Published in final edited form as:

ACS Chem Biol. 2016 January 15; 11(1): 172–184. doi:10.1021/acscchembio.5b00517.

First-in-Class Inhibitors of Sulfur Metabolism with Bactericidal Activity against Non-Replicating *M. tuberculosis*

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Abstract

Development of effective therapies to eradicate persistent, slowly replicating *M. tuberculosis* (*Mtb*) represents a significant challenge to controlling the global TB epidemic. To develop such therapies, it is imperative to translate information from metabolome and proteome adaptations of persistent *Mtb* into the drug discovery screening platforms. To this end, reductive sulfur metabolism is genetically and pharmacologically implicated in survival, pathogenesis, and redox homeostasis of persistent *Mtb*. Therefore, inhibitors of this pathway are expected to serve as powerful tools in its preclinical and clinical validation as a therapeutic target for eradicating persisters. Here, we establish a first functional HTS platform for identification of APS reductase (APSR) inhibitors, a critical enzyme in the assimilation of sulfate for the biosynthesis of cysteine and other essential sulfur-containing molecules. Our HTS campaign involving 38 350 compounds led to the discovery of three distinct structural classes of APSR inhibitors. A class of bioactive compounds with known pharmacology displayed potent bactericidal activity in wild-type *Mtb* as well as MDR and XDR clinical isolates. Top compounds showed markedly diminished potency in a conditional APSR mutant, which could be restored by complementation with *Mtb* APSR. Furthermore, ITC studies on representative compounds provided evidence for direct engagement

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.5b00517. Detailed methods, figures, and tables (PDF)

Author Contributions

P.B.P. developed LUM and FP assays; conducted pilot screenings; performed secondary, counterscreening, and whole-cell assays in *Msm*; performed ITC studies, qRT-PCR, and thiol content measurements; and wrote the manuscript. A.B. performed whole-cell *Mtb* assays and EMSE measurements. L.E.P.-R. performed 38 350 compound HTS, dose-response, and counterscreen. F.M. and P.C. guided the pilot screens. L.E.P.-R. and F.M. analyzed the HTS data. T.S. guided assay implementation and screening for the HTS. L.S. guided the compound selection and delivery for HTS. V.G. synthesized the prioritized in-house hits. A.S. guided whole-cell *Mtb* assays and EMSE measurements. K.S.C. conceived and guided the entire project.

Notes

The authors declare no competing financial interest.

of the APSR target. Finally, potent APSR inhibitors significantly decreased the cellular levels of key reduced sulfur-containing metabolites and also induced an oxidative shift in mycothiol redox potential of live *Mtb*, thus providing functional validation of our screening data. In summary, we have identified first-in-class inhibitors of APSR that can serve as molecular probes in unraveling the links between *Mtb* persistence, antibiotic tolerance, and sulfate assimilation, in addition to their potential therapeutic value.

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB) and kills more human beings each year than any other single infectious agent.¹ One in three people in the world are infected with *Mtb*. In 90–95% of infected individuals, *Mtb* resides in a dormant, slow-growing state in immune cell aggregates, called granulomas. Current anti-TB drugs primarily target active replicating *Mtb* and do not effectively kill these dormant bacteria, known as “persisters.”² The failure to clear bacteria in latent-TB infection (LTBI) represents a vast reservoir for potential reactivation and transmission of TB, and complex treatment regimens drive the emergence of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Mtb* strains.³ Thus, there is an acute need for new and effective therapies targeting persister *Mtb* populations to help end the global TB epidemic.

In mycobacteria and other pathogens, the sulfate assimilation pathway (Figure 1a) provides reduced sulfur for biosynthesis of a myriad of vital metabolites including cysteine, methionine, enzyme cofactors, and mycothiol (MSH), a major lowmolecular weight antioxidant (Figure 1b).^{4–6} Transcription and proteomic analyses have consistently identified genes involved in sulfate assimilation being upregulated in response to oxidative stress, nutrient starvation, and dormancy adaptation, which all model fundamental aspects of persistent *Mtb* survival.^{7–10} Moreover, mutagenesis studies in both macro-phage and mouse TB infection models show that disabling genes within the sulfate assimilation pathway severely attenuates virulence and survival of *Mtb*, especially during chronic infection.^{11,12} These and other studies clearly indicate that the sulfate assimilation pathway plays a fundamental role in latency adaptation, survival, and pathogenesis of *Mtb*. Based on a unified mechanism for bactericidal action of antibiotics involving generation of destructive reactive oxygen species (ROS), targeting bacterial systems and pathways that remediate ROS damage has recently emerged as a prospective strategy to potentiate the bactericidal

action of antibiotics.^{13,14} Although this unified mechanism has been recently disputed,^{15,16} it is strongly supported and extended by several independent findings demonstrating that tolerance to antibiotics depends on the ability of bacteria to defend itself against ROS.^{17–19} Specific to *Mtb*, it has been shown that persister *Mtb* subpopulations show differential sensitivity to antibiotic generated ROS and can be eradicated by stimulating ROS production.²⁰ Recently, ROS-mediated bactericidal action of vitamin C in *Mtb* was shown to be greatly potentiated in MSH-deficient *Mtb*.²¹ As indicated earlier, sulfate assimilation provides MSH, which functions as a major antioxidant defense system in *Mtb*. Therefore, disruption of sulfur-mediated redox homeostasis by inhibiting sulfate assimilation represents an attractive strategy to kill drug-tolerant subpopulations of *Mtb*.

The importance of sulfate assimilation in persistence and antibiotic tolerance of *Mtb* necessitates the development of small molecules for characterizing essential enzymes in this pathway and validating them as novel anti-TB targets. To date, no inhibitors have been reported for any enzyme in the sulfate assimilation pathway. APSR is a critical enzyme that lies at a metabolic branch-point of sulfur assimilation in *Mtb* and catalyzes the first committed step in sulfate reduction.²² In this reaction, activated sulfate in adenosine-5'-phosphosulfate (APS) is reduced to sulfite (SO_3^{-2}) and byproduct AMP (Figure 2a).²³ Disruption of the gene encoding APSR (*CysH*) attenuates virulence and persistence in a murine model of TB infection. A strong connection between APSR, *Mtb* survival, and oxidative stress in granulomatous lesions has also been established by the restored virulence of *CysH* *Mtb* in animals that are deficient in phagocytic enzymes producing reactive nitrogen and oxygen species, such as nitric oxide synthase (*NOS*) and NADPH oxidase (*NOX*).¹² From a future translational perspective, APSR is an attractive therapeutic target for the anti-TB drug development as no APSR homologue has been identified in humans. Therefore, APSR inhibitors will serve as powerful chemical tools, not only in dissecting the vital link between reductive sulfur metabolism, *Mtb* persistence, and antibiotic tolerance but also to further validate APSR as a clinically relevant anti-TB target.²⁴

