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Identification of a small molecule with activity against drug-resistant and persistent tuberculosis

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A cell-based phenotypic screen for inhibitors of biofilm formation in mycobacteria identified the small molecule TCA1, which has bactericidal activity against both drug-susceptible and -resistant Mycobacterium tuberculosis (Mtb) and sterilizes Mtb in vitro combined with rifampicin or isoniazid. In addition, TCA1 has bactericidal activity against nonreplicating Mtb in vitro and is efficacious in acute and chronic Mtb infection mouse models both alone and combined with rifampicin or isoniazid. Transcriptional analysis revealed that TCA1 down-regulates genes known to be involved in Mtb persistence. Genetic and affinity-based methods identified decaprenyl-phosphoryl-D-ribofuranose dprE1 and moeW, enzymes involved in cell wall and molybdenum cofactor biosynthesis, respectively, as targets responsible for the activity of TCA1. These in vitro and in vivo results indicate that this compound functions by a unique mechanism and suggest that TCA1 may lead to the development of a class of antituberculosis agents.

Significance

The global problem of TB has worsened in recent years with the emergence of drug-resistant organisms, and new drugs are clearly needed. In a cell-based high-throughput screen, a small molecule, TCA1, was discovered that has activity against replicating and nonreplicating Mycobacterium tuberculosis. It is also efficacious in acute and chronic rodent models of TB alone or combined with frontline TB drugs. TCA1 functions by a unique mechanism, inhibiting enzymes involved in cell wall and molybdenum cofactor biosynthesis. This discovery represents a significant advance in the search for new agents to treat persistent and drug-resistant TB.


The authors declare no conflict of interest.

Data deposition: The atomic coordinates and structure factors reported in this paper have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4KWS).

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rophytic, nonpathogenic mycobacteria that also forms in vitro biofilms (9) that induce drug tolerance (10), is amenable to high-throughput screening. Therefore, the primary cell-based screen was based on the inhibition of biofilm formation in *M. smegmatis*. We found that the in vitro biofilm, visualized as a pellicle that grows at the air–liquid interface, covers the whole surface of the well in a 384-well plate after formed, affording a high signal-to-noise ratio for positive hits. A diverse library of 70,000 heterocycles was screened (SI Materials and Methods), which afforded 17 compounds with minimum inhibitory concentrations (MIC<sub>50</sub> values) of less than 10 μM in a biofilm inhibition assay. Two classes of compounds were identified: the first class inhibits the growth of mycobacteria under biofilm culture conditions, whereas the second class inhibits the formation of biofilms without significant growth inhibition. These hit compounds from the primary screen were then tested for their ability to inhibit in vitro biofilm growth in virulent *Mtb* H37Rv using a scaled-up 24-well assay as previously described (11). Two compounds, C7 and TCA1, were found to also inhibit biofilm formation by *Mtb* H37Rv (Fig. 1A). TCA1, which displayed potent inhibitory activity against *Mtb* under both biofilm and planktonic culture conditions, was selected for additional studies.

**In Vitro Bactericidal Activity.** TCA1 shows selective inhibitory activity against bacterial growth—it is inactive against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, suggesting that the target for its bactericidal activity is specific to the genus *Mycobacterium* (Fig. 1B). Interestingly, the activities of TCA1 against *M. smegmatis*, *M. bovis bacillus Calmette–Guérin*, and *Mtb* are 20- to 150-fold higher in biofilm medium (MIC<sub>50</sub> = 0.03, 0.04, and 0.01 μg/mL, respectively) than 7H9 medium (MIC<sub>50</sub> = 4.5, 3, and 0.19 μg/mL, respectively). This observation underscores the variable efficacy of a drug in different growth media (3), which in part, may result from the expression of distinct target genes and metabolic pathways. TCA1 is bactericidal with an MIC<sub>50</sub> values of 2.1 μg/mL in solid medium. To evaluate the bactericidal activity of TCA1 against *Mtb* compared with the two frontline TB drugs INH and RIF, we performed a 21-d kinetic killing assay using comparable levels of each of the three drugs (20× MIC<sub>50</sub> of each of the three drugs). TCA1 is active by itself against exponentially growing virulent *Mtb* in 7H9 media, with a more than 3 log reduction in the number of bacilli over a treatment period of 21 d. Treatment with INH or RIF resulted in a comparable drop in cfu over the first 7 d of treatment, but the subsequent outgrowth of bacilli detected in INH- and RIF-treated cultures is absent in TCA1-treated cultures. Furthermore, TCA1 combined with either RIF or INH is able to sterilize an *Mtb* culture in ~3 wk (Fig. 2A); removal of drug after 3 wk of combination drug treatment did not lead to *Mtb* outgrowth.

