Targeting mycobacterium protein tyrosine phosphatase B for antituberculosis agents

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Protein tyrosine phosphatases are often exploited and subverted by pathogenic bacteria to cause human diseases. The tyrosine phosphatase mPTPB from *Mycobacterium tuberculosis* is an essential virulence factor that is secreted by the bacterium into the cytoplasm of macrophages, where it mediates mycobacterial survival in the host. Consequently, there is considerable interest in understanding the mechanism by which mPTPB evades the host immune responses, and in developing potent and selective mPTPB inhibitors as unique antituberculosis (antiTB) agents. We uncovered that mPTPB subverts the innate immune responses by blocking the ERK1/2 and p38 mediated IL-6 production and promoting host cell survival by activating the Akt pathway. We identified a potent and selective mPTPB inhibitor I-A09 with highly efficacious cellular activity, from a combinatorial library of bidentate benzofuran salicylic acid derivatives assembled by click chemistry. We demonstrated that inhibition of mPTPB with I-A09 in macrophages reverses the altered host immune responses induced by the bacterial phosphatase and prevents TB growth in host cells. The results provide the necessary proof-of-principle data to support the notion that specific inhibitors of the mPTPB may serve as effective antiTB therapeutics.

combinatorial chemistry | pathogen-host interaction | phosphatase inhibitor | signaling mechanism

Protein tyrosine phosphatases (PTPs) are a large family of signaling enzymes involved in the regulation of virtually all aspects of eukaryotic biology, including growth and differentiation, cell motility, metabolism, apoptosis, and the immune responses (1, 2). Malfunction of PTP activity has been linked to cancer, diabetes, and immune dysfunctions (3). The importance of PTPs in cellular physiology is further underscored by the fact that they are often exploited and subverted by pathogenic bacteria to cause human diseases. For instance, YopH, the PTP from *Yersinia*, inhibits bacterial phagocytosis by removing phosphates from $p130^{Cas}$ and other focal adhesion proteins (4), while the *Salmonella* tyrosine phosphatase SptP dephosphorylates host AAA+ ATPase valosin-containing protein (VCP) to promote development of its intracellular replicative niche (5).

Tuberculosis (TB) is the world's leading cause of mortality due to infectious disease, with 2 million deaths each year, and approximately one-third of the world's population is infected with *Mycobacterium tuberculosis* (*Mtb*), the causative agent of this disease (6). Although onerous treatments are available, *Mtb*'s ability to survive for extended periods of time in the host requires prolonged drug treatment (six to nine months), thus resulting in low compliance. Furthermore, the prevalence of multidrug-resistant (MDR) and extremely drug resistant (XDR) mycobacteria and the AIDS epidemic heighten the need for new and improved therapeutics to speed up the course of treatment and combat TB infections (7). An emerging and unique drug discovery strategy is to identify and target virulence factors essential for pathogen survival and replication (8).

Mtb is an intracellular pathogen whose primary target cells are macrophages. Although macrophages play a central role in host defense, recognizing and destroying invading pathogens, *Mtb* is able to survive and replicate within this hostile environment. The genome of *Mtb* encodes for two PTPs, termed mPTPA and mPTPB, but lacks any tyrosine kinases (9). Both mPTPA and mPTPB are secreted by *Mtb* into the cytoplasm of the macrophage, but mPTPB is restricted in strains that cause TB in humans or in animals (10). Given the absence of endogenous tyrosine phosphorylation within *Mtb*, mPTPA and mPTPB likely modify macrophage proteins to manipulate host-pathogen interactions. Interestingly, genetic deletion of mPTPB blocks intracellular survival of *Mtb* in interferon-γ (IFN-γ) activated macrophages and severely reduces the bacterial load in a clinically relevant guinea pig model of TB infection (11). These findings led to the hypothesis that mPTPB might mediate mycobacterial survival in macrophages by targeting host cell processes, although the underlying molecular basis is not understood. Moreover, the importance of mPTPB for *Mtb* survival in macrophages also suggests that specific inhibition of mPTPB activity may augment intrinsic host signaling pathways to eradicate TB infection.

In this study, we have defined the biochemical mechanism by which mPTPB evades the host immune responses for the benefit of *Mtb* survival in macrophages. We also describe a salicylic acid-based combinatorial library approach, which led to the identification of a potent and selective mPTPB inhibitor with highly efficacious cellular activity. This proof-of-concept mPTPB inhibitor mimics genetic deletion of mPTPB and prevents *Mtb* growth in host macrophages, validating the concept that chemical inhibition of mPTPB may be therapeutically useful for improved TB treatment.

