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Article

Mycolic Acid Cyclopropanation is Essential for Viability, Drug Resistance, and Cell Wall Integrity of *Mycobacterium tuberculosis*

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SUMMARY

Mycobacterium tuberculosis **infection remains a major global health problem complicated by escalating rates of antibiotic resistance. Despite the established role of mycolic acid cyclopropane modification in pathogenesis, the feasibility of targeting this enzyme family for antibiotic development is unknown. We show through genetics and chemical biology that mycolic acid methyltransferases are essential for *M. tuberculosis* viability, cell wall structure, and intrinsic resistance to antibiotics. The tool compound dioctylamine, which we show acts as a substrate mimic, directly inhibits the function of multiple mycolic acid methyltransferases, resulting in loss of cyclopropanation, cell death, loss of acid fastness, and synergistic killing with isoniazid and ciprofloxacin. These results demonstrate that mycolic acid methyltransferases are a promising antibiotic target and that a family of virulence factors can be chemically inhibited with effects not anticipated from studies of each individual enzyme.**

INTRODUCTION

Human infection with *Mycobacterium tuberculosis* continues to cause unrelenting suffering. Although infection with *M. tuberculosis* is a curable with prolonged multidrug antibiotic therapy, the drug regimens are often toxic or difficult to complete. In the developing world, curative therapy for *M. tuberculosis* is difficult to execute, leading to two million deaths per year worldwide (Dye, 2006). Infection with antibiotic-resistant *M. tuberculosis* is an increasing problem and requires more prolonged antibiotic therapy for cure (World Health Organization, 2008). In the case of extensively drug-resistant tuberculosis, therapy is often impossible (Gandhi et al., 2006). This dire reality has prompted a significant worldwide effort to discover new drugs to treat *M. tuberculosis* infection. Although any new agent active against *M. tuberculosis* would be welcome, of particular interest are new drugs that would allow shortening of tuberculosis chemotherapy through rational targeting of gene products important for persistent infection. An increasing number of potential drug targets in *M. tuberculosis* are cell wall biosynthetic enzymes, including enzymes involved in mycolic acid biosynthesis and modification.

Mycolic acids are α-alkyl, β-hydroxy fatty acids that are produced by all mycobacteria and are the signature lipid of the hydrophobic mycobacterial cell wall. Mycolic acid biosynthesis has been intensely studied due to the unique structure of these lipids and their importance for tuberculosis antibiotic therapy and *M. tuberculosis* pathogenesis (Barry et al., 1998). *M. tuberculosis* and *Mycobacterium bovis* produce three major mycolic acid types: alpha mycolate, methoxymycolate, and ketomycolate. The chemical structures of these lipids shown in Figure 1 were determined over years of exhaustive study (Barry et al., 1998; Watanabe et al., 2002, 2001). Although the core mycolate structure is conserved among mycobacteria, only pathogenic slow growing mycobacteria produce significant amounts of cyclopropanated mycolic acids. Alpha mycolic acids contain two *cis* cyclopropane rings on the meromycolate chain. Oxygenated mycolates contain either a distal methoxy or ketone group and a proximal *cis* or *trans* cyclopropane ring. The cyclopropane rings and methyl branches of these lipids are synthesized by a family of S-adenosyl methionine-dependent methyltransferases. The enzymes of this family are highly homologous both in primary sequence and tertiary structure (Huang et al., 2002). Despite this structural similarity, genetic deletion of each methyltransferase in *M. tuberculosis* has revealed highly specific biosynthetic roles of each enzyme. PcaA and MmaA2 are required for alpha mycolate cyclopropanation (Glickman, 2003; Glickman et al., 2000), CmaA2 for *trans* cyclopropanation of the oxygenated mycolates (Glickman et al., 2001), MmaA4 and MmaA3 for distal functionality of the oxygenated mycolates (Behr et al., 2000; Dubnau et al., 2000), and MmaA1 for methyl branch formation preceding the cyclopropanation step by CmaA2 (M.S.G., unpublished data).

M. tuberculosis strains deficient for cyclopropanation have revealed an important role for this lipid modification in pathogenesis. Loss of *pcaA* in *M. tuberculosis* causes an early growth defect in the lungs, defective persistence during late infection, and failure to activate macrophage innate immune responses (Glickman et al., 2000; Rao et al., 2005). In contrast, loss of *cmaA2* results in hypervirulence and hyperinflammatory innate immune activation in macrophages (Rao et al., 2006) and loss of *mmaA4* causes excessive IL-12 production (Dao et al., 2008).

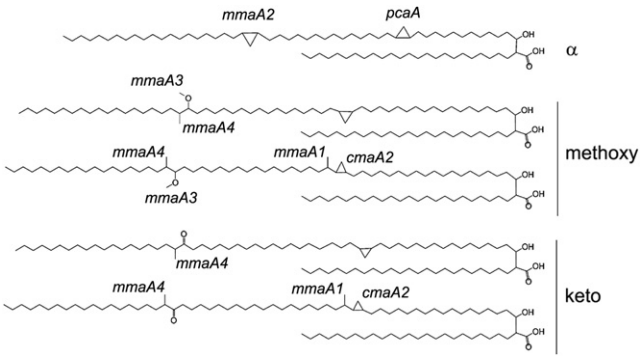

Figure 1. Chemical Structures of the Major Mycolic Acids of *M. tuberculosis* and BCG-R

Cyclopropane rings and methyl branches are shown and annotated with the methyltransferase responsible for their synthesis. BCG-P lacks methoxymycolates due to a mutation in *MmaA3*.

These phenotypes are due to altered inflammatory activity of cyclopropane-deficient trehalose dimycolate, implicating cyclopropanation as an immunomodulatory lipid modification (Dao et al., 2008; Rao et al., 2005, 2006). However, these results do not clearly define mycolic acid methyltransferases as an attractive drug target in *M. tuberculosis* because the consequences of complete cyclopropane loss are unknown and targeting of CmaA2 alone in preference to other methyltransferases might be deleterious to the host.

In this study we address the suitability of mycolic acid methyltransferases as an *M. tuberculosis* drug target. We show that the mycolic acid methyltransferase enzyme family can be targeted by a single tool compound and that this inhibition has pleiotropic effects on *M. tuberculosis* cells, including loss of cell wall integrity and eventual cell death.

