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**N-methylation of a bactericidal compound as a resistance mechanism in *Mycobacterium tuberculosis***

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The rising incidence of antimicrobial resistance (AMR) makes it imperative to understand the underlying mechanisms. *Mycobacterium tuberculosis* (Mtbc) is the single leading cause of death from a bacterial pathogen and estimated to be the leading cause of death from AMR. A pyrido-benzimidazole, 14, was reported to have potent bactericidal activity against Mtbc. Here, we isolated multiple Mtbc clones resistant to 14. Each had mutations in the putative DNA-binding and dimerization domains of rv2887, a gene encoding a transcriptional repressor of the MarR family. The mutations in rv2887 led to markedly increased expression of rv0560c. We characterized Rv0560c as an oxidoreductase-dependent methyltransferase that N-methylates 14, abolishing its mycobactericidal activity. An Mtbc strain lacking rv0560c became resistant to 14 by mutating decaprenylphosphoryl-β-D-ribose 2-oxidase (DprE1), an essential enzyme in arabinogalactan synthesis; 14 proved to be a nanomolar inhibitor of DprE1, and methylation of 14 by Rv0560c abrogated this activity. Thus, 14 joins a growing list of DprE1 inhibitors that are potently mycobactericidal. Bacterial methylation of an antibacterial agent, 14, catalyzed by Rv0560c of Mtbc, is a previously unreported mechanism of AMR.

### antimicrobial resistance | transcription factor | methyltransferase | arabinogalactan synthesis

The World Health Organization recently announced success in meeting the millennium development goal of reversing the rise in worldwide incidence of tuberculosis (TB) by 2015 (1). Nonetheless, TB mortality remains high, with 1.5 million deaths in 2013 alone, of which 0.2 million were estimated to be caused by multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (Mtbc). MDR TB is defined as TB caused by Mtbc resistant to isoniazid and rifampin, two of the four frontline TB drugs. MDR TB requires over 20 mo of treatment with toxic second-line drugs (2). Extensively drug-resistant (XDR) TB, which involves additional resistance to second-line drugs, has been detected worldwide. A recent study with a 5-y follow-up of patients with XDR TB in South Africa found that only 5% were cured, 73% died, and 10% who failed treatment but survived discharged to the community with Mtbc-positive sputum, that is, with contiguous XDR TB (3). There is an urgent need to deepen our understanding of mechanisms of antimicrobial resistance (AMR). Elucidation of AMR mechanisms will help in the development of new anti-TB drugs. For example, a structural modification to a drug candidate could avoid resistance, or combination therapy could include an inhibitor of an enzyme that inactivates the drug, when β-lactamase inhibitors are coadministered with β-lactams.

AMR in Mtbc and other bacterial pathogens is heritable or phenotypic (4). Heritable AMR was observed in Mtbc soon after the use of the first TB drug, streptomycin, and led to the introduction of combination chemotherapy to the practice of medicine (5). Unlike many other bacterial pathogens, mycobacteria do not readily exchange genetic material to spread resistance-causing genes; instead, accumulation of spontaneous mutations allows selection for drug resistance. Clinically, resistance to most frontline TB drugs is caused by mutations in the activation pathways of prodrugs (e.g., *katG* mutations can confer resistance to isoniazid) and/or mutations in the target (e.g., *rpoB* mutations can confer resistance to rifampicin) (6). Additional mechanisms of AMR have been described in Mtbc and the fast-growing *Mycobacterium smegmatis* (7). Some are non-specific, such as increased expression of the efflux pumps Tap and InhBAC (8, 9). Others involve direct modification and inactivation of the drug, such as expression of the β-lactamase BlaC that hydrolyzes β-lactams (10) and the acetyl transferase Eis that acetylates and inactivates kanamycin (11). Target modification plays a role in macrolide resistance through methylation of 23S rRNA by the Erm family of methyl transferases (12). However, to the best of our knowledge, inactivation of an antibiotic by its methylation has not been reported.

The goal of this study was to determine the mechanism of resistance to and mode of action of 14 (Fig. L4), which was recently published online by the PNAS open access option.

**Significance**

**Better understanding of the mechanisms used by bacteria to counter antibacterial agents is essential to cope with the rising prevalence of antimicrobial resistance.** Here, we identified the mechanism of resistance of *Mycobacterium tuberculosis* to an antimycobacterial cyano-substituted fused pyrido-benzimidazole. Clones bearing mutations in a transcription factor, Rv2887, markedly up-regulated the expression of rv0560c, a putative methyltransferase. Rv0560c N-methylated the pyrido-benzimidazole in vitro and in *Mycobacterium tuberculosis*, abrogating its bactericidal activity. Resistant mutants selected in the absence of rv0560c led to the identification of the target of the compound, the essential oxidoreductase, decaprenylphosphoryl-β-D-ribose 2-oxidase (DprE1). Methylation of an antibacterial compound is a previously uncharacterized mode of antimicrobial resistance.