Here, we have developed a robust HTS platform that constitutes a combination of HTS assays for identification and validation of APSR inhibitors and implemented them in screening a selection of 38 350 compounds carefully chosen from a ~640 000 compound library. These efforts represent a first HTS campaign to target an essential member of the sulfate assimilation pathway in *Mtb*. Rigorous secondary and counter-screening of 398 primary hits yielded seven compounds representing three different structural classes to have promising bactericidal activity against nonreplicating *Mtb*. Significantly, a class of bioactive compounds with known pharmacology displayed potent bactericidal activity in drug-sensitive and drug-resistant *Mtb*, providing a promising opportunity for development of repurposed anti-TB compounds. A conditional *CysH* *Msm* mutant showed markedly diminished sensitivity for active compounds, which was restored nearly completely by complementation with *Mtb* *CysH*. Additionally, isothermal titration calorimetric (ITC) studies confirmed direct APSR engagement of selected compounds. The most potent compounds were then functionally validated, showing a significant decrease in cellular levels of key sulfur metabolites as well as inducing a significant oxidative shift in *Mtb* MSH

redox potential (E_{MSH}) by employing a novel redox biosensor we have recently developed.²⁵

RESULTS AND DISCUSSION

Design and Optimization of HTS Assays

Luminescence (LUM) HTS Assay—APSR reduces substrate APS to SO_3^{-2} and byproduct AMP.²³ We started with the development and validation of a luminescence-based biochemical HTS assay configured to detect the byproduct AMP using an AMP-Glo platform by Promega (Figure 2a). This assay quantitatively monitors the concentration of AMP with the luminescence signal being linearly proportional to AMP over a wide concentration range (0.01–6.0 μM ; Figure 2b). After initial concept validation, we began with a systematic assay optimization study in a 384 well-plate using Z' factor and signal-to-background ratio (S/B) as statistical indicators of assay performance.²⁶ We first determined the optimal concentrations of APS and APSR required for a balance between robust detection signal and maximal sensitivity to inhibitors. We identified 0.3 μM as the optimal APS concentration (~6-fold lower than apparent $K_m^{\text{APS}} = 2 \mu\text{M}$). APSR concentration was subsequently optimized (2 nM) to give linear reaction progress (Figure 2c), where 20% of APS is converted to product within the reaction time course. Next, we verified that assay bioreagents were stable on ice for an extended period of time (10 h). Tolerance to DMSO (0.6–5%) and Brij-35 detergent (0.006–0.1%) was also evaluated, and no significant deterioration in assay performance was observed. Finally, a plate uniformity study was run over three days to assess uniformity and separation of the signals, using the DMSO concentration (0.33%) to be used in the HTS and automated liquid dispensers. Given the lack of available control inhibitor at this stage, assay mixtures without APSR served as the positive control (100% inhibition or “high control”). Clear separation between “high” and “low” controls (DMSO, 0% inhibition) was observed with high reproducibility over a period of three days (Z' factor = 0.87 ± 0.03 ; S/B = 21.3 ± 1.5). Given the excellent performance and robustness of the 384-well plate luminescence assay, it was readily miniaturized to a 1536 well-plate assay with no deterioration in the assay performance indicators (Figure 2d). The fully optimized 1536-well plate homogeneous LUM assay involves only four liquid transfer steps, in a total volume of 7.5 μL (Table S1). While our optimized 1536-well plate LUM assay was being validated and utilized in an HTS of 38 350 compounds, a comparatively low-throughput (96-well plate) kinetic assay for measurement of plant APSR activity was reported utilizing the luciferase-based AMP detection strategy.²⁷

LUM Assay Counterscreen—Luminescence assays are widely used in HTS campaigns due to their high sensitivity and low susceptibility to compound interferences.²⁸ However, the most common apparent activity observed in luminescence assays is from the inhibition of luciferase enzyme and/or luminescence quenching. Therefore, we conceived a LUM counterscreen that substituted APSR and APS with the product AMP, added in a concentration (90 nM) equivalent to 30% substrate conversion (Figure S1a). Other critical parameters and assay protocols remained the same as the parent LUM assay.

Fluorescence Polarization (FP) HTS Assay—Another essential requirement of a robust HTS platform is the availability of a secondary HTS assay possibly using an orthogonal detection technique for confirmation and prioritization of primary screening hits.²⁸ Therefore, we developed and optimized a secondary HTS assay that detects AMP using the Transcreeper fluorescence polarization immunoassay (FPIA) platform.²⁹ This assay utilizes a red-shifted fluorophore (AMP-AlexaFluor 633 tracer) and antibody that binds AMP with very high affinity and specificity. The assay signal is generated when AMP displaces the tracer bound to an antibody leading to a decrease in FP signal (Figure S1b,c). This assay is designed to operate in the far-red region to minimize interference from compounds with autofluorescence properties.²⁸ For optimization of this assay, the polarization signal in response to the detection of byproduct AMP served as our positive signal (mP_{+ve}), while the unstimulated reaction devoid of APSR served as our negative signal (mP_{-ve}). The enzyme reaction parameters optimized for the luminescence assay could be readily extended to the FP assay without further optimization. Other assay conditions were optimized to maximize the detection window ($mP = |mP_{+ve} - mP_{-ve}|$) and Z' factor. The assay was found to be extremely robust in the plate uniformity studies conducted in duplicate over a three day period in 384-well plates (Figure S1d and Table S2).

Validation of HTS Assays in Pilot Screens

The LUM assay in a 384-well plate was validated in a pilot screen using the commercially available library of pharmacologically active compounds (LOPAC) constituting 1280 compounds. A triplicate study conducted on three different days showed high reproducibility (Figure 3a), with an average Z' factor of 0.85 ± 0.03 . Using a calculated cutoff of 26.5% ($AVG_{sample} + 3 \cdot SD_{sample}$), 18 hits were identified corresponding to a hit rate of 1.4% (Table S3). High reproducibility in hit identification is also indicated by a linear correlation between two independent trials (Figure 3b). Each of the 18 hits was then retested in a 10-point; 3-fold serial dilution concentration–response study. Of these, 17 displayed a full concentration–response curve (CRC), and one compound displayed a partial CRC. All 18 hits were retested in the LUM assay counterscreen to eliminate false-positive hits. Of the 18 hits, three (~17%) were found to be active in the counterscreen, identifying them as false positives due to primary assay interference (Table S3 and Figure 3c). An inactive control compound did not show any activity in either assays, as expected (Figure 3c).

