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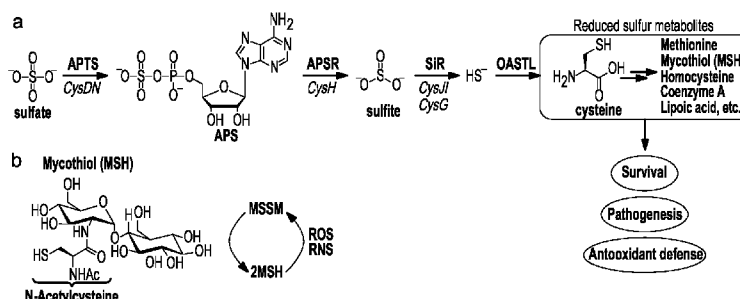
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(54) Title: INHIBITORS OF SULFUR METABOLISM WITH POTENT BACTERICIDAL ACTIVITY AGAINST MDR AND XDR M. TUBERCULOSIS

**FIG. 1**

(57) Abstract: An HTS platform for identification of APS reductase (APSR) inhibitors, a crucial enzyme in reductive sulfate assimilation, was developed, and a class of bioactive compounds displaying potent bactericidal activity in wild-type *Mtb* as well as multidrug-resistant (MDR) or extensively drug-resistant (XDR) tuberculosis clinical isolates was identified. A class of N-methylellipticinum analogs was shown to have potent antibacterial bioactivity against these drug-resistant TB strains. The invention thus provides a method of treatment of a patient afflicted with a tuberculosis infection such as an MDR or XDR strain, with an N-methylellipticinum compound that is an inhibitor of adenosine phosphosulfate reductase (APSR).

**Inhibitors of Sulfur Metabolism with Potent Bactericidal Activity against MDR and XDR
M. tuberculosis.**

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the priority of U.S. provisional application serial number 62/243,229, filed October 19, 2015, the disclosure of which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under GM087638 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

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 low-molecular 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 macrophage 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 serve as a major antioxidant defense system in *Mtb*. Therefore, disruption of sulfur-mediated redox homeostasis by inhibiting sulfate assimilation is also an attractive strategy to kill drug-tolerant subpopulations of *Mtb*.

Importance of sulfur metabolism in persistence and antibiotic tolerance of *Mtb* necessitates 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 ΔCysH *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

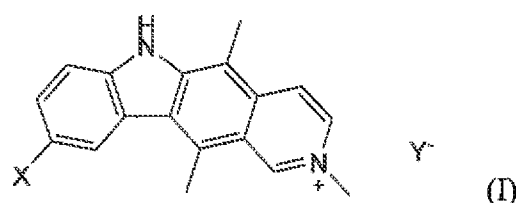
SUMMARY

From a future translational perspective, APSR is an attractive therapeutic target for the anti-TB drug development as no APSR homolog 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 constitute a combination of HTS assays for identification and validation of APSR inhibitors and implemented it 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 7 compounds representing three different structural classes to have promising bactericidal activity against non-replicating *Mtb*. Significantly, ellipticine and its derivatives with known pharmacology, showed potent bactericidal activity in drug-sensitive and drug-resistant *Mtb*, providing a promising opportunity for development of repurposed anti-TB compounds. Conditional $\Delta CysH$ *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 to show significant decrease in cellular levels of key sulfur metabolites as well as induce significant oxidative shift in *Mtb* MSH redox potential (E_{MSH}) by employing a novel redox biosensor we recently developed.²⁵

Accordingly, the invention provides, in various embodiments, a method of treatment of a patient afflicted with a tuberculosis (TB) infection, comprising administering to the patient an effective dose of an N-methylellipticinium compound that is an inhibitor of adenosine phosphosulfate reductase (APSR). The tuberculosis infection can be a multiple drug resistant (MDR) tuberculosis infection, an extremely drug resistant (XDR) tuberculosis infection, or a latent-TB infection (LTBI).

For example, the wherein the N-methylellipticinium compound can be a compound of formula (I)



wherein X is hydrogen, halo, (C1-C4)alkyl, or (C1-C4)alkoxy, and Y is a pharmaceutically acceptable counterion. The term N-methylellipticinium as used herein refers to the metho salt of ellipticine or a 9-substituted analog as shown in formula (I).

The invention can further provide a method of treatment of a tuberculosis infection wherein the N-methylellipticinium compound co-administered to the patient in conjunction with an effective dose of a second anti-tuberculosis drug; e.g., wherein the N-methylellipticinium compound, such as of formula (I), potentiates the effectiveness of the second anti-tuberculosis drug.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Depicts significance of sulfur metabolic pathway in *Mtb* persistence. a) Reductive branch of *Mtb* sulfate assimilation pathway showing biosynthesis of reduced sulfur-containing biomolecules. Sulfur, available to mycobacteria within the host as 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 as antioxidant defense in *Mtb*.

Fig. 2. Depicts Design, Optimization and Miniaturization of LUM Assay. a) Schematic of the LUM assay employing 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 - 40 nM in presence of APS (300 nM) in 50 mM bis-tris propane buffer (pH 7.4) containing 1 μM 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 1,536-well plate as indicated by the assay performance indicators Z' and signal:baseline (S:B) ratio.

Fig. 3. Depicts 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 cut-off line). The compounds were screened at 9.25 μM 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 is shown.

Fig. 4. Depicts no cytotoxicity of whole-cell assay active compounds to mammalian cells. Cytotoxicity of compounds 1-10 in non-carcinogenic VERO monkey kidney cell line as measured in MTT assay. The % cell viability was plotted against compound concentration. The data for compound 5 was fit to four parameter sigmoidal dose-response function using GrapPad Prism 5.

Fig. 5. Depicts inability of ellipticinium compounds to cause DNA Damage or intercalate with DNA. a) Measuring compound concentration-dependent increase in relative expression of *RecA* mRNA in *Msm* upon treatment with either 0, 1.7 μ M (1xMBC), 3.4 μ M (2xMBC), 6.8 μ M (4xMBC) and 13.6 μ M (8xMBC) concentration of compound 8. Mitomycin C (0.2 μ g/mL), a known compound to cause DNA damage in *Msm*,³⁹ was used as a positive control. Comparative C_T method with housekeeping gene *SigA* as a reference gene was used to measure the relative expression of *RecA*. b) Testing whole-cell active ellipticinium compounds (8-10) for DNA intercalation properties. Grey shaded area marks the concentration of compounds ranging from MBC – 5 $\times$ MBC values, highlighting less than 20 % DNA intercalation at this concentration range.

Fig. 6. Depicts direct target engagement and proposed mode of interaction of whole-cell assay active compounds with APSR. a) ITC measurement of compound 8 binding to APSR. (top) ITC titration showing time-dependent deflection of heat signal after each injection of 8 (600 μ M) into microcalorimetric cell containing 30 μ M MtbAPSR (black) or buffer (red). ITC experiments were carried out at 25 $^{\circ}$ 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 independent model using NanoAnalyze software to obtain binding and thermodynamic parameters. (inset) Graphical representation of thermodynamic parameters for interaction between 8 and MtbAPSR. b) Docking of 8 with subunit C of PaAPSR x-ray crystal structure (PDB entry 2GOY) 45 using 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 inter-digitated salt bridge interaction between D66 of APSR and quarternary N2 of 8, and H-bonding interaction between S225 of PAPR and indole (N6) proton of 8.

Fig. 7. Depicts target specificity and functional validation of whole-cell assay actives. a) Bactericidal activity of compounds **8-10** in non-replicating (NR) WT *Msm* (●), NR $\Delta CysH$ *Msm* (●) and complemented $\Delta CysH$ (pMSGS: *Mtb CysH*) *Msm* (●). Survival of mycobacteria was determined by CFU measurements and the data were fit to 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 decrease in cellular concentration of three different bimane-labelled reduced thiols produced *via* reductive sulfur assimilation. *N*-Ethylmaleimide (NEM) treatment of cell extract prior to mono-bromobimane 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 reduced state due to high MSH:MSSM ratio. A reduction in the MSH levels results in an oxidative shift in E_{MSH} measured as increase in the 390:490 nm excitation ratio. (right) H37Rv *Mtb* expressing Mrx1-roGFP2 incubated with vehicle or compounds at five 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 decrease in MSH levels.

Figure 8. Depicts kill kinetics of compound **8**. Nutrient-starved WT *Msm* were incubated with Compound **8** at 0.01 (○), 0.04 (◆), 0.16 (▼), 0.63 (▲), 2.50 (■), and 10.0 (●) μM concentration and a 5 μL aliquot at periodic interval was serially diluted to 1:200 in PBST (with thorough mixing) and a 20 μL aliquot from this diluted sample was then plated on to a 12-well 7H11 plates. The plates were incubated for 3 days and colonies were counted to measure CFU/mL.

Fig. 9. Depicts transcriptional response of mycobacteria to compound **8** treatment. a) Heat-map representation of sulfate transport and activation genes with mean expression fold changes >2 in *Msm* at 3 h and 8 h post compound **8** treatment. b) Heat-map representation of selected genes representing iron-sulfur proteins with mean expression fold changes >2 in *Msm* at 3 h and 8 h post compound **8** (10 μM , 5XMBC) treatment.

Fig. 10. Depicts isolation and whole genome sequencing of resistant mutant of *Msm* to compound **8**. a) Comparative dose-response study of compound **8** with WT and resistant mutant of *Msm*. b) Sequence alignment of MetN protein from WT and resistant mutant (*Msm_6X*) displaying mutation in the C-terminal tail as a result of a deletion of a single nucleotide. c) Cartoon depicting the consequences of mutation in the three genes leading to compound resistance: 1. MetN encoding a protein from methionine import channel; 2. TetR encoding regulatory protein that governs expression of RND-type drug efflux channels and; 3. Gene encoding CypP450 hydroxylase responsible for drug metabolism.