Results and Discussion

Role of mPTPB in Promoting Mycobacteria Survival in the Macrophage. IFN-γ is the predominant inflammatory cytokine responsible for macrophage's antimicrobial activity against diverse intracellular pathogens. However, IFN-γ is unable to turn on macrophages to restrict or eliminate virulent *Mtb*, suggesting that *Mtb* may interfere with cellular signal transduction pathways that are activated by IFN-γ and therefore avoids being killed within the macrophage. Unfortunately, how *Mtb* evades the host immune responses remains unknown. Given the observation that *Mtb*-lacking mPTPB are unable to survive in macrophages and in guinea pig (11), it has been hypothesized that mPTPB may

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promote mycobacterial survival in the host by targeting the IFN-γ mediated signaling pathway, although the exact mechanism by which this occurs has not been elucidated. Since mPTPB functions within the macrophage, we established Raw264.7 mouse macrophage cell lines stably expressing mPTPB in order to investigate the role of mPTPB in host cell biology. As expected, treatment of the Raw264.7 cells with IFN-γ resulted in the activation of cytoplasmic JAK2 kinase, signal transducer and activator of transcription 1 (STAT1), as well as MAP kinases ERK1/2, p38, and JNK (Fig. 1*A*). In addition, IFN-γ stimulation also led to a dramatic increase in the production of interleukin-6 (IL-6) (Fig. 1*B*), a key cytokine secreted by the macrophage that is important for upregulating microbicidal activity in macrophages (12) and also initiates the systemic immune response to *Mtb*. Interestingly, expression of mPTPB in activated macrophages attenuated the IFN-γ induced IL-6 production, while little effect on IL-6 level was observed when the catalytically inactive mPTPB/C106S was introduced to the macrophage (Fig. 1*B*). The results indicate that mPTPB supports mycobacteria survival by blocking the bactericidal immune responses in host cells and that its phosphatase activity is essential for virulence. To determine the mechanism by which mPTPB abrogates IFN-γ stimulated IL-6 production, we measured the status of JAK2, STAT1, ERK1/2, p38, and JNK phosphorylation in Raw264.7 cells expressing either wild-type or the C106S mutant mPTPB (Fig. 1*C*). No significant change was noted on the level of JAK2, STAT1, and JNK phosphorylation upon mPTPB expression, indicating that they are not direct targets of mPTPB. In contrast, large decreases in ERK1/2 (1.9-fold) and p38 (2.1-fold) phosphorylation were observed in mPTPB cells when compared to cells expressing either the vector control or the catalytically inactive mutant mPTPB. Since ERK1/2 and p38 are known to promote IL-6 gene expression (13, 14), mPTPB likely blocks the IFN-γ stimulated IL-6 production by down-regulating ERK1/2 and p38 activity.

In addition to suppression of the INF-γ mediated bactericidal responses, other host cell processes that are inhibited by pathogenic mycobacteria include apoptosis (10). Apoptosis of infected macrophages alerts the immune system to bacterial invasion. Thus, as an important component of the innate immune defense machinery against pathogenic mycobacteria, macrophage apoptosis is desirable to prevent colonization of intracellular pathogens (15). To investigate whether mPTPB affects INF-γ induced macrophage apoptosis, the apoptotic profiles of the control and mPTPB transfected Raw264.7 cells were analyzed by flow cytometry. Compared to unstimulated cells, macrophages undergo increased apoptosis under continuous INF-γ stimulation (Fig. 2*A*). Strikingly, mPTPB expression more than doubled the amount of viable cells as evidenced by increased annexin V-negative/PI-negative cells (from 12.0 to 31.8%, average of two experiments), whereas the catalytically inactive mutant mPTPB/C106S had no effect on macrophage apoptosis. To provide further evidence that mPTPB promotes host cell survival, we also measured the activity of Akt, an enzyme critical for cell survival, and caspase 3, which executes cell death programs. Consistent with the observed increase in host cell survival, macrophages expressing mPTPB displayed a 2.1-fold increase in Akt phosphorylation and a 2.3-fold reduction in caspase 3 activity (Fig. 2*B* and *C*). These findings reveal that mPTPB prevents macrophage cell death by activating Akt and blocking caspase 3 activity. The ability of *Mtb* to keep its host cell alive should ultimately favor bacterial survival and replication inside the host. Although the exact mechanism by which mPTPB activates Akt awaits further investigation, it is noteworthy that Akt has also been shown to be important for intracellular growth of unrelated pathogens such as *Salmonella typhimurium* and *Mtb* (16). Taken together, the results from the above experiments indicate that mPTPB functions to overcome host defense mechanisms during infection by attenuating the bactericidal immune responses and promoting macrophage survival.