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The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The RNASeq data described in SI Appendix, Table S1 have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE77556).

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identified as bactericidal to replicating Mtb (13). We identified the mechanism of resistance as N-methylation of compound 14 by a previously uncharacterized methyltransferase, Rv0560c. After the resistance mechanism was incapacitated by genetic deletion of Rv0560c, selection of additional mutants at a far lower frequency allowed us to identify the target of 14 as decaprenylphosphoryl-β-D-ribose 2-oxidase (DprE1), an essential enzyme involved in arabinogalactan synthesis.

Results

Sterilizing Activity of 14 in Vitro. Earlier work showed that 14 inhibits Mtb growth with an IC₉₀ of 0.39 μM and that the compound is bactericidal under conditions that support bacterial replication (13). In the present studies, we found that 14’s mycobactericidal activity was dose- and time-dependent, with a multilog reduction in colony-forming units observed at 0.78 μM after a brief exposure of 3 d and further colony-forming unit reduction to below the limit of detection by 8 d (Fig. 1B). A recent study reported that some antmycobacterial compounds with potent activity in vitro were inactive in vivo because glycerol was used as the carbon source in the in vitro experiments (14). However, we observed no change in the IC₉₀ of 14 when glycerol, dextrose, or acetate served as the carbon source (SI Appendix, Fig. S1).

Resistance to 14 Is Associated with Mutations in rv2887. To elucidate the mode of action of 14, we isolated resistant clones after incubation of Mtb at 4-, 5-, 10-, or 20-fold the IC₉₀ of 14 and observed a frequency of resistance of 1–3 × 10⁻⁶ (SI Appendix, Fig. S2A). Whole genome resequencing of four resistant clones (highlighted in Table 1) found one gene, rv2887, to be mutated in common. Rv2887 is annotated as a nonessential, putative transcription repressor of Escherichia coli linked to the multiple antibiotic resistance (mar) phenotype. Resequencing of rv2887 in 16 additional resistant clones revealed 12 additional SNPs leading to missense or nonsense mutations (Table 1).

We used Phyre 2 (15) to build a homology model of Rv2887 that predicted an α₁–α₂–α₃–α₄–β₁–loop–β₂–α₅–α₆ topology, as observed in crystal structures of E. coli MarR and its homologs (Fig. 1C) (16). The N-terminal α₁–α₂ helices and C-terminal α₅–α₆ helices of a MarR monomer interact with the corresponding regions observed in crystal structures of 16 unique MarR and its homologs linked to the multiple antibiotic resistance phenotype. Rv2887 has the winged helix–turn–helix DNA binding domain found in the MarR family of transcription factors (SI Appendix).

Mtb has at least six other genes that belong to this family, namely, rv0042c, rv0737, rv0880, rv1049, rv1404, and rv2327. These proteins share homology with MarR, the transcriptional repressor of Escherichia coli linked to the multiple antibiotic resistance (mar) phenotype. Resequencing of rv2887 in 16 additional resistant clones revealed 12 additional SNPs leading to missense or nonsense mutations (Table 1).

Table 1. Characterization of Mtb clones resistant to 14

<table>
<thead>
<tr>
<th>Strain name*</th>
<th>IC₉₀ of 14, μM</th>
<th>SNP in rv2887</th>
<th>Mutation in Rv2887</th>
<th>Other SNPs</th>
<th>Fold change in IC₉₀ of RIF</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.39</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1A</td>
<td>12.5</td>
<td>G242A</td>
<td>R81Q</td>
<td>—</td>
<td>—</td>
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<tr>
<td>2A</td>
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<td>C274T</td>
<td>P92S</td>
<td>None in DprE1</td>
<td>0.5</td>
</tr>
<tr>
<td>5A</td>
<td>12.5</td>
<td>T122C</td>
<td>L41P</td>
<td>Rv0516c:L69M, plcB:R190R, 3418802 A &gt; G</td>
<td>1</td>
</tr>
<tr>
<td>6A</td>
<td>25</td>
<td>G62A</td>
<td>R21Q</td>
<td>Rv1619:Y457H, Rv2173/idsA2:5201P</td>
<td>1</td>
</tr>
<tr>
<td>8A</td>
<td>&gt;25</td>
<td>G400T</td>
<td>C-term 6 AA deletion and addition of 61 AA</td>
<td>Rv0560c:S140A</td>
<td>1</td>
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<td>Q64P</td>
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<tr>
<td>183A</td>
<td>12.5</td>
<td>Insertion of 14 nts after G125 leading to stop codon after AA45</td>
<td>Stop codon after 45AA</td>
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<tr>
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<td>25</td>
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<td>G88E</td>
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<td>L133P</td>
<td>None in DprE1</td>
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</table>

*The names of strains analyzed by whole genome resequencing are in bold. RIF, rifampin.

