell Repository of the National Centre for Cell Science (Pune, India). THP-1 monocytes were differentiated into macrophages by the addition of 30 nM phorbol 12-myristate 13-acetate. For cytotoxicity assays, cells were diluted to a density of 5 × 10⁶/ml in fresh medium, and 100 µl was aliquoted into 96-well flat bottom plates (Nunc). After 24 h of incubation at 37 °C, cells were overlayed with RPMI medium containing drugs (0.625–50 µM) along with DMSO control in triplicate wells. After 96 h postincubation, cellular viability was assayed by measuring lactate dehydrogenase activity in culture supernatants using Cytoxtox96 nonradioactive cytotoxicity kit as per manufacturer’s recommendations (Promega Corporation, Madison, WI). These assays were performed three independent times, and concentration causing 50% cytotoxicity (TC₅₀) was calculated.

Mycobacterial Growth Inhibition Assays—Minimum inhibitory concentration (MIC₉₀) against M. tuberculosis H₃₇Rv and M. bovis BCG for these primary hits was determined using standard procedures. Bacterial cells were incubated at 37 °C in the presence of varying concentration of drugs for 14 days. MIC₉₀ was determined as the lowest concentration of drug inhibiting visible growth. To determine whether the observed growth inhibition by SerB2 specific inhibitors was bactericidal or bacteriostatic, mycobacterial cultures were grown till an OD₆₀₀ use of 0.1 and subsequently exposed to drugs at 10 × MIC₉₀ concentration. For bacterial enumeration, samples were withdrawn at designated time points, and 100 µl of 10-fold serial dilutions were plated on MB-7H11 plates and incubated at 37 °C for 3 weeks. For intracellular killing experiments, THP-1 cells were seeded at a density of 5 × 10⁵/well in a 24-well plate (Nunc) in RPMI medium supplemented with 10% FBS. Macrophages were infected with bacteria at a multiplicity of infection of 1:10 in triplicate wells for 4 h, washed with antibiotic free RPMI medium, and overlaid with RPMI medium containing 200 µg/ml amikacin for 2 h. Subsequently, macrophages were washed twice with antibiotic free RPMI medium and overlaid with RPMI medium supplemented with 10% FBS. After 24 h postinfection, cells were overlaid with RPMI medium containing drugs at either 4 × or 16 × MIC₉₀ concentration for 4 days. For bacterial enumeration, macrophages were lysed by the addition of 1 × PBS, 0.1% Triton X-100, and the number of intracellular bacteria was determined by plating 100 µl of 10-fold serial dilutions on MB-7H11 plates.

Molecular Docking Studies—All programs used for molecular docking studies were from Suite 2012 from Schrodinger. SerB1 and SerB2 models and HPSP crystal structure (Protein Data Bank code 1L8L) were optimized for docking studies with protein preparation wizard. These structures were finally minimized by converging it to a root mean square deviation (r.m.s.d.) tolerance of 0.3 Å using the OPLS 2005 force field. Three-dimensional ligand structures were generated using LigPrep module. Ligands were fitted to SerB1 and SerB2 models and HPSP crystal structure using the GLIDE extra precision method (35). Images were obtained and visualized through the Maestro interface.

RESULTS

Bioinformatic and Homology Modeling Studies—The enzymes involved in L-serine biosynthesis are highly conserved in various mycobacterial species (Fig. 1A and Table 2). M. tuberculosis PGDH and PSAT enzymes catalyzing the first two steps of L-serine biosynthesis have been biochemically well characterized (13–15). Phylogenetic analysis among PSP proteins from various organisms revealed that PSP proteins from actinobacteria and helicobacter species were more similar to each other in comparison with enterobacterial and human PSP enzymes (Fig. 1B). As shown in Fig. 1B, both SerB1 and SerB2 proteins were distantly related to each other and HPSP enzyme, with which they shared an overall sequence similarity of 18 and 27%, respectively. Multiple sequence alignment among PSP enzymes from various microorganisms revealed that these proteins share an overall homology of 15% among themselves, and both SerB1 and SerB2 possessed HAD specific motifs (motifs I–III; Fig. 2A). In these enzymes, motif I DXXE motif II (S/T)XX and motif III KXX–30G(S)/D(S)XXX(D/N) are involved in coordination of the Mg²⁺ ion and phosphoprotein intermediate (17–20).