Fig. 11. Depicts tissue distribution of ellipticinium compounds **8-10**. a) Mean tissue concentrations of compounds **8-10** in mice ($n = 3$) after a single IV dose of 1 mg/kg. b) Mean tissue concentration of compound **8** in mice ($n = 3$) after a single IP dose of 25 mg/kg.

DETAILED DESCRIPTION

Development of effective therapies to eradicate persistent, slowly-replicating *M. tuberculosis* (*Mtb*) is a massive challenge in control of 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, sulfur metabolic pathway 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. We have established a first functional HTS platform for identification of APSR reductase (APSR) inhibitors, a crucial enzyme in reductive sulfate assimilation. Our HTS campaign involving 38,350 compounds led to the discovery of 3 distinct structural classes of APSR inhibitors. Ellipticines, class of bioactive compounds with known pharmacology, displayed potent bactericidal activity in replicating and non-replicating wild-type *Mtb* as well as multiple drug resistant (MDR) and extremely drug resistant (XDR) clinical isolates. Top hits showed markedly diminished potencies in a conditional Δ APSR mutant which could be restored nearly completely by complementation with *Mtb* APSR. ITC studies on representative compounds provided evidence for direct APSR engagement. Finally, potent APSR inhibitors significantly decreased the cellular levels of key sulfur metabolites and also induced oxidative-shift in mycothiol redox potential of live *Mtb*, thereby providing functional validation. 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.

Development and Validation of HTS Assays. APSR reduces substrate APS to SO_3^{2-} and byproduct AMP.²³ We developed and optimized 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). We identified 0.3 μM as the optimal APS concentration (~six-fold lower than apparent $K_m^{\text{APS}} = 2 \mu\text{M}$). APSR concentration was subsequently optimized (2 nM) to give a linear reaction progress (Figure 2c). When plate uniformity studies were performed over a period of three days, a clear separation between “high” and “low” controls (DMSO, 0% inhibition) was observed with high reproducibility over the 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 1,536 well-plate assay with no deterioration in the assay performance indicators (Figure 2d). The fully optimized 1,536-well plate homogenous LUM assay involves only four liquid transfer steps, in a total volume of 7.5 μL . While our optimized 1,536-well plate LUM assay was being validated and utilized in 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.²⁶ 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. Other critical parameters and assay protocol remained the same as the parent LUM 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.²⁷ Therefore, we developed and optimized a secondary HTS assay that detects AMP using Transcreeper fluorescence polarization immunoassay (FPIA) platform.²⁸ The assay was found to be extremely robust in the plate uniformity studies conducted in duplicate over a 3 day period in 384-well plates. Both LUM (1536-well plate) and FP (384-well plate) assays were validated in pilot screens using the commercially available library of pharmacologically active compounds (LOPAC) constituting 1,280 compounds. Same hits were

reproducibly identified in both assays demonstrating that these assays can be used in orthogonal fashion to identify and confirm APSR inhibitors.

High-Throughput Screen. After validation of HTS assays and the work-flow, the most critical task, perhaps, is the selection of compounds for screening.²⁹ 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),³⁰ ~5,000 compounds from the rule of 5 diversity (RO5) library, ~2,000 compounds from the in-house natural product diversity library, ~7,000 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 μ M concentration in the 1,536-well LUM assay with an average Z' factor of 0.91 ± 0.05 (Figure 3a). 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 cut-off of 21.9 % inhibition ($AVG_{sample} + 3 * SD_{sample}$), 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. The primary hits were then funneled through a battery of secondary and counterscreening assays for confirmation and validation of hits. To start with, all available 398 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 $IC_{50} \leq 10 \mu$ M, with 24 compounds (~6 %) showing $IC_{50} \leq 1 \mu$ M, 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 4 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 3b). A total of 25 compounds representing clusters A-F were selected for further follow-up studies. 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.³¹ 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.³²

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* lie in a slowly replicating state that closely mimic the persistent state of bacilli in human disease. Therefore, we started with evaluating the potential of APSR inhibitors in killing non-replicating *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.³³ Among the panel of twenty-eight compounds, five compounds (1-5) representing four different structure clusters showed promising bactericidal activity against non-replicating *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 (LC_{90}) of 63 μ M, however, its cytotoxicity was ~5-fold lower compared to its bactericidal activity against the non-replicating *Mtb* (MBC = 12.5 μ M) (Figure 4).

Interestingly, compound 5 is a plant alkaloid, ellipticine, which is a known antineoplastic agent with cytostatic activity specific to tumor cells.³⁴ 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.³⁴ 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.^{34,35} Additionally, 9-hydroxyellipticine, an active metabolite of ellipticine, has also been linked with selective inhibition of RNA polymerase I transcription.³⁶ 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 non-replicating *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 2). 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 non-replicating *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 non-replicating *Mtb*^{37,38}. On the contrary, 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 7H9 medium with inorganic sulfate as sole source of sulfur (Table 3). Given their excellent bactericidal activity against the replicating and non-replicating 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 4). Subsequently, compound **8** was also tested in additional MDR and XDR clinical isolates from the actual TB-patients and it killed these bacteria with identical potency to that of the WT bacteria (Table 5). A currently used anti-TB drug isoniazid (INH), on the other hand, was ineffective in killing these drug resistant strains. Given the promising bactericidal activity shown by these compounds in WT as well as MDR and XDR strains of *Mtb*, a representative compound was also tested for synergy in bactericidal action with other anti-TB drugs (Table 6). Significantly, compounds **8** was found to have synergistic effect with clofazimine and rifampicin anti-TB drugs, increasing their bactericidal potency by 32-fold and 8-fold respectively, while it showed additive effect with INH. Thus, these compounds exhibit potential to be used along with other anti-TB drugs and enhance their action.

Interestingly, 9-hydroxyellipticine and ellipticine-*N*²-oxide have been characterized to be the major active metabolites of ellipticine (**5**) as a result of its 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.³⁵ In this context, compounds **8-10** bearing substituents at *C*-9 position and a quaternary nitrogen at *N*-2 position cannot be oxidized to form these metabolites. This strong chemical evidence is further validated by very poor anti-cancer activity exhibited by **8-10** compared to that of the parent compound **5** 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 5a). On the contrary, mitomycin C, known to induce DNA damage in mycobacteria,³⁹ 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.⁴⁰ 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 5b), 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 6a and Table 2). As an expected trend from the whole-cell evaluation, we measured ~ 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 probable increase in electrostatic or hydrogen bonding interactions between **8** and APSR (Figure 6a). Our APSR kinetics studies in 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.⁴¹ 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)⁴² using AutoDock (AD4) computational tool.⁴³ *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.⁴⁴ 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 6b). 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. 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 $\Delta CysH$ mutant of *Mtb* lacks the ability to survive under *in vitro* conditions (7H9 medium), but its growth can be restored partially by addition of cysteine or methionine.¹² This suggested to us that addition of methionine may 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 non-replicating *Mtb* grown in presence of 2 mM methionine (Table 1). This modest rescue effect may also be due to reduced uptake of methionine similar to other xenobiotics in non-replicating *Mtb*.⁴⁵ An alternate way to assess target specificity was to test compounds directly in $\Delta CysH$ mutant cultured in presence of cysteine or methionine. The $\Delta CysH$ 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. Presence of methionine also increases the survival of nutrient-starved $\Delta CysH$ *Msm*, albeit only partially. Therefore, we decided to use $\Delta CysH$ *Msm* to expedite the testing of our compounds. After verifying that the APSR inhibitors have comparable potencies in the non-replicating wild-type (WT) *Msm* and non-replicating WT *Mtb*, we proceeded with testing the activity of these compounds in $\Delta CysH$ *Msm* (Table 1 & Figure 7a). There was a significant decrease in the bactericidal activity of compounds in $\Delta CysH$ *Msm* compared to that of WT *Msm*. Compounds **1-5** showed no measureable bactericidal activity in $\Delta CysH$ *Msm*, while compounds **8-10** showed 24-62 fold reduction in bactericidal activity. Significantly, the lost potencies of **8-10** against $\Delta CysH$ *Msm*

were restored nearly completely by complementation with *Mtb* CysH, thereby indicating that APSR is likely the major target for the bactericidal effect of these compounds (Figure 7a).

Functional Validation. Methods for measuring the intracellular levels of thiols in bacterial cells including mycobacteria are well established.^{46, 47} We next measured the content of specific thiol products downstream to APSR in the reductive sulfate assimilation pathway in response to compound treatment. As expected, we found 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 7b). 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 7c). 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 hours (Figure 7c). Compounds 4, 8, 9 and 10 that inhibit APSR and display high potency in killing non-replicating *Mtb*, included a significant oxidative shift in the E_{MSH} of *Mtb*. Since short treatment duration (3 h) was used to measure 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 E_{MSH} value of ~245 mV compared to the vehicle treated *Mtb* (E_{MSH} = 280 mV). Compound 6, which poorly inhibits APSR and does not kill non-replicating *Mtb* effectively, showed no change in the E_{MSH} of *Mtb*. These data clearly indicate that the identified compounds kill non-replicating *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.⁴⁸ Encouragingly, the metabolic vulnerability of persistent bacilli in maintaining low respiration has recently been exploited to show *in vitro* sterilizing effect in persistent *Mtb*.^{49, 50} A growing body of evidence have 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.⁴⁸

Global transcriptional analysis after compound treatment. We next examined the global transcriptional response of *Msm* to compound 8, focusing on the changes in the gene expression profile during the initial phase of treatment (3 h and 8 h) (Figure 9). Among the genes showing the strongest down-regulation at 3h and 8h time points were the genes related to sulfate transport and activation (Figure 9a). In the hindsight, the downregulation of these genes is an expected consequence of accumulation of APS and sulfate after inhibition of APSR. Interestingly, among the genes that were significantly upregulated were the genes that encoded iron sulfur proteins (Figure 9b). Genes for many essential proteins bearing Fe-S cluster as a cofactor were found to be highly upregulated like molybdoprotein oxidoreductase (*mopB*), phthalate 4,5-dioxygenase (*pth2*), Vanillate O-demethylase oxidoreductase (*VanB*), etc. The upregulation of genes for Fe-S bearing proteins is a likely consequence of inhibition of cysteine biosynthesis. Cysteine also serves as a substrate for reduced sulfur required for synthesis of Fe-S protein in addition to the synthesis of various essential reduced sulfur metabolites. These data thereby validate inhibition of APSR and reduced sulfur metabolism by ellipticinium compounds. Moreover, it also suggests that decrease in biosynthesis of Fe-S bearing proteins may be a likely cause of bactericidal effect of these compounds.