RESULTS

Deletion of *cmaA2* from BCG Pasteur Is Only Possible after Complementation with a Functional *mmaA3*

Previous work from our laboratory and others has shown that mycolic acid methyltransferases are individually nonessential for growth in vitro (Behr et al., 2000; Dubnau et al., 2000, 1998; Glickman, 2003; Glickman et al., 2001, 2000), including the *trans* cyclopropane synthase of the oxygenated mycolates CmaA2 (Glickman et al., 2001). As we have shown that deletion of *cmaA2* from *M. tuberculosis* produces a hypervirulent strain (Rao et al., 2006), we attempted to delete this gene from BCG Pasteur (BCG-P) to study its effect on BCG immunogenicity. To delete *cmaA2*, we infected BCG-P with a temperature-sensitive specialized transducing phage designed to replace the entire *cmaA2* coding sequence with a hygromycin resistance gene. To our surprise, despite our prior deletion of *cmaA2* from *M. tuberculosis* using the same technique, we were unable to obtain hygromycin-resistant transductants on multiple attempts (data not shown). We have successfully deleted multiple genes from BCG-P using specialized transduction (data not shown; Glickman et al., 2000; Makinoshima and Glickman, 2005), indicating that our failure to obtain a *cmaA2* knockout was not due to a general failure of this technique in this strain. BCG-P, along with many other BCG substrains, lacks methoxymycolates due to a point mutation in *mmaA3*, the gene encoding the methyltransferase that adds the methoxy group to methoxymycolates (Behr et al., 2000). BCG Russia (BCG-R) has a functional MmaA3 and therefore synthesizes methoxymycolates. We were able to delete *cmaA2* from BCG-R using the same specialized transducing phage that failed in BCG-P (Figure 2A), indicating that lack of methoxymycolates may be the factor preventing *cmaA2* deletion in BCG-P. To test this idea, we complemented BCG-P at the chromosomal *attB* site with the *mmaA3* gene (BCG-P *attB::mmaA3*). As previously

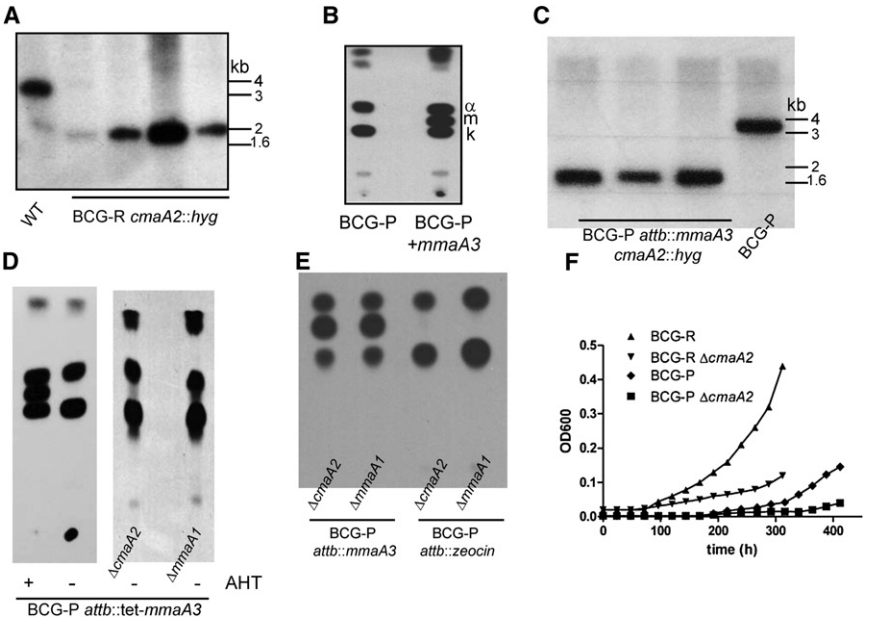

Figure 2. Genetic Analysis of Mycolic Acid Methyltransferase Synthetic Phenotypes

(A) Southern blot showing the deletion of *cmaA2* from BCG-R. Genomic DNA was cut with EcoRI and probed with a DNA fragment flanking *cmaA2*. The predicted size for wild-type is 3.8 kb and for Δ*cmaA2* is 1.8 kb.

(B) Radio TLC from BCG-P and BCG-P *attB*::*mmaA3*, showing the appearance of methoxymycolates in the complemented strain. α, alpha mycolates; m, methoxymycolates; k, ketomycolates.

(C) Southern blot showing deletion of *cmaA2* from BCG-P *attB*::*mmaA3*. Fragment sizes are as in (A).

(D) BCG-P was complemented at the *attB* site with *mmaA3* gene under an AHT-sensitive promoter and mycolic acids were analyzed from strains grown with (+AHT) and without (−AHT) inducer. After the deletion of *cmaA2* or *mmaA1* from this strain (while supplementing with AHT), AHT was withdrawn and mycolic acids were prepared.

(E) Radio TLC showing the removal of *mmaA3* by zeocin marker exchange (see text for details). Mycolates from parent strains and zeocin-resistant transformants were analyzed by TLC.

(F) Growth curve (represented as OD_{600} measurements) at 32.5°C of BCG-P (◆), BCG-P Δ*cmaA2* (MGM1919; ■), BCG-R (▲), and BCG-R Δ*cmaA2* (MGM295; ▼).

reported (Behr et al., 2000; Belley et al., 2004; Dubnau et al., 1998), BCG-P *attB::mmaA3* synthesized methoxymycolates (Figure 2B). Transduction of BCG-P *attB*::*mmaA3* with the *cmaA2* knockout phage resulted in successful deletion of *cmaA2* (Figure 2C). To confirm that the function of *cmaA2* in BCG is to synthesize *trans* cyclopropane rings, as previously shown in *M. tuberculosis* (Glickman et al., 2001), we isolated total mycolic acids from the BCG *attB*::*mmaA3* *cmaA2*::hyg strain and analyzed them by nuclear magnetic resonance (NMR). *Trans* cyclopropane rings were not detected in the Δ*cmaA2* strain, confirming that the function of CmaA2 is identical in BCG and *M. tuberculosis* (see Figure S1A available online). This data suggested that null mutations in *mmaA3* and *cmaA2* may be synthetically lethal for slow growing mycobacteria.

To further confirm synthetic lethality, we created an *mmaA3* depletion strain that expressed *mmaA3* from a tetracycline-inducible promoter. This strain displayed anhydrotetracycline (AHT)-dependent synthesis of methoxymycolates (Figure 2D, left). Using specialized transduction, *cmaA2* and *mmaA1* were deleted from this strain in the presence of AHT. Southern blotting confirmed successful deletion of *cmaA2* and *mmaA1* (data not shown) and NMR of total mycolic acids from these strains confirmed lack of *trans* cyclopropane rings (Figures S1B–S1C) To our surprise, depletion of *mmaA3* by AHT withdrawal did not result in cell death (data not shown). Examination of these strains revealed minimal methoxymycolate production without AHT (Figure 2D, right), ruling out constitutive expression of *mmaA3* due to a mutation in the tetracycline repressor. This result could indicate that *mmaA3*/*mmaA1* and *mmaA3*/*cmaA2* are not synthetically lethal. However, the tetracycline regulation is somewhat leaky, which could allow survival due to the low level methoxymycolate production that is visible in Figure 2D. To completely remove the *mmaA3* cassette from the Δ*mmaA1* and Δ*cmaA2* strains, we used marker exchange (Pashley and Parish, 2003). A zeocin marked vector replaced the *mmaA3* cassette after transformation and the resulting zeocin-resistant transformants lacked methoxymycolates (Figure 2E), indicating that sequential deletion of *cmaA2*/*mmaA3* or *mmaA1*/*mmaA3* is possible. Taken together, one interpretation of these genetic studies is that compensatory changes in membrane fluidity in the tetracycline depletion and zeocin strains allowed isolation of the double mutants, possibly due to the order in which the genes were deleted. To test whether the *cmaA2*/*mmaA3* double mutants have alterations in membrane fluidity that would support this model, we grew these strains in low temperature. We found that deletion of *cmaA2* from BCG-R moderately impaired growth at 32° (Figure 2F). Strikingly, BCG-P grew poorly at 32° and inactivation of *cmaA2* in BCG-P abolished growth (Figure 2F). These findings support the genetic data indicating that loss of *cmaA2*/*mmaA3* impairs viability due to alteration in membrane fluidity.