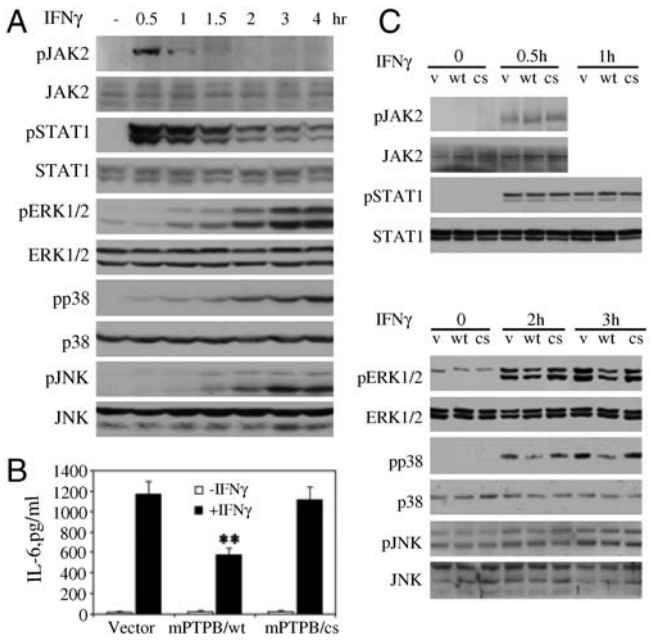


Fig. 1. mPTPB blocks INF-γ stimulated IL-6 production by down-regulating ERK1/2 and p38 activity. (*A*) JAK2, STAT1, ERK1/2, p38, and JNK are activated by INFγ. (*B*) Effect of mPTPB on INFγ-mediated IL-6 production. Data are expressed as means ± SD of three independent experiments ($^{**}P < 0.01$ by Student's *t* test). (*C*) mPTPB has no effect on INFγ stimulated JAK2, STAT1, and JNK phosphorylation, but impairs ERK1/2 and p38 activation.

Identification of a Potent and Selective mPTPB Inhibitor. In view of the importance of mPTPB for mycobacteria survival in the host, mPTPB represents an exciting target for antiTB drug development. Specific inhibition of mPTPB could provide a unique means of therapy with minimal side effects on the host, given the phylogenetic distance between *Mtb* and human PTPs. Moreover, since mPTPB is secreted into the cytosol of host macrophages, drugs targeting mPTPB are not required to penetrate the waxy mycobacterial cell wall. Accordingly, there is increasing interest in developing mPTPB inhibitors (17–21). However, the common architecture of the PTP active site (i.e., pTyr-binding pocket) poses a significant challenge for the acquisition of selective PTP inhibitors. Moreover, the highly positively charged pTyr-binding pocket impedes the development of inhibitors possessing favorable pharmacological properties. Thus although several compounds have been reported to exhibit inhibitory activity against mPTPB, inhibitors with robust biochemical selectivity and in vivo activity have proved elusive.

An effective strategy for the acquisition of active site-directed, potent, and selective PTP inhibitors is by tethering a nonhydrolyzable pTyr mimetic to an appropriately functionalized moiety in order to engage both the active site and a unique nearby subpocket (22). Unfortunately, most of the pTyr surrogates described in the literature are not drug-like and thus lack cell membrane permeability. Through in silico virtual screening, we discovered that the natural product salicylic acid can serve as a pTyr surrogate (23). We further demonstrated that naphthyl and polyaromatic salicylic acid derivatives exhibit enhanced affinity for PTP relative to the corresponding single ring compounds (23, 24). Consequently, we sought to develop bicyclic salicylic acid-based PTP inhibitors that carry sufficient polar and nonpolar interactions with the active site and yet possess favorable pharmacological properties.

Fig. 3 depicts a focused library-based strategy for the acquisition of potent and selective mPTPB inhibitory agents that are