Isolation of resistant mutants and whole genome sequencing. Mutants of *M. smegmatis* resistant to compound 8 were selected *in vitro* by subjecting bacteria to sub-inhibitory concentrations of compound 8 and sequential sub-culturing. The MIC of compound 8 in resistant mutant was verified to be 6-fold higher than that in susceptible *Msm*, thus confirming successful identification of resistant mutant (Figure 10a). The genomes of resistant *Msm* strain (*Msm_6XR*), as well as the parental *Msm*, were sequenced to near-completion. Point mutations that conferred resistance to compound 8 were identified by comparative analysis of the genome sequences. Among the three genes conferring resistance, one of them encodes protein MetN, which is a part of the ABC transporter complex MetNIQ involved in methionine import. The resistant mutant shows single nucleotide deletion in MetN with 98.7% frequency, thereby mutating the C-terminal 8 amino acids compared to WT (Figure 10b). The crystal structure of *Msm* MetNI is not known, however, the crystal structure of MetNI from *E. coli* has been reported.⁵¹ The C-terminal tail positioned into the cytoplasm serves as a binding site for intracellular methionine and is involved in trans-inhibition of MetNI channel. Hence, mutation in C-terminal tail is likely to suppress this negative feedback inhibition mechanism and keep the

channel constantly open for methionine import. Subsequently, the imported methionine can serve as an alternative source of reduced sulfur through de novo cysteine biosynthetic pathway.

Therefore, this mutation helps resistant bacteria survive by overcoming the shortage of reduced sulfur due to inhibition of APSR, further validating APSR as a target for these compounds. The other prominent mutations were observed as single nucleotide variations in TetR regulator at 100% frequency and cytochrome P450 hydroxylase at 91.7 % frequency. TetR regulator regulates the expression of RND-type drug efflux channels, and therefore, is commonly mutated to acquire resistance, while the cytochrome P450 hydroxylases are involved in drug metabolism.

Evaluation of antibiotic spectrum. *Mtb*APSR inhibitors identified here were also tested against a variety of gram-positive and gram-negative pathogens. Compounds 8-10 showed excellent activity against many gram-positive and gram-negative bacteria (Table 7). Similar to *Mtb*, other pathogenic bacteria are dependent on reduced sulfur metabolites for their survival and possess sulfonucleotide reductases (SRs) in the form of APSR or PAPS reductase (PAPR). SRs can also be categorized into those that bear Fe-S cluster and those that don't. Interestingly, species with SRs bearing Fe-S cluster were susceptible for killing by the *Mtb*APSR inhibitors, with the exception of *P. aeruginosa*. On the other hand, the SRs that do not bear Fe-S cluster, were not found to be susceptible to killing by compounds 8-10. Overall, this data suggested that the APSR inhibitors identified by us can also be developed into broad spectrum antibiotics.

Preliminary Pharmacokinetic evaluation. To investigate the potential of ellipticinium compounds 8-10 as anti-TB therapeutics, we next evaluated various pharmacokinetic parameters. The compounds showed good aqueous solubility and high metabolic stability, as indicated by long $t_{1/2}$ values and low hepatic clearance (CL_{int}) (Table 8). Moreover, the compounds showed low toxicity when tested in 60 different mammalian cancer cell lines. Significantly, their cytotoxicity concentrations (CC_{99}) were thousands of fold higher than their MBC_{99} concentrations, thereby providing a wide safety index (Table 8). Finally, preliminary *in vivo* pharmacokinetic studies were performed by dosing mice with compounds 8-10 by IV route at 1 mg/kg dose and measuring the drug concentration in plasma and tissue distribution in key organs after 1 h. Although, the plasma levels were found to be low, the compounds were found to distribute in target organs like lungs and spleen at or above their MBC concentrations after 1 h (Figure 11a). The tissue levels of compound 8 were also tested by the IP route. Compound 8 was found to be at concentrations greater than 10-fold of their MBC concentration in target

organs like lungs and spleen after an hour of 25 mg/kg IP injection. Therefore, the compounds so far have shown favorable pharmacokinetics.

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.⁵¹ For instance, fluoroquinolone anti-TB agents serve as the classic example of a class of repurposed drugs in treatment of TB infection.⁵² Given the similarities in their pharmacological action, the repositioning of anticancer agents as antibacterial agents has recently emerged as an attractive strategy.⁵³ This is especially true when the antibacterial activity of the candidate compound is more potent than its anticancer activity, thereby providing sufficient safety index. Our HTS with 38,350 compounds and follow-up studies have identified at least 3 different structural classes of compounds as APSR inhibitors.

The structural class of compounds that shows 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.⁵⁴ This selectivity has been attributed to their preferential uptake and intracellular accumulation,⁵⁵ which was further found to be dependent on membrane potential of target cells.⁵⁶ Given their cell-specific cytostatic properties, the high potencies displayed by ellipticiniums 8-10 in killing non-replicating *Mtb* was interesting. *In vitro* studies have shown that the membrane potential of *Mtb* is compromised in their persistent state.⁵⁷ In the hindsight, this could possibly explain the potent bactericidal activity of compounds 8-10 in non-replicating *Mtb*. Our ITC studies and whole-cell evaluation using conditional $\Delta CysH$ *Msm* mutant and its complement strain suggested APSR to be the major target for these compounds in mycobacteria. Interestingly, these compounds displayed MBC values ranging from 1.5- 6.0 μ M in drug-sensitive as well as drug-resistant *Mtb*, which is significantly lower than the LC_{50} values of 59-74 μ M against tumorigenic mammalian cells reported for these compounds. Significantly, the bactericidal activity shown by compound 8 (MBC₉₉ = 1.7 μ M; 0.70 μ g/mL) is comparable to the *in vitro* activity of investigational drug PA-824 or other compounds effective against non-replicating *Mtb*.³⁸ Therefore, ellipticinium compounds show tremendous promise as a scaffold for development of effective LTBI therapy.

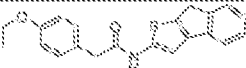
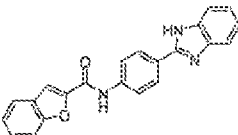
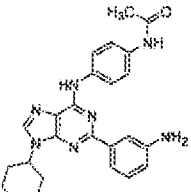
Although the fused-heteroaromatic nature of these compounds may appear less desirable

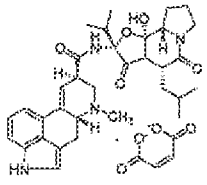
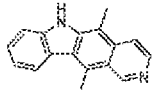
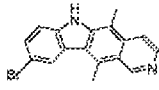
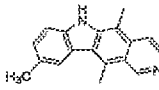
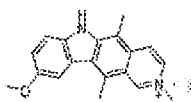
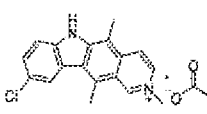
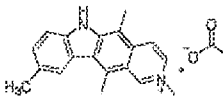
as a medicinal chemistry lead, it should be noted that the active-site of APSR is tailor-made for occupying substrate (APS) constituting a flat and planar adenine-base. Interestingly, 4 out of our top 6 hit clusters (Figure 3b) constitute fused aromatic rings despite inclusion of 5,000 compounds from RO5 diversity library with rich Fsp³ character in our screening collection. This clearly indicates that compounds mimicking the structural features of substrate are inherently preferred as APSR inhibitors. Parallels can be drawn with kinase inhibitors where majority of clinically used as well as investigational compounds are flat and aromatic to mimic the adenine portion of ATP.⁵⁸

In addition to serving as lead scaffolds for 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 *Mtb* sulfur metabolic pathway as a therapeutic target for eradication of persistent *Mtb*. Additionally, existence of APSR in other pathogens enables similar use of APSR inhibitors in dissecting sulfur metabolic pathways across species.

TABLES

Table 1. Bactericidal activity of APSR inhibitors against non-replicating WT *Mtb*, WT *Msm* and $\Delta CysH$ *Msm*.

Comp ID	Structure	LUM Assay; <i>IC</i> ₅₀ (μ M)	Non-replicating WT <i>Mtb</i> H37Rv; MBC ^a (μ M)		Non-replicating <i>Msm</i> mc ² :155; MBC ^a (μ M)	
			(-) Met	(+) Met ^b	WT	$\Delta cysH^b$
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
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

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

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

^cMBC values determined by CFU measurement on 7H11 plates.

Table 2. Depicts measurement of binding and thermodynamic parameters for selected inhibitors to *Mtb*APSR at 25°C.^[a]

Compound	K_d	ΔH	$T\Delta S$	ΔG		
	ID	(μ M)	(kJ/mol)	(kJ/mol)		(kJ/mol)
[a]All	1	3.9 ± 1.9	-32.5 ± 3.5	-2.5 ± 1.5	-30.1 ± 5.0	ITC
	5	12.0 ± 2.8	-12.0 ± 2.8	15.7 ± 0.6	-27.7 ± 0.2	
	6	41.4 ± 0.3	-5.4 ± 2.1	19.5 ± 1.9	-24.9 ± 0.2	
	8	0.8 ± 0.2	-52.1 ± 3.5	-16.4 ± 2.4	-35.7 ± 5.9	
	9	2.6 ± 1.7	-45.5 ± 3.3	-13.3 ± 2.2	-32.2 ± 1.8	
	2-(Methylthio) ADP	No Measurable Binding				

experiments were performed in 50 mM bis-tris propane buffer containing 0.02 % Brij-35 (pH 7.4).