The best templates for homology modeling for SerB1 and SerB2 proteins were identified by PSI-BLAST analysis using Protein Data Bank. The closest homolog for SerB1 protein was PSP enzyme from Bordetella pertussis (Protein Data Bank code 3FVV) with 34% sequence identity, 41% query coverage (consisting only the HAD superfamily domain region), and an E-value of 7 e⁻¹⁶. We were unable to build full-length structure of SerB1 because homologous template with high sequence coverage was not available. The closest homolog for SerB2 protein was SerB protein from Mycobacterium avium (Protein Data Bank code 3P96) with 84% sequence identity, 99% query coverage, and an E-value of 0.0. The superimpositions of SerB1- and SerB2-modeled proteins over 3FVV and 3P96, respectively, resulted in backbone r.m.s.d. of 0.449 and 0.451 Å, respectively. The superimpositions of SerB1 and SerB2 models over HPSP crystal structure resulted in r.m.s.d. of 6.216 and 2.565 Å, respectively (Fig. 2B). These computational studies predicted 11 α-helices and 7 β-sheets for SerB1 domain region and 16 α-helices and 15 β-sheets for SerB2 protein (Fig. 2B). The Ramachandran plot, a measure of the stereochemical parameters of modeled structures, revealed that 87% and 92% of the amino acid residues were in the favored region for SerB1 and SerB2 built models, respectively (data not shown). In addition, Verify_3D and Errat scores for SerB1 model were 81.02 and 85.85%, respectively, whereas these values were 96.21 and 94.06%, respectively, for SerB2 model. Overall, these results suggested that both models were of acceptable quality and suitable for molecular docking studies. Molecular docking of O-phospho-L-serine using Discovery Studios 2.5 revealed that amino acid residues Asp-127 (motif I), Thr-234 (motif II), Lys-279, and Asp-302 (motif III) in the case of SerB1 protein; residues Asp-185 (motif I), Ser-273 (motif II), Lys-318, and Asp-341 (motif
III) in the case of SerB2 protein; and residues Asp-20 (motif I), Ser-109 (motif II), Lys-158, and Asp-179 (motif III) in the case of HPSP are part of their respective substrate binding pockets (Fig. 2C). In addition to these above mentioned critical interacting residues, docking studies also revealed that Val-186 and Ser-188 of SerB2 enzyme might also interact with O-phospho-L-serine.

**Expression and Purification of M. tuberculosis Phosphoserine Phosphatases**—To biochemically characterize PSP enzymes, pET28b-serB1 and pET28b-serB2 were transformed into BL-21 (DE3, plysS), and expression of recombinant protein in transformants was induced by addition of 1 mM IPTG. As shown in Fig. 3A, His6-SerB2 was expressed at high levels in soluble fraction and purified at levels >95% using nickel-nitrilotriacetic acid chromatography with a total yield of 8 mg/liter. However, we observed that expression of His6-SerB1 in E. coli transformants was very minimal and only detectable by immunoblot analysis using α-His6 antibody. As expected, both His6-SerB1 and His6-SerB2 migrated at their predicted molecular masses of 42.0 and 45.0 kDa, respectively, on 10% SDS-PAGE (data not shown). To achieve better expression, serB1 was also cloned in other prokaryotic expression vectors such as pMALc2x or pGEX4T-1. However, as observed in the case of His6-SerB1, expression of both MBP-SerB1 and GST-SerB1 in IPTG-induced transformants was very minimal and nondetectable in Coomassie Brilliant Blue-stained 10% SDS-PAGE (data not shown).

**Biochemical Characterization of SerB2 Enzyme**—To determine kinetic parameters for SerB2 enzyme, steady state kinetics was performed by varying O-phospho-L-serine concentration in the presence of 1 μM His6-SerB2 enzyme. Conversion of O-phospho-L-serine to L-serine by SerB2 followed Michaelis-Menten kinetics with a $K_m$ of 92.68 μM and $k_{cat}$ of 8.83 min$^{-1}$ (Fig. 3B). The catalytic efficient constant ($k_{cat}/K_m$) for SerB2 enzyme was 0.0952 min$^{-1}$ μM. These kinetic constants were observed to be lower in comparison with those obtained for PSP enzymes characterized from either *Hydrogenobacter thermophilus* ($K_m$ of 1.6 mM), *P. gingivalis* ($K_m$ of 2.0 mM and 2.6 mM for phosphoserine peptides), or *Pseudomonas aeruginosa* ($K_m$ of 207 μM) (26, 36, 37). We also observed that maximal SerB2 activity was observed in the initial 5 min and that inclusion of 0.01% Triton X-100 enhanced SerB2 activity by 15–20% (Fig. 3B).
High Throughput Screen Identifies PSP Specific Inhibitor

TABLE 2

The locus of genes for enzymes involved in L-serine biosynthesis in various mycobacterial genomes.

|        | PGDH               | SerB1          | SerB2          |
|--------|--------------------|----------------|----------------|
| PSP    | PGM                | PGDH           | SerB1          |
| spp.   | spp.               | spp.           | spp.           |
| M. tuberculosis | BCG_0548c | BCG_0548c | BCG_0548c |
| M. bovis    | BCG_0548c | BCG_0548c | BCG_0548c |
| MMAR_0814, MMAR_3747 |
| M. marinum | BCG_0548c | BCG_0548c | BCG_0548c |
| M. leprae  | BCG_0548c | BCG_0548c | BCG_0548c |
| MUL_3691  |
| M. ulcerans| BCG_0548c | BCG_0548c | BCG_0548c |
| M. africanum| BCG_0548c | BCG_0548c | BCG_0548c |
| M. smegmatis| BCG_0548c | BCG_0548c | BCG_0548c |
| M. xenopi | BCG_0548c | BCG_0548c | BCG_0548c |
| M. canettii| BCG_0548c | BCG_0548c | BCG_0548c |
| M. colombiense| BCG_0548c | BCG_0548c | BCG_0548c |
| M. intracellulare| BCG_0548c | BCG_0548c | BCG_0548c |