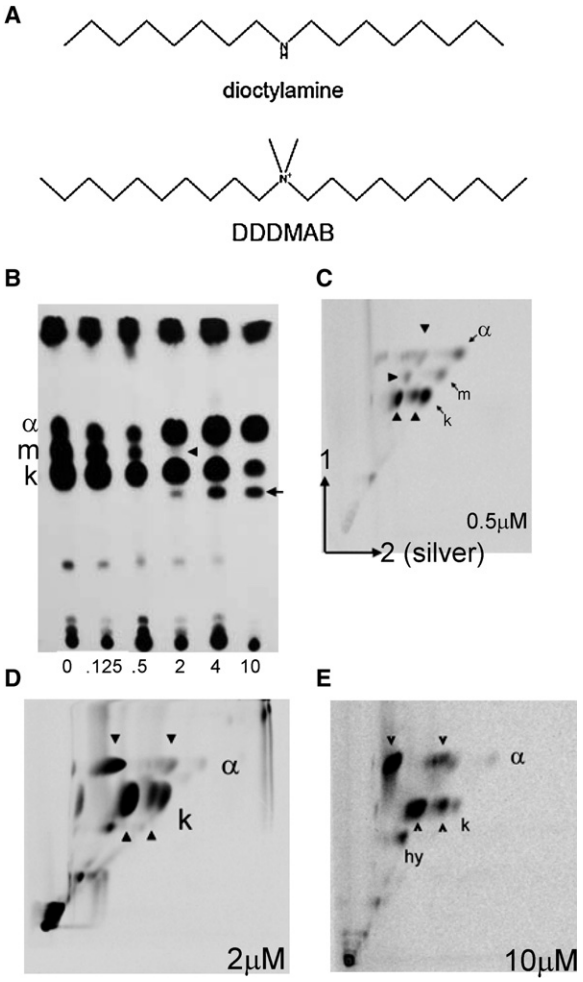

Figure 3. Dioctylamine Inhibits Multiple Pathways of Mycolic Acid Modification

(A) Chemical structures of dioctylamine and DDDMAB. DDDMAB was previously identified in the active site of crystals of CmaA2.

(B) One-dimensional TLC of mycolates isolated from BCG-R treated with the concentration of dioctylamine (μM) indicated under each lane. Alpha mycolate (α), methoxymycolate (m), and ketomycolate (k) are indicated at the left edge. The arrowhead marks the position of methoxymycolate, which is absent from the 2 μM sample. The arrow marks hydroxymycolate.

(C) Two-dimensional Argentation TLC of BCG-R treated with 0.5 μM dioctylamine. The sample was separated without silver (dimension 1) and then with silver (dimension 2). The silver dimension retards lipids on the basis of unsaturation. Two unsaturated derivatives of alpha mycolate are visible, as well as unsaturated ketomycolates and methoxymycolates (arrowhead).

(D and E) Two-dimensional Argentation TLC of BCG-R treated with 2 μM (D) and 10 μM (E) dioctylamine. Unsaturated derivatives of the alpha mycolates and ketomycolates are marked with arrowheads, as are the positions of mature alpha mycolate (α), ketomycolate (k), and hydroxymycolate (hy).

Dioctylamine Inhibits Multiple Pathways of Mycolic Acid Modification

To investigate the phenotypic consequences caused by loss of mycolic acid modification, we sought a chemical inhibitor of these enzymes. Dioctylamine was recently identified as an inhibitor of *Escherichia coli* CFAS, an enzyme that cyclopropanates the membrane fatty acids of *E. coli* (Grogan and Cronan, 1997; Guianvarc'h et al., 2006). The IC_{50} of dioctylamine for CFAS was 4 μM. Dioctylamine is chemically similar to didecyldimethylammonium bromide (DDDMAB), which we have previously crystallized in the active site of CmaA2 (Huang et al., 2002) (see Figure 3A for structures), suggesting that dioctylamine might also inhibit mycolic acid methyltransferases by acting as a substrate mimic. To test whether dioctylamine is an inhibitor of cyclopropanation, we grew BCG-R (which has a mycolic acid

profile highly similar to that of *M. tuberculosis*) with ^{14}C acetic acid and in escalating concentrations of dioctylamine ranging from 0.125 to 10 μM or vehicle control. After 6 hr, ^{14}C-labeled mycolic acid methyl esters were prepared and analyzed by radio thin layer chromatography (TLC). We observed that 2 μM dioctylamine completely inhibited methoxymycolate production, consistent with loss of MmaA3 function (see arrowhead in Figure 3B). In addition, treatment with 4 and 10 μM dioctylamine caused accumulation of a mycolic acid migrating slower than ketomycolate (see arrow in Figure 3B), consistent with the previously reported hydroxymycolate that accumulates in the absence of functional MmaA3 (Quemard et al., 1997). This polar lipid migrated slower than authentic epoxymycolate from *Mycobacterium smegmatis* (data not shown), confirming its likely identity as hydroxymycolate. At 10 μM, there is substantial but incomplete inhibition of ketomycolate biosynthesis (compare 0.125 to 10 μM in Figure 3B). The observed mycolic acid profiles with dioctylamine treatment are consistent with loss of MmaA3 and MmaA4 function, suggesting that dioctylamine inhibits these enzymes.

Whereas inhibition of MmaA3 and MmaA4 leads to loss of an entire mycolate class and is therefore easily detected on one-dimensional TLC, chemical inhibition of other cyclopropane synthases produces unsaturated lipids that are identical in polarity to their parent lipids and therefore not detectable on one-dimensional TLC. This phenotype was previously demonstrated in *M. tuberculosis* strains lacking the mycolic acid cyclopropane synthases PcaA, MmaA2, and CmaA2 (Glickman, 2003; Glickman et al., 2001, 2000). To test the inhibition of these enzymes by dioctylamine, we performed two-dimensional argentation TLC on mycolic acids isolated from dioctylamine-treated BCG-R. In this technique, lipids are first separated by polarity and then by degree of any type of unsaturation in the second (silver) dimension. After treatment of BCG-R with 0.5 μM dioctylamine, we observed two unsaturated derivatives of the alpha mycolate, consistent with inhibition of alpha mycolate cyclopropanation (Figure 3C, arrowhead to the left of mature alpha). We also observed unsaturated derivatives of methoxymycolates and ketomycolates, consistent with impaired cyclopropanation of the proximal position of these lipids (Figure 3C). Cells treated with 2 μM dioctylamine produced no mature alpha mycolate and two species of unsaturated alpha mycolate, the more abundant of which was more fully retarded (arrowheads in Figure 3D). The ketomycolate appeared as three species, mature keto and two unsaturated species, likely *cis* and *trans* unsaturated. At 10 μM, almost no mature ketomycolate was observed (Figure 3E). These data strongly indicate that dioctylamine is a dose-dependent inhibitor of multiple mycolic acid methyltransferases. These TLC data, combined with extensive prior genetic characterization of mycolic acid modifications, indicate that dioctylamine inhibits the lipid modifications performed by MmaA4, MmaA3, PcaA, MmaA2, and CmaA2.