Table 3. Depicts bactericidal activity of ellipticine analogs against replicating WT H37Rv *Mtb* grown in 7H9 medium with or without added methionine (2 mM)

Compound ID	Replicating WT H37Rv <i>Mtb</i> ; MIC (μM)	
	(-) Met	(+) Met
8	1.5	6
9	3	12
10	6	50

Table 4. Depicts bactericidal activity of ellipticine analogs against non-replicating MDR and XDR clinical isolates of *Mtb*.

Compound ID	Non-replicating <i>Mtb</i> ; MIC (μM)		
	WT (H37Rv)	MDR (Jal 2287)	XDR (MYC 431)
6	>100	>100	>100
7	>100	>100	>100
8	1.5	6	1.5
10	3	12.5	1.5

Table 5. Depicts bactericidal activity of compound 8 against replicating MDR and XDR clinical isolates of *Mtb*.

MDR and XDR <i>Mtb</i> Strains	MIC INH (μg/mL)	MIC comp. 8 (μg/mL)
H37Rv (WT)	0.4	1.25
BND320	>100	0.66
JAL1934	>100	1.25

<u>JAL2261</u>	<u>>100</u>	<u>1.25</u>
<u>JAL 2278</u>	<u>>100</u>	<u>2.5</u>
<u>MYC431</u>	<u>>100</u>	<u>2.5</u>

Table 6. Synergistic bactericidal activity of compound 8 with other anti-TB drug

<u>Drug</u>	<u>Decrease in [8]</u>	<u>Decrease in [drug]</u>	<u>FICI Score</u>	<u>Interaction type</u>
<u>Clofazimine</u>	<u>2-fold</u>	<u>32-fold</u>	<u>0.5</u>	<u>Synergistic</u>
<u>Isoniazid</u>	<u>4-fold</u>	<u>2-fold</u>	<u>0.75</u>	<u>Additive</u>
<u>Rifampicin</u>	<u>4-fold</u>	<u>8-fold</u>	<u>0.5</u>	<u>Synergistic</u>

Table 7. Activity of APSR inhibitors against other pathogenic organisms.

<u>Species</u>	<u>Fe-S cluster</u>	<u>MIC (ug/mL)</u>			<u>Linezolid</u>	<u>Tetracycline</u>
		<u>8</u>	<u>9</u>	<u>10</u>		
<u>Mycobacteria</u>						
<u>Mycobacterium tuberculosis</u>	<u>Present</u>	<u>0.6</u>	<u>1.0</u>	<u>1.0</u>	<u>=</u>	<u>=</u>
<u>Mycobacterium smegmatis</u>	<u>Present</u>	<u>1.2</u>	<u>1.0</u>	<u>1.4</u>	<u>=</u>	<u>=</u>
<u>Gram-positive aerobes</u>						
<u>Staphylococcus aureus (MSSA)</u>	<u>Present</u>	<u>16</u>	<u>8</u>	<u>16</u>	<u>2</u>	<u>0.5</u>
<u>Staphylococcus aureus (MRSA)</u>	<u>Present</u>	<u>>32</u>	<u>16</u>	<u>>32</u>	<u>2</u>	<u>>32</u>
<u>Enterococcus faecalis (VSE)</u>	<u>NA</u>	<u>>32</u>	<u>8</u>	<u>32</u>	<u>2</u>	<u>16</u>

<i>Enterococcus faecalis (VRE)</i>	NA	32	16	32	2	0.25
<i>Streptococcus pneumoniae (PISP)</i>	Present	8	8	8	1	0.12
<i>Streptococcus pyogenes</i>	NA	2	1	2	2	0.25
<i>Streptococcus agalactiae</i>	NA	4	2	2	1	32
<i>Streptococcus mutans</i>	NA	0.5	0.5	0.25	1	0.5
<u>Gram-negative aerobes</u>						
<i>Escherichia coli ATCC 25922</i>	Absent	>32	16	>32	>32	0.5
<i>Pseudomonas aeruginosa</i>	Present	>32	>32	>32	>32	2
<i>Vibrio cholera</i>	Absent	>32	>32	>32	4	0.25
<i>Salmonella typhimurium</i>	Absent	>32	>32	>32	>32	2
<i>Haemophilus influenza</i>	Present	8	8	4	8	8
<i>Moraxella catarrhalis</i>	Present	0.5	0.25	0.25	4	0.12

Table 8. Preliminary *in vitro* pharmacokinetics and toxicity profiling of APSR inhibitors.

<u>Parameter</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>Control</u>
<u>Physicochemical Properties</u>				
<u>Molecular weight (Da)</u>	418	356	335	:-
<u>Solubility (pH 7.4) (μM)</u>	71	≥100	≥100	:-
<u>logD</u>	1.97	2.69	2.66	:-
<u>Metabolic stability (Human/Mice)</u>	<u>Sunitinib</u>			

<u>T_{1/2} (min)</u>	<u>78.9/23.2</u>	<u>67.1/53.0</u>	<u>44.6/6.0</u>	<u>70.8/13.8</u>
<u>CL_{int} (μl min⁻¹ mg⁻¹)</u>	<u>8.8/29.8</u>	<u>10.3/13.1</u>	<u>15.5/116.1</u>	<u>9.8/50.0</u>
<u>Cytotoxicity (μM)</u>				<u>Ellipticine</u>
<u>Avg. CC₅₀ in 60 cell lines</u>	<u>5900</u>	<u>7395</u>	<u>6593</u>	<u>1554.2</u>
<u>MBC₉₉ in WT Mtb H37Rv</u>	<u>1.5</u>	<u>3.0</u>	<u>3.0</u>	<u>12.5</u>
<u>Safety Index (SI)=CC₅₀/MBC₉₉</u>	<u>3933</u>	<u>2465</u>	<u>2195</u>	<u>124</u>

Examples

Preparation of Reagents for HTS Assays. *Mtb*APSR and *E.coli* thioredoxin (*Ec*Trx) were expressed and purified as described previously and stored at -80 °C in 50 mM Tris-HCl, 150 mM NaCl (pH 7.4) buffer containing 5 mM DTT as single-use portions.^{1,2} Immediately before their use, *Mtb*APSR and *Ec*Trx were buffer exchanged in to assay buffer (50 mM bis-tris propane, 0.02% of Brij-35 detergent, pH 7.4 at RT) using pre-equilibrated P-30 and P-6 Bio-Gel micro Bio-Spin columns (BioRad), respectively. Purity and molecular mass of proteins was determined using SDS page electrophoresis and intact mass analysis using LTQ XL™ Linear Ion Trap Mass Spectrometer. Adenosine 5'-phosphosulfate (APS) was obtained in ≥ 95 % purity from BIOLOG Life Science Institute (Bremen, Germany) and re-purified using ion-exchange liquid chromatography to ≥ 99 % purity. A freshly made solution of molecular biology grade DTT (Invitrogen) was used in HTS assays to prevent auto-oxidation of DTT.

Additional Secondary Screening Assays

HRP-PR Assay: This assay was performed following protocol described earlier³ with some modifications. Briefly, 5 μL assay buffer (50 mM bis tris propane, 0.02 % Brij-35, pH 7.4) containing 1.6 mM DTT was dispensed in to 384-well black plates (Griener, Cat. No. 781101) using 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 384-well pin-tool unit to achieve final assay concentration range of 100-0.005 μM. The first and last two columns were pinned with DMSO as low control. The first two columns were then dispensed with 5 μL of 200 μM H₂O₂ in assay buffer as high control while the remaining wells were dispensed with 5 μL of assay buffer. The

plates were incubated at RT for 15 min followed by addition of 10 μ L of detection mixture (120 μ g/mL HRP and 200 μ g/mL PR in assay buffer). After incubation at RT for another 5 min, the HRP reaction was terminated by dispensing 5 μ L 1N NaOH. The plates were centrifuged, incubated for 5 min and read for absorbance at 610 nm on Envision plate reader (PerkinElmer). The data were analyzed as described previously for the FP assay.

PDI Inhibitors Assay: The assay was performed using PDI inhibitor assay kit (Abcam) following manufacturer's protocol, except that the human PDI in assay kit was substituted by *Ec*Trx.

Fluorescent Intercalator Displacement Assay: The assay was performed using the standard protocol described earlier.⁴

Mode of Inhibition Studies: Mode of inhibition of compounds was determined using a continuous assay for sulfite detection recently reported by our laboratory that employs a fluorescent Lev-Cou probe to measure sulfite produced in APSR reaction.⁵ Briefly, parallel APSR reactions were setup in a 1 mL clear quartz cuvette constituting *Mtb* APSR (100 nM), DTT (25 μ M), *E. coli* Trx (10 μ M) and Lev-Cou probe (5 mM) in 10 mM HEPES buffer at pH 7.5. The APSR reactions were initiated by adding APS at 1, 3, 6, 12, 24, 48, 192 or 138 μ M concentrations in presence of inhibitor **8** at either 0, 17 and 34 μ M concentration. The reactions were monitored by recording the fluorescence (Exc. at 350 nm and Emi. 450 nm) every 3 min at RT. Initial velocity (V_0) was measured and was plotted *versus* substrate concentration to obtain K_m and V_{max} at different inhibitor concentration using standard Michaelis Menten equation. The double reciprocal plot of V_0 and substrate concentration provided the linear Lineweaver–Burk relationship at each inhibitor concentration that intersected on the Y-axis providing evidence for competitive inhibition.