As expected, SerB2 displayed substrate preference for O-phospho-L-serine over O-phospho-L-threonine (Fig. 3D). We did not observe any Pi release even at 80 μM of O-phospho-L-threonine (Fig. 3D). The amino acid residues of SerB2 enzyme predicted to interact with O-phospho-L-serine were mutated, cloned into either PET28b or pMALC2x, and purified as His6- or MBP-tagged proteins. We observed that mutation of aspartic acid at position 185 and lysine at position 318 completely abolished SerB2 activity (Fig. 3E). As shown in Fig. 3E, mutation of aspartic acid at position 341, serine at position 273, and valine at position 186 abolished SerB2 activity by 80, 60, and 50%, respectively, as compared with wild type protein activity.

To examine the changes in secondary structural content (such as α-helix, β sheet, or random coil) of SerB2 upon amino acid changes, both wild type and mutant proteins were analyzed using far-UV CD spectroscopy between wavelength range of 195 and 250 nm. As shown in Fig. 3F, SerB2 showed characteristic spectra of a protein consisting of a mixture of secondary structure elements such as α helix and β sheet. We observed that mutant proteins S273A and D341G showed similar spectra as that of the wild type protein. However, D185G and K318E proteins showed decreased and increased secondary structural content, respectively, in comparison with that of the wild type protein (Fig. 3F). These data suggest that reduced enzymatic activity of S273A and D341G proteins was not due to changes in their secondary structures but could be due to their altered interaction with O-phospho-L-serine as predicted by molecular docking. However, a decrease in activity of both D185G and K318E proteins could be due to the combined effect of both altered structure and interaction with O-phospho-L-serine.

Optimization of Assay Conditions for HTS—To optimize the assay conditions for HTS to identify novel SerB2 inhibitors, the influence of various parameters such as cations and buffer pH on its activity was evaluated in saturating O-phospho-L-serine concentration. We observed that inclusion of divalent ions such as Mg2+ and Mn2+ significantly enhanced SerB2 activity in comparison with inclusion of Ca2+, Zn2+, and Fe3+ in the assay buffer. The optimum SerB2 activity was observed upon inclusion of 5 mM Mg2+ or Mn2+ ions in the assay buffer (Fig. 4A). In our assay conditions, increasing Fe3+ ion concentration in the buffer did not affect SerB2 activity, whereas significant reduction in activity was observed upon inclusion of Zn2+ ion in assay buffer in a dose-dependent manner (Fig. 4A). We also observed a slight decrease in SerB2 dephosphorylating activity upon increasing concentration of Ca2+ ion in the assay buffer (Fig. 4A). To determine pH optima for SerB2 enzyme, assays were performed in buffers of pH ranging from 6.0–9.0 and optimum activity was achieved at pH 7.5 (Fig. 4B). Therefore, the optimum conditions for HTS assays were 100 mM Tris, pH 7.5, 5 mM MgCl2, 5 mM DTT, and 0.01% Triton X-100. For further optimization and validation of HTS assays, SerB2 activity was determined in the presence of increasing concentration of known PSP inhibitors such as DL-AP3 and sodium orthovandate (26, 33, 34). We observed that both DL-AP3 and sodium orthovandate inhibited SerB2 activity by 50% at the concentration of 458 and 670 μM, respectively (data not shown). The IC50 values obtained for
these two inhibitors for SerB2 enzyme were comparable with those obtained for other PSP enzymes (26, 33, 34).

**HTS Assays and Identification of Inhibitors for SerB2 Enzyme**—In our preliminary screening, we determined the percentages of inhibition for each compound (belonging to either NCI diversity set or mechanistic set or natural product set) at 100 μM concentration, and the compounds that inhibited SerB2 activity by at least 50% were considered to be primary hits. In our initial assays, the majority of the compounds were inactive, whereas 21 compounds inhibited SerB2 activity by at least 50% in vitro (Fig. 4, C and D). We observed that SerB2 enzymatic activity was inhibited by >80%, 60–80%, and 40–60% in the presence of 5 compounds, 4 compounds, and 12 compounds, respectively (Fig. 4, C and D). However, in our repeat in vitro assays, only 10 of these 21 compounds inhibited SerB2 enzyme by at least 50% at 100 μM concentration. These 10 active scaffolds were structurally different from known PSP inhibitors (Fig. 5). As expected, these primary hits inhibited SerB2 activity in a dose-dependent manner with at least 50% inhibition observed at the highest concentration. As shown in Table 3, CID 4 and CID 8 were most potent in our in vitro SerB2 inhibition assays with an IC50 value of 4.12 ± 1.2 and 3.89 ± 1.2 μM, respectively. The IC50 values for remaining primary hits varied between 10 and 40 μM with CID 2 showing least inhibitory activity in our in vitro enzymatic assays (Table 3). To determine specificity of these primary hits, we next evaluated their ability to inhibit either *M. tuberculosis* PstP (Rv 0018c) or alkaline phosphatase enzyme. Both PstP and alkaline phosphatase were suitable control for these assays because they belong to different phosphatase families. We observed that CID 1, CID 5, CID 6, CID 7, CID 8, CID 9, and CID 10 exhibited similar inhibition patterns on both PstP and alkaline phosphatase enzymes (Table 3).