The assay buffer used for LUM and FP assays has a reducing agent in the form of dithiothreitol (DTT) at a 5 mM concentration. Use of a strong reducing agent such as DTT is essential to maintaining a pool of reduced thioredoxin that provides reducing equivalents for the APSR reaction, and also to prevent aerial oxidation of APSR during the course of the assay. However, inclusion of DTT in HTS assays for proteins with redox-active cysteines have been associated with the identification of redox-cycling compounds (RCCs) as false positive hits.³⁰ RCCs can produce H_2O_2 in the presence of DTT, which nonspecifically inhibits proteins with redox-active thiols. However, production of H_2O_2 by RCCs has been found to be greatly dependent on the assay concentration of DTT, with the concentrations 5 mM resulting in highly diminished production of H_2O_2 .³¹ Since both LUM and FP assays use DTT in 5 mM concentration, the probability of identifying RCCs as false positives is relatively low. Moreover, such interference, if any, can be readily eliminated using a

horseradish peroxidase–phenol red (HRP-PR) H_2O_2 detection assay reported earlier as a counterscreen.³¹ All 18 LOPAC hits were tested in the HRP-PR counterscreen. The paranaphthoquinone compound NSC 95397, a known RCC identified as a hit, expectedly showed generation of H_2O_2 in the HRP-PR assay performed at 0.8 mM DTT concentration (Figure S2a).

Similar to the LUM assay, the FP assay in 384-well was validated in a pilot screen with the LOPAC library at a compound concentration of 10 μM (Figure S2b). The FP assay also proved to be highly robust, as indicated by the excellent Z' factor in a triplicate study (average Z' factor = 0.79 ± 0.04). This screen reproducibly identified 10 hits with a hit rate of 0.8% (cutoff = 19.51%; Table S3). Importantly, all 10 hits identified in the FP assay were among the 18 hits identified in the LUM assay, thus demonstrating the utility of FP assay as secondary assay in confirming the activity of primary hits from the LUM assay and prioritizing them for further studies. Importantly, 2-(methylthio)ADP trisodium confirmed as falsepositive hit interfering with the LUM assay format (Figure 3c), did not show any activity in the FP assay as expected. In summary, these pilot screens demonstrate that both LUM and FP assays are robust, sensitive, and ready to be implemented in HTS of large compound libraries to identify APSR inhibitors. Our follow up CRC and counterscreening studies demonstrate that compounds interfering with the LUM assay as well as the RCCs can be effectively eliminated as false positives.

High-Throughput Screen

After validation of HTS assays and the work-flow, the most critical task, perhaps, is the selection of compounds for screening.³² Based on various selection criteria, we compiled a collection of 38 350 compounds carefully chosen from the ~640 000 compound Scripps Drug Discovery Library (SDDL) that has excellent diversity and representation of chemical space. This selection included ~22 000 compounds cured of pan assay interferences (PAINS),³³ ~5000 compounds from the rule of five diversity (RO5) library, ~2000 compounds from the in-house natural product diversity library, ~7000 compounds from the Scripps clinically relevant collection, and ~2000 compounds from the Scripps in-house kinase inhibitor collection. The selected 38 350 compounds were screened at 9.25 μM concentration in the 1536-well LUM assay with an average Z' factor of 0.91 ± 0.05 (Figure 4a). The compound NSC 95397, which inactivates APSR by oxidation, served as a positive control. Defining a hit as a compound showing greater than or equal to the calculated cutoff of 21.9% inhibition ($\text{AVG}_{\text{sample}} + 3 \cdot \text{SD}_{\text{sample}}$), we identified a total of 403 hits corresponding to a hit rate of 1.05%. Out of the 403 hits, 398 were available for retesting.

Confirmatory Assays and Scaffold Analysis

The primary hits were then funneled through a battery of secondary and counterscreening assays for confirmation and validation of hits (Figure S3). To start with, all 398 available hits were retested in a triplicate 10-point, 3-fold serial dilution concentration–response format in the primary LUM assay. Significantly, a total of 160 hits (~40%) displayed IC_{50} 10 μM , with 24 compounds (~6%) showing IC_{50} 1 μM , thus identifying multiple structural classes of compounds as potential APSR inhibitors. All 398 hits were also tested in the LUM assay counterscreen. The LUM assay interfering compound 2-(methylthio) ADP

trisodium served as a control compound in this screen. A total of four compounds among the top 160 compounds showed activity in the LUM counterscreen ruling them out as false-positive hits. The remaining 156 compounds were clustered into various structural classes using cheminformatic tools followed by manual selection. The structure clusters A–F constituted compounds with high potency for APSR inhibition (Figure 4b). A total of 25 compounds representing clusters A–F were selected for further follow-up studies (Table S4). Although identity and purity of primary hits was verified at an earlier stage, it was desirable to source fresh samples of the compounds at this stage of follow-up studies.³⁴ Among the chosen set, 22 compounds were available through commercial sources, while the remaining six representing cluster B and E were chemically synthesized using literature reported methods (Table S4).³⁵ All synthesized or freshly acquired compounds were arrayed in a 384-well plate and subjected to testing in a 10-point dose–response format LUM assay showing reproducible activity. Their APSR inhibitory potential was also independently verified in the secondary FP assay (Table S4). None of the selected compounds showed activity in the HRP-PR counterscreen for RCCs, thus excluding the possibility of nonspecific APSR inactivation by these compounds. Finally, all compounds were also counterscreened for their potential to inhibit LUM assay by inhibiting thioredoxin activity using the ProteoStat PDI assay. All selected compounds were found to be inactive in this assay, indicating their ability to specifically inhibit APSR in the biochemical assays.