Whole-Cell Assays: Mycobacterial Nutrient Starvation: Mycobacteria were nutrient starved in PBS buffer following standard protocol reported earlier.⁶ Briefly, seed stocks of WT *Mtb* (H37Rv), drug-resistant clinical isolates MDR (Jal 2287) and XDR (MYC 431), WT *Msm* (Mc²:155), Δ CysH *Msm* and Δ CysH (*pMSGS Mtb CysH*) complement strain were thawed and grown in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC (Aldrich), 0.1% glycerol and 0.1% Tween 80 until the mid-log phase ($OD_{600} = 0.6$). The cultures for Δ CysH *Msm* conditional mutant were supplemented with sterile-filtered methionine (2 mM) at all stages. Bacilli were then diluted 1:100 in 100 ml of 7H9 media and grown until mid-log phase, pelleted, washed and re-suspended in 1X PBS buffer containing 0.1% Tween 80 (pH 7.4) at final $OD_{600} =$

0.2. The cultures were starved at 37 °C without shaking for 2 (*Msm* cultures) or 7 days (*Mtb* cultures). The starvation cultures were periodically tested for viability and oxygen depletion as reported earlier.⁶

MBC Determination by Alamar Blue Assay: The nutrient-starved mycobacterial cultures were then plated (final OD₆₀₀ = 0.1) in black, clear-bottom, 96-well plates (Costar; Cat. No. 3603) with test compounds in a concentration-response format (final concentration range = 100 – 0.005 µM) in a total volume of 200 µL. Wells containing only PBST served as negative control, while wells containing bacilli with no test compound served as positive control. Plates were incubated for 3 (*Msm* cultures) or 5 days (*Mtb* cultures) at 37 °C in a humidity chamber to avoid evaporation⁷ followed by addition of 20 µL Alamar Blue reagent (Life Technologies). The plates were incubated for another 16 hours at 37 °C and the fluorescence signal (Ex. 530 nm; Em. 590 nm) was measured using spectraMaxM3 microplate reader (Molecular devices) in a top-reading mode. The % inhibition values were calculated as described above and plotted against compound concentration. The data was fit to Gompertz function⁸ using GraphPad Prism 5 software to calculate MBC values.

Kill Kinetics: For kill kinetics study, the nutrient starved WT *Msm* (final OD₆₀₀ = 0.1) in PBST buffer were plated in clear, flat-bottom, 96-well plates and incubated with test compound 8 in a 4-fold, 6-point serial dilution format with a starting concentration of 10 µM and a total assay volume of 200 µL. At the interval of 0, 1, 2, 3, 4, 5, 6, and 7 days incubation at 37 °C, a 5 µL of culture from each well was serially diluted to 1:200 in PBST (with thorough mixing) and a 20 µL aliquot from this diluted sample was then plated on to a 12-well 7H11 plates. The plates were incubated for 3 days and colonies were counted to calculate CFU.

Measurement of Cysteine and MSH Content in *Msm*: The cell extraction for thiol analysis was performed using the protocol described earlier with slight modification to .⁹ Briefly, a seed stock of WT *Msm* (Mc²:155) was thawed and grown in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC (Aldrich), 0.1% glycerol and 0.1% Tween 80 until the mid-log phase. Bacilli were then diluted 1:100 in 500 ml of 7H9 media supplemented with 10% OADC (Aldrich), 0.1% glycerol and 0.1% Tween 80 and grown until OD₆₀₀ = 0.58. Following this, the cultures were divided into 25 mL pre-weighed falcon tubes and pelleted to harvest ~100 mg of wet weight of *Msm*. The cell pellets were then re-suspended in 25 mL of PBST buffer (OD₆₀₀ = 0.6), inoculated with varying concentrations of compounds (0, 0.5 x MBC, 1 x MBC, 2 x MBC, 4 x MBC and 5

x MBC) and incubated at 37 °C for 12 hours. The cultures were then pelleted to remove compound, re-suspended in 1 mL of fresh PBST buffer, transferred to a pre-weighed 1.5 mL microcentrifuge tube and pelleted. The pellets were re-suspended in warm (60 °C) 50% acetonitrile in 20 mM HEPES (Sigma) buffer (pH 8.0) and 2 mM monobromobimane (mBB; Sigma) was added to each tube. The tubes were then incubated at 60 °C for 15 min in dark and vortexed intermittently. The cell extracts were then acidified with 5 μ L of 5N methanesulfonic acid (Sigma) and the proteins and cellular debris were pelleted by centrifugation for 10 min at maximum speed. The supernatants were transferred to a fresh microcentrifuge tube and used for HPLC analysis. Control samples were prepared by allowing the cell extract to react with 5 mM N-ethylmaleimide (Sigma) for 15 min at 60 °C followed by addition of 2 mM mBB and incubation for additional 15 min at 60 °C.

HPLC analysis to measure relative concentrations of mBB-labelled cysteine and MSH was performed using the methods reported earlier with minor variations.^{10, 11} Briefly, 20 μ L of 5-fold diluted sample of the cell extract was injected onto Ultrasphere ODS 5 μ m analytical column (Beckman). Solvent A was 0.25% aqueous acetic acid titrated to pH 3.55 with concentrated NaOH and solvent B was methanol. The gradient used was as follows: 0 min, 10% B; 5 min, 10% B; 15 min, 18% B; 30 min, 27% B; 32 min, 100% B; 34 min, 10% B. Fluorescence detection of R-S-BBm derivatives was accomplished with a 390 nm excitation and 475 nm emission filter.

E_{MSH} Measurements in Mtb: The measurement of intracellular mycothiol redox potential (E_{MSH}) was performed as reported earlier.¹² Briefly, H37Rv *Mtb* expressing Mrx1-roGFP2 was grown in 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.¹²

RNA Extraction and qRT-PCR to Measure DNA Damage: RNA was extracted by using the RiboPure-Bacteria Kit (Ambion) following the manufacturer's protocol. Briefly, WT *Msm* grown in 7H9 medium to mid-log phase were divided aseptically into 10 mL portions in culture tubes, and incubated with varying concentrations of compound 8 (0, 1 x MBC, 2 x MBC, 4 x MBC and 8 x MBC) or 0.2 µg/mL of mitomycin C as a positive control (a potent mycobacterial DNA damaging agent)¹³ at 37 °C for 12 hours. The cultures were then pelleted to remove compound, washed with PBST buffer, re-suspended in 350 µl of RNAWIZ solution (Ambion) and transferred to a 0.5 ml skirted screw-capped microcentrifuge tube containing 250 µl of ice-cold zirconia beads. Tubes were mounted on to a horizontal vortex adapter and vortexed at maximum speed for 10 min. Remaining steps were performed according to the manufacturer's instructions. All RNA samples were treated with DNase I (Ambion) to remove trace amounts of genomic DNA. RNA yield was evaluated with the Nanodrop (Thermo Scientific) while RNA quality was examined using Agilent 2100 Bioanalyzer. First strand cDNA synthesis was accomplished using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reactions (RT-qPCR) were performed using the DyNAmo SYBR Green qPCR kit (NEB) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions. *Msm* sigA gene (MSMEG_2758; a mycobacterial sigma factor) was used as a reference gene for the relative quantification method. At the end of the PCR, melting curve analysis was performed to verify the product specificity. Fold changes in expression levels were calculated using the $2^{-\Delta\Delta C_T}$ statistical method.

Global Transcriptional Analysis: WT *Msm* grown in 7H9 medium to mid-log phase were divided aseptically into two 60 mL portions in 250 mL culture flasks, and incubated with 10 µM of compound 8 (10 x MBC) or DMSO (negative control) at 37 °C. A 10 mL aliquot from each flask was removed into 15 mL falcon tube at 3 hour and 8 hour interval. The cultures were then pelleted to remove compound, resuspended in PBST and pelleted again. The cell pellets were fast-frozen in liquid nitrogen and then subjected to extraction of total RNA as explained above. RNA yield was evaluated with the Nanodrop (Thermo Scientific) while RNA quality was

examined using Agilent 2100 Bioanalyzer. The RNA was then subjected to transcriptional analysis using RNA-seq analysis.

Mammalian Cell Toxicity: VERO and HeLa cell lines (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), respectively, with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/L streptomycin, 0.5 mg/L gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM HEPES. Confluent cells were trypsinized, counted and seeded in clear, flat-bottomed 96-well plates (Costar; Cat. No. 3598) at 1×10^4 cells/well. The cells were allowed to adhere for 12 hours followed by addition of test compounds in a 10-point, 3-fold serial dilution concentration-response format to achieve final concentration range of 100 – 0.005 μ M. The plates were incubated at 37°C for 2 days, the medium was removed, cells were washed once with PBS buffer, and 100 μ L of fresh DMEM medium lacking phenol red (Gibco) was added. This was followed by addition of 10 μ L of MTT (Life Technologies) at a final concentration of 1 mM and incubation at 37 °C for 4 hours. The medium was removed and 100 μ L of DMSO was added to each well. The plates were incubated at 37 °C for 10 min with shaking. The absorbance signal at 540 nm was read using spectraMaxM3 microplate reader (Molecular devices). The % cell viability was plotted against compound concentration and the data were fit to a four-parameter equation describing a sigmoidal dose-response curve using GraphPad Prism software to calculate the apparent LC_{90} values.

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While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements will be apparent to those skilled in the art without departing from the spirit and scope of the claims.

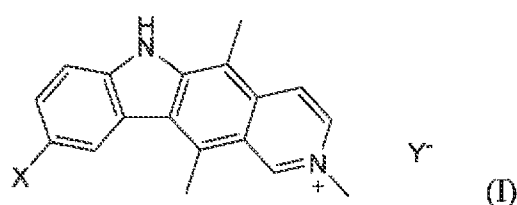
All patents and publications referred to herein are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference in its entirety.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method of treatment of a patient afflicted with a tuberculosis (TB) infection, comprising administering to the patient an effective dose of an N-methylellipticinium compound that is an inhibitor of adenosine phosphosulfate reductase (APSR).
2. The method of claim 1, wherein the tuberculosis infection is a multiple drug resistant (MDR) tuberculosis infection, an extremely drug resistant (XDR) tuberculosis infection, or a latent-TB infection (LTBI).
3. The method of claim 1, wherein the N-methylellipticinium compound co-administered to the patient in conjunction with an effective dose of a second anti-tuberculosis drug.
4. The method of claim 3, wherein the N-methylellipticinium compound potentiates the effectiveness of the second anti-tuberculosis drug.
5. The method of claim 1, wherein the N-methylellipticinium compound is a compound of formula (I)



wherein X is hydrogen, halo, (C1-C4)alkyl, or (C1-C4)alkoxyl, and Y is a pharmaceutically acceptable counterion.