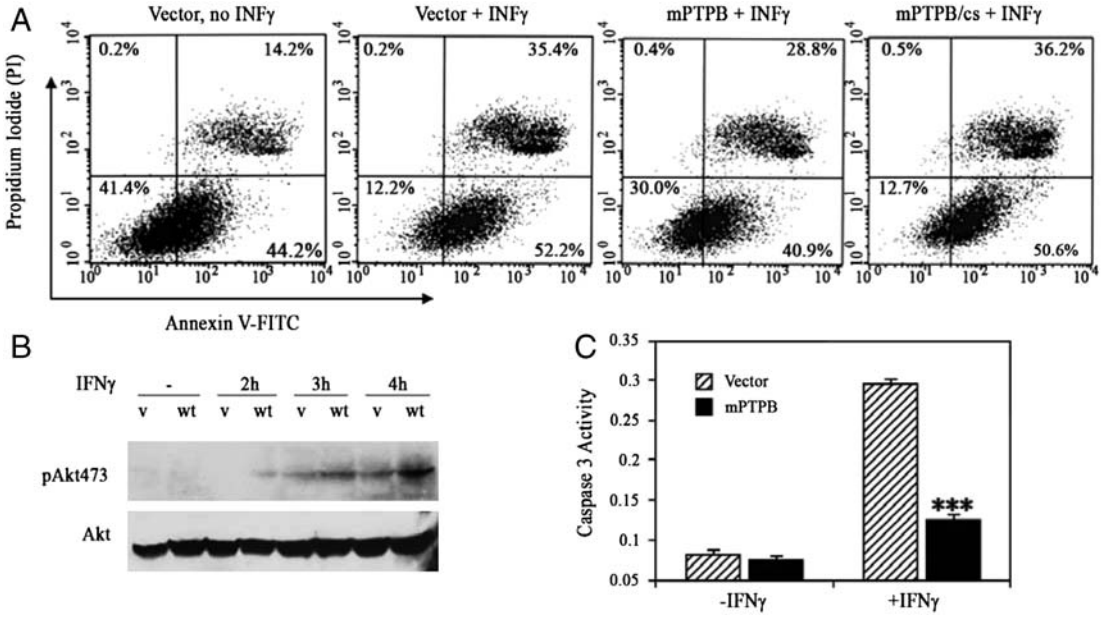


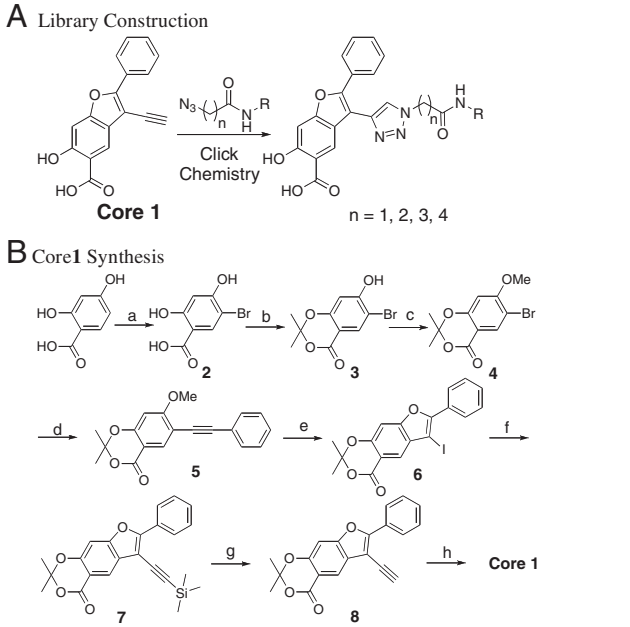
Fig. 2. Effect of mPTPB on macrophage survival. Macrophages expressing mPTPB have a higher propensity for cell survival (*A*), an elevated level of Akt phosphorylation (*B*), and a marked reduction in caspase 3 activity (*C*). Data are expressed as means ± SD of three independent experiments (***$P < 0.001$ by Student's *t* test).

capable of bridging both the active site and an adjacent peripheral site. As a proof-of-concept, we chose benzofuran salicylic acid as our active site-directed pTyr surrogate. Benzofuran derivatives are of considerable interest because of their widespread occurrence among natural products and their physiological properties (25). Thus, the library contains (a) a benzofuran salicylic acid core to engage the active site and (b) four alkyl linkers of one to four methylene units to tether the pTyr surrogate to (c) a structurally diverse set of 20 amines, aimed at capturing additional interactions with adjacent pockets surrounding the active site. The benzofuran salicylic acid core **1** was prepared from a commercially available compound 4-hydroxysalicylic acid that, upon regioselective bromination, afforded 5-bromo-4-hydroxysalicylic acid (**2**). This compound was selectively protected in the presence of acetone and trifluoroacetic anhydride/trifluoroacetic acid to furnish dioxanone **3**, which then reacted with CH_3I to give the methylation product **4**. Compound **4** was coupled with phenylacetylene in the presence of $Pd(PPh_3)_4$ to furnish **5**, which was then subjected to I_2 induced cyclization. Coupling of the iodination product **6** with ethynyltrimethylsilane gave compound **7**. Core **1** was obtained by desilylation and deacetylation of **7**.