FIGURE 2. A, multiple sequence alignment among PSP proteins. Multiple sequence alignment among PSP proteins from various microorganisms was performed using Clustal Omega software. Highly conserved residues among PSP enzymes from various bacterial species are shaded in black, whereas light gray shading denotes a level of conservation among these proteins. The accession numbers for these proteins are EFC04663, *Staphylococcus aureus*; YP_177732, *M. tuberculosis* SerB1; EDN61796, *Saccharomyces cerevisiae* AAA97284, *E. coli*; ZP_18026828, *Vibrio cholerae* YP_005780390, *Helicobacter pylori*; NP_217558, *M. tuberculosis* SerB2; YP_002342576, *Neisseria meningitidis*; YP_004510496, *P. aeruginosa*; and NP_253647, *P. aeruginosa*.

B and C, superimposition of modeled structures of SerB1, SerB2, and HPSP (Protein Data Bank code 1L8L). B, the built SerB1 model (green), SerB2 model (pink), and HPSP (yellow) were superimposed and visualized using PyMOL. C, the binding site for O-phospho-L-serine in superimposed models is magnified. The critical amino acid residues of SerB1 (green), SerB2 (pink), and HPSP (yellow) predicted to interact with O-phospho-L-serine have been highlighted.

### Table 3

| Compound | IC50 (μM) |
|----------|-----------|
| CID 4    | 4.12 ± 1.2|
| CID 8    | 3.89 ± 1.2|
| CID 2    | >40        |
| CID 3    | >40        |
| CID 9    | >40        |
| CID 10   | >40        |

As expected, these primary hits inhibited SerB2 activity in a dose-dependent manner with at least 50% inhibition observed at the highest concentration. As shown in Table 3, CID 4 and CID 8 were most potent in our in vitro SerB2 inhibition assays with an IC50 value of 4.12 ± 1.2 and 3.89 ± 1.2 μM, respectively. The IC50 values for remaining primary hits varied between 10 and 40 μM with CID 2 showing least inhibitory activity in our in vitro enzymatic assays (Table 3). To determine specificity of these primary hits, we next evaluated their ability to inhibit either *M. tuberculosis* PstP (Rv 0018c) or alkaline phosphatase enzyme. Both PstP and alkaline phosphatase were suitable control for these assays because they belong to different phosphatase families. Alkaline phosphatase is a hydrolase responsible for removing phosphate group from various molecules including nucleotides, proteins, and alkaloids. PstP belongs to PP2C phosphatase family that strictly requires Mn2+ ion for binding. We observed that CID 1, CID 5, CID 6, CID 7, CID 8, CID 9, and
CID 10 failed to significantly inhibit PstP enzyme even at 100 μM concentration, whereas CID 2, CID 3, and CID 4 displayed equal inhibitory activity (~80–90%) for both SerB2 and PstP enzymes, in vitro (Fig. 6A). We observed that CID 1, CID 5, CID 6, CID 7, CID 8, CID 9, and CID 10 inhibited enzymatic activity of SerB2 and PstP enzymes by ~70 and 0–20%, respectively. We also observed that none of these inhibitors were able to inhibit alkaline phosphatase activity even at 100 μM concentration (Fig. 6A). We next performed SPR experiments to confirm binding of CID 1, CID 7, CID 8, CID 9, and CID 10 with SerB2 enzyme. The protein immobilization over the flow cell varied between 8,000 and 10,000 RU, and binding responses were recorded at 1 mM drug concentration. At this tested concentration, all the compounds showed increase in RU indicative of interaction with SerB2; however, the increase in RU varied for different compounds (Fig. 6B). We observed that CID 1, CID 7, and CID 8 displayed higher binding to SerB2 enzyme in comparison with CID 9 and CID 10 (Fig. 6B).