6. The method of claim 5 wherein X is chloro, methyl, or methoxyl, and Y is acetate or chloro.

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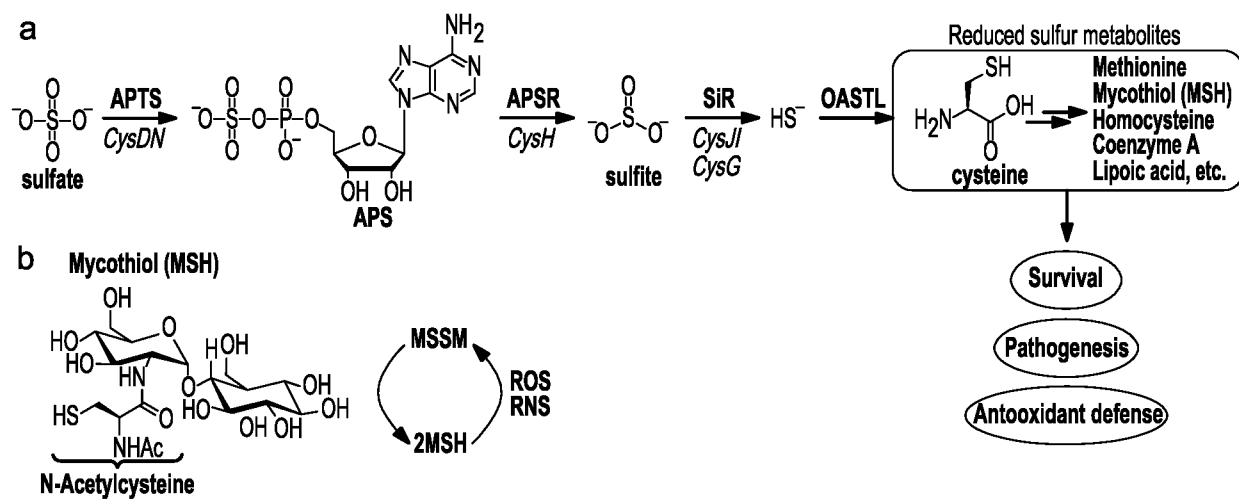


FIG. 1

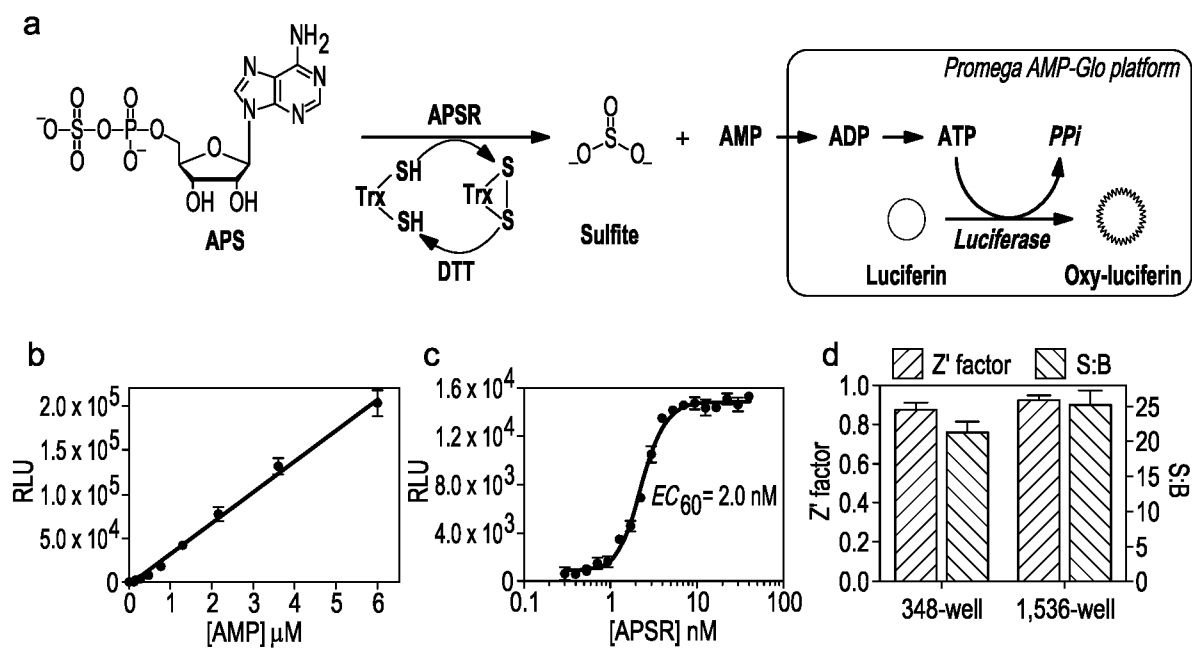
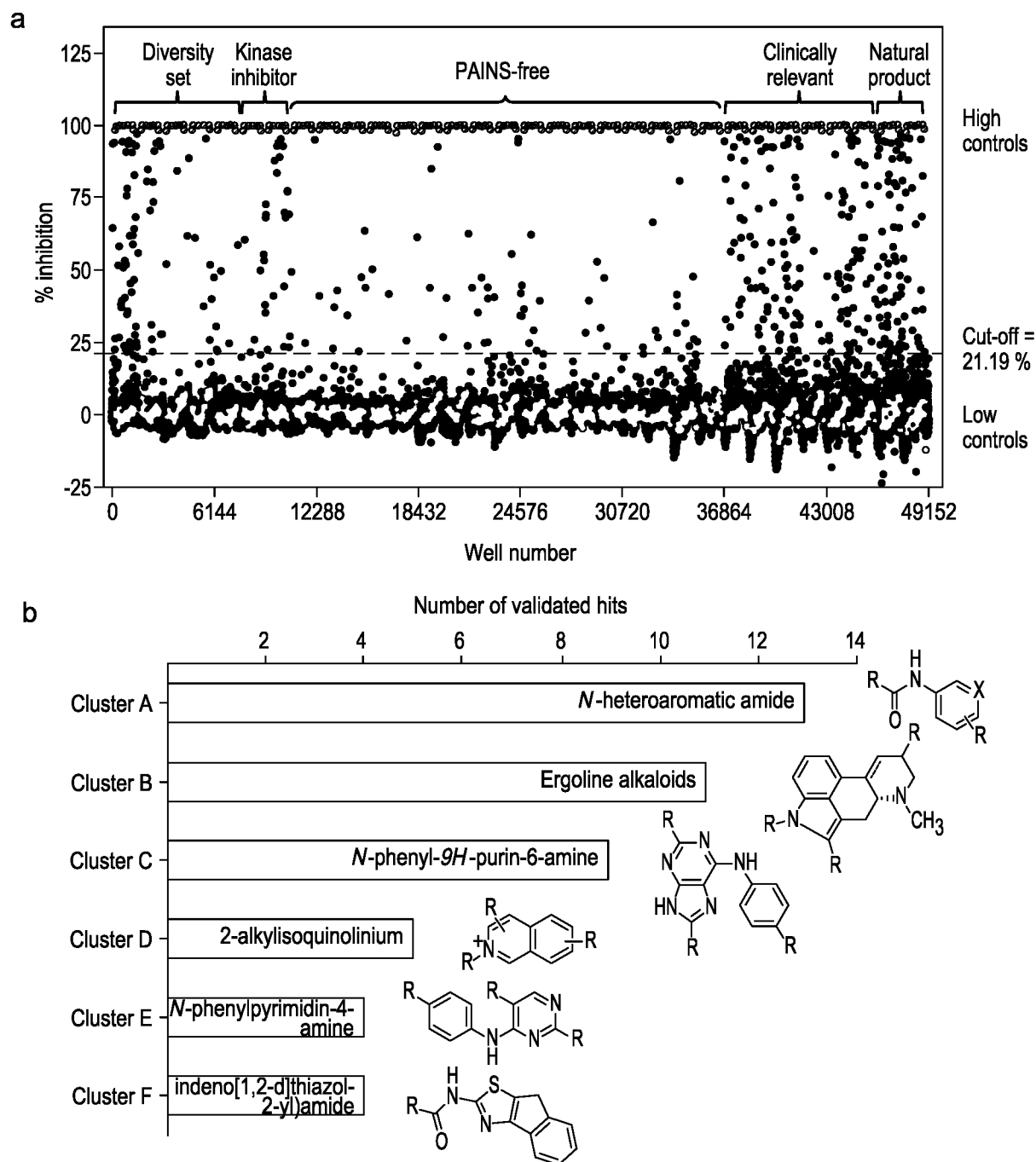


FIG. 2

**FIG. 3**

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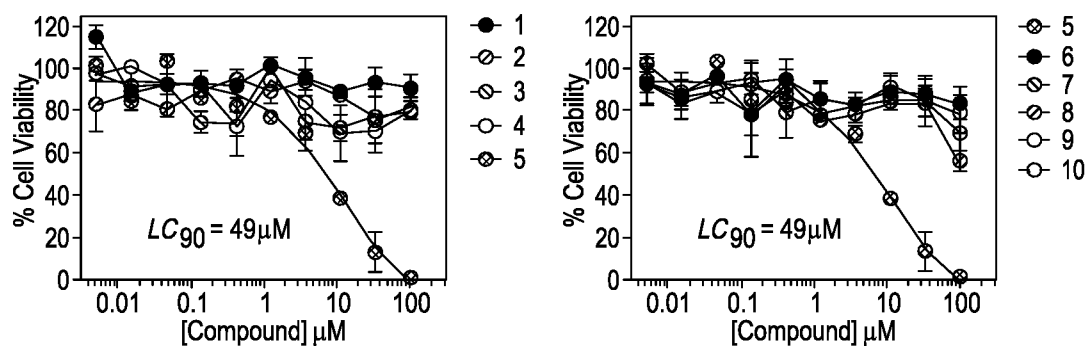