Whole-Cell Activity in *Mtb*

Pharmacological studies using the mouse model of TB infection have validated the essentiality of APSR in survival and pathogenesis of *Mtb*, specifically during the chronic phase of infection.¹² In this chronic phase of mice infection, *Mtb* exist in a slowly replicating state that closely mimics the persistent state of bacilli in human disease. Therefore, we started with evaluating the potential of APSR inhibitors in killing nonreplicating *Mtb* by employing a well-studied nutrient starvation model that mimics many important features of *Mtb* persistence, including arrested growth and tolerance to anti-TB drugs.³⁶ Among the panel of 28 compounds, five compounds (1–5) representing four different structure clusters showed promising bactericidal activity against nonreplicating *Mtb* with the minimum bactericidal concentration (MBC) values in low-micromolar range (Table 1). As a primary step in evaluating their specificity against non-replicating *Mtb*, all five compounds were tested for their cytotoxicity to mammalian cell lines. Compounds **1–4** showed no measurable cytotoxicity in VERO and HeLa cell lines to the highest tested concentration of 100 μ M. Compound **5** was cytotoxic in VERO cells with a lethal concentration (LC₉₀) of 63 μ M; however, its cytotoxicity was ~5-fold lower compared to its bactericidal activity against the nonreplicating *Mtb* (MBC = 12.5 μ M; Figure S4).

Interestingly, compound **5** is a plant alkaloid, ellipticine, which is a known antineoplastic agent with cytostatic activity specific to tumor cells.³⁷ Ellipticines (**5** and its derivatives) have attracted significant clinical interest due to their limited toxic effects, perhaps due to their selectivity for rapidly dividing tumor cells and their complete lack of hematologic and hepatic toxicity.³⁷ The exact mechanism of action of ellipticines was not clearly understood until recently, when these compounds were shown to form covalent adducts with DNA after being activated by multiple cytochrome (CYP) P450 enzymes, expressed at elevated levels

in cancer cells.^{37,38} Additionally, 9-hydroxyellipticine, an active metabolite of ellipticine, has also been linked with selective inhibition of RNA polymerase I transcription.³⁹ These mechanisms underline the specificity of ellipticine and its derivatives in cancer cells. Given the cytostatic property of ellipticines along with their cancer cell specificity, the bactericidal activity exhibited by **5** in nonreplicating *Mtb* was intriguing. Our data from biochemical assays demonstrate inhibition of APSR activity by **5**, suggesting its physical interaction with APSR. However, as an additional validation of target engagement, we investigated the binding of **5** to APSR by ITC. Compound **5** bound to APSR with a binding affinity (K_d) of 12 μ M, suggesting direct engagement of the target APSR by **5** (Table S5). To build on this potential result and explore this scaffold for APSR inhibition further, we performed a brief structure–activity relationship (SAR) by testing a total of eight ellipticine analogs readily available to us from the NCI compound collection. An interesting trend emerged from the SAR studies where compounds **8–10** bearing substitutions at both C-9 and N-2 positions were tremendously effective in killing nonreplicating *Mtb* with MBC values ranging from 1.5 to 3.0 μ M (0.62–1.02 μ g/mL). Significantly, we noticed that the bactericidal activity of compounds **8–10** fall in the range of potent *in vitro* bactericidal activity shown by an investigational anti-TB compound PA-824 and other compounds known to kill nonreplicating *Mtb*.^{40,41} By contrast, compounds **6** and **7** with only C-9 substitution showed greatly diminished activity against the persistent *Mtb* (Table 1). As expected, these compounds were also found to be active against replicating WT *Mtb* grown in a 7H9 medium with inorganic sulfate as a sole source of sulfur (Table S6). Given their excellent bactericidal activity against the replicating and nonreplicating WT *Mtb*, selected ellipticine analogs were also tested against non-replicating MDR (Jal 2287) and XDR (MYC 431) clinical isolates of *Mtb*. Again, compounds **8** and **10** substituted at both C-9 and N-2 positions showed significant bactericidal activity in MDR and XDR clinical isolates, while compounds **6** and **7** with no N-2 substitution remained ineffective (Table S7). Interestingly, 9-hydroxyellipticine and ellipticine-*N*²-oxide have been characterized to be the major active metabolites of ellipticine (**5**) as a result of oxidative hepatic metabolism. Incidentally, 9-hydroxyellipticine is widely considered as the pharmacologically active form of ellipticine, while ellipticine-*N*²-oxide can undergo Polonovski rearrangement as a major route to form carbenium species responsible for covalent adduct formation with DNA.³⁸ In this context, compounds **8–10** bearing substituents at the C-9 position and a quaternary nitrogen at the N-2 position cannot be oxidized to form these metabolites. This strong chemical evidence is further validated by very poor anticancer activity exhibited by **8–10** compared to that of the parent compound **5** (Table S8) when screened against 60 different cancer cell lines at NCI. Nonetheless, to rule out DNA damage as a mechanism for the bactericidal effect of these compounds in mycobacteria, we measured the upregulation of DNA-damage response gene *RecA* by qRT-PCR. We observed no upregulation of *RecA* in response to treatment with **8** (Figure S5a). In contrast, mitomycin C, known to induce DNA damage in mycobacteria,⁴² showed significant upregulation of *RecA* normalized to the housekeeping gene *SigA*. These data suggest that DNA damage by covalent DNA adduct formation is not the mechanism by which **8–10** display their bactericidal activity in mycobacteria. To evaluate if **8–10** can still intercalate with DNA in the range of their MBC concentration, a standard fluorescence-displacement assay involving calf-thymus DNA and ethidium bromide was employed.⁴³ Hearteningly, none of the compounds showed >5% decrease in fluorescence intensity (FI) at

their MBC concentration, or >20% decrease in **FI** at concentrations 5 times the MBC concentration (Figure S5b), thereby indicating the possibility of APSR being the major target for their bactericidal action.

Direct Target Engagement

Similar to **5**, ITC binding measurements were used to study the direct target engagement by other hits including **8** (Figure 5a and Table S5). As an expected trend from the whole-cell evaluation, we measured a ~15-fold increase in binding affinity of compound **8** compared to the parent compound **5**. Interestingly, this increase in binding affinity was a direct result of increase in the binding enthalpy suggesting a probable increase in electrostatic or hydrogen bonding interactions between **8** and APSR (Figure 5a). Our APSR kinetics studies in the presence of inhibitor **8** employing a continuous assay for sulfite detection confirmed that the mode of inhibition is competitive and that these inhibitors occupy the catalytic pocket of APSR (Figure S5a).⁴⁴ To obtain initial structural insights into the mode of binding, we performed docking of **8** into the crystal structure of *P. aeruginosa* APSR (*Pa*APSR)⁴⁵ using the AutoDock (AD4) computational tool.⁴⁶ *Pa*APSR and *Mtb*APSR are related by 27.2% sequence identity and 41.4% sequence similarity, with a particularly high sequence homology in the substrate binding site.⁴⁷ Compound **8** preferentially docked into the substrate-binding pocket of *Pa*APSR that is lined with several negatively charged amino acid residue side-chains at physiological pH. Based on the distance measurements, the quaternary nitrogen (*N*²-methyl) of **8** lies within the charge–charge interaction range of *Pa*APSR residue D66, while the indole (*N*⁶) proton of **8** falls in the hydrogen bonding distance range of the S225 residue side chain oxygen of *Pa*APSR (Figure 5b). Significantly, sequence alignment shows that both D66 and S225 residues are conserved in *Mtb*APSR, suggesting that these studies can be readily extended to *Mtb*APSR (Figure S5b). These results indicate that the positively charged quaternary nitrogen of ellipticinium derivatives **8–10** plays a significant role in their enhanced affinity for *Mtb*APSR compared to the parent compound **5**.