Mycolic Acid Methyltransferases Are Direct Targets of Dioctylamine

The data presented above strongly indicate that dioctylamine inhibits multiple pathways of mycolic acid cyclopropanation and methylation. However, this effect could be due to direct inhibition of mycolic acid methyltransferase enzymes or an indirect effect. To prove that the methyltrasferases are direct dioctylamine targets, we overexpressed hemagglutinin-tagged versions of MmaA1, MmaA3, CmaA2, PcaA, MmaA2, and MmaA4 on a multicopy episomal plasmid and tested the effect on dioctylamine sensitivity. The expression of these proteins was confirmed by western blotting in *M. smegmatis* and BCG-R (Figure S2). Overexpression of MmaA4 strongly reversed the inhibition of ketomycolate synthesis observed with dioctylamine (Figure 4A). Similarly, overexpression of CmaA2 reversed the accumulation of unsaturated ketomycolates seen with dioctylamine treatment (Figure 4B). Overexpression of MmaA2 and PcaA also reversed the accumulation of unsaturated alpha mycolate in dioctylamine-treated cells. In MmaA2-expressing cells, the predominant alpha mycolate that accumulated was fully saturated (white arrowhead in Figure 4C), whereas in PcaA-expressing cells, the alpha mycolate was mixture of monounsaturated and dicyclopropanated lipids (Figure 4C). Although diunsaturated alpha mycolate is the major lipid in dioctylamine-treated cells (black arrowhead in Figure 4C), none of this lipid was visible in the PcaA overexpressor. In contrast, although the lack of methoxymycolate in dioctylamine-treated cells is consistent with loss of MmaA3 function, overexpression of MmaA3 did not reverse this effect (Figure S3). This suggests that MmaA3 may be an indirect target of dioctylamine, possibly through other mycolic acid methyltransferases.

Dioctylamine Inhibits the Methyltransferase Activity of CmaA2 In Vitro

To examine the in vitro activity of *M. tuberculosis* mycolic acid methyltransferases and their inhibition of dioctylamine, we developed a new enzymatic assay based on a colorimetric assay that detects conversion of S-adenosylhomocysteine (SAH) to homocysteine by SAH hydrolase (Lozada-Ramirez et al., 2006). This assay is diagrammed in Figure S4. Using this assay, we screened unsaturated fatty acids, including oleic, nervonic, arachidonic, and *cis*-11,14-eicosadienoic acid as substrates of *M. tuberculosis* CmaA2. Although none of these substrates are close in structure to the authentic substrate (i.e., a long chain acyl-ACP), we were able to demonstrate cyclopropanation of double bonds and determine kinetic parameters using a Lineweaver-Burk plot (Figure S5B). Of these, Eicosadienoic acid (K_m = 16.8 μM and k_{cat} = 0.03 s^{-1}) was the best candidate to test the inhibitory effect of dioctylamine. An accurate IC_{50} was not obtainable because we had to use a relatively large amount of protein (4 μM) in order to observe the reaction progress. However, the inhibition from dioctylamine was obvious as it reduced the activity of CmaA2 in a concentration-dependent manner (Figure S5A). When 2 μM dioctylamine was added to the reaction, the rate was reduced by 50%, indicating a fairly potent inhibition against the enzyme and consistency with dioctylamine inhibition of the cyclopropane fatty acid synthase from *E. coli*. These data demonstrate that dioctylamine is a direct inhibitor of mycolic acid methyltransferases.

The Structure of CmaA2 with Bound Dioctylamine Reveals the Molecular Basis for Activity

To further demonstrate that dioctylamine is a direct inhibitor of mycolic acid methyltransfer and explore the molecular basis for its activity, we solved the structure of the CmaA2 protein in complex with the compound. The structure was solved by molecular replacement at 2.67 Å resolution (Table 1). The overall fold is largely similar to the previously reported structure of

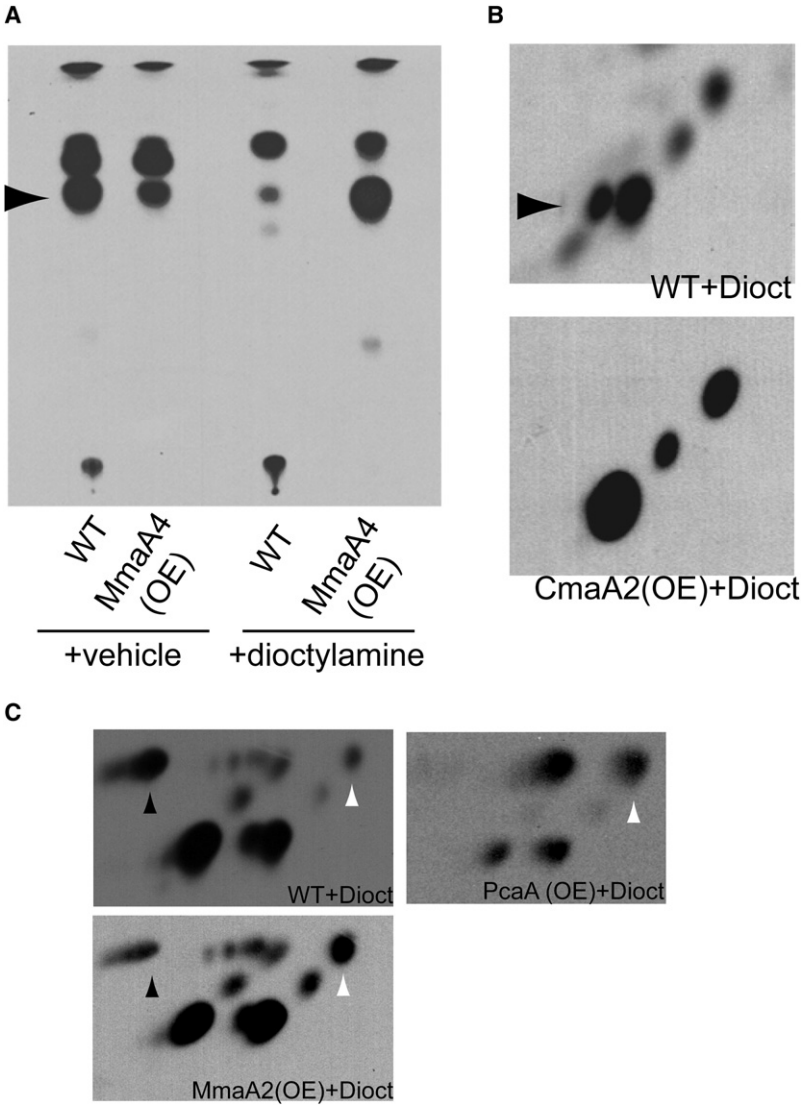

Figure 4. Mycolic Acid Methyltransferases Are Direct Targets of Dioctylamine

(A) Wild-type BCG-R (plus empty vector) or BCG-R carrying a multicopy plasmid expressing MmaA4 (MmaA4 OE) were treated with vehicle (left lanes) or dioctylamine (right lanes) and radiolabeled mycolic acids were analyzed by TLC.

(B) Wild-type BCG-R (plus empty vector) (top panel) or BCG-R carrying a multicopy plasmid expressing CmaA2 (CmaA2 OE) (bottom panel) were treated with dioctylamine and radiolabeled mycolates were analyzed by two-dimensional argentation TLC.