FIG. 4

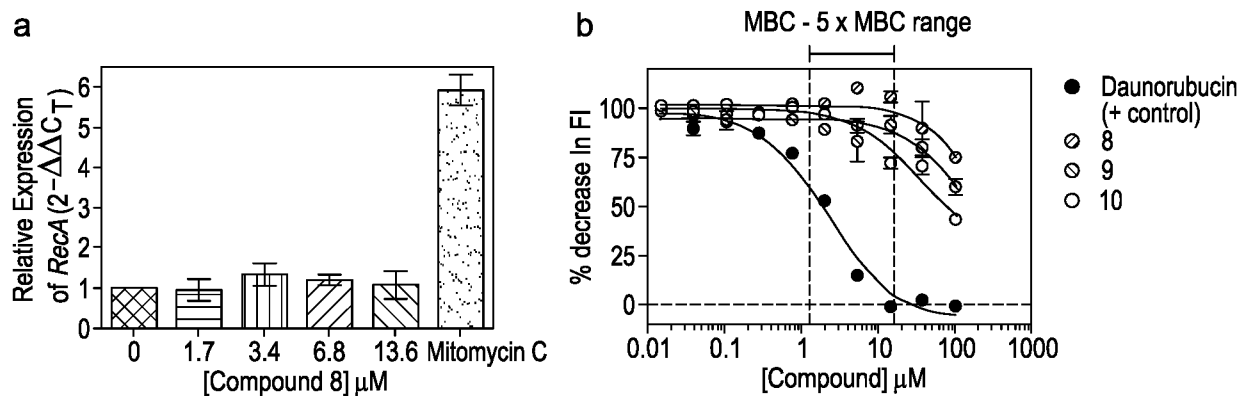


FIG. 5

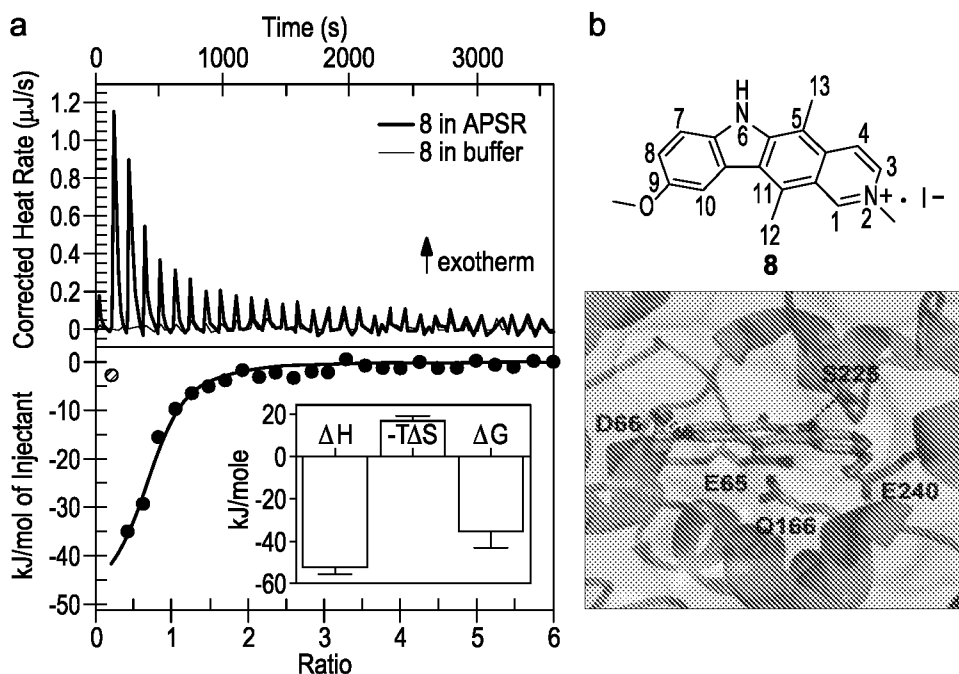


FIG. 6

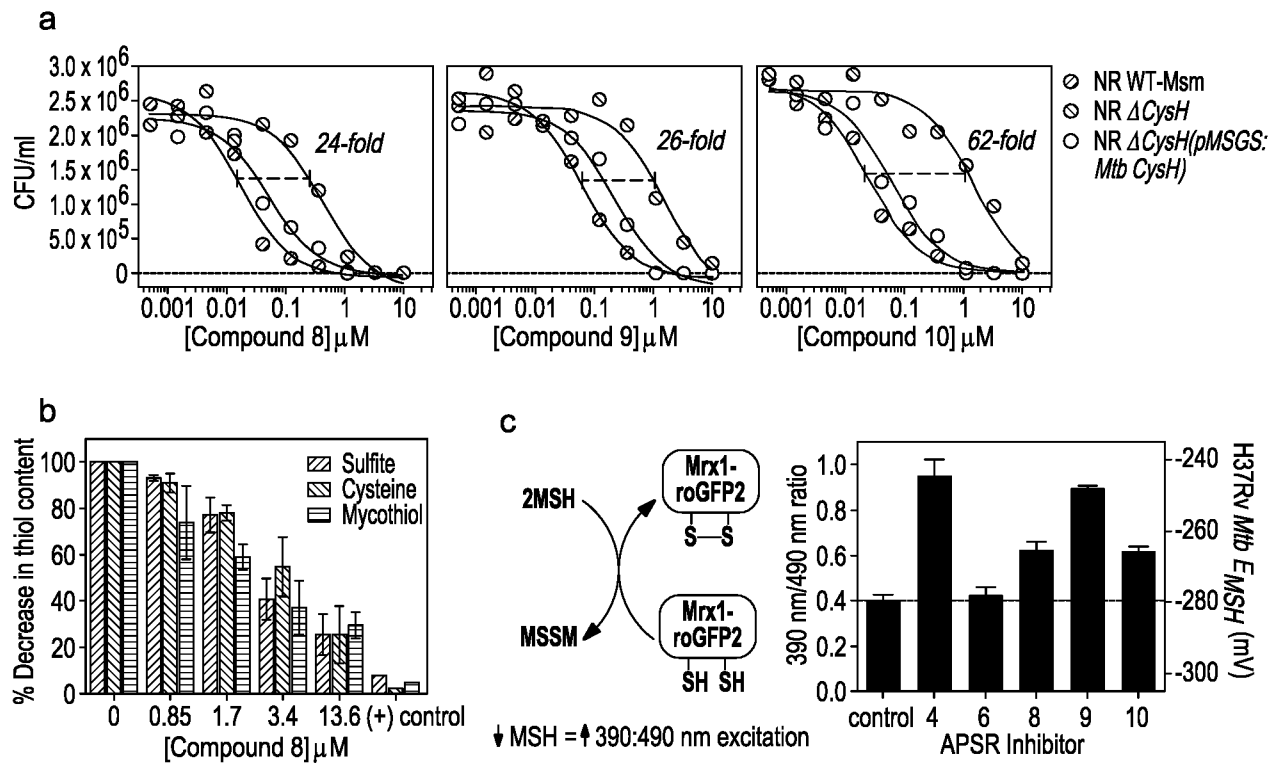


FIG. 7

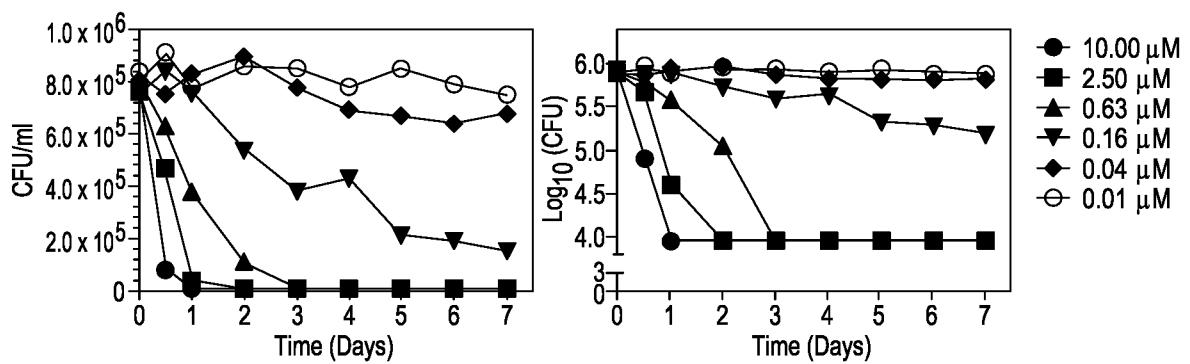


FIG. 8

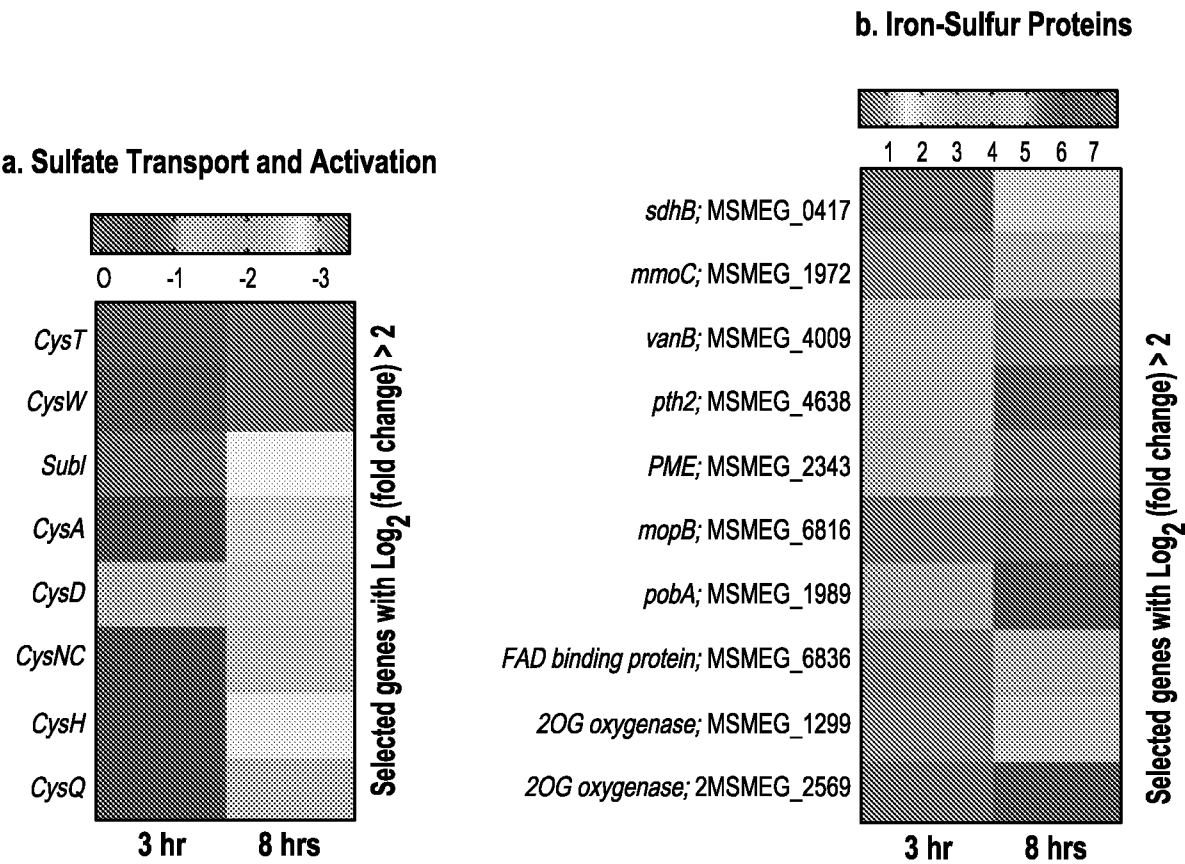
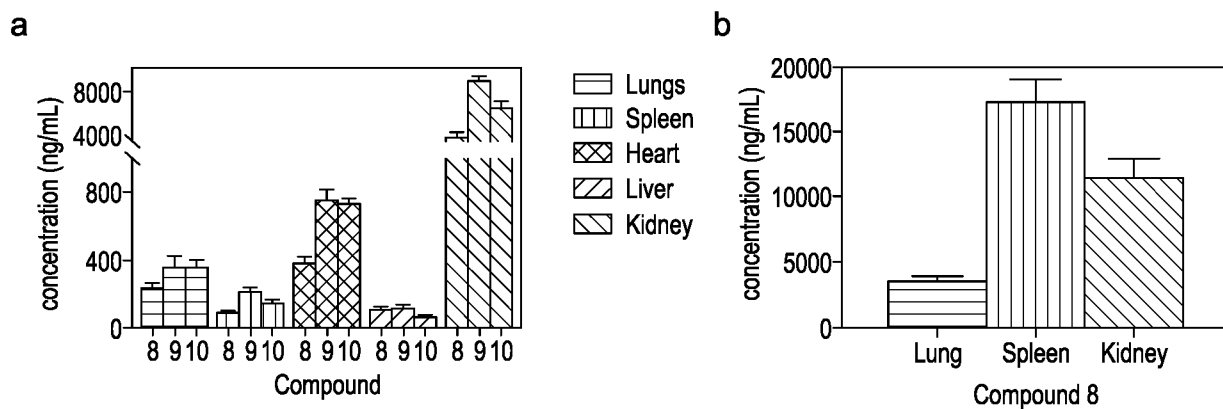
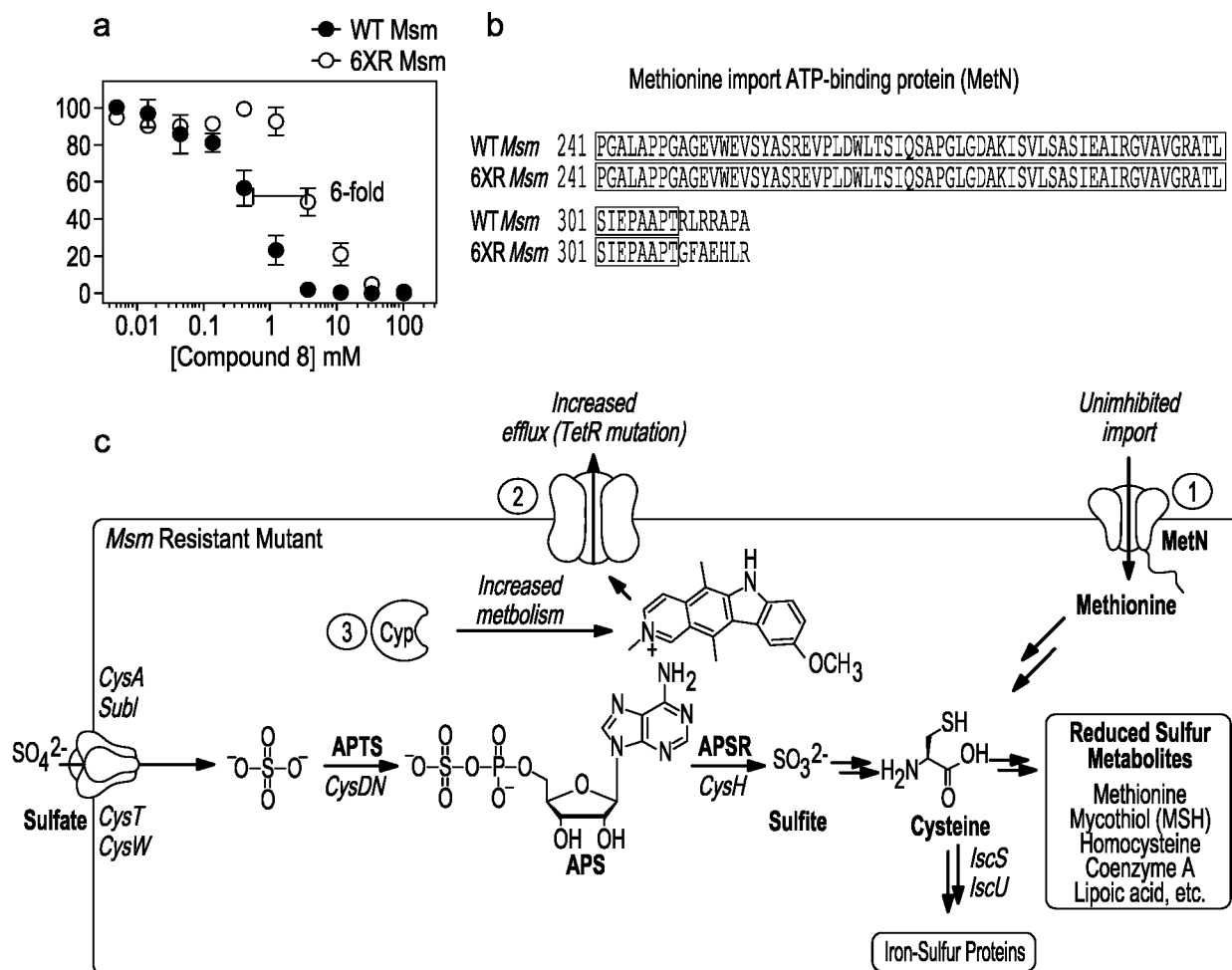


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/56228

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/437; C07D 471/04; A61P 31/06 (2016.01)

CPC - C07D471/04; A61K31/437

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 31/437; C07D 471/04; A61P 31/06 (2016.01)

CPC: C07D471/04; A61K31/437

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/285Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar, PubWEST tuberculosis, infection, treat, N-methylellipticinium, adenosine phosphosulfate reductase, inhibitor, drug resistant, latent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006/0286574 A1 (ROMESBERG et al.) 21 December 2006 (21.12.2006) para [0174], [0198], [0258], [0260], [0269], [0271], [0275]	1-6