Cellular On-Target Activity

It has been shown that the *CysH* mutant of *Mtb* lacks the ability to survive under *in vitro* conditions (7H9 medium), but its growth can be restored partially by the addition of cysteine or methionine.¹² This suggested to us that the addition of methionine might partially desensitize the bacilli against the bactericidal effect of APSR inhibitors. Accordingly, we observed modest but measurable 2–4 fold reduction in the bactericidal activity for six out of eight compounds when tested against nonreplicating *Mtb* grown in the presence of 2 mM methionine (Table 1). The modest rescue effect may also be due to reduced uptake of methionine, similar to other xenobiotics in nonreplicating *Mtb*.⁴⁸ An alternate way to assess target specificity was to test compounds directly in *CysH* mutant cultured in the presence of cysteine or methionine. The *CysH* mutant with similar dependence on methionine or cysteine has also been reported for *Mycobacterium smegmatis* (*Msm*),²² an organism that is commonly used as a surrogate for the slow-growing and pathogenic *Mtb* H37Rv. The presence of methionine also increases the survival of nutrient-starved *CysH Msm*, albeit only partially (Figure S7). Therefore, we decided to use *CysH Msm* to expedite the testing

of our compounds. After verifying that the APSR inhibitors have comparable potencies in the nonreplicating wild-type (WT) *Msm* and nonreplicating WT *Mtb*, we proceeded with testing the activity of these compounds in *CysH Msm* (Table 1 and Figure 6a). Notably, a significant decrease in the bactericidal activity of compounds in *CysH Msm* compared to that of WT *Msm* was observed. Compounds **1–5** showed no measurable bactericidal activity in *CysH Msm*, while compounds **8–10** showed **24–62** fold reduction in bactericidal activity. Significantly, the lost potency of **8–10** against *CysH Msm* was almost completely restored by complementation with *Mtb* CysH, indicating that APSR is likely the major target accounting for the bactericidal effect of these compounds (Figure 6a).

Functional Validation

Methods for measuring the intracellular levels of thiols in bacterial cells, including mycobacteria, are well established.^{49,50} Accordingly, we next measured the content of specific thiol products downstream of APSR in the reductive sulfate assimilation pathway in response to compound treatment. As expected, we found a significant decrease in the intracellular levels of sulfite (a direct product of APSR catalysis), cysteine, and MSH when treated with **8**, thus providing functional validation (Figure 6b). We have recently developed a genetically encoded, redox-sensitive probe Mrx1-roGFP, to quantitatively measure the MSH redox potential (E_{MSH}) within *Mtb*.²⁵ (Figure 6c). This novel redox biosensor exclusively responds to the total MSH concentration within live *Mtb*. H37Rv *Mtb* expressing this biosensor was used to measure the perturbations in E_{MSH} after incubation with the compounds for 3 h (Figure 6c). Compounds **4**, **8**, **9**, and **10** that inhibit APSR and display high potency in killing nonreplicating *Mtb* induced a significant oxidative shift in the E_{MSH} of *Mtb*. Since short treatment duration (3 h) was used to measure the E_{MSH} of *Mtb*, the observed increase in oxidative stress can be attributed to changes in MSH concentration rather than mycobacterial killing. The highest oxidative shift was induced by compounds **4** and **9** with an E_{MSH} value of ~245 mV compared to the vehicle treated *Mtb* (E_{MSH} = 280 mV). Compound **6**, which inhibits APSR poorly and does not kill nonreplicating *Mtb* effectively, showed no change in the E_{MSH} of *Mtb*. These data clearly indicate that the identified compounds kill nonreplicating *Mtb* by inducing MSH concentration dependent oxidative stress in *Mtb*.

A monumental goal in the effective control of tuberculosis is to eradicate the present 2-billion person reservoir of persistent *Mtb*.³ This goal can only be achieved by discovery and development of new drugs with sterilizing effect against the persistent bacilli. Identification of such effective sterilizing drugs is simply not possible without translating information about the metabolome and proteome adaptations in persistent *Mtb* into the drug discovery screening platforms.⁵¹ Encouragingly, the metabolic vulnerability of persistent bacilli in maintaining low respiration has recently been exploited to show an *in vitro* sterilizing effect in persistent *Mtb*.^{52,53} A growing body of evidence has also pinpointed the extreme susceptibility of the persistent *Mtb* to interference with its cellular redox state, thus making redox homeostasis a promising target for killing persistent bacilli.⁵¹

Here, we target an essential enzyme in sulfur metabolism, APSR, that is genetically and pharmacologically implicated in redox homeostasis, survival, and virulence of persistent

Mtb.^{12,22} Importantly, APSR lacks a human homologue, and its isoforms are present in many pathogenic bacteria including *P. aeruginosa*, *E. coli*, and *S. typhimurium*, making it an attractive target for broad-spectrum antibiotic development.⁴ We report a first HTS platform for discovery of APSR inhibitors comprised of 1536-well LUM and 384-well FP assays, which report APSR activity with high sensitivity and can be employed orthogonally to screen for APSR inhibitors. Although more elegant in many ways, the target-based HTS strategy for discovery of antibacterial compounds has gained some disrepute.⁵⁴ The primary reason for doubt is the inability of biochemical hits to reproduce the same level of potency in the whole cells, which is often ascribed to their poor cell-wall penetration properties. However, drug efflux is increasingly being discovered as a factor responsible for diminished efficiency of anti-TB compounds⁵⁵ and has been shown to be successfully overcome by cotreatment with efflux pump inhibitors or by chemical modification.⁵⁶