Cell Cytotoxicity, Antimycobacterial Activity, and HPSP Inhibition Studies—In our lactate dehydrogenase-based cell viability assays, we observed that primary hits CID 1, CID 5, CID 6, CID 7, CID 8, and CID 10 that specifically inhibited SerB2 enzyme in vitro were nontoxic to THP-1 macrophages even at 50 μM concentration (Table 3). Scaffolds such as CID 2, CID 3, and CID 4 that inhibited both PstP and SerB2 activity by >90% were highly cytotoxic to THP-1 macrophages with TC_{50} values of 5, 5, and 2.5 μM, respectively (Table 3). A similar pattern of cell cytotoxicity for these scaffolds was also observed in Vero cell lines (Table 3). Next, these inhibitors were also evaluated for their ability to inhibit mycobacterial growth in vitro. In our MIC_{99} determination assays, most of these compounds possessed modest activity (ranging from 2 to 25 μM) against both *M. tuberculosis* H_{37}Rv and *M. bovis* BCG Danish strains (Table 3). As shown in Table 3, CID 5, CID 6, CID 8, and CID 10 were less active against mycobacteria in vitro in our whole cell-based assays, which might be attributed to lower intracellular concentration of drugs because of (i) their poor penetration, (ii) their effluxing out by various pumps, or (iii) their modification by intracellular enzymes. The most potent inhibitors in our whole cell-based assays were CID 1 and CID 7, both of which displayed MIC_{99} values of 2 μM against both *M. tuberculosis* and *M. bovis* BCG in vitro.

Next we performed kill kinetics assays in vitro by exposing actively growing mycobacteria to 10× MIC_{99} concentration of either CID 1 or CID 7 or isoniazid. As shown in Fig. 6C, both CID 1 and CID 7 were bactericidal in their mode of killing with ~10-fold killing observed after exposure of mycobacteria for 3 days. In our time kill experiments, 7 days of exposure to CID 1 and CID 7 led to approximately ~80- and 20-fold killing, respectively (Fig. 6C). As expected, no significant growth inhi-
bition was observed in the presence of DMSO during the course of experiment (Fig. 6C). Because *M. tuberculosis* is an intracellular pathogen, we next evaluated the ability of both CID 1 and CID 7 to kill bacteria in THP-1 macrophages at either 4× or 16× MIC<sub>99</sub> concentration. In our macrophage experiments, both CID 1 and CID 7 were able to arrest *M. bovis* BCG replication in a dose-dependent manner. As shown in Fig. 6D, at 4 days after drug exposure, ~100- and 50-fold intracellular killing was observed in the presence of 16× MIC<sub>99</sub> concentration of CID 7 and CID 1, respectively.

Cross-reactivity of these inhibitors with the human homolog would be a major concern in further validation of SerB2 as a drug target. Therefore, we compared the ability of these active and noncytotoxic primary hits to inhibit HPSP enzyme *in vitro* at 100 μM concentration. As shown in Fig. 7A, both CID 7 and CID 10 were nonselective PSP inhibitors,
inhibiting both HPSP and SerB2 enzymes to a similar extent of 70% at 100 μM concentration. However, the rest of the compounds CID 1, CID 5, CID 8, and CID 9 were highly specific in their ability to inhibit SerB2 enzyme in comparison with HPSP enzyme even at 100 μM concentration (Fig. 7A). These results suggest that despite the presence of a human homolog, subtle differences exist between secondary structures of these two enzymes, which could be explored

### TABLE 3
Chemical properties and activities of SerB2 inhibitors identified in the study

| CID No. | NSC number | Molecular weight | IC$_{50}$ | MIC$_{90}$ | TC$_{50}$ (THP-1/Vero) | T-i values | GLIDE binding energy | kcal/mol |
|---------|------------|------------------|----------|-----------|------------------------|------------|----------------------|----------|
| 1       | NSC227186  | 697              | 16.84 ± 1.6 | 2.34 ± 0.4 | >50                    | >21        | -6.0                 |
| 2       | NSC104129  | 455              | 37.57 ± 1.8 | 9.37 ± 1.8 | >50                    | 5          | -4.9                 |
| 3       | NSC672904  | 354              | 11.68 ± 1.4 | 25         | 5                      | 0.2        | -4.5                 |
| 4       | NSC693172  | 414              | 4.12 ± 1.2  | 12.5       | 2.5                    | 0.2        | ND $^a$              |
| 5       | NSC71948   | 1171             | 25.73 ± 1.4 | 50 ± 14.3  | >50                    | >1         | -5.4                 |
| 6       | NSC204656  | 376              | 25.37 ± 2.4 | >200       | >50                    | ND         | ND                   |
| 7       | NSC93739   | 338              | 31.49 ± 1.4 | 2.34 ± 0.4 | >50                    | >21        | -5.14                |
| 8       | NSC76027   | 1058             | 3.89 ± 1.2  | 150 ± 28.8 | >50                    | >0.3       | -4.44                |
| 9       | NSC305798  | 4043             | 33.0 ± 1.2  | 125 ± 28.8 | >50                    | 0.25       | -5.0                 |
| 10      | NSC165701  | 2954             | 29.7 ± 2.8  | 200        | >50                    | >0.4       | -4.0                 |

$^a$ ND, not determined.

