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 (Mtb) 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 Mtb. Here, we isolated multiple Mtb 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 S-adenosyl-L-methionine-dependent methyltransferase that N-methylates 14, abolishing its mycobactericidal activity. An Mtb 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 nonomolar 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 Mtb, is a previously unreported mechanism of AMR.

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.
Earlier work showed that 14 of 14 when glycerol, dextrose, or acetate served as the carbon source. 12.5 T122C L41P Rv0516c:L69M, plcB:R190R, rv2327 α 10 of 14 and Warrier et al. of 14, of RIF MarR and its homologs > 0.39 G — rv0737 Escherichia coli Mutation in Rv2887 Other SNPs G in 16 ad— 1 rv1404 12.5 G242A R81Q Rv0516c:L69M, plcB:R190R, 25 G62A R21Q Rv1619:Y457H, rv0880 12.5 T398C L133P None in DprE1 1 12.5 G119T C40F None in DprE1 1 12.5 G170A R57Q None in DprE1 1 12.5 G232C A78P None in DprE1 1 12.5 Insertion of 14 nts after G125 leading to stop codon after AA45 Stop codon after 45AA None in DprE1 1 12.5 A191C Q64P None in DprE1 1 12.5 C274T P92S None in DprE1 0.5 12.5 C274T P92S None in DprE1 0.5 12.5 C274T P92S None in DprE1 0.5 12.5 C274T P92S None in DprE1 0.5 12.5 C274T P92S None in DprE1 0.5 12.5 C274T P92S None in DprE1 0.5

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 × 10⁻⁷ (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 factor. Amino acid sequence analysis predicted that 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).

We used Phyre 2 (15) to build a homology model of Rv2887 that predicted an 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 α₂–α₃ helices and C-terminal α₅–α₆ helices of a MarR monomer interact with the corresponding regions 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-α-D-ribose 2-oxidase (DprE1), an essential enzyme involved in arabinogalactan synthesis.

Table 1. Characterization of Mtb clones resistant to 14

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<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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<td>—</td>
<td>—</td>
<td>—</td>
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<td>R81Q</td>
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<td>&gt;25</td>
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*The names of strains analyzed by whole genome resequencing are in bold. RIF, rifampin.
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 ß-loop-ß sheet domain, is predicted to bind the promoter region of the *marR*IB 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 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 5A), by oligonucleotide-mediated recombinering (22), conferred resistance specifically to 14 (Fig. 2C and SI Appendix, Fig. S2C). However, introduction of the *rv2887* 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 *rv2887* was overexpressed in the resistant clones 1A and 8A. Overexpression of Rv2887 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 and 17 down-regulated genes, relative to the wild-type strain (SI Appendix, Table S1). The gene with the largest increase in expression—400-fold—was *rv0560c*, annotated as a putative, nonessential benzoquinone methyltransferase. The gene *rv0559c*, which is predicted to be in the same operon as *rv0560c*, was up-regulated by ~15-fold in vehicle-treated resistant clones. Two genes transcribed in the opposite direction from the *rv0560c* operon, *rv0557* and *rv0558*, were also up-regulated in the resistant clones, by ~threefold and ~20-fold, respectively. Unlike *E. coli* marR, which is self-regulated, *rv2887* expression was not altered in the resistant clones. The down-regulated genes included the tRNA and noncoding RNA transcripts *asnT*, *argL*, and *mpr11* (SI Appendix, Table S1), but no protein-coding transcripts.

The RNAseq results thus directed our attention to the cluster of four protein-coding genes up-regulated in the *rv2887* mutants, *rv0557*–*rv0560c*. Quantitative reverse transcriptase (qRT) PCR (Fig. 2B) confirmed that *rv0560c*, *rv0559c*, and *rv0558* were significantly up-regulated (>fivefold) in the resistant clones, whereas *rv0557* was not. Finally, *rv0560c* 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 *rv0559c* and *rv0558*. Based on these data, we predicted a model in which Rv2887 acts as a repressor of *rv0560c* and loss-of-function mutations in Rv2887 lead to derepression and thus increased expression of *rv0560c*.