Development of new antibiotics is an arduous, lengthy, and a very costly endeavor. Moreover, the usage of new antibiotics is likely to be of limited duration due to the problem of drug resistance. Therefore, the strategy of repurposing existing bioactive compounds for antibacterial application is gaining increasing popularity.⁵⁷ For instance, fluoroquinolone anti-TB agents serve as the classic example of a class of repurposed drugs in treatment of TB infection.⁵⁸ Given the similarities in their pharmacological action, the repositioning of anticancer agents as antibacterial agents has recently emerged as an attractive strategy.⁵⁹ This is especially true when the antibacterial activity of the candidate compound is more potent than its anticancer activity, thereby providing a sufficient safety index. Our HTS with 38 350 compounds and follow-up studies have identified at least three different structural classes of compounds as APSR inhibitors. The structural class of compounds that shows the most potent bactericidal activity against WT as well as MDR and XDR clinical isolates of *Mtb* are the ellipticinium compounds **8–10**. Ellipticinium compounds are known for their selective cytostatic activity against the brain tumor cell lines of glial origin as identified in the *in vitro* screen conducted by NCI.⁶⁰ This selectivity has been attributed to their preferential uptake and intracellular accumulation,⁶¹ which was further found to be dependent on the membrane potential of target cells.⁶² Given their cell-specific cytostatic properties, the high potencies displayed by ellipticiniums **8–10** in killing nonreplicating *Mtb* was interesting. *In vitro* studies have shown that the membrane potential of *Mtb* is compromised in their persistent state.⁶³ In hindsight, this could account for the potent bactericidal activity of compounds **8–10** in nonreplicating *Mtb*. Our ITC studies and whole-cell evaluation using conditional *CysH Msm* mutant and its complement strain suggest that APSR is the major target for these compounds in mycobacteria. Interestingly, these compounds display MBC values ranging from 1.5 to 6.0 μM in drug-sensitive as well as drug-resistant *Mtb*, which is significantly lower than the LC_{50} values of 59–74 μM against tumorigenic mammalian cells reported for these compounds (Table S7). Significantly, the bactericidal activity shown by compound **8** ($\text{MBC}_{99} = 1.7 \mu\text{M}$; 0.70 $\mu\text{g/mL}$) is comparable to the *in vitro* activity of investigational drug PA-824 or other compounds effective against nonreplicating *Mtb*.⁴¹ Therefore, ellipticinium compounds show tremendous promise as a scaffold for development of effective LTBI therapy. Although the fused-heteroaromatic nature of these compounds may appear less desirable as a medicinal chemistry lead, it should be noted that the active site of APSR is tailor-made for an occupying substrate (APS)

constituting a flat and planar adenine-base. Interestingly, four out of our top six hit clusters (Figure 4b) constitute fused aromatic rings despite inclusion of 5000 compounds from RO5 diversity library with rich Fsp³ character in our screening collection. This clearly indicates that compounds mimicking the structural features of the substrate are inherently preferred as APSR inhibitors. Parallels can be drawn with kinase inhibitors where a majority of clinically used as well as investigational compounds are flat and aromatic to mimic the adenine portion of ATP.⁶⁴

In addition to serving as lead scaffolds for the development of potential anti-TB therapeutics, the APSR inhibitors identified here can serve as molecular probes in unraveling the links between *Mtb* persistence, antibiotic tolerance, and sulfate assimilation pathway. Altogether, they are expected to serve as powerful tools in preclinical and clinical validation of the *Mtb* sulfur metabolic pathway as a therapeutic target for the eradication of persistent *Mtb*. Additionally, the existence of APSR in other pathogens enables similar use of APSR inhibitors in dissecting sulfur metabolic pathways across species.

METHODS

Primary High Throughput Screening

Reagents for HTS assays were prepared as detailed in the SI. The HTS of 38 350 compounds was executed using a 1536-well LUM assay on the automated GNF/ Kalypsys robotic platform of the Scripps Research Institute Molecular Screening Center (Jupiter, FL). A stepwise protocol as outlined in Table S1 was followed. Briefly, 1.5 μ L of assay buffer containing 4 nM *Mtb*APSR, 2 μ M Trx, and 10 mM DTT was dispensed into 1536-well plates (Corning; Cat. No. 7254) using a liquid dispenser. Compounds in 10 mM DMSO stock solution were then robotically pinned using a 1536-well PinTool unit at a final assay concentration of 9.25 μ M. Positive (compound NSC 95397 at 100 μ M, high signal, 100% inhibition) and negative controls (DMSO only, low signal, 0% inhibition) were located in columns 3 and 46, respectively ($n = 24$ each). The plates were allowed to incubate at RT (RT) for 15 min. Next, the APSR reaction was initiated by dispensing 1.5 μ L of assay buffer containing 0.6 μ M APS, followed by an incubation period of 5 min. The AMP produced in the APSR reaction was then detected using the AMP-Glo assay platform (Promega) following the manufacturer's protocol. This involved dispensing of 1.5 μ L of AMP-Glo reagent I, incubation at RT for 15 min followed by dispensing of 3 μ L of AMP-Glo detection reagent, and incubation at RT for 50 min. The plates were then read for luminescence signal with the ViewLux reader (PerkinElmer). HTS data were analyzed using Assay Explorer software (Symyx). The Z' values were calculated for each plate from the high and low controls using a standard equation.²⁶ Percent inhibition for each compound was calculated from the median values of high and low controls as described before.⁶⁵ A hit was defined as a compound showing inhibition greater than or equal to the calculated cutoff of 21.9% ($AVG_{\text{sample}} + 3 \cdot SD_{\text{sample}}$). The purity and chemical identity of all 403 hits was verified using LC/MS. A total of 398 hits were available for retesting in a concentration–response format in 1536 well plates using the same protocol listed in Table S1. For concentration–response studies, compounds were arrayed in a 10-point, 3-fold serial dilution using 2.5 mM stock solution in DMSO and pinned to 1536-well plates (as described above)

to provide final compound concentrations in the well ranging from 46.24 to 0.001 μM . All 398 hits were also tested in a concentration–response format in the 1536-well plate LUM assay counterscreen. The compound 2-(methylthio)ADP trisodium (Aldrich) at 100 μM served as a high control and DMSO as a low control. For each compound, triplicate % inhibition values were plotted against compound concentration. The data were fit to a four-parameter equation describing a sigmoidal dose–response curve with adjustable baseline using Assay Explorer software to calculate the apparent IC_{50} values.