To increase potency and selectivity, the strategically positioned alkyne in the benzofuran salicylic acid core was tethered to 80 azide-containing diversity elements (20 discrete amines with four alkyl linkers of one to four methylene length), using click chemistry or the Cu(I)-catalyzed [3 + 2] azide-alkyne cycloaddition reaction (Fig. 3). The click chemistry offers an expedient way to connect two components together with high yield and purity under extremely mild conditions (26). More importantly, the cycloaddition reaction can be conducted in aqueous solution in the absence of deleterious reagents, thus allowing direct screening and identification of hits from the library. In fact, click chemistry has found increasing applications in lead discovery and optimization for a number of enzymes including the PTPs (27–31). The azide-containing building blocks were synthesized in a one-pot procedure, in which alkyl or aryl amines were reacted with the acyl chloride linkers in N,N-Dimethylformamide (DMF), followed by S_N2 reaction with sodium azide to generate the corresponding azides. To construct the 80-member library, each azide was coupled with the alkyne containing core **1** in a mixed solvent of ethanol and water (7:3) and the click reaction was initiated by a catalytic amount of Cu(I), which was generated by reacting $CuSO_4$ with sodium ascorbate (*SI Appendix*). After 48 hr, the products were collected by simple centrifugation. All products were assessed by liquid chromatography–mass spectrometry and determined to be at least 70–100% pure and were used directly for screening without further purification.

The ability of the library compounds to inhibit the mPTPB-catalyzed hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) was assessed at pH 7 and 25 °C. Out of the 80-member library, 3 compounds displayed measurable inhibitory activity at ~10 μM concentration. Resynthesis of the hits confirmed that they were genuine inhibitors for mPTPB with IC_{50} values in the low μM range (*SI Appendix*). Compound I-A09 (Fig. 4*A*) appeared to be the most potent and selective for mPTPB with an IC_{50} of 1.26 ± 0.22 μM and was selected for further characterization. Kinetic analysis indicated that I-A09 is a reversible and noncompetitive inhibitor for mPTPB with a K_i of 1.08 ± 0.06 μM (Fig. 4*B*). To determine the specificity for I-A09, its inhibitory activity toward mPTPA and a panel of mammalian PTPs including cytosolic PTPs, PTP1B, TC-PTP, SHP2, Lyp and FAP1, the receptor-like PTPs, CD45, LAR, and PTPα, the dual specificity phosphatases VHR, VHX, Cdc14, and the low molecular weight PTP, were measured. As shown in Table 1, I-A09 is highly selective for mPTPB, exhibiting a 61-fold preference over mPTPA and greater than an 11-fold preference for mPTPB vs. all mammalian PTPs examined. Together, the results show that I-A09 is among the most potent and specific mPTPB inhibitors reported to date.

mPTPB Inhibitor I-A09 Recapitulates the Phenotype of mPTPB Gene Deletion. Our ultimate goal is to develop potent and specific mPTPB inhibitors as antiTB agents. Given the excellent potency and selectivity of I-A09 toward mPTPB, we proceeded to evaluate its cellular efficacy in Raw264.7 macrophages engineered to express mPTPB. As shown above, Raw264.7 cells expressing mPTPB display decreased ERK1/2 activity and INF-γ stimulated IL-6 production. Moreover, macrophages containing mPTPB also have a higher propensity for cell survival due to Akt activation and suppression of caspase 3 activity (Figs. 1 and 2). Consequently, one would predict that inhibition of mPTPB activity should reverse the effects of the bacterial phosphatase on cellular signaling and restore the cell's full capacity to secrete IL-6 and



Conditions: a) Br_2, AcOH, r.t., 94%; b) TFAA, TFA, Acetone, r.t., 55%; c) MeI, K_2CO_3, DMSO, r.t., 95%; d) Phenylacetylene, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, 70°C, 72%; e) I_2, $NaHCO_3$, MeCN, 40°C, 40%; f) Trimethylsilylacetylene, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, r.t., 85%; g) TBAF, THF, r.t., 89%; h) LiOH, THF/H_2O, 80°C, 71%.

Fig. 3. Strategy for the construction of a benzofuran-based salicylic acid library using click chemistry.

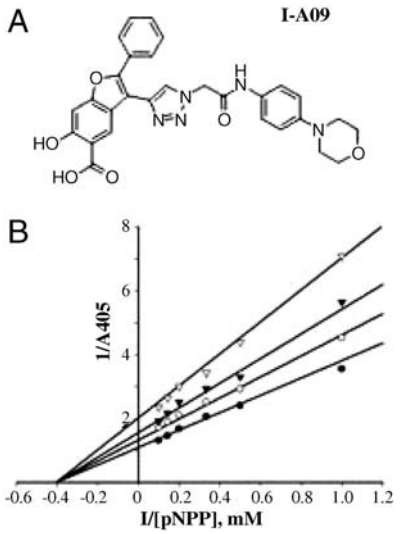


Fig. 4. I-A09 is a noncompetitive inhibitor of mPTPB. (*A*) Structures of I-A09. (*B*) Lineweaver-Burk plot for I-A09-mediated mPTPB inhibition. I-A09 concentrations were 0 (•), 0.25 (○), 0.5 (▼), and 1.0 μM (▽), respectively.