We also tested the activity of TCA1 on drug-resistant *Mtb*. RIF resistance is a marker for MDR-TB (90% of RIF-resistant strains are also MDR) and typically requires 18–24 mo of treatment. TCA1 by itself was active against a clinical strain that is resistant to RIF (because of a mutation in *rpoB*), and more importantly, combined with INH, it sterilized the cultures within 1 wk (Fig. 2B). Removal of both drugs after 3 wk of treatment did not result in outgrowth. TCA1 was also found to be bactericidal
against a strain with a mutation in katG (resulting in resistance to INH) (Fig. 2C). Finally, we also tested TCA1 against an XDR-TB strain, mc²8013, which is resistant to 10 TB drugs, including all frontline drugs (Table S1). TCA1 showed potent bactericidal activity against the XDR-TB strain (5 log cfu reduction in 3 wk) (Fig. 2D). The lack of cross-resistance to TCA1 in any of these drug-resistant strains suggests that TCA1 functions by a distinct mechanism.

We next tested the activity of TCA1 against nonreplicating Mtb in a nutrient starvation assay, a widely used in vitro model of the Mtb dormancy phenotype (12, 13). Under these conditions, Mtb enters a nonreplicating state and has been shown to become tolerant to drugs without acquiring heritable drug resistance-inducing mutations (13). TCA1 shows bactericidal activity against nonreplicating Mtb at a concentration of 7.5 μg/mL (40× MIC₅₀ in 7H9 medium), reducing cfu by 3 logs in 3 wk (Fig. 2E). Under the same assay conditions, RIF (40× MIC₅₀ in 7H9 medium) showed less bactericidal activity than TCA1. We also tested the activity of TCA1 in an intramacrophage cell culture system to determine whether it is active against intracellular mycobacteria, because in the mouse model of infection and in humans, Mtb is believed to reside mainly in macrophages. TCA1 was found to be quite potent in an intracellular cfu assay with an MIC₅₀ value of 0.6 μg/mL [MIC₅₀ (RIF) = 2.7 μg/mL, MIC₅₀ (INH) = 0.2 μg/mL] (SI Materials and Methods). Finally, TCA1 shows no cytotoxicity against five mammalian cell lines (Huh7, 293T, K562, HepG2, and Vero cells) at the highest concentration tested (100 μM for Vero cells and 25 μM for others); an hERG assay indicated that TCA1 has no activity at 30 μM (Tables S2 and S3).

**Fig. 2.** In vitro activity of TCA1. (A) Kill kinetics of Mtb by TCA1 (3.75 μg/mL) alone or combined with INH (1 μg/mL) or RIF (2 μg/mL) compared with RIF (2 μg/mL) and INH (1 μg/mL) alone in 7H9 medium. Activity of TCA1 (3.75 μg/mL) against (B) an RIF-resistant Mtb strain and (C) an INH-resistant Mtb strain in 7H9 medium. (D) Kill kinetics of an XDR-TB strain by TCA1 (7.5 μg/mL) in 7H9 medium. (E) Activity of TCA1 against nonreplicating Mtb under nutrient starvation conditions. (F) Quantitative PCR analysis of expression ratios of selected genes from TCA1-treated (3.75 μg/mL) and untreated (0.1% DMSO) Mtb.