Secondary Screening

The 384-well plate FP assay was used orthogonally to screen hits from a primary screen using the protocol listed in Table S2. Briefly, 2.5 μL of assay containing 4 nM *Mtb*APSR, 2 μM Trx, and 10 mM DTT was dispensed into 384-well black plates (Griener; Cat. No. 784076) using an FRD-IB liquid dispenser (Aurora). Compounds at 10 mM concentration in DMSO were arrayed in a 10-point, 3-fold serial dilution, concentration–response format, and pinned using the Biomex NXP instrument (Beckman) equipped with a 384-well pin-tool unit to achieve a final assay concentration range of 100–0.005 μM . A positive control (compound NSC 95397 at 100 μM , high signal, 100% inhibition) was located in columns 1 and 2, while negative control (DMSO only, low signal, 0% inhibition) was located in columns 23 and 24, respectively ($n = 24$ each). The plates were allowed to incubate at room RT for 15 min. Next, the APSR reaction was initiated by dispensing 2.5 μL of assay buffer containing 0.6 μM APS followed by an incubation period of 10 min. The APSR reaction was stopped using 2.5 μL of 800 mM MES buffer at pH 6.5. The AMP produced in the APSR reaction was then detected using the Transcreeper AMP detection platform (BellBrook Laboratories). This involved dispensing of 2.5 μL of AMP detection mixture (16 nM AlexaFluor 633 tracer and 13 $\mu\text{g}/\text{mL}$ AMP antibody in 50 mM tris buffer, pH 7.5) followed by incubation at RT for 90 min. The plates were then read for FP signal (Ex. = 620 nm; Em. (P) = 688 nm; Em. (S) = 688 nm) with Envision plate reader (PerkinElmer). The HTS data were analyzed using GraphPad prism software using a similar protocol. For each compound, triplicate % inhibition values were plotted against compound concentration. The data were fit to a four-parameter equation describing a sigmoidal dose–response curve using GraphPad Prism software to calculate the apparent IC_{50} values. The counterscreens for RCCs (HRP-PR assay), thioredoxin inhibition, and DNA intercalation were performed as described in the SI.

ITC Binding Studies

A Nano-ITC low-volume (TA Instrument) sample cell was rinsed with freshly degassed ITC buffer (50 mM bis tris propane, 0.02% Brij-35, pH 7.4). It was then loaded with 220–250 μL of 60 μM *Mtb*APSR in ITC buffer. The 50 μL Nano-ITC syringe was rinsed with ITC buffer and loaded with 45–50 μL of 600 μM test compound. The instrument temperature was set to 25 $^{\circ}\text{C}$ with a stirring speed of 300 rpm. A total of 15–30 injections of test compound (first inj. = 1–2 μL ; remaining inj. = 1.5–3 μL each, separation between inj. = 120–180 s) were made into the sample cell containing *Mtb*APSR. The dilution control for test compounds was performed by substituting *Mtb*APSR in the sample cell with ITC buffer. The raw ITC data were analyzed using NanoAnalyze Software. The integrated data were corrected for

compound dilution and fit to the independent model (NanoAnalyze software) in order to determine the binding and thermodynamic parameters.

MBC Determination by CFU Measurement

MBC values for the potent APSR inhibitors were determined by measuring colony forming units (CFU) on 12-well 7H11 agar plates. The nutrient starvation of WT *Msm*, *CysH Msm* mutant, and *CysH (pMSGS/Mtb CysH)* complement¹² strains was performed as detailed in the SI. Nutrient-starved bacilli (final OD₆₀₀ = 0.1) in PBST buffer were plated in clear, flat-bottom, 96-well plates and incubated with test compounds in a 3-fold, 11-point serial dilution format with a starting concentration of 10 μ M and a total assay volume of 200 μ L. After incubation for 3 days at 37 °C, a 5 μ L culture from each well was serially diluted to 1:200 in PBST, and a 20 μ L aliquot from this diluted sample was then plated onto 12-well 7H11 plates. The plates were incubated for 3 days, and colonies were counted to calculate CFU. The MBC values were reported as the lowest concentration of compounds that resulted in a 2-log reduction in CFU compared to the CFU measured for wells without compound treatment. The MBC could also be calculated by plotting CFU against the concentration of a compound in a semilog plot and fitting the data to a standard four parameter equation to calculate MBC₉₉ values using Graphpad Prism 5.

E_{MSH} Measurements in *Mtb*

The measurement of intracellular E_{MSH} was performed as reported earlier.²⁵ Briefly, H37Rv *Mtb* expressing Mrx1-roGFP2 was grown in the presence or absence of test compounds (5 $\times$ MBC) in 7H9 medium at OD₆₀₀ of 0.6–0.8 for 3 h. The bacilli were harvested, washed twice with PBS, and treated with 10 mM N-ethylmaleimide (NEM) for 5 min followed by fixation with 4% PFA for 15 min at RT. Treatment with NEM is necessary to block the redox state of roGFP2. Bacilli were subjected to flow cytometry (BD FACS Verse Flow cytometer, BD Biosciences) after washing thrice with PBS. The ratio of emission (510/10 nm) after excitation at 405 and 488 nm was calculated. Data were analyzed using the FACSuite software. For each experiment, the minimal and maximal fluorescence ratios were also determined, which correspond to 100% sensor reduction and 100% sensor oxidation, respectively. Cumene hydroperoxide (1 mM) was used as the oxidant and DTT (10 mM) as the reductant. Finally, these observed ratios were used to calculate E_{MSH} using the Nernst equation as described earlier.²⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health (GM087638 to K.S.C.), the Department of Biotechnology (DBT), Ministry of Science and Technology, India (BT/PR5020/MED/29/454/ 2012 to A.S.), and the Wellcome-DBT India Alliance (500034/ Z/09/Z to A.S.) for financial support. We also thank H. Paritala for helpful discussions.

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Figure 1.

Significance of sulfur metabolic pathway in *Mtb* persistence. (a) Reductive branch of *Mtb* sulfate assimilation pathway showing biosynthesis of reduced sulfur-containing biomolecules.⁶ Sulfur, available to mycobacteria within the host as an inorganic sulfate, is activated through adenylation to APS, a reaction catalyzed by ATP sulfurylase (ATPS). APS is reduced by APS reductase (APSR) to sulfite (SO_3^{2-}) and subsequently to sulfide (HS^-) by sulfite reductase (SiR). Sulfide is incorporated into O-acetylserine (OAS) to form cysteine by OAS (thiol)lyase (OASTL). Ultimately, cysteine is used for the biosynthesis of proteins, MSH, and other essential biomolecules required for survival, pathogenesis, and antioxidant defense in persistent *Mtb*. (b) Mycothiol, a major low molecular weight thiol antioxidant, and its primary function in maintaining redox homeostasis.⁶⁶

Figure 2.