Fig. 1. Activity of 14 and the rv2887 mutations identified in 14-resistant Mtb clones. (A) Structure of 14. (B) Kinetics of bactericidal activity of 14 against Mtb as measured by cfu counts on postexposure days 1, 3, 5, 8, and 15. (C) Phyre 2 generated homology model of Rv2887 highlighting the 16 unique mutations identified in Mtb clones resistant to 14. The secondary structure elements are color coded as described: blue, α₁ helix; light blue, α₂ helix; light green, α₃ helix; dark green, α₄ helix; orange, β₁-loop–β₂ region; dark red, α₅ helix; and red, α₆ helix. The dashed line demarcates the predicted dimerization and DNA-binding domains.
in a second monomer to form the functional dimer (16). Previous studies with E. coli mutant strains showed that point mutations or deletions in the N or C termini of MarR led to loss of repressor activity (17, 18). Nine of the 16 unique mutations in our resistant clones mapped to the homologous regions of Rv2887 (Fig. 1C and Table 1). The winged helix fold in MarR, formed by the third and fourth helices and the β-loops–β-sheets domain, is predicted to bind the promoter region of the marR/ID operon in E. coli, and the remaining seven unique mutations in our resistant clones mapped to the homologous domain of Rv2887 (Fig. 1C and Table 1). Three of the mutated residues identified in Rv2887—R81, G99, and Q64—are homologous to MarR residues reported to be essential for DNA binding in E. coli, where they correspond to R86, G104, and G69, respectively (17, 19). R86 and G104 are among the most highly conserved residues among MarR homologs across different species (16, 17, 19). Thus, each of the Rv2887 mutations identified in the resistant clones is likely to cause complete or partial loss of the DNA binding function of Rv2887.

Specific Role of Rv2887 in Mediating Resistance to 14. E. coli mutants lacking MarR and MarA are resistant to diverse antibiotics and other stresses (20, 21). To determine if Rv2887 fulfilled a similar role in Mtb, we tested susceptibility of two resistant clones, 1A and 8A, to the TB drugs rifampin, isoniazid, ethambutol, moxifloxacin, and streptomycin and did not observe increased resistance or susceptibility relative to wild-type strain (SI Appendix, Fig. S2B). Replacement of the wild-type allele of Rv2887 in Mtb with mutant allele carrying the point mutations found in one of these two resistant clones, namely R81Q (as found in clone 1A) or deletion of six amino acids at the C terminus (as found in clone 8A), by oligonucleotide-mediated recombineering (22), conferred resistance specifically to 14 (Fig. 2A and SI Appendix, Fig. S2C). However, introduction of the r2887 mutation found in resistant clone 8A into wild-type Mtb did not lead to a level of resistance as high as that displayed by clone 8A itself (IC50 > 25 μM). This data suggested that additional mutations in clone 8A might contribute to its high level of resistance. Whole genome resequencing of clone 8A revealed an additional SNP leading to S140A mutation in Rv0560c, whose significance emerged in studies described below. To confirm the role of Rv2887 in resistance to 14, wild-type r2887 was overexpressed in the resistant clones 1A and 8A. Overexpression of r2887 increased the susceptibility of both clones to 14 (Fig. 2A).

Genes and Pathways Regulated by Rv2887. Based on similarity of Rv2887 to the transcriptional repressor MarR, we hypothesized that loss-of-function mutations in Rv2887 lead to derepression of the target of 14 or of genes linked to the target pathway. RNASeq gene expression profiles of the wild-type strain and the resistant clones 1A and 8A were generated after 4 h of exposure to 14 or vehicle (DMSO). In the absence of compound treatment, resistant clones 1A and 8A had a common set of 13 up-regulated genes—10 elements and predicted a putative repressor binding site (23). We determined if recombinant Rv2887 (∼molar equivalent of Rv2887) could bind the 70-bp region spanning the promoter of an IdeR-regulated gene, mbtB (24). Our observation that Rv2887 acts as a repressor of mbtB gene expression suggested an additional role in Mtb, we tested susceptibility of two resistant clones, 1A and 8A; wild-type strains carrying the two Rv2887 mutations, R81Q and C-terminal deletion, found in clones 1A and 8A; and three strains, WT, 1A, and 8A, that express r2887 constitutively (WT o/e r2887, 1A o/e r2887, and 8A o/e r2887). o/e, overexpression. (B) Relative change in expression of r0560c, r0559c, r0558, and r0557 in wild-type Mtb H37Rv (WT) treated with 3.9 μM of 14 and two resistant clones, 1A and 8A, that were vehicle-treated for 4 h. The baseline of 1 indicates expression level in Mtb H37Rv treated with vehicle control. (C) Native PAGE gels of wild-type Rv2887 or mutant Rv2887, with the point mutation R81Q, incubated with a 70-bp DNA sequence spanning the promoter of r0560c (560c-prom).