FIGURE 6. A, SerB2, PstP, and alkaline phosphatase inhibition by primary hits. SerB2, PstP, and alkaline phosphatase activity assays in the presence of primary hits were performed as described under “Experimental Procedures.” The values depicted in this panel are the means ± S.E. from three independent experiments. B, SPR experiments to confirm binding of inhibitors to SerB2 enzyme. The binding of CID 1, CID 7, CID 8, CID 9, and CID 10 was evaluated by SPR. The experiment was done in duplicate, and the data shown are representative of two separate experiments. The inset shows the sensorgram obtained upon passing various inhibitors over the SerB2 immobilized surface. C, time kill curves of CID 1, CID 7, and INH against mycobacteria in liquid cultures. Early logarithmic cultures of M. bovis BCG were exposed to either CID 1, CID 7, or isoniazid at 10× MIC$_{90}$ concentrations, and bacterial enumeration was performed by plating 100 μl of 10-fold serial dilutions on MB-7H11 plates at days 3 and 7 post-exposure. D, intracellular activity of CID 1 and CID 7 against bacteria in infected macrophages. Intracellular bacterial numbers in THP-1 macrophages after 4 days post-exposure to either CID 1 or CID 7 at 4× or 16× MIC$_{90}$ concentration were determined by lysing macrophages in 1× PBS, 0.1% Triton X-100 and plating 100 μl of 10-fold serial dilutions on MB-7H11 plates.
further for identification of inhibitors with more potency and specificity toward SerB2 enzyme.

**Molecular Docking Studies of Scaffolds on M. tuberculosis SerB2 Model Protein and HPSP Enzyme—**Molecular docking studies were performed for CID 1 (clorobiocin), novobiocin, CID 5, CID 7 (rosaniline), CID 8, CID 9, and CID 10 using SerB2 built model and HPSP protein as described under “Experimental Procedures.” The binding free energies for interaction of these inhibitors with SerB2 protein ranged from $-4.44$ to $-5.14$ kcal/mol, which is comparable with the binding energy for interaction of O-phospho-L-serine with SerB2 ($-6.89$ kcal/mol; Table 3). Because amino coumarins are clinically utilized antibiotics with tolerable toxicity, we were particularly intrigued by the ability of clorobiocin to inhibit SerB2 enzyme in vitro. This class of compounds that includes novobiocin, clorobiocin, and coumermycin is known to inhibit DNA topoisomerase and heat shock proteins by binding to their nucleotide binding pockets (38, 39). Interestingly, novobiocin, a compound structurally similar to clorobiocin, did not inhibit SerB2 activity even at 10-fold higher concentration in our in vitro assays (Fig. 7B). Molecular docking of a SerB2 built model with clorobiocin and novobiocin predicted that the difference in ability of these structural analogs to inhibit SerB2 enzyme could be attributed to (i) interaction of the Asp-341 residue of SerB2 enzyme with clorobiocin via hydrogen bond formation and (ii) better fit of the pyrrole ring of clorobiocin in comparison with the substituted amino group of novobiocin in the substrate binding pocket. In addition, Lys-361 and Arg-365 residues of SerB2 enzyme might interact with clorobiocin via hydrogen bond formation and Asp-187 and Glu-197 residues were observed to be closely associated (2.2 and 2.8 Å, respectively) with clorobiocin (Fig. 7, C and D). In concordance with our in vitro activity results, molecular docking of clorobiocin in HPSP revealed that Asp-179 (identical to Asp-341 in SerB2) is interacting with O-phospho-L-serine but not with clorobiocin (Figs. 2C and 7E). In addition, further analysis revealed that the conserved FDVDST motif forms a different secondary structure (right-handed helix in case of HPSP) as compared with...
SerB2 protein, which might block the accessibility of clorobiocin to the substrate binding pocket of HPSP (Fig. 7E).

Molecular docking studies revealed that CID 5, CID 7, CID 8, CID 9, and CID 10 also interact with critical Asp-341 and Asp-187 residues of SerB2 protein (Fig. 8, A–E). In addition to these interactions, CID 5 is also interacting with Val-186 and Glu-197 via hydrogen bond and salt bridge interactions. Molecular docking studies revealed that CID 7 might also interact with Glu-197 and Val-186 residues of SerB2 protein, and there might be some electrostatic interactions with Glu-214, because it resides in close proximity of 3.8 Å (Fig. 8B). As shown in Fig. 8C, we observed that CID 8 might also interact with the Lys-361 residue of SerB2 enzyme through π-cation interaction, whereas CID 9 might interact with Glu-194, Ser-188, and Val-186 residues of SerB2 protein through hydrogen bond formation (Fig. 8D). As shown in Fig. 8E, CID 10 might also be possibly interacting with Glu-197 and Glu-214 residues through electrostatic interaction because these two residues seem to be in close contact (2.23 and 1.99 Å, respectively). Despite the lack of structural similarities among these primary hits, these scaffolds possess a substructure that fits well in the SerB2 modeled protein. Molecular docking of CID 7 with HPSP crystal structure revealed that Asp-20, Asp-22, Asp-179, and Ala-71 might interact with rosaniline via hydrogen bond formation, and binding free energy for this interaction was $-5.7$ kcal/mol (Fig. 9B). In concordance with our in vitro activity assays, we observed that CID 5, CID 8, and CID 9 are not interacting with known HPSP critical residues, thereby explaining their inability to inhibit HPSP enzyme (Fig. 9, A, C, and D). Molecular docking of CID 10 with HPSP revealed that it might interact with Val-56 and Thr-182 residues via hydrogen bond formation and with Lys-158 through cation–π interaction (Fig. 9E). Even though Val-56 and Thr-182 of HPSP are not conserved with SerB2 enzyme, Lys-158 is a conserved active site residue between SerB2 and HPSP (as per pair wise sequence alignment studies).