Design, optimization, and miniaturization of HTS assays. (a) Schematic of the LUM assay employing the AMP-Glo platform (Promega) to detect AMP produced in the APSR reaction. (b) Signal linearity for AMP detection in the luminescence assay. (c) Optimization of APSR assay concentration to provide linear reaction progress during the course of assay at fixed APS concentration. APSR assay concentration was varied from 0.3 to 40 nM in the presence of APS (300 nM) in 50 mM bis-tris propane buffer (pH 7.4) containing 1 μ M thioredoxin (Trx) and 5 mM DTT and incubated at RT for 10 min. The AMP produced by APSR at different concentrations was then measured in the LUM assay. (d) Robustness of LUM assay in 384- and 1536-well plates as indicated by the assay performance indicators Z' and signal/baseline (S/B) ratio.

Figure 3.

Validation of HTS work-flow in pilot screen. (a) Activity scatterplot for the LOPAC screen (triplicate study) using the LUM assay shows clear separation between high (blue) and low (green) controls and reproducible identification of the same 18 hits (red dots above the calculated cutoff) in three different trials. The compounds were screened at a 10 μM concentration. (b) Comparison of two LOPAC screening replicates with linear correlation indicating good reproducibility. (c) Dose-response and counterscreening of primary hits identified in the LOPAC screen. Ellipticine inhibits the LUM assay with an apparent IC_{50} of 11.1 μM but is inactive in the LUM counterscreen. 2-Thiomethyladenoside diphosphate trisodium, a false-positive hit, is active in both the LUM assay and its counterscreen. β -

Chloro-L-alanine hydrochloride, a “non-hit” control compound showing no activity in both the assays.

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Figure 4.

HTS of 38 350 compounds. (a) Activity scatterplot showing clear separation between high (blue) and low (green) controls and identification of 403 primary hits (red dots above cutoff line). The compounds were screened at 9.25 μ M concentration. (b) Confirmed hits after dose-response and counterscreening ($n = 160$) were clustered using chem-informatic tools as well as manual culling. Selected clusters A–F with hits displaying potent APSR inhibition and their general structure are shown.

Figure 5.

Evaluating target-engagement and specificity of whole-cell assay active compounds. (a) ITC measurement of compound **8** binding to APSR. (Top) ITC titration showing time-dependent deflection of the heat signal after each injection of **8** (600 μM) into a microcalorimetric cell containing 30 μM *Mtb*APSR (black) or buffer (red). ITC experiments were carried out at 25 °C in 50 mM bis-tris propane buffer, 0.02% Brij-35, pH 7.4. (Bottom) The integrated calorimetric data after correction for dilution of **8** was fit to the independent model using NanoAnalyze software to obtain binding and thermodynamic parameters. (Inset) Graphical representation of thermodynamic parameters for interaction between **8** and *Mtb*APSR. (b) Docking of **8** with subunit C of *Pa*APSR X-ray crystal structure (PDB entry 2GOY)⁴⁵ using the AutoDock program. It preferentially docks into the APSR-active site lined by amino acid residues bearing negatively charged side chains (D66, E65, E240). Distance measurements predict interdigitated salt bridge interaction between D66 of APSR and quaternary N^2 of **8**, and H-bonding interaction between S225 of PAPR and indole (N^6) proton of **8**.

Figure 6.

Target specificity and functional validation of whole-cell assay actives. (a) Bactericidal activity of compounds **8–10** in nonreplicating (NR) WT *Msm*, NR *CysH*, *Msm*, and complemented *CysH* (pMSGS: *Mtb*, *CysH*) *Msm*. Survival of mycobacteria was determined by CFU measurements, and the data were fit to a standard four parameter logistic curve to calculate the MBC_{99} values using GraphPad Prism. (b) Determination of thiol content in compound treated *Msm*. WT *Msm* treated with varying concentrations of **8** corresponding to 0, 0.5, 1, 2, and 8 times its MBC concentration (1.7 μM) were analyzed for a decrease in cellular concentration of three different bimane-labeled reduced thiols produced via reductive sulfur assimilation. *N*-Ethylmaleimide (NEM) treatment of cell extract prior to monobromobimane served as a positive control. (c) Measurement of mycothiol redox potential (E_{MSH}) in compound treated live *Mtb*. (Left) Schematic showing coupling of Mrx1-roGFP2 probe to MSH levels inside *Mtb*. Under normal conditions, Mrx1-roGFP2 is maintained in a reduced state due to a high MSH/MSSH ratio. A reduction in the MSH levels results in an oxidative shift in E_{MSH} measured as an increase in the 390:490 nm excitation ratio.²⁵ (Right) H37Rv *Mtb* expressing Mrx1-roGFP2 incubated with vehicle or compounds at 5 times their MBC concentrations for 3 h followed by measurement of biosensor response by flow cytometry. The graph showing 390/490 nm excitation ratios normalized to the ratios of fully reduced bacilli set as 0.1 and fully oxidized bacilli as 1. Full reduction and oxidation achieved by treating bacilli with 10 mM DTT and 1 mM CHP, respectively. Increase in 390/490 nm excitation ratio upon treatment with compounds indicates a decrease in MSH levels.

Table 1
Bactericidal Activity of APSR Inhibitors against Nonreplicating WT *Mtb*, WT *Msm*, and *CysH Msm*

Comp ID	Structure	LUM Assay; IC_{50} (μ M)	Non replicating WT <i>Mtb</i>		Non replicating <i>Msm</i>	
			(-) Met	(+) Met ^b	mc ² :155;MBC ^a (μ M)	WT <i>cysH^b</i>
1		1.1	6	12	43 ^c	>100 ^c
2		4.3	25	50	46	>100
3		0.6	100	>100	62.7	>100
4		1.5	6	12	38.1 (33) ^c	>100
5		4.2	12.5	12.5	87.1	>100
6		47	>100	>100	>100	>100
7		32	>100	>100	>100	>100

Comp ID	Structure	LUM Assay; IC_{50} (μ M)	Non replicating WT <i>Mtb</i> H37Rv; MBC ^a (μ M)		Non replicating <i>Msm</i> mc ^c :155; MBC ^a (μ M)	
			(-) Met	(+) Met ^b	WT	<i>cysH</i> ^b
8		0.5	1.5	6	1.7 ^c	42.5 ^c
9		2.4	3	3	3.9 ^c	101 ^c
10		2.5	3	3	2.5 ^c	156 ^c

^a Unless otherwise noted, MBC values were determined using Alamar Blue assay.

^b Methionine (Met) in 2 mM concentration was used during growth, nutrient starvation (PBST Buffer), and compound incubation (PBST Buffer).

^c MBC values determined by CFU measurement on 7H11 plates.