Y	VISTICA et al. 'Role of Membrane Potential in the Accumulation of Quaternized Ellipticines by Human Tumor Cell Lines', THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, 1996, Vol.279, No.2, pp. 1018-1025. abstract; pg 1996, col 1, para 1	1-6
A	DUGUE et al. 'Covalent Binding of Elliptinium Acetate (NSC 264137) to Nucleic Acids of L1210 Cells in Culture', CANCER RESEARCH, 1986, Vol.46, pp. 3828-3833. Fig 1	1-6
A	COSCONATI et al. 'Structure-Based Virtual Screening and Biological Evaluation of Mycobacterium tuberculosis Adenosine 5'-Phosphosulfate Reductase Inhibitors', J Med Chem. 2008, Vol.51(21), pp. 6627-6630. [pages 1-12] Entire Document	1-6
A	TEWEY et al. 'Intercalative Antitumor Drugs Interfere with the Breakage-Reunion Reaction of Mammalian DNA Topoisomerase II', THE JOURNAL OF BIOLOGICAL CHEMISTRY, 1984, Vol.259, No.14, pp. 9182-9187. Entire Document	1-6
A	WO 2013/182519 A1 (UNIVERSITAET BASEL) 12 December 2013 (12.12.2013) Entire Document	1-6

☐ Further documents are listed in the continuation of Box C.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

25 November 2016

Date of mailing of the international search report

23 DEC 2016

Name and mailing address of the ISA/US

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