(C) Wild-type BCG-R (plus empty vector) (upper left) or BCG-R carrying a multicopy plasmid expressing PcaA (PcaA OE) (upper right) or MmaA2 (MmaA2 OE) (lower left) were treated with dioctylamine and radiolabeled mycolic acids were analyzed by two-dimensional argentation TLC. The black arrowhead indicates alpha mycolate with two double bonds and the white arrowhead indicates mature alpha mycolate with two cyclopropane rings.

CmaA2 complexed with DDDMAB and SAH (PDB code 1KPI) (Huang et al., 2002), showing an rmsd of 0.315 Å after superimposition (Figure 5A). Moreover, the lipophilic ligands in the two structures are similarly oriented in the binding site of the mycolic acyl substrate. Like DDDMAB, dioctylamine adopts a U-shape conformation with the nitrogen pointing to the previously revealed SAM binding pocket and the two aliphatic chains extending to the catalytic site entrance. Modeling SAM into the active site by superimposing the MmaA4-SAM structure (PDB code 2FK8) with CmaA2-SAH and dioctylamine complex, we see the nitrogen of dioctylamine is approximately 3.1 Å away from the active methyl group of SAM, indicating that the nitrogen is situated at the position of the substrate double bond to be modified. Whereas the hydrophobic binding pocket binds the aliphatic chains of DDDMAB and dioctylamine similarly, the ammonium or amine nitrogens differ substantially in their interaction with the protein (Figure 5B). For both of the species, the residues within 5 Å distance from nitrogen include Tyr24, Tyr41, Gly145, Glu148, and Tyr247. Tyr41 stabilizes the ammonium of DDDMAB through cation-π interaction. In contrast, when dioctylamine binds to the same site, its nitrogen drifts 0.5 Å away from the face of Tyr41. Gly145 tilts toward the nitrogen at the same time, with its backbone oxygen forming a van der Waals interaction with the nitrogen atom at a 3.2 Å distance. This distance is 4.0 Å in the structure in complex with DDDMAB, which is likely the result of the steric repulsion from the two methyl groups of the ammonium. Thus, this structural analysis of the dioctylamine-CmaA2 interaction predicts that amine-based inhibitors of these will be more potent than ammonium-based inhibitors.

Dioctylamine Is a Growth Inhibitor of BCG and *M. tuberculosis*

To test the hypothesis that simultaneous inhibition of multiple mycolic acid methyltransferases is lethal to mycobacteria, we grew BCG-R and *M. tuberculosis* Erdman in 7H9 media or 7H10 plates supplemented with different concentrations of dioctylamine. We found that *M. tuberculosis* growth was partially inhibited by 4 μM and completely inhibited by a 6 μM (Figure 6A). Similar results were obtained with *M. tuberculosis* grown on solid media containing dioctylamine (Figure 6B) and with BCG-R in liquid and solid media (data not shown). We did find that there was a considerable inoculum effect, and at higher inoculums the inhibitory effect was seen at higher concentrations of dioctylamine (data not shown). As the growth inhibitory concentration of dioctylamine is similar to the concentration active against mycolic acid methyltransferases, this data, coupled with the genetic data presented above, strongly suggest that mycolic acid modification may be an essential function for slow growing mycobacteria.

To further substantiate that growth inhibition effect of dioctylamine on mycobacteria is indeed related to its effect on cyclopropanation, we used another tool compound, hexadecyltrimethylammonium bromide (CTAB). This compound, which

Table 1. Data Collection and Refinement Statistics for CmaA2 in Complex with Dioctylamine

Data collection	
Space group	$I4_122$
Unit cell dimensions (Å)	a = b = 106.86 c = 224.98
Molecules/asymmetric unit	1
Wavelength (Å)	0.9795
Resolution (Å)	50–2.68
Completeness (%)	99.7 (98.1)
No. of reflections	287,172
I/σI	20.1 (1.62)
R_{sym}	0.0413 (0.76)
Refinement statistics	
Resolution (Å^2)	38.74–2.68
No. of reflection work	18,719
No. of protein atoms	2,380
No. of water molecules	41
No. of heteroatoms	21
R_{cryst} (%)	22.29
R_{free} (%)	24.30
Rmsd bond lengths (Å)	0.012
Rmsd angles (Å)	1.157
Mean temperature factor (Å^2)	61.33

has some detergent properties, was previously found to crystallize in the active site of CmaA1 and shares an ammonium head group similar to DDMABB, suggesting that it should be a weaker inhibitor than dioctylamine if growth inhibition is attributable to binding to mycolic acid methyltransferases. Consistent with this structure-activity prediction, we found CTAB to be 5-fold less active than dioctylamine in inhibiting mycobacterial growth (Figure 6C). CTAB was also approximately 5-fold less active than dioctylamine in inhibiting methoxymycolate synthesis in vivo (Figure 6D).

To further demonstrate that inhibition of growth by dioctylamine is the direct result of inhibition of multiple mycolic acid methyltransferases, we overexpressed these enzymes and tested for resistance to dioctylamine. None of the strains overexpressing a single enzyme (either MmaA1, MmaA2, MmaA3, MmaA4, CmaA2, or PcaA) were resistant to dioctylamine (data not shown), which is consistent with the nonessentiality of these gene products in prior genetic experiments. We then overexpressed simultaneously, from a single plasmid, six different enzymes of this family: MmaA1, MmaA2, MmaA3, MmaA4, CmaA2, and PcaA. The strain overexpressing these six enzymes (mgm1950) grew three times as fast as the control strain (mgm1910) in 6.5 μM dioctylamine, indicating partial resistance (Figures 6E and 6F). This data indicates that dioctylamine kills *M. tuberculosis* by inhibiting multiple methyltransferases.

Mycolic Acid Modification Is Required for Acid Fastness and Intrinsic Resistance to Antibiotics

The activity of dioctylamine against mycolic acid methyltransferases allowed us to probe the physiologic function of this unique lipid modification in a way not possible using the previously isolated single gene mutants or, as in prior studies, using overexpression of individual enzymes (Yuan et al., 1998). To test whether cyclopropanation contributes to antibiotic resistance, we tested sublethal concentrations of dioctylamine in combination with sublethal concentrations of isoniazid (INH), ciprofloxacin, and kanamycin. We found strong synergy between dioctylamine and both INH and ciprofloxacin (Figures 7A and 7B), but no synergy with kanamycin (data not shown). To show that this synergy is related to increased drug penetration in dioctylamine-treated cells, we measured the uptake of radiolabeled ciprofloxacin in bacteria treated with 4 μM dioctylamine. We found a significant increase in ciprofloxacin penetration into dioctylamine-treated cells, an effect that was most evident after 5 min, but was sustained after 40 min (Figure 7C). These results suggest that the mechanism of synergy between these two antibiotics facilitated uptake of ciprofloxacin by dioctylamine's effects on the mycobacterial membrane. A similar mechanism may explain synergy between INH and dioctylamine, although it is possible that the INH synergy is also a result of their combined effect on mycolic acid synthesis.

Acid fastness of mycobacteria, a hallmark of this genus, is thought to reflect the hydrophobicity and structure of mycolic acids in the cell wall. This idea is supported by prior findings that *M. tuberculosis* lacking *kasB* display both loss of acid fastness and attenuation (Bhatt et al., 2007). We tested the effect of 4 μM dioctylamine on the acid fastness of BCG-R and found that most of the bacteria lost their acid fastness (Figure 7D), despite only partial growth inhibition.