undergo apoptosis in response to INF-γ stimulation. Indeed, treatment of mPTPB expressing Raw264.7 macrophages with 5–10 μM I-A09 restored INF-γ induced ERK1/2 activation and IL-6 production (Fig. 5). In addition, I-A09 normalized Akt and caspase 3 activities and rescued the INF-γ induced apoptosis in mPTPB cells to the same extent as the vector control cells (Fig. 6). To ensure that the cellular activity displayed by I-A09 is not due to nonspecific effects, we also evaluated a structurally related but inactive compound I-C07 (*SI Appendix*), which exhibits no inhibitory activity against mPTPB and the panel of mammalian PTPs at 50 μM concentration. As expected, I-C07 failed to reverse the biochemical and functional perturbations introduced by mPTPB (Figs. 5 and 6). Thus the ability of I-A09 to block the mPTPB-mediated cellular signaling is unlikely due to off-target effects. Remarkably, I-A09 inhibited mPTPB in intact cells with similar potency as that toward isolated enzyme, whereas previous PTP inhibitors have shown 100- to 10,000-fold loss of potency between biochemical and cellular assays. The results indicate that I-A09 is cell permeable and capable of blocking mPTPB activity in macrophages.

To provide further proof-of-principle data to support the notion that specific inhibitors of mPTPB may be effective antiTB agents, we tested I-A09 against the classical *Mtb* Erdman strain in a model of active TB infection performed in a mouse macrophage J774A.1 cell line (32). Cultures of macrophages infected with actively growing *Mtb* were treated with 10 μM I-A09 starting on Day 0 and the cultures were allowed to incubate for a further seven days before assessment of the remaining bacterial load in the cells (Fig. 7). During the treatment period the bacterial load in the control cultures increased over 15-fold. Consistent with the genetic observation that deletion of mPTPB impairs the ability of *Mtb* to survive in activated macrophages (11), I-A09 was able to further potentiate the effect of INF-γ to reduce the bacterial load by 90%. Interestingly, resting macrophages treated with I-A09 also exhibited nearly 90% reduction of *Mtb* relative to untreated control cells. By contrast, treatment of macrophages with 10 μM I-C07 had no effect on TB growth compared with the control cells. To eliminate the possibility that the observed decrease in bacterial load was a result of compound cytotoxicity, we determined that macrophage viability was unaffected by the presence of I-A09 or I-C07 at concentrations up to 100 μM in the culture medium. We also found the MIC (minimum inhibitory

Table 1. Selectivity of I-A09 against a panel of PTPs

PTP	IC_{50} (μM)
mPTPB	1.26 ± 0.22
mPTPA	77.3 ± 5.1
PTP1B	19 ± 1.5
TC-PTP	22 ± 2.5
SHP2	26.4 ± 6.6
FAP1	30.9 ± 6.6
Lyp	14 ± 1.8
VHX	21.5 ± 5.1
VHR	14.2 ± 2.4
LMWPTP	40.9 ± 4.5
Cdc14A	>100 μM
PTPα	>100 μM
LAR	>100 μM
CD45	>100 μM

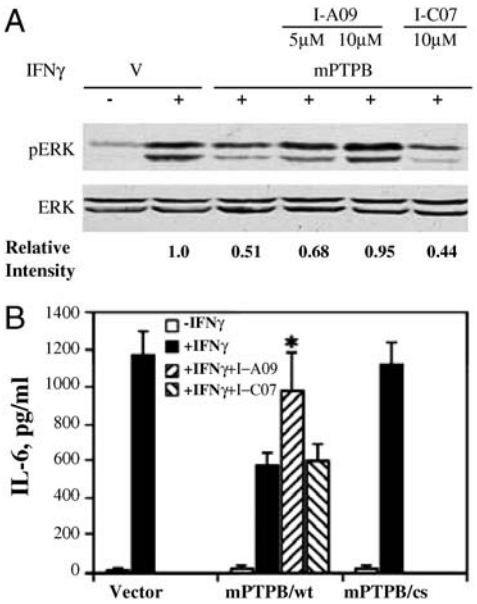


Fig. 5. Compound I-A09 restores ERK1/2 activity (*A*) and IL-6 production (*B*) in activated macrophages. Cells overexpressing mPTPB have decreased ERK1/2 activity and secrete lower levels of IL-6, and these can be reversed by treatment with the mPTPB inhibitor I-A09 but not with a structurally related inactive compound (I-C07). Data in (*B*) are expressed as means ± SD of three independent experiments ($^{*}P < 0.05$ by Student's *t* test).

concentration) values for I-A09 and I-C07 on extracellular *Mtb* H37Rv and *Mtb* Erdman to be >100 μM, indicating the lack of bactericidal activity of these compounds. Thus, I-A09 blocks intracellular TB growth in the macrophage, presumably by impairing mPTPB's ability to overcome host defense mechanisms.