Validation of SerB2 Small Molecule Interactions Predicted by Molecular Docking Studies—To validate SerB2-small molecule interactions predicted by molecular docking, Lys-361, Arg-365, Glu-214, and Asp-187 were mutated to alanine residue as described under “Experimental Procedures.” As shown in Fig. 10A, mutation of Asp-187 and Glu-214 reduced SerB2 activity by 50% as compared with wild type protein, whereas the enzymatic activity of K361A-SerB2, R365A-SerB2, and E197A-SerB2 were almost similar to that of wild type protein. Far-UV CD studies revealed that mutation of these amino acids did not significantly alter the folded state of these mutant proteins except for E197A-SerB2 where we observed increase in secondary structure content (Fig. 10B). These results suggest that
reduction in activity of D187A-SerB2 and E214A-SerB2 could be due to their altered interaction with O-phospho-L-serine (Fig. 10, A and B). In our enzymatic assays, we observed that mutation of Asp-187 and Asp-341 prevented efficient binding of CID 1, CID 5, CID 7, CID 8, CID 9, and CID 10 to the binding pocket of these mutant proteins (Fig. 10, C–H). We also observed that mutation of Glu-197 to alanine residue reduced the ability of CID 1, CID 5, and CID 7 to inhibit dephosphorylation activity of SerB2 enzyme. As shown in Fig. 10 (E and F), Val-191 and Lys-361 were important for interaction of SerB2 with CID 7 and CID 8, respectively. In concordance with our docking studies, we report that Val-186 is important for interaction of SerB2 with CID 9, whereas Glu-214 is critical for interaction of CID 7 and CID 10 with SerB2 enzyme (Fig. 10, E, G, and H).

DISCUSSION

The development of novel inhibitors for essential and conserved M. tuberculosis pathways is one plausible solution to shorten duration of TB chemotherapy and eradicate drug-resistant TB. The advent of new computational methods, combinatorial synthetic chemistry approach and whole cell- and target-based HTS assays, have led to identification of several antitubercular scaffolds that are currently being evaluated in different stages of clinical trial. Numerous studies have shown that M. tuberculosis strains deficient in enzymes involved in various amino acid biosynthetic pathways are compromised in their ability to infect mice in comparison with the ability of parental strain (40–42). In addition, several of these enzymes involved in amino acid biosynthesis are being currently explored for development of more potent antitubercular scaffolds (43–48). L-Serine biosynthesis is an attractive and unexplored anti-microbial drug target because L-serine is not only involved in protein synthesis but also acts as precursor for various cellular metabolites (15, 49–54). The enzymes involved in L-serine biosynthetic pathway are widely conserved across various mycobacterial species including Mycobacterium leprae, an organism that has undergone massive gene decay, thereby suggesting that these enzymes are indispensable and are essential for survival of bacteria in the host.

This is the first study where a detailed biochemical characterization of PSP homolog from M. tuberculosis has been performed. Phylogenetic and sequence alignment analysis revealed that both SerB1 and SerB2 proteins are distantly related to each other and share an identity of 18 and 27%, respectively, with HPSP enzyme. Multiple sequence alignment analysis revealed that HAD specific motifs responsible for Mg2+ ion binding, phosphoprotein formation, and stabilization are present in both SerB1 and SerB2. Despite several attempts, we were unable to express SerB1 in detectable amounts as either His6-tagged, GST-tagged, or MBP-tagged proteins. We observed that SerB2 had a substrate preference for O-phospho-L-serine over O-phospho-L-threonine and displayed lower kinetic constants in comparison with PSP enzymes previously characterized from H. thermophiles, P. gingivalis, and P. aeruginosa (26,
The optimum SerB2 activity was observed in the pH range of 7.0–8.5 and in the presence of 5 mM Mg\(^{2+}\) or Mn\(^{2+}\) ions. The activity of SerB2 enzyme was unaltered by the inclusion of Fe\(^{3+}\) ion in the assay conditions. However, inclusion of Zn\(^{2+}\) ion in the assay buffer abolished SerB2 enzymatic activity in a dose-dependent manner that might be attributed to disruption of the SerB2 secondary structure, thereby preventing binding of O-phospho-L-serine to its active site. We also observed reduction in SerB2 activity upon inclusion of Ca\(^{2+}\) ion in the assay buffer, which might be attributed to disruption of nucleophilic attack by the conserved Asp residue (motif I) on the phosphate group of O-phospho-L-serine as reported earlier in the case of HPSP (22). The activity of SerB2 enzyme was enhanced by 15–20% by inclusion of nonionic detergent in assay conditions that might be attributed to stabilization of SerB2 secondary structure. Molecular docking and in vitro enzymatic assays using purified mutant proteins revealed that amino acid residues Asp-185, Asp-187, Val-186, Ser-273, Lys-318, Glu-214, and Asp-341 are critical for SerB2 dephosphorylation activity.