The RNASeq results thus directed our attention to the cluster of four protein-coding genes up-regulated in the r2887 mutants, r0555–r0560c. Quantitative reverse transcriptase (qRT) PCR (Fig. 2B) confirmed that r0560c, r0559c, and r0558 were significantly up-regulated (∼fivefold) in the resistant clones, whereas r0557 was not. Finally, r0560c was found to be up-regulated by ∼eightfold and ∼40-fold as detected by qRT-PCR (Fig. 2B) and RNA Seq (SI Appendix, Table S1), respectively, in wild-type Mtb exposed to 3.9 μM (∼10x IC50) of 14 for 4 h. In contrast, exposure of wild-type Mtb to 14 did not affect expression of r0559c and r0558. Based on these data, we predicted a model in which Rv2887 acts as a repressor of r0560c and loss-of-function mutations in Rv2887 lead to derepression and thus increased expression of r0560c.

Rv2887 Binds to the Promoter Region of r0560c. Previous characterization of the promoter region of r0560c identified the putative −35 and −10 elements and predicted a putative repressor binding site (23). We determined if recombinant Rv2887 (SI Appendix, Fig. S3A) could bind the 70-bp region spanning the reported promoter region of r0560c (560c-prom) (SI Appendix, Table S2). Migration of the oligonucleotide in the gel was retarded by ≥1 molar equivalent of Rv2887 (Fig. 2C). Quantitative analysis of band intensities indicated ≥90% reduction in the unbound DNA at 1.5 molar equivalent of Rv2887 (SI Appendix, Fig. S4). Rv2887 did not bind to a 36-bp region corresponding to the promoter region of an IdeR-regulated gene, mbtB (mbtB-prom) (SI Appendix, Fig. S4). Our observation that Rv2887 regulates
expression of Rv0560c is supported by a recent genome-wide analysis of regulatory interactions in Mtb using ChIP-Seq- and RNA-Seq-based assays (25). Introduction of the R81Q mutation into Rv2887 abolished its ability to bind 560c-prom DNA (Fig. 2C and SI Appendix, Fig. S4), providing direct evidence that the mutation present in resistant clone 1A impacts ability of Rv2887 to bind the promoter region of rv0560c. We also examined the impact of mutations in 560c-prom DNA sequence, both those in palindromic regions and those previously reported to affect repression (23), on binding by Rv2887 in vitro, and observed a mild effect at ≥2 molar equivalents of Rv2887 (SI Appendix, Fig. S4).

**Rv0560c Methylates and Inactivates 14.** We hypothesized that up-regulation of rv0560c, rv0558, or rv0559c could be contributing to the resistance phenotype of the clones, 1A and 8A. We tested susceptibility of wild-type Mtb individually overexpressing each of these genes to 14. Mtb became 16-fold more resistant to 14 when rv0560c was overexpressed, whereas overexpression of rv0558 and rv0559c had no impact on sensitivity to 14 (Fig. 3 and SI Appendix, Fig. S5). Because Rv0560c is reportedly non-essential (26), we reasoned that it was unlikely to be the target of 14. Instead, given that increased expression of Rv0560c conferred resistance to 14 on the wild-type strain, we hypothesized that Rv0560c inactivates 14, directly or indirectly. Moreover, given that the least resistant mutant was clone 8A, which carried mutations in both rv2887 and rv0560c, we predicted that the S140A mutation in Rv0560c might augment the ability of Rv0560c to inactivate 14.

Based on the putative methyltransferase activity of Rv0560c, we tested recombinant Rv0560c and S140A-Rv0560c (SI Appendix, Fig. S3B) for methyltransferase activity with 14 as the candidate substrate. After overnight coincubation of 14 with δ-adenosyl-L-methionine (SAM) and Rv0560c, mass spectrometry coupled to liquid chromatography (LC-MS) analysis detected an additional peak with an m/z of 363.18, which is 14 mass units (CH₂ group) greater than that of 14 (SI Appendix, Fig. S6). We then examined the kinetics of methylation of 14 by Rv0560c and S140A-Rv0560c by taking advantage of the characteristic blue shift at 380 nm observed in the absorption spectrum of 14 upon methylation (SI Appendix, Fig. S7A). Initial velocity of the methylation reaction, as reflected by the absorbance change at 380 nm, depended on the concentration of Rv0560c (SI Appendix, Fig. S7B). Although wild-type Rv0560c can modify 14, the substrate turnover measured by k_cat was increased by 2.4-fold for S140A-Rv0560c (Fig. 3B). Enhancement was even greater when δ-adenosyl-L-homocysteine (SAH) was depleted by two coupled enzymes, 5'-methylthioadenosine/δ-adenosylhomocysteine nucleosidase (MTAN) and adenine deaminase (SI Appendix, Fig. S7C). SAH, the common byproduct of methylation, is often characterized as a pan-inhibitor of methyltransferases and can be rapidly degraded inside cells by other metabolic enzymes, as mimicked by the addition of the coupling enzymes mentioned above (27). Given that S140 is predicted to be near a putative SAM binding site, the increased methylation activity of S140A-Rv0560c relative to wild-type Rv0560c may be attributed to increased k_cat/k_M, decreased SAH inhibition, or a combination of both.