**Rv2887 Binds to the Promoter Region of *rv0560c*.** Previous characterization of the promoter region of *rv0560c* 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 promoter region of the *rv0560c* (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 50% 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, *mfbB* (mfbB-prom) (SI Appendix, Fig. S4) (24). 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 rrv0560c. 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 rrv0560c, 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 rrv0560c was overexpressed, whereas overexpression of rv0558 and rv0559c had no impact on sensitivity to 14 (Fig. 3A 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 most resistant mutant was clone 8A, which carried mutations in both rrv0560c and rrv0562c, 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 coinubation of 14 with S-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 kcat/Rrv0560c was increased by 2.4-fold for S140A-Rv0560c (Fig. 3B). Enhancement was even greater when S-adenosyl-L-homocysteine (SAH) was depleted by two coupled enzymes, 5'-methylthioadenosine/S-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 S140A is predicted to be near to a putative SAM binding site, the increased methyltransferase activity of S140A-Rv0560c relative to wild-type Rv0560c may be attributed to increased kcat/Km, 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 methylated 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 (~20x 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-arabinose (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 (Fig. 4B). Preincubation of 14 with Rv0560c in the presence of SAM led to a 36-fold increase in 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), G17C (32), E221Q (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 benzoxazine 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. Wingler 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, deacetylation, and hydrolysis (34). Previous studies have shown that some proportion of intrabacterial para-aminosalicylic 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, rRNA, 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 salicylate, membrane depolarizers (carbonyl cyanide m-chlorophenyl hydrazone, valinomycin, or dinitrophenol), the detergent SDS, and the respiratory inhibitors chlorpromazine and thiordizane, can up-regulate rv0560c expression (www.tbdb.org/expressionHistory.shtml?gn=Rv0560c). We hypothesize that this occurs by the dissociation of Rv2887, the repressor, from the rv0560c promoter for this reason. This theory is corroborated by the very low number of rv0560c transcripts in untreated, wild-type Mtb (SI Appendix, Table S1), compared with the marked up-regulation of rv0560c in Mtb strains carrying mutations in Rv2887 (Fig. 2B) and demonstration of a direct interaction of Rv2887 with the promoter region of rv0560c in a mobility shift assay (Fig. 2C). Although PAS, chlorpromazine, and thiordizane have been reported to up-regulate rv0560c gene expression, we did not observe increased resistance to these compounds in the clones 1A and 8A, which have markedly higher rv0560c 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 Rv1404, have been shown to respond to xenobiotics in a phylogenetically relevant manner. 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 methyltransferases, 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 bactericidal 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% tylooxol, 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 rv0560c (Δrv0560c) 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, L368, 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#STK717481). Compound 14-IA 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 reagents 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 Resazarin Microtiter Assay was used to measure IC50 of compounds against strains carrying the Y314H, E221Q, G248S, and C387S, L368P, and G17C 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 tylooxol (0.02%), and spread on 7H11 agar plates. Plates were incubated at 37 °C for 21–25 d, and colonies were counted.

Generation of Constructs for Constitutive Expression of Genes in Mtb and for Expression and Purification Proteins from E. coli. Details of the cloning strategy and the primers used for cloning are included in SI Appendix.

Isolation of Mtb Clones Resistant to 14 and Whole Genome Sequencing. Log phase culture of Mtb H37Rv, LP, or Δrv0560c 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 lysozyme 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 rv2887 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 Resistance to 14 into Wild-Type Mtb. From 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 kit (LS protocol; 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 samples. 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, rvS57, 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.

EMSA 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% non-denaturing Tris-borate-EDTA (TBE) buffer gelled for 1.75 h at 110V in a TBE gel. The gels were stained with a 0.5% (w/v) SYBR Gold nucleic acid stain (Life Technologies) in 1:10 TBE buffer on ice. The gels 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 μM MTAN, and 2 nM adenine deaminase were allowed to equilibrate at 37 °C in 10 mM phosphate buffer saline (2.7 mM KCl, 138 mM NaCl) with 10 mM MgCl2, pH 7.4. The reaction was initiated by adding 670 nM wild-type enzyme or 600 nM S140A-Rv0560c. For substrate dependence, 96 μM SAM was used in the reaction mixture. The initial velocity was converted to units of concentration using an extinction coefficient (ε380nm of (13,200 M−1 cm−1) for 14. The initial velocities were calculated and plotted using Prism. The kinetic parameters were also tested in the absence of the coupling enzymes using the same setup.

NMR-Based Determination of Methylation Site in 14. The methylation reaction was set up on a 15-μL scale with 25 μM 14, 96.5 μM SAM, 0.6 μM S140A-Rv0560c, 25 nM MTAN, and 2 nM adenine deaminase at 37 °C for about 15 h. The formation of the product was confirmed using LC-MS (363 m/z [M+H]+). The methylated product was extracted using chloroform and purified using reverse phase column chromatography (Kb-Prep C18, 5 μm) with a 5–95% gradient of acetonitrile/water with 0.1% TFA (flow rate 10 μL/min; retention time 12.2 min). The product was lyophilized and dissolved in 500 μL CDCl3 for identifying the site of methylation using NMR spectroscopy. All NMR spectra were acquired on a 600-MHz Bruker AV III. A 1H-13C COSY (ns = 8) experiment was used to assign the 1H spectra of 14 and 14-A, with literature mining to confirm assignment of C-6 and C-9 protons of 14 and 14-A. The gradient NOESY experiments for both 14 and 14-A were performed with a mixing time of 0.8 s (ns = 32).

Quantification of Intracellular Amounts of 14 and 14E. Rv37H wild-type strain (Rv), Rv strain constitutively expressing rv0560c, resistant clones, 1A and 1B, H37Rv London Pride (LP), and ∆rv0560c strains were grown to log phase and inoculated on filters at a density of 3.5–5 × 109 bacteria per milliliter. The filters were transferred onto 7H10 agar plates and incubated at 37 °C for 5–7 d to allow bacterial growth. The filters were then exposed to 7.8 μM of 14 in 7H9 medium (without tyloxapol) for 24 h, and the metabolites were extracted with a mixture of acetonitrile:methanol:water (40:40:20) as described (46). The extracellular medium was also collected for analysis. LC-MS (46) was used to detect 14 (m/z of 349.16 in the positive mode) and me-14 (m/z of 363.18 in the positive mode) in the lysates and extracellular medium. BCA assay (Thermo Scientific) was used to measure protein amounts in the samples to normalize to sample biomass.

Enzyme Assay for DprE1. Compounds were dispensed in a black 384-well low-volume microplate (Greiner Bio-One) using a Hewlett Packard HP 3D30 digital dispenser (Tecan Group Ltd.). Precolumn mix (5 μL) containing 10 μM Rv0560c and 300 μM SAM in assay buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 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 compounds. 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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