**TCA1 Is Efficacious in Acute and Chronic Mtb Infection Mouse Models.** We next examined the activity of TCA1 in a mouse model of Mtb infection. We first determined the physical and pharmacokinetic characteristics of TCA1. It is stable to proteolytic activity in human or mouse plasma for up to 4 h. Moreover, a GSH trapping assay indicated that no GSH adduct was formed, and TCA1 has no inhibitory activity against four CYP enzymes. After i.v. administration, TCA1 exhibited a low clearance and steady-state volume of distribution, with an elimination half-life of 0.73 h. After oral administration of 20 and 50 mg/kg in solution formulation, TCA1 showed a high Cmax (2,122 and 5,653 nM, respectively), moderate exposure with oral bioavailability ranging from 19% to 46%, and a half-life of 1.8 h (Tables S2 and S3).

We first performed the in vivo efficacy experiments in an acute infection model with a low dose of TCA1. BALB/c mice were infected with a low dose of Mtb H37Rv (~200 bacilli); 2 wk after infection, mice were treated with TCA1 (40 mg/kg), INH (25 mg/kg), or RIF (10 mg/kg) for 4 wk (dosed 1 time/d for 5 d/wk). The doses of INH and RIF are consistent with those doses published in the literature (14). After 4 wk of treatment with TCA1, the cfu dropped 0.5 log in lung and 1.5 logs in spleen, which is comparable with the potency of RIF but less than the potency of INH (Fig. S1). The gross pathology and histopathology also showed significant improvement in both tissues (Table S4). We also tested the in vivo efficacy of TCA1 (40 mg/kg) combined with INH (25 mg/kg) or RIF (10 mg/kg). In the acute infection model, TCA1+INH and TCA1+RIF showed nearly a 2 and 3 log cfu reduction in lung, respectively, and a more than 3 log cfu reduction in spleen (Fig. 3A and B). There is a greater cfu drop in the lungs of mice treated by the combination of TCA1 and INH relative to 

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the combination of INH and RIF. We next tested the drug in a mouse model of chronic TB infection. Mice were challenged with a low-dose aerosol infection, and treatment was initiated 4 wk after infection. Similar combination treatments were efficacious in the chronic infection model as well (Fig. 3C and D).

Because the mice were able to tolerate 40 mg/kg TCA1, we increased the dose to 100 mg/kg using a similar protocol in the acute infection model. After 4 wk, the cfu dropped nearly 2 logs in lungs and more than 3 logs in spleen, showing that the in vivo bactericidal activity of TCA1 is dose-dependent (Fig. 3A and B). The mice again showed no obvious adverse effects or weight loss after 4 wk of treatment. We also tested the drug in the chronic infection model at 100 mg/kg. Again, TCA1 showed efficacy in both lung (1 log cfu reduction) and spleen (1.4 log cfu reduction) (Fig. 3C and D). These results show that the in vitro efficacy of TCA1 is recapitulated in vivo, suggesting that the in vitro mycobacterial biofilm is a useful phenotype to identify compounds effective against Mtb in vivo either alone or combined with existing TB drugs.

### Mechanism of Action Studies of TCA1.

To gain insight into the mechanism of action of TCA1, we treated Mtb H37Rv with TCA1 (3.75 μg/mL) in 7H9 media and carried out genome-wide transcriptional analysis. Similar to INH and ethambutol (15), cell wall and fatty acid biosynthetic genes are affected by TCA1 treatment, suggesting that TCA1 likely interferes with these pathways. Unlike other known TB drugs, 10 of 86 genes differentially down-regulated compared with the DMSO control are genes previously implicated in TB dormancy, stress response, and RIF susceptibility. These genes include *r3130c–r3134c, fdaA*, and *hoxP* (members of the *dos* regulon), *cysD*, and *r3288c–r3290c* (members of the *sigF* regulon). The microarray results were confirmed by quantitative PCR (Fig. 2F). Most of these genes are part of the dormancy regulon controlled by *dosR* (16) and up-regulated under hypoxic conditions or by nitric oxide exposure. For example, *fdaA*, a low-redox potential electron carrier, is highly up-regulated in Mtb under hypoxic conditions (17) but significantly down-regulated (>20-fold) in response to TCA1 treatment. Likewise, *r3130c* is induced (>300-fold) under multiple stress conditions (18) but down-regulated (>30-fold) by TCA1. This down-regulation of genes involved in dormancy and drug tolerance seems to be unique to TCA1 and suggests that TCA1 may potentially sensitize Mtb to killing by antibiotics.