In summary, we have shown that mPTPB suppresses the innate immune responses by blocking the ERK1/2 and p38 mediated IL-6 production and promoting host cell survival by activating the Akt pathway. We identified a potent and selective mPTPB inhibitor I-A09 with highly efficacious cellular activity, from a combinatorial library of bidentate benzofuran salicylic acid derivatives assembled by click chemistry. We demonstrated that inhibition of mPTPB with I-A09 in macrophages reverses the perturbation of host immune responses induced by the bacterial phosphatase and prevents growth of the TB bacterium in host cells. Ideally, I-A09 or its related analogs will potentially provide an innovative therapeutic starting point for the treatment of TB, including MDR and XDR forms, that is not only complementary, but also synergistic with current drugs, particularly those that focus directly on the TB bacterium. It is anticipated that such combination therapy will result in the shorter duration of treatment and recovery time for the TB patient.

Materials and Methods

Materials. Recombinant mouse IFN-γ was purchased from PeproTech Inc. Anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-JNK, anti-phospho-JAK2, anti-phospho-Akt473, and anti-phospho-STAT1 antibodies were purchased from Cell Signaling.

Cell Culture and Transfection. Raw264.7 mouse macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen), penicillin (50 units/mL), and streptomycin (50 μg/mL) under a humidified atmosphere containing 5% CO_2 at 37 °C. mPTPB and mPTPB/C160S were subcloned into pcN-HA expression vectors, respectively. Raw 264.7 cells were seeded at 40% confluency in an antibiotic-free medium and grown overnight, HA-tagged mPTPB, HA-tagged mPTPB/C160S, or pcN-HA empty vectors were transfected into cells by electroporation at 800 μF and 280 V. After 24 hr of transfection, 0.5 mg/ml G418 was added to the culture medium. Stable clones were picked after two weeks of selection.

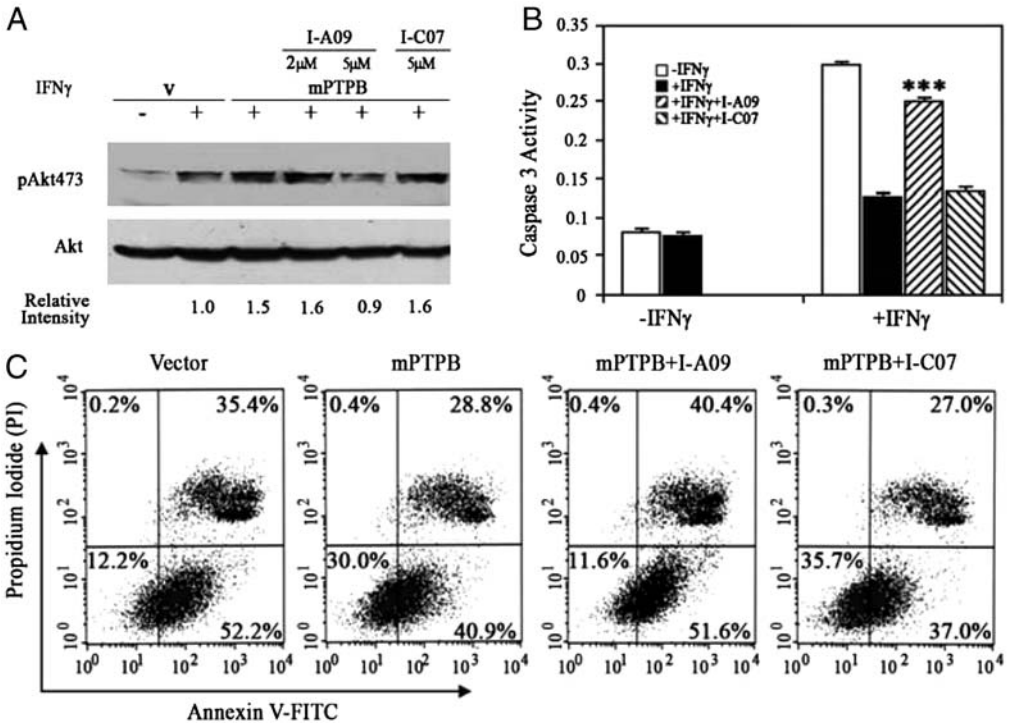


Fig. 6. Inhibition of mPTPB with I-A09 blocks Akt activation (*A*) and promotes caspase 3 activation (*B*) and macrophage apoptosis (*C*). Cells overexpressing mPTPB have increased Akt activity, lower caspase 3 activity, and higher propensity for cell survival, and these can be reversed by treatment with the mPTPB inhibitor I-A09 but not with a structurally related inactive compound (I-C07). Data in (*B*) are expressed as means ± SD of three independent experiments ($^{***}P < 0.001$ by Student's *t* test).