DISCUSSION

Cyclopropanation is a common membrane modification in bacteria and plants (Bao et al., 2002; Grogan and Cronan, 1997). In most cases, the physiologic function of cyclopropanation is poorly defined, although cyclopropanation clearly has substantial effects on membrane fluidity in model systems (McGarrity and Armstrong, 1981; Nagamachi et al., 1991). Pathogenic mycobacteria have an extensive set of cyclopropane modifications that decorate the signature lipid of the mycobacterial cell wall, the mycolic acid. Recent studies have strongly implicated mycolic acid cyclopropanation in *M. tuberculosis* pathogenesis through its effect on the immunomodulatory properties of trehalose dimycolate (Rao et al., 2005, 2006). However, these studies did not clearly establish mycolic acid methyltransferases as an attractive drug target. Here we demonstrate that chemical inhibition of multiple mycolic acid methyltransferases is lethal to mycobacteria and causes pleiotropic alterations in cell envelope structure and drug susceptibility. These studies provide strong scientific basis for targeting mycolic acid cyclopropanation as an antibiotic strategy against *M. tuberculosis*.

Our findings also reveal novel physiologic roles of cyclopropanation in mycobacteria. Although some prior studies have indicated a role for cyclopropanation in membrane fluidity and permeability (George et al., 1995; Yuan et al., 1998), the exact role of cyclopropanation in the physiology of pathogenic mycobacteria was unclear. A prior study indicated that the antitubercular thiacetazone may affect cyclopropanation, but this study concluded that the growth inhibitory effect of this drug was

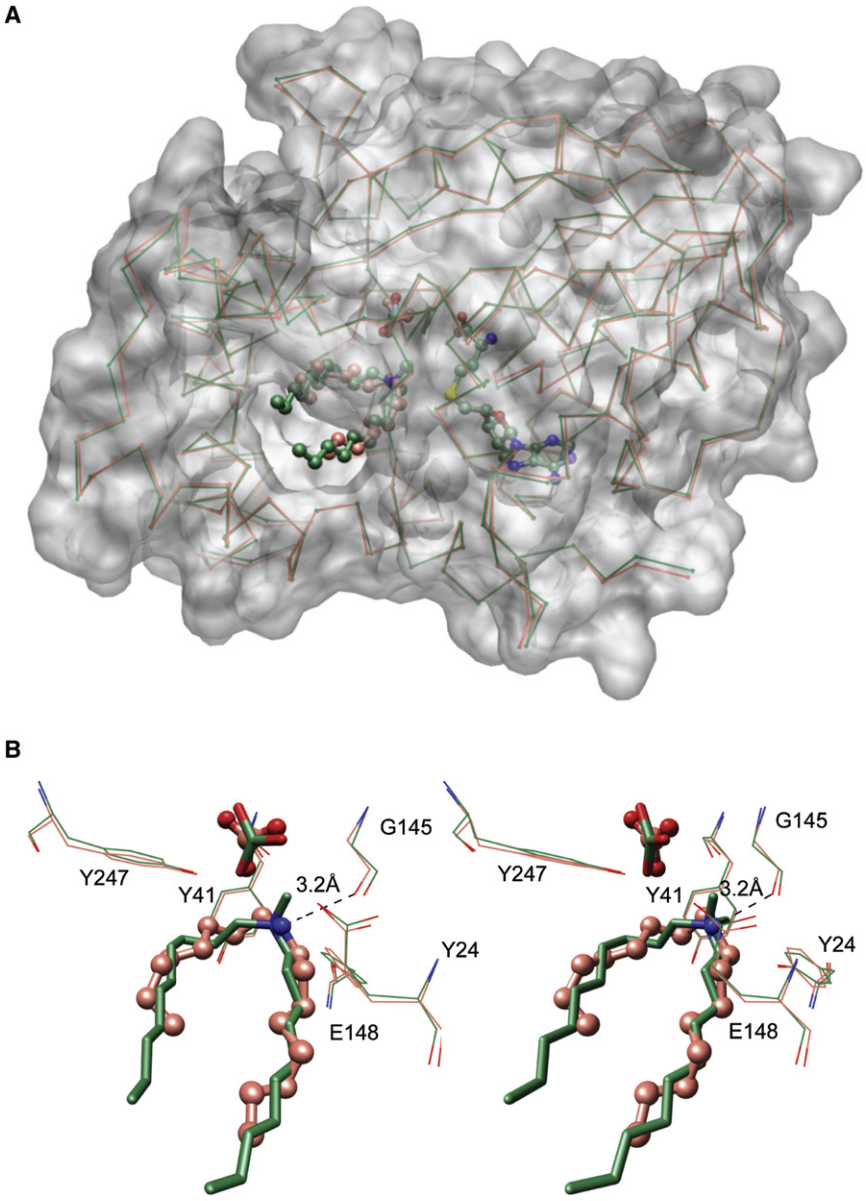

Figure 5. Structures of *M. tuberculosis* CmaA2 in Complex with Dioctylamine and DDDMAB/SAH

(A) The α-Carbon traces and ligands of the two structures are shown with dioctylamine colored pink and DDMAB/SAH colored green. The two structures show similar overall folding. All the ligands including bicarbonate are represented as ball and stick. The surface of the protein is shown with transparency. (B) Stereo diagram of the active site residues in proximity to dioctylamine or DDDMAB nitrogens. Dioctylamine and the cognate bicarbonate are represented as ball and stick, while DDDMAB and the cognate bicarbonate are represented as stick. The specific van der Waals interaction between the backbone oxygen of Gly145 and the nitrogen of dioctylamine is shown as dotted line.

unrelated to its effect on cyclopropanation (Alahari et al., 2007). This conclusion was based on the difference between the minimum inhibitory concentration of thiacetazone and the concentration required to affect cyclopropanation. In a more recent paper, the authors show that MmaA4 is required for activity of thiacetazone and that deletion of *mmaA4* confers resistance, rather than hypersensitivity, to the drug (Alahari et al., 2009). Using dioctylamine as a tool compound, we suggest that pharmacologic inhibition of this enzyme class causes loss of viability, a novel and unanticipated finding. Our finding that mycolic acid methyltransferases are essential for viability suggests an unanticipated role for mycolic acid modification in some essential cellular process. The most likely cause of this essentiality is lethal dysregulation of membrane fluidity leading to impaired protein localization or cell division. Although an off-target effect of dioctylamine causing cell death is possible, we believe this is unlikely because: (1) cell death is observed at the same concentration required to inhibit multiple methyltransferases in vivo; (2) overexpression of multiple enzymes can partially reverse the growth defect; (3) the structure of CmaA2 protein with dioctylamine both reveals that this enzyme is a direct target and that dioctylaimine is acting as a substrate mimic rather than a general SAM antagonist. This makes nonspecific inhibition of other SAM-dependent methyltransferases (such as DNA methyltransferases) with nonlipid substrates unlikely. The essential physiologic role of mycolic acid methyltransferases is an area of interest that will be the subject of future investigations.