To further explore the mechanism of action of TCA1, a TCA1-resistant mutant that carries the cosmid (MSMEG_6379–MSMEG_6384) was isolated by selection of *M. smegmatis* transformed with a genomic cosmid library and grown in biofilm formation medium. Overexpression of each gene in this cosmid revealed that MSMEG_6382, which is homologous to *r3790* in the Mtb genome, confers high-level resistance to TCA1 (>20× MIC<sub>50</sub>) in both *M. smegmatis* and Mtb. We also managed to isolate spontaneous resistant mutants of *M. smegmatis* and Mtb, although the spontaneous mutation rate to TCA1 resistance is extremely low (10<sup>−8</sup> to 10<sup>−9</sup>). Whole-genome sequencing of the genomic DNA of the resistant mutants revealed that they all have...
a single-point mutation, resulting in the amino acid replacement Tyr321Cys in *M. smegmatis* and Tyr314Cys in *Mtb* (Fig. 4A). Rv3790 encodes DprE1, a component of the essential decaprenyl-phosphoryl arabinose 2′-epimerase DprE1/DprE2 required for cell wall arabinan biosynthesis. Indeed, TCA1 suppressed the activity of *M. smegmatis* DprE1 in membrane and cell envelope enzymatic fractions in a dose-dependent manner (Fig. 4B). DprE1 was previously identified as the target of the benzothiazinones (BTZs) and a nitro-triazole molecule (3, 14). Both scaffolds contain active nitro-moieties and are believed to covalently modify Cys387 on activation. We performed a competitive binding assay using a fluorescently labeled BTZ analog. TCA1 potently competed with BTZ in binding to DprE1, suggesting that the binding site of TCA1 overlaps with BTZ (Fig. S2). However, TCA1 does not have an active nitro-moietiy, and the Tyr314Cys mutant strain that is resistant to TCA1 is sensitive to BTZ, suggesting that the binding mechanism of TCA1 is different from these nitro-heterocycles.

To determine the molecular basis by which TCA1 inhibits DprE1, we determined the crystal structure of the enzyme bound to TCA1 (Table S5). The overall structure of the DprE1–TCA1 complex is largely unaltered compared with the structure of the ligand-free protein with the same crystal symmetry (19). The enzyme, which is structurally related to the vanillyl-alcohol oxidase family of flavoproteins (20), consists of an FAD binding and a substrate binding domain, with the flavin moiety of FAD positioned at the interface between the two domains (Fig. 4C, Left). The substrate binding domain includes two disordered loop regions (residues 269–283 and 316–330) that leave the active site open and accessible for inhibitors. TCA1 binds in the central cavity of the enzyme, adjacent to the isoalloxazine ring of FAD, in a boomerang-like conformation, with the thiophene moiety inserted deeply into the bottom of the active site (Fig. 4C, Right). The benzothiazole ring is oriented roughly parallel to the isoalloxazine of FAD. Noncovalent interactions between TCA1 and DprE1 are shown. Residues within a 4-Å radius of the inhibitor (violet) are shown as sticks, with FAD in yellow. Dashed lines indicate the shortest contacts (yellow, hydrophobic/van der Waals; orange, polar) between the residues and the inhibitor. Trp230, located within 4 Å of the carbamate moiety of TCA1, has been omitted for clarity.