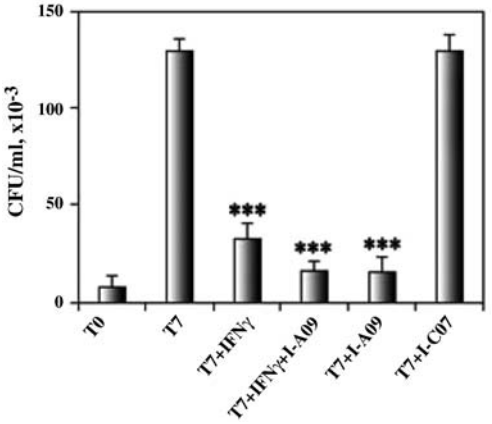


Fig. 7. Compound I-A09 reduces bacterial load in infected macrophages. Mouse macrophages were exposed to infectious *Mtb* and the infection was allowed to establish until the bacterial load approached 10,000 CFU/ml. Parallel cultures were treated with IFN-γ, mPTPB inhibitor I-A09 (10 μM), or both substances. After a further seven days the cultures were washed, lysed, and bacterial load was determined by standard methods. Results are presented as means ± SD of three independent experiments (***$P < 0.001$ by Student's t test).

IL-6 Enzyme-Linked Immunosorbent Assay (ELISA). mPTPB transfected Raw264.7 cells were seeded in a 12-well plate at a density of 4×10^4 cells/well. The following day cells were treated with mPTPB inhibitor I-A09 and negative control compound I-C07 at 10 μM, respectively, for 1 hr, then stimulated with IFN-γ (200 U/ml). After 24 hr of incubation, supernatants were collected, cleared by centrifugation, and assayed for IL-6 release using mouse IL-6 ELISA kit (eBioscience) and a plate reader. The ELISA was performed according to the manufacturer's instructions. Briefly, 200 μl/well of supernatant were added into the 96-well plate that had been coated with IL-6 capture antibody. After 24 hr of incubation at 4 °C, the plate was aspirated and washed, and then the biotin conjugated IL-6 detection antibody was added into the wells. After 1 hr of incubation at room temperature, the avidin-HRP and tetramethylbenzidine (TMB) substrate were added into the plate subsequently for chemiluminescent reaction. The IL-6 production in supernatant was quantitatively calibrated using the IL-6 standard curve, which is determined following the same procedures as above using recombinant IL-6 standard instead of supernatant samples.

Macrophage Assay. Inhibition of growth of *M. tuberculosis* Erdman (ATCC 35801) in a macrophage cell culture was assessed as previously described (32). J774A.1 cells were grown to confluency in 75 cm^2 cell culture flasks in DMEM medium containing 10% FBS. Using a cell scraper, the cells were detached, centrifuged at 200 × g for 5 min at room temperature and the pellet suspended to a final concentration of $1–3 \times 10^5$ cells/ml. One ml aliquots of cell suspension were distributed into 24-well plates (Falcon Multiwell 24 well) containing 13 mm cover slips (Nalge Nunc International), and the plates were incubated at 37 °C in a 5% CO_2 incubator overnight. Frozen bacterial cultures were thawed, sonicated for 15 s, and diluted to a final concentration of $1–3 \times 10^5$ CFU/ml with DMEM and 1 ml of the dilution dispensed to each well of a new 24-well plate. J774.1 cells on coverslips were transferred to the 24-well plates containing *M. tuberculosis* Erdman and the plates incubated at 37 °C for 1 h to allow for phagocytosis. Coverslips were rinsed with HBSS to remove the extracellular bacteria, and the coverslips were transferred to new 24-well plates with 1 ml of fresh media in each well. Cultures were incubated at 37 °C under 5% CO_2 for 16 h, then transferred those of coverslips to 1 ml per well fresh media containing the test compounds at 10 μM and amikacin (to prevent growth of any extracellular bacilli) at 20 μg/ml. Interferon-γ (Sigma, 087k1288) was added at 50 U/ml. All experimental conditions were set up in triplicate. At T_0 (for untreated controls) and after seven days the incubation medium was removed and macrophages were lysed with 200 μl of 0.25% SDS. After 10 min of incubation at 37 °C, 200 μl of fresh media were added. The contents of the wells were transferred to a microtube, sonicated (Branson Ultrasonics model 1510, Danbury, CT) for 15 s, and 1:1, 1:10, 1:100, and 1:1,000 dilutions were plated on 7H11 (Difco) agar plates. Colonies were counted after incubation at 37 °C for two to three weeks.

Details on combinatorial library synthesis, mPTPB expression and purification, screening, and kinetic characterization of mPTPB inhibitors, immunoblotting, flow cytometric analysis, caspase activity, EC_{50}, and MIC measurements are provided in *SI Appendix*.

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