Despite the importance for PSP enzymes in biosynthesis of L-serine, these enzymes have not been extensively studied as an antimicrobial drug target except for a report where dihydroquinolinone derivatives were shown to inhibit SerB enzymes from \textit{P. gingivalis} (55). In the present study, HTS was performed using 2300 compounds belonging to a library received from National Cancer Institute Developmental Therapeutic Program, and we identified 10 scaffolds that inhibited dephosphorylation activity of SerB2 enzyme. The interaction of some of these chemical entities with SerB2 protein was further confirmed using SPR studies. A subset of these primary hits inhibited \textit{M. tuberculosis} growth in vitro and displayed low cytotoxicity toward both THP-1 and Vero cell lines. The best two chemical entities in our primary hits, clorobiocin and rosaniline with a therapeutic index (\(T_i\), ratio of TC\(_{50}\)/MIC\(_{99}\) values) of...
>21, were evaluated for their ability to kill mycobacteria in liquid cultures and in infected macrophages. Both chlorobiocin and rosaniline were bactericidal in their mode of killing and inhibited bacterial growth in infected macrophages in a dose-dependent manner. NSC 76027, the most potent compound in our in vitro enzymatic assays with an IC50 value of 3.8 μM, was highly specific for SerB2 enzyme but displayed MIC99 value of 150 μM against both M. tuberculosis H37Rv and M. bovis BCG, which might be attributed to its poor penetration or intracellular stability. NSC 693172 displayed an IC50 value of 4 μM in our enzymatic assays but was highly cytotoxic in THP-1 cells, which might be due to its ability to inhibit other phosphatases in a nonspecific manner. Therefore, future experiments would involve designing of their structural analogs with more potent in vitro SerB2 specific and intracellular activity.

To the best of our knowledge, this is the first study where we show that chlorobiocin inhibits SerB2 enzyme of M. tuberculosis, a non-ATP binding protein in addition to its other known bacterial targets such as DNA gyrase, heat shock protein, and UDP-galactose-4′-epimerase (38, 39, 56). Interestingly, novobiocin, a close structural analog of chlorobiocin failed to inhibit SerB2 enzymatic activity even at 10-fold higher concentration, which might be attributed to hydrogen bond formation between Asp-341 residue of SerB2 protein with chlorobiocin and a better fit of its pyrrole ring in the SerB2 substrate binding pocket. Similar subtle differences were also observed in inhibition of UDP-galactose 4′-epimerase by chlorobiocin and novobiocin, respectively (56). Further experiments to solve crystal and co-crystal structure of SerB2 enzyme either alone or with chlorobiocin would be useful to understand such subtle differences in the ability of these aminocoumarins to inhibit SerB2 enzyme. We propose that use of scaffolds like chlorobiocin rather than novobiocin might be more effective for eradication of drug-resistant TB. Molecular docking of CID 5, CID 7, CID 9, and CID 10 in the SerB2 built model revealed that these scaffolds interact with common critical residues Asp-187 and Asp-341 of SerB2 protein via hydrogen bond formation. But we did not observe much difference in the far-UV CD spectra for K361A-SerB2, E214A-SerB2, D187A-SerB2, D341G-SerB2, and wild type protein, we speculate that the observed reduced inhibition for these mutant proteins could be due to loss of interaction of these mutant proteins with their respective scaffolds. However, the altered folded state of E197A-SerB2 could have contributed to the loss of inhibition observed in the case of CID 1, CID 5, and CID 7.

Validation of enzymes with human homologs is hampered by lack of safe and highly specific inhibitors. Interestingly except CID 7 and CID 10 all other scaffolds were highly specific in their ability to inhibit M. tuberculosis SerB2 enzyme. As expected, we did not observe any significant interactions between CID 5, CID 8, and CID 9 with HPSP enzyme, therefore explaining their ability to specifically inhibit SerB2 enzyme. Because RNAi-mediated inhibition of PSAT and PSP enzymes reduces tumor formation in breast cancer, we propose that nonspecific inhibitors (NSC 93739 and NSC 165701) could be exploited further for treating such disorders or infections caused by P. gingivalis (57). Based on our observations, future experiments would involve (i) screening of more libraries to identify novel PSP inhibitors and (ii) structure–activity relationship studies involving these SerB2 specific scaffolds in an attempt to design analogs that display enhanced potency, specificity toward SerB2 enzyme, and better intracellular activity. These findings suggest that despite being widely conserved, enzymes involved in energy metabolism can be targeted to combat the problem of drug resistance in intracellular pathogens. Collectively, these results demonstrate feasibility of HTS to obtain novel PSP inhibitors that would be useful for development of anti-mycobacterial agents.

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