**Fig. 4.** TCA1 is a DprE1 inhibitor. (A) Sequence alignment of DprE1 of *M. smegmatis* and *Mtb*. A Y321C (Y314C in *Mtb*) mutation was identified in both *M. smegmatis* and *Mtb* strains resistant to TCA1. (B) Inhibition of DprE1 by TCA1 in the cell-free assay for decaprenyl phosphoryl arabinose (DPA) production analyzed by TLC and autoradiography. *M. smegmatis* membrane or cell envelope fractions were incubated with (Left) phospho-[14C]-ribose diphosphate and 25 μg/mL TCA1 or BTZ043 or (Right) TCA1 in a dose–response fashion. Both TCA1 and BTZ043 potently inhibit conversion of the substrate DPR to the product DPA by DprE1/DprE2 epimerase. (C) Molecular surface of *Mtb* DprE1 with the FAD domain in light blue and the substrate binding domain in beige. The surface areas in pale green and magenta indicate the positions of (Left) Cys387 and Tyr314. (Right) Noncovalent contacts between TCA1 and DprE1 are shown. Residues within a 4-Å radius of the inhibitor (violet) are shown as sticks, with FAD in yellow. Dashed lines indicate the shortest contacts (yellow, hydrophobic/van der Waals; orange, polar) between the residues and the inhibitor. Trp230, located within 4 Å of the carbamate moiety of TCA1, has been omitted for clarity.
substituting this tyrosine with cysteine renders DprE1 insensitive to TCA1. Superimposition of the structures of DprE1 bound to the BTZ analog (CT325) (19) and TCA1 showed that the binding sites of these two inhibitors overlap significantly.

The above results suggest that DprE1 is a relevant target for the bactericidal activity of TCA1 against replicating bacteria, similar to BTZ. However, there are some clear distinctions between TCA1 and BTZ. First, BTZ is not active against nonreplicating Mtb (14), whereas TCA1 is active against replicating and nonreplicating Mtb. Second, the gene expression profiles of Mtb treated by two compounds are also very different—TCA1 down-regulates persistence genes that are usually up-regulated in Mtb-dormant models, whereas BTZ does not (14). The Mtb strain overexpressing DprE1 is resistant to TCA1 in 7H9 medium but still sensitive to TCA1 in the nutrient starvation model (Fig. 5A). Moreover, TCA1 still potentiates INH or RIF on this DprE1-overexpressing strain (Fig. 5B). These results suggest that TCA1 could potentially act on an additional mycobacterial target.

Because TCA1 had diminished activity against the DprE1 (Y314C) mutant in normal growth medium, it is possible that a second TCA1 target is not essential for Mtb growth under conditions of optimal growth. This complication makes the selection of relevant mutants more difficult, and therefore, affinity-based methods were used to identify additional potential targets. Among a group of analogs of TCA1, we found that a pyridyl analog, TCA17, has very similar in vitro activity to the activity of TCA1. TCA17 was immobilized on a resin through a linker moiety (TCAP1) (Fig. 1C) and used in a pull-down experiment with cell lysates from Mtb. A 35-kDa band was identified on an SDS/PAGE after silver staining, which disappeared in the presence of 50 μM TCA1 as a competitor. MS identified the band as MoeW, a protein involved in the biosynthesis of the molybdenum cofactor (MoCo) (Fig. S3) with homologs in only a few bacterial genomes. To confirm the binding of TCA1 to MoeW, we overexpressed moeW in E. coli (which lacks an moeW gene homolog in its genome) and treated this strain with a photoaffinity probe analogous to TCA1 (TCAP2) (Fig. 1C) followed by UV irradiation and cell lysis. As shown in Fig. S4, a band with the size of MoeW is present on an SDS/PAGE gel for the sample from the moeW-induced strain and absent in the uninduced control sample. These results show that TCA1 scaffold directly binds to MoeW.