Although dioctylamine is unlikely to be a useful antimicrobial due to host toxicity (data not shown), our data provide strong support for targeting mycolic acid methyltransferases for inhibitor development and provides a structural framework for optimizing active site binding through structure guided design. The structures of CmaA2 reveal different binding determinants for ammonium or amine-based inhibitors. The ammonium ion mimics the carbocation intermediate of the cyclopropanation reaction and is stabilized by cation-π interaction. In contrast, through mimicking an electron-donating double bond, the amine nitrogen atom of dioctylamine forms a van der Waals interaction with Gly145. This interaction will not occur in a mycolate substrate and provides a structural explanation for the successful competition of dioctylamine with the natural mycolate substrates. Moreover, this interaction may contribute more than cation-π interaction to enhancing inhibitor binding, evident from our finding that acylamine compounds are more potent than ammonium compounds (CTAB) at inhibiting cyclopropanation. This indicates that amine-based inhibitors may serve as a template for further drug development.

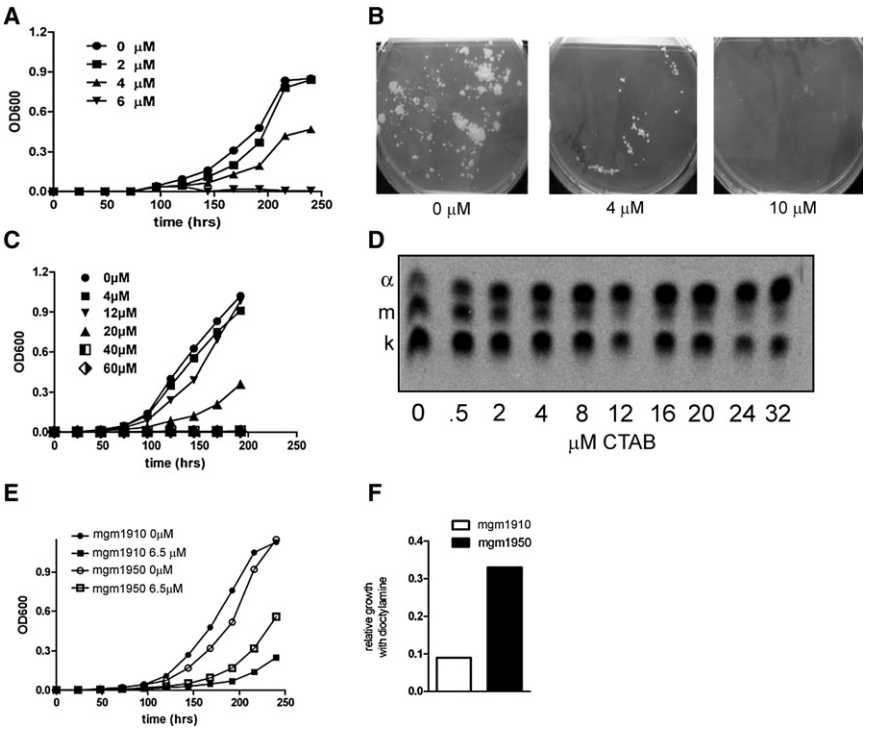

Figure 6. Dioctylamine Inhibits Growth of *M. tuberculosis*

(A) *M. tuberculosis* Erdman was grown in 7H9 media, supplemented with 0 (vehicle)(●), 2 (■), 4 (▲), and 6 μM (▼) dioctylamine. OD_{600} measurements were taken daily. A representative experiment out of five is shown.

(B) *M. tuberculosis* Erdman was grown on 7H10 plates, impregnated with 0, 4, or 10 μM dioctylamine. The plates were kept at 37°C with 5% CO_2 for 4 weeks.

(C) BCG-R was grown in the indicated concentrations of CTAB and bacterial growth was measured by OD.

(D) Radiolabeled mycolic acids from BCG-R treated with the indicated concentrations of CTAB (μM) were separated by one-dimensional TLC.

(E) BCG-R overexpressing six methyltransferases (MmaA1-4, PcaA, and CmaA2) (MGM1950) is more resistant to dioctylamine than BCG-R with vector alone (MGM1910). A representative experiment out of four is shown.

(F) The ratio of growth in the presence of dioctylamine to without dioctylamine at the 192 hr time point from the experiment in (E).

SIGNIFICANCE

The prevailing model of rational antimicrobial development envisions a one-to-one relationship between target and inhibitor (Mdluli and Spigelman, 2006; Payne et al., 2007). Inhibitors are optimized for specificity for a single protein target, rather than a group of protein targets. The disadvantage of this strategy is that mutation in a single target arises easily, generating drug resistance, as has been observed with many antimicrobials, including antimycobacterials. The findings presented here introduce a variation on the inhibitor-target relationship by demonstrating that multiple members of a highly conserved enzyme class can be simultaneously targeted by a single compound, thereby achieving physiologic effects not possible with inhibition of any single member of the enzyme group. As such, we propose that the mycolic acid methyltransferases may be an Achilles heel of the mycobacterial cell wall as their conserved protein structure but diverse biosynthetic functions allows a single inhibitor to have pleiotropic effects on the organism. In

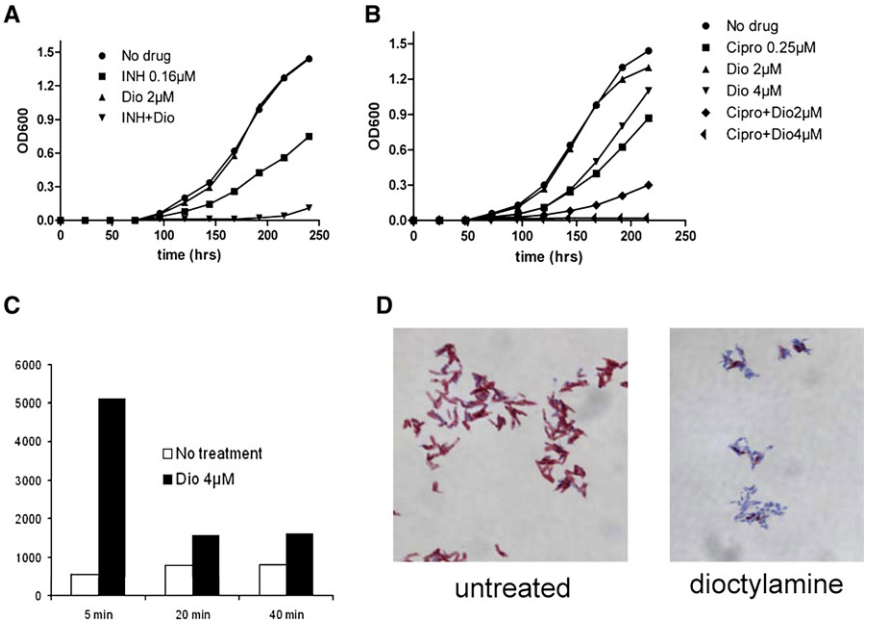

Figure 7. Mycolic Acid Methyltransferases Are Required for Drug Resistance and Acid Fastness of Mycobacteria

(A) BCG-R was treated with INH, dioctylamine (Dio), or both at the indicated concentrations and growth was measured by OD at 600 nm.

(B) BCG-R was treated with ciprofloxacin (Cipro), dioctylamine (Dio), or both at the indicated concentrations and growth was measured by OD at 600 nm.

(C) Accumulation of ^{14}C ciprofloxacin in untreated (white bar) or dioctylamine treated (4 μM; black bar) at the indicated time points after ^{14}C ciprofloxacin addition. The y axis is counts per minute.