We then sought to determine the site of methylation on 14 using NMR. Comparison of 1H-NMR spectra of 14 and its methylation product, me-14, revealed the nascent methyl group at 4.1 ppm (Fig. 3C). The 1H-1H COSY [number of scans (ns) = 8] allowed us to assign all of the peaks in 1H spectra of 14. A gradient NOESY experiment was set up with the optimal mixing time of 0.8 s for the characteristic methyl group of me-14 (ns = 32). An NOE cross-peak between 4.1 ppm and the doublet around 7.3 ppm (C-6 proton) indicated that N-5 is the site of methylation (Fig. 3C). Similar assays were conducted with an analog of 14, 14-IA (SI Appendix, Fig. S8), which has a methyl group at N-5 and was inactive against Mtb (Fig. 3D). As expected, a characteristic NOE cross-peak was observed between the C-6 doublet of the analog 14-IA at 7.3 ppm and the N-5 methyl proton at 4.1 ppm (SI Appendix, Fig. S8). We conclude that Rv0560c is an N-methyltransferase that modifies 14 at the N-5 position.

We used Rv0560c to generate me-14 in vitro, extracted it from the reaction mixture, and tested it against wild-type Mtb. Like the synthetically prepared analog 14-IA, me-14 did not inhibit growth of Mtb (Fig. 3D), confirming that resistance to 14 can arise by its inactivation upon N-methylation by Rv0560c.
Methylation Reduces the Level of 14 in Resistant Clones. We next sought to relate the foregoing in vitro observations to the mechanism of resistance within mutant Mtb. To do this, we treated bacteria with 7.8 μM (~20× IC₉₀) of 14 for 24 h and measured intrabacterial levels of 14 and me-14 in resistant clones and in wild-type Mtb overexpressing rv0560c. There was no significant increase in the amount of me-14 in the resistant clones relative to the wild-type strain (H37Rv North strain, Rv), either inside the bacteria or in the extracellular medium (Fig. 3E and SI Appendix, Fig. S9). However, the amount of parent compound, 14, was reduced by fivefold in the resistant clones and by twofold in the rv0560c-overexpressing strain (Fig. 3E). The unchanged levels of me-14 in these cells might be due to the brief exposure to a large excess of 14. Moreover, these observations raise the possibility that me-14 may be subject to further modifications in Mtb. In contrast, levels of me-14 in a Δrv0560c strain were 20-fold lower than in the wild-type control strain (H37Rv London Pride strain, LP), accompanied by a ~10-fold increase in 14 (Fig. 3E). Both differences were reversed by constitutive expression of rv0560c in the knockout strain (Fig. 3E). Collectively, these studies established that Rv0560c catalyzes the methylation of 14 within Mtb.

DprE1 Mutations Confer Resistance to 14 in the Absence of Rv0560c. To circumvent selection of resistant clones carrying rv2887 or rv0560c mutations, we isolated resistant clones in the Δrv0560c background. The observed frequency of resistance to 14 in Δrv0560c strain was 100-fold lower than for wild-type Mtb and ranged between 0.5 × 10⁻⁹ and 8.5 × 10⁻⁹ (SI Appendix, Fig. S10A). Three of the four analyzed clones carried a point mutation, P116S, in dprE1, an essential gene involved in synthesis of decaprenylphosphoryl-β-D-arabinoside (DPA) (SI Appendix, Fig. S10B). Introduction of this mutation into wild-type Mtb by replacing the wild-type allele with oligonucleotide-mediated recombineering led to a 16-fold increase in IC₉₀ (Fig. 4A). These data indicated that DprE1 could be the main target of 14, and this possibility was tested as described in Inhibition of DprE1 by 14. Whole genome resequencing of four clones identified common mutations in rv0678, the putative regulator of the MmpL5-S5 transporter system, in all four clones (SI Appendix, Fig. S10B) (28). Hence, increased efflux of 14 through MmpL5 might occur in these resistant clones in addition to reduced inhibition of DprE1.

Inhibition of DprE1 by 14. Using a previously reported fluorescence-based assay (29) to monitor inhibition of pure, recombinant Mtb DprE1 by 14, we observed an IC₉₀ of 70 nM against DprE1 from the recombinant Mtb (Fig. 4B). Preincubation of 14 with Rv0560c in the presence of SAM led to a 36-fold increase in the IC₉₀ (Fig. 4B). Thus, 14 is a potent inhibitor of DprE1, and methylation by Rv0560c markedly reduced this activity. Based on the proximity of the P116 residue to the flavin adenine dinucleotide (FAD) cofactor-binding site in DprE1, we hypothesized that displacement of FAD by 14 might be the mechanism of its DprE1 inhibition. We monitored release of FAD in the presence of 14, but no release was detected (SI Appendix, Fig. S11A). A 20-fold increase in FAD fluorescence would have been observed if there was release (29); instead, binding of 14 to DprE1 caused a slight quench in FAD fluorescence (SI Appendix, Fig. S11A). We analyzed DprE1 by mass spectrometry after incubation with 14 and the substrate farnesylphosphoryl-β-D-ribose (FPR) to monitor the formation of covalent adducts by the inhibitor, but none were detected (SI Appendix, Fig. S11B). This data is also consistent with the inhibition assay data, which showed no evidence of time-dependent inhibition.