MoeW is predicted to contain an FAD/NAD binding domain by protein sequence analysis, but its function has yet to be determined. The gene encoding MoeW is only conserved in Mtb and bacillus Calmette–Guérin and not M. smegmatis or other mycobacterial species, although it is homologous to moeB, another gene involved in MoCo biosynthesis pathway and conserved in all mycobacteria species and many other bacteria (21).
molybdenum—using enzymes identified to date contain MoCo. MoCo is essential for the nitrate respiratory and assimilatory function of Mtb nitro-reductase. Some of these nitro-reductases have been found to be involved in the response of Mtb to hypoxia and nitric oxide (21, 22). To determine if TCA1 can block the biosynthesis of MoCo, we analyzed cell extracts from Mtb treated with TCA1 by detection of dephosphorylated MoCo Form A using HPLC with fluorescence detection. Indeed, TCA1 (7.5 μg/mL) completely abolished the formation of MoCo in Mtb (Fig. 5 C–F). It is known that MoCo is indispensable for nitrate assimilation by Mtb and thus, essential for Mtb to survive in media that uses nitrate as the only nitrogen source (designated as nitrate media). We generated an Mtb strain overexpressing moeW and found it to confer resistance to TCA1 in nitrate media over 30 d of treatment (Fig. 5G). These results clearly show that TCA1 asserts its activity against Mtb by inhibition of MoCo biosynthesis through interaction with MoeW. The Mtb strain overexpressing moeW conferred resistance to TCA1 in a nutrient starvation model over 21 d of treatment as well (Fig. SH), but the lower level of resistance to TCA1 in the nutrient starvation model than nitrate media suggests that the mechanism of action of TCA1 is more complicated under the former condition. Nevertheless, the biochemical and genetic results clearly show that MoeW is a relevant target of TCA1.

Conclusion

We have developed a cell-based screen involving the growth of mycobacteria as an in vitro biofilm (a pellicle). The natural mode of growth of Mtb in liquid culture in the absence of detergent is as a pellicle at the liquid–air interface. Indeed, bacillus Calmette–Guérin is grown as a pellicle for vaccine production. This assay allowed us to identify a potent inhibitor TCA1 against both replicating and nonreplicating Mtb as well as drug-resistant Mtb. TCA1 functions by a unique mechanism involving down-regulation of persistence genes and inhibition of both cell wall and MoCo biosynthesis. Moreover, TCA1 showed excellent in vivo efficacy in both acute and chronic TB infection mouse models, suggesting that this compound may serve as a lead for the development of a class of drugs against persistent and drug-resistant Mtb. Indeed, we have subsequently identified a compound with good serum half-life that has excellent activities under both aerobic and anaerobic conditions (MIC50 values are 0.3 and 1.5 μg/mL, respectively). Future work will focus on additional improvements in the in vivo activity of this molecule and detailed mechanistic studies, including attempts to isolate additional resistant mutants under varied growth conditions. This work underscores the power of cell-based phenotypic screens to uncover molecules with mechanisms of action that provide unique approaches to the treatment of human disease.