Recent studies have described six mutations in DprE1, namely C387S (30), Y314H (31), L368P (32), G248S (29), and G248S (29), associated with resistance to inhibition of the enzyme by diverse molecular scaffolds. We interrogated the roles of these residues in the interaction of 14 with DprE1 by measuring the IC₉₀ of 14 against Mtb strains carrying these point mutations (Fig. 4C). The greatest resistance to 14 (~16- to 32-fold increase in IC₉₀) was seen in the strains in which either P116 or E221 were mutated. A milder effect (~sixfold to eightfold increase in IC₉₀) was observed when L368 or G248 were mutated. The covalent inhibitor BTZ043 was inactive only when C387, the key residue with which it forms a covalent bond with DprE1, was mutated. The activity of TCA1 was dramatically reduced when Y314 or P116 were mutated. None of these mutations affected the activity of ethambutol, isoniazid, and rifampin. Finally, overexpression of DprE1 conferred resistance to 14, as it did to the DprE1 inhibitors TCA1 and BTZ043 (Fig. 4C).

Discussion

We have uncovered N-methylation of antimicrobial compounds as a novel mechanism of AMR. Mtb exploits this mechanism to counter the antimycobacterial action of a potent pyrido-benzimidazole, 14, with bactericidal activity against replicating Mtb. The nonessential gene rv0560c, annotated as a putative benzoquinone methyltransferase, was found to inactivate 14 by catalyzing the transfer of a methyl group from SAM to the N-5 position. Mtb clones resistant to 14 up-regulated expression of rv0560c by incurring mutations in the transcriptional repressor, Rv2887. Winglee et al. (33) recently reported a role for Rv2887 in Mtb’s resistance to imidazo[1,2-a]pyridine-4-carbonitrile (MP-III-71), a compound with structural similarity to 14. That study also documented up-regulation of rv0560c in the setting of resistance-conferring mutations in rv2887, but did not directly implicate Rv0560c in resistance nor establish a biochemical mechanism for mycobacterial resistance to MP-III-71.

Methylation now joins a list of covalent modifications of antibacterial compounds by which bacteria can manifest AMR.
including acetylation, phosphorylation, dephosphorylation, and hydrolysis (34). Previous studies have shown that some proportion of intrabacterial para-amino salicylic acid (PAS) is methylated in PAS-sensitive Mtb treated with PAS (35). However, methylation of PAS by Mtb has not been observed to cause PAS resistance (36). Bacterial methyltransferases of the Erm family contribute to AMR by modifying the target of macrolides, RNA, but those methyltransferases do not act on the macrolides themselves. Rv0560c-mediated inactivation of 14 is, to our knowledge, the first instance of compound methylation as a resistance mechanism in a bacterial species.

It is unclear how Mtb senses 14 such that a consequence is the relief of repression of rv0560c. One hypothesis is that 14 directly binds to Rv2887. Alternatively, an endogenous metabolite associated with DprE1 inhibition may accumulate as a consequence of its inhibition and bind to Rv2887, initiating a response to the stress of cell wall synthesis blockade. Diverse stresses, including sialylate, membrane depolarizers (carbonyl cyanide m-chlorophenyl hydrazone, valinomycin, or dinitrophenol), the detergent SDS, and the respiratory inhibitors chlorpromazine and thioridazine, can up-regulate rv0560c expression (www.tbdb.org/expressionHistory.html?gn=Rv0560c). We hypothesize that this occurs by the dissociation of Rv2887, the repressor, from the r1049 selects for the region. This theory is corroborated by the very low number of r1049c transposants in untreated, wild-type Mtb (SI Appendix, Table S1), compared with the marked up-regulation of r0560c in Mtb strains carrying mutations in Rv2887 (Fig. 2B) and demonstration of a direct interaction of Rv2887 with the promoter region of r0560c in a mobility shift assay (Fig. 2C).

Although PAS, chlorpromazine, and thioridazine have been reported to up-regulate r0560c gene expression, we did not observe increased resistance to these compounds in the clones 1A and 8A, which have markedly higher r0560c transcripts (SI Appendix, Fig. S12). It is unclear how Rv0560c, a putative cytoplasmic enzyme, inactivates 14 before 14 targets DprE1, whose activity has been localized to the Mtb cell wall (37). There may be an uptake mechanism for 14 that delivers most of it from the extracellular medium to the cytosol before it can reach DprE1 in the periplasm, or perhaps marked increase in expression of Rv0560c results in some of the enzyme becoming periplasmic.

Two other MarR homologs in Mtb, Rv1049 (MosR) and Rv1404c, have been shown to respond to physiologically relevant stresses. For example, exposure to hydrogen peroxide or nitric oxide led to dissociation of MosR from DNA and subsequent up-regulation of the putative oxidoreductase Rv1050 (38). Rv1404 was involved in Mtb’s response to acid and hypoxia by up-regulating 10 genes, including two putative methyl transferases, Rv1403c and Rv1405c (39).

The P116S mutation in DprE1 that we found to confer resistance to 14 is different from the mutations that confer resistance to several other DprE1 inhibitors. Covalent inhibitors of DprE1 exemplified by BTZ043 select for the C387S substitution in DprE1 because they require Cys-387 to form adducts. TCA1 requires Tyr-314 for its inhibition of DprE1. Compound 14 was active against Mtb clones carrying both the C387S and Y314H mutations, indicating that its interaction with DprE1 occurs by a mechanism distinct from that of BTZ043 and TCA1. In contrast, 14 selects for the P116S mutation in DprE1. Pro-116 is adjacent to the FAD binding pocket in the DprE1 active site. Compound 14 is predicted to be largely planar, similar to the isoalloxazine ring system of the FAD. Although 14 did not displace FAD from the enzyme, it might engage in a stacking interaction with the FAD in the substrate-binding site, thus inhibiting enzymatic turnover by competing with the substrate.

In conclusion, our analysis of Mtb’s resistance mechanism to the inhibitor, 14, revealed that a bacterial compound could be inactivated by methylation. To the best of our knowledge, this is the first report of N-methylation as a strategy for bacterial drug resistance. The target of 14 is DprE1, the essential oxidoreductase involved in DPA synthesis, leading to inhibition of cell wall synthesis and death of mycobacteria. Knowledge of the resistance mechanism provides a mechanistic rationale to overcome resistance to this class of antimycobacterial agents. We established that the mycobacterial enzyme catalyzing this reaction, Rv0560c, can serve as an N-methyl transferase. It remains for further study to identify the physiologic roles of Rv2887 and Rv0560c in Mtb not exposed to xenobiotics.

Methods

Materials. Mtb strains were grown in Difco Middlebrook 7H9 (BD Biosciences) medium with 0.2% glycerol, 10% (vol/vol) OADC (BD Biosciences) or ADN (0.5% BSA; Roche, 0.2% dextrose, 0.085% NaCl) and 0.02% tyloxapol, or plated on 7H11 agar (BD Biosciences) supplemented with 10% (vol/vol) OADC (BD Biosciences) and 0.5% glycerol; 7H10 agar (BD Biosciences) supplemented with 10% (vol/vol) ADN and 0.5% glycerol was used to grow Mtb strains on PVDF filters (Durapore) to quantify intrabacterial abundance of 14. Mtb H37Rv North strain (Rv) was used as the wild-type strain, unless otherwise specified. The strain deficient in r0560c (Aprv0560c) and its wild-type background strain, H37Rv London Pride (LP), were a kind gift from Tanya Parish, Infectious Disease Research Institute, Seattle, and the strains carrying the point mutations C387S (30), L368P, and G17C (32) in DprE1 were a kind gift from Stewart Cole, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland. Compound 14 was provided by GSK or purchased from Vitas Lab (Cat#STK177481). Compound 14-lA was provided by GSK. Rifampin, isoniazid, TCA1 (life chemicals), and BTZ-043 (Selleckchem) were obtained from commercial vendors. MTAN was purified as described (40), and adenine deaminase was purchased from G-Biosciences. All other agents were purchased from Sigma-Aldrich unless otherwise specified.

IC50 and Colony-Forming Unit Assays. Mtb strains were grown to log phase and diluted to an optical density (OD600) of 0.005 in 7H9 medium. Then 200 μL/well of the Mtb suspension was dispensed in 96-well plates (Costar), and compounds were added. OD600 was read after 10–14 d of incubation at 37 °C with 20% O2, 5% CO2. IC50 was defined as the concentration that led to ≥90% growth inhibition. The Resazurin Microtiter Assay was used to measure IC50 of compounds against strains carrying the Y314H, E221Q, G248S, and Y314H mutations in DprE1, as described (41). Colony-forming unit assays were set up after exposure of a single-cell suspension of Mtb in 7H9 medium at an OD600 of 0.005 to various concentrations of 14. At each time point, the samples were mixed, diluted 10-fold in PBS with tyloxapol (0.02%), and spread on 7H11 agar plates. Plates were incubated at 37 °C for 21–25 d, and colonies were counted.