Materials and Methods

High-Throughput Screen for Inhibitors of Biofilm Formation Inhibition. A diverse chemical library (~70,000 compounds) was used for the primary screen. This in-house compound library was created based on a chemoinformatic analysis of scaffold chemical diversity, historical proprietary screen hit rates (>300 Mio data points from the high throughput (HTS) database), and commercial availability; 105 M. smegmatis cells were plated in 384-well plates in biofilm formation medium of M63 salts minimal medium supplemented with 2% glucose, 0.5% Casamino Acids, 1 mM MgSO4, and 0.7 mM CaCl2. Rif and TMC207 were used as positive controls, and DMSO (0.1%) was used as a negative control. Cells were treated with 10 μM compound and incubated for 3 d, and the OD of each well was determined with an EnVision Multilabel Reader. The average Z’ and coefficient values are 0.512 and 8.7%, respectively. We used a high-stringency cutoff (threefold inhibition) to pick hits that are most likely growth inhibitors (hit rate = 0.03%) and a low-stringency cutoff (twofold inhibition) to include hits that inhibit biofilm formation without significant growth inhibition (hit rate = 0.17%).
Affinity-Labeling Proteomics and Photo-Affinity Labeling. H37Ra cells were lysed with homogenization buffer (60 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, 25 mM Mops, pH 7.2, 15 mM MgCl₂, 1 mM DTT, protease inhibitors, 0.5% Nonidet P-40). Cell lysates were centrifuged at 16,000 × g for 20 min at 4 °C, and the supernatant was collected. Total protein concentration in the supernatant was determined by a BCA protein assay kit (Pierce). The lysates (1 mg) were then added to the affinity resin (30 µL) and the loading buffer (50 mM Tris HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, protease inhibitors, 0.1% Nonidet P-40) was added to a final volume of 1 mL (for the competition experiment, TCA1 was added to a final concentration of 50 µM). After rotating at 4 °C for 1 h, the mixture was centrifuged at 16,000 × g for 1 min at 4 °C, and the supernatant was removed. The affinity resin was then washed five times with cold loading buffer and eluted by boiling with Laemmli sample buffer (Invitrogen) at 95 °C for 3 min. Samples were loaded and separated on a 4%–20% Tris-glycine gel (Invitrogen). The gel band was extracted and analyzed by proteomics. For the photo-affinity experiments, E. coli cells expressing MoeW and native cells were lysed, and the photo-affinity probe was added to cell lysates. The complexes in vivo and at room temperature were harvested, and the pellet was resuspended in extraction solution (2 mL; 10 mM sodium ascorbate). The reactions were terminated by the addition of prechilled acetone (0.5 mL), placed at −20 °C for 30 min, and centrifuged at 16,000 × g for 10 min at 4 °C to precipitate proteins. The pellet was washed two times with 200 µL prechilled methanol, resuspended in 25 µL 1× standard reducing SDS loading buffer, and heated for 10 min at 95 °C. Samples were loaded for separation by SDS/PAGE and then visualized by in-gel fluorescent scanning.

In Vitro Assays in Nitrate-Only Media. An Mtb culture was resuspended under nitrogen-limiting conditions (22) (a basal medium [1 L basal medium contains 1 g KH₂PO₄, 2.5 g Na₂HPO₄, 2 g K₂SO₄, 2 mL trace elements; 1 L trace elements contained 40 mg ZnCl₂, 200 mg FeCl₃·6H₂O, 10 mg CuCl₂·4H₂O, 10 mg MnCl₂·4H₂O, 10 mg Na₂B₄O₇·10H₂O, 10 mg (NH₄)₂MoO₄·4H₂O) supplemented with NaNO₃ as the sole source of nitrogen, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 10% ADS, 0.2% glycerol and 0.05% Tween 80) and incubated for 24 h. 7.5 µg/mL TCA1 was then added to the culture and incubated for 30 d. CFU assay was used to determine the bacterial viability at each time point. All experiments were carried out in triplicate.

MoCo Inhibition Assay. The synthesis of MoCo Form A dephospho was carried out according to the procedures described. The 1H-NMR spectrum matches what has been previously reported with slight modifications (21, 30). 100 µL Mtb culture was harvested, and the pellet was resuspended in extraction solution (2 mL; 10 mM sodium ascorbate). The cells were lysed and centrifuged at 16,000 × g; the supernatant was collected and treated with acidic iodine solution at 95 °C for 25 min, and the excess iodine was removed by adding sodium ascorbate. After centrifugation, the solution was neutralized with ammonium hydroxide and then concentrated and dephosphorylated using calf intestinal phosphatase (NEB) at 37 °C for 3 h. HPLC analysis was performed using Agilent C18 column (150 × 4.6 mm, 10-µm particle size) with gradient elution by buffer A (50 mM ammonium acetate) and buffer B (MeOH; 97% A to 93% A in 14 min and 97% B wash from 15 to 22 min). Fluorescence detection was set at 370/450 nm.

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