Isolation of Mtb Clones Resistant to 14 and Whole Genome Sequencing. Log phase culture of Mtb H37Rv, LP, or Aprv0560c strains were plated on 7H11 agar with 4-, 5-, 10-, or 20-fold the IC50 of 14. Colonies visible after 3–5 wk were counted to measure frequency of resistance. These colonies were subjected to a second round of selection and then grown up in 7H9 medium with the corresponding concentration of 14 as a selection pressure. DNA was extracted using cetyltrimethylammonium bromide and lysis buffer as described (42). The genomes of the resistant mutants characterized in this study were sequenced using an Illumina HiSeq 2500. The read length was between 54 bp and 125 bp (depending on sequencing run), and paired-end data were collected. Genome sequences were assembled by a comparative assembly method using custom-developed scripts for mapping reads and building contigs to identify indels, as described (43). Previously sequenced genomes of the parental strain (local stocks of Mtb H37Rv) for each of the mutants were used as reference sequences for the comparative assembly and for calling SNPs and other polymorphisms. Targeted sequencing (Macrogen Corp.) of dprE1 and r2887 was performed after PCR amplification of the genes using the primers listed in SI Appendix, Table S2.

Transfer of SNPs Encoding Resistance to 14 into Wild-Type Mtb. Transfer of the SNPs encoding the R81Q and C-terminal deletion mutations in Rv2887 and the P116S, E221Q, G248S, and Y314H mutations in DprE1 into wild-type Mtb was performed by oligonucleotide-mediated recombineering as described (22),
using the oligonucleotides listed in SI Appendix, Table S2. Details of the method are included in SI Appendix. RNAseq and qRT-PCR. Log-phase culture of Mtb H37Rv-North (Rv) and the clones resistant to 14, 1A and 8A, were treated with 3.9 µM of 14 or with vehicle control (1% DMSO) for 4 h. RNA was extracted and the mRNA was enriched using the MicroExpress kit (Ambion). The cDNA libraries were synthesized according to instructions of the TruSeq RNA LT kit (Illumina), and RNA sequencing was performed with HiSeq2000/1000 (Illumina). The sequenced reads were aligned to the Mtb H37Rv complete genome (National Center for Biotechnology Information database) using Burrows-Wheeler alignment tool (44). The transcript abundances were measured in reads per kilobase of exon per million mapped reads (RPKM) using the Cufflinks package (45). Up- or down-regulated genes were identified based on the criteria that expression levels differed by >2-fold relative to control and RPKM > 10 in all data sets. All data are available at the GEO database (accession no. GSE77556). Reverse transcriptase qScript cDNA synthesis kit from Quanta Biosciences-generated cDNA was used for PCR amplification (PerfeCTa qPCR FastMix UNG from Quanta Biosciences) and quantification of rv0560c, rv0559c, rv0558, rv0557, and rv2887 expression levels with sigA expression as the normalization control for cDNA input. Primer and probe sequences are listed in SI Appendix, Table S2.

EMS Assay. Purified Rv2887 (wild type or R81Q) was incubated with 50 nM of a 70-mer spanning the promoter region of rv0560c (wild type or mutant; SI Appendix, Table S2) or 36-mer spanning mbtB promoter in binding buffer (pH 8) with 20 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 0.25 mg/mL BSA, and 10% glycerol for 10 min at room temperature. The samples were run in a 12% nondenaturing Tris-borate-EDTA (TBE) buffered gel for 1.75 h at 110V in TBE buffer. All lanes were stained with 1:10,000 SYBR Gold nucleic acid stain (Life Technologies) in 1X TBE buffer for 10 min at room temperature. Before loading the samples, the gel was prerun at 100 V for 30 min on ice. DNA bands were visualized with UV light using the Genius Bioimaging system (Syngene), and images were captured. ImageJ was used to quantify the intensity of the DNA bands (area under the peaks formed by bands). Values were normalized to control samples without Rv2887 protein and plotted against the protein/DNA ratio.

Methylation of 14 by rv0560c. Initially, methylation of 14 by wild-type Rv0560c and S140A-Rv0560c was monitored using a coupled spectrophotometric assay as described (27). However, because of overlap in the absorption spectra of 14, the initial velocities could not be measured at 260 nm. Instead, the reaction was monitored at 380 nm due to the blue shift in the absorption spectrum of 14 upon methylation. Upon confirmation of a dependence of the monitored initial velocity on the enzyme concentration, steady-state kinetic analysis of wild-type Rv0560c and S140A-Rv0560c was performed in a 96-well plate format using a SpectraMax M2 microplate reader. For SAM dependence, 25 µM 14, 25 nM MTAN, and 2 nM adenine deaminase were allowed to equilibrate at 37 °C in 10 mM phosphate buffer saline (2.7 mM NaCl, 100 mM NaClI, 100 µM Tween-20, 2 µM FAD, and 4 µM BSA) was added, and the reactions were incubated for 6.5 h at 25 °C to allow covalent modification of substrates. Substrate mix (5 µL) containing 1 mM FPR and 50 µM resazurin in assay buffer was then added (both are final assay concentrations). The DprE1 reactions were initiated immediately by adding enzyme mix (5 µL) containing 50 nM DprE1 in assay buffer and monitored spectrophotometrically using a Tecan Safire2 instrument (Tecan Group Ltd.). The DprE1 enzyme assay and data analysis have been described in detail (29).

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