(D) Untreated or dioctylamine (4μM)-treated cells were examined by acid fast staining using the modified Kinyoun technique.

addition, our data and that from another recent study (Arora et al., 2009) indicate that targeting multiple enzymes simultaneously is feasible and we speculate that this strategy may delay the emergence of resistance to a mycolic acid methyltransferase inhibitor as mutation of multiple enzymes would be required. Rather than seeking a clinically useful inhibitor that specifically targets one enzyme in the class, we would seek an inhibitor with broad activity against mycolic acid methyltransferases with the goal of mimicking the effects observed in this study, not least of which are cell death and the enhancement of activity of other established antimycobacterials through facilitated drug penetration.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

M. smegmatis mc^2155 was grown in 7H9 liquid media supplemented with 0.05% Tween 80, 0.5% glycerol, and 0.5% dextrose. BCG-R (kindly provided by M. Behr), BCG-P, and *M. tuberculosis* Erdman were grown in 7H9 media supplemented with 0.05% Tween 80, 0.5% glycerol, and 10% OADC (for *M. tuberculosis*) or ADS (for BCG). Growth on plates was done on 7H10 plates supplemented with glycerol and OADC or ADS at the same concentrations.

Growth in 7H9 media was monitored by optical density (OD) at 600 nM measurement. Antibiotic concentrations were 20 μg/ml for kanamycin, 50 μg/ml for hygromycin, and 12.5 μg/ml for zeocin.

Construction of Mutant Strains

Deletion of *mmaA1* and *cmaA2* was done as described previously (Glickman et al., 2001; Makinoshima and Glickman, 2005). Briefly, a temperature-sensitive mycobacteriophage (phAE87) was used to introduce a hygromycin-resistance cassette interrupting the candidate gene at 39°C. The bacteria were plated on 7H10 plates with hygromycin. The resulting colonies were analyzed by southern blotting using a probe flanking the gene of interest. Removal of *mmaA3* previously introduced via the *attB* site on the pMV306kan plasmid (marker exchange) was done be electroporating a pMV306zeo (pMV306kan with a *zeocin-R* cassette replacing the kanamycin-R) and plating the bacteria on plates supplemented with zeocin.

Mycolic Acid Preparation and Analysis

Mycobacteria were grown in 7H9 media to an OD_{600} of 0.3–0.5 in a volume of 50–200 ml. ^{14}C-labeled acetic acid was added to the media at a concentration of 1 μCi/ml, and the bacteria were allowed to grow for 6 hr (in the presence of dioctylamine) or 24 hr (without dioctylamine) more. The bacteria were harvested, and mycolic acids were extracted as previously described (Glickman et al., 2001). Single dimension separation of mycolic acid classes was done on a normal phase silica gel HPTLC plate (Analtech) and run in a 95:5 mixture of hexanes/ethyl acetate for five to six developments. Two-dimensional separation was done by immersing 90% of the TLC plate in 10% silver nitrate, activating at 130°C for 20 min, running the sample along the line with no silver nitrate (first dimension), rotating the TLC plate 90°, and running for five to six additional developments into the silver nitrate impregnated area. After completion, autoradiograms were developed using a Kodak BioMax Transcreen LE intensifying screen at −80C. NMR analysis was done as previously described (Glickman et al., 2001). Bacteria were grown to an OD_{600} of 0.8, in a volume of 400–500 ml. Bacteria were harvested and mycolic acids were prepared as before and resuspended in d-chloroform for the NMR analysis.

Dioctylamine and CTAB Preparation and Use

Dioctylamine was purchased from Sigma-Aldrich. One hundred microliters was diluted in 400 μl of DMSO and the volume was complemented with 33 ml of 100% ethanol to produce a stock solution in final concentration of 10 mM. The vehicle solution of DMSO and ethanol alone was used as control. CTAB was purchased from Sigma-Aldrich and a stock solution of 10 mM in water was prepared.

Radiolabeled Ciprofloxacin Permeability

^{14}C-ciproflixacin (15 mCi/mmol) was purchased from Moravek biochemicals. Permeability testing was done as described previously (Chevalier et al., 2000; Liu et al., 1996). Fifty milliliters total volume of BCG-R was grown with or without 4 μM dioctylamine to an OD_{600} of 0.5. The cells were concentrated and resuspended in 1 ml to 1.5×10^{10} cfu/ml and left to rotate at 8 rpm at 37°C for 45 min. Carbonyl cyanide 3-chlorophenyl hydrazone (Sigma-Aldrich) was added to a final concentration of 0.1 mM for 30 min, ^{14}C-ciprofloxacin was added to a concentration of 25 μM, and cells were left rotating at 37°C. Samples of 200 μl were removed at 5, 20, and 40 min. Each sample was washed five times with PBS, filtered through a 0.45 μm GF/C filter on a Unifilter-96 plate (Perkin-Elmer), washed again, and left to dry overnight. Radioactivity was measured by scintillation reader (Packard).

Production of *M. tuberculosis* CmaA2

Cloning, protein expression, and purification of CmaA2 were performed similarly as previously reported (Huang et al., 2002), except that a modified pET28b vector was used to incorporate TEV cleavage sequence right ahead of the N terminus of the protein. The protease TEV was consequently used to remove the His_6 tag.

Crystallization and Data Collection

2 mg mL^{-1} CmaA2 in 25 mM Tris-HCl (pH 8.0) was incubated in ice with 50 μM dioctylamine for 20 min. The protein was then concentrated to 10 mg mL^{-1} and crystallized at 18°C by hanging drop vapor diffusion. Each drop contained an equal volume of the protein solution and reservoir solution (2 M ammonium sulfate and 0.1 M CAPS [pH 9.0]). The crystal of binary CmaA2 was flash-frozen in liquid nitrogen using cryoprotectant paratone. The diffraction data were collected at beamline 19-ID at the Advanced Photon Source, Argonne National Laboratory, and then was processed and reduced using HKL2000 (Otwinowski and Minor, 1997).

Structure Determination and Model Refinement

The structure of CmaA2 was solved by molecular replacement using Molrep (Vagin and Teplyakov, 1997) in CCP4. CmaA2-SAH-DDDMAB (PDB code 1KPI) with all nonprotein molecules removed, was used as the search model. The crystal was in a space group of I_{422}. There was one protein molecule in each unit cell with dimensions of a = b = 106.86 Å, c = 224.98 Å, and $\alpha = \beta = \gamma = 90°$. A single solution for the molecular replacement was obtained. After rigid-body and restrain refinement by Refmac5 (Murshudov et al., 1997) in CCP4, the R_{cryst} and R_{free} were 24.6% and 26.8%, respectively. These values were reduced to 23.5% and 26.0% once dioctylamine and bicarbonate ion were manually built in the model by examining the $F_o - F_c$ map in XtalView (McRee, 1999). The final model containing residues 9–302 as well as 41 water molecules was obtained after further cycles of model building and PHENIX (Zwart et al., 2008) refinement yielding R factors of 22.3% and 24.3%. The full crystallization statistics are given in Table 1.

Enzymatic Assay for CmaA2

All assays were performed in the presence of a 100 mM (pH 7.5) phosphate buffer. Twenty-five micromoles of SahH, 250 μM NAD, and unsaturated fatty acid were preequilibrated for more than 5 min. After 400 μM DTNB was added, the solution was blanked and added to preincubated 4 μM CmaA2/100 μM SAM to start the reaction. To examine the inhibition by dioctylamine, the dioctylamine/DMSO solution was added together with DTNB to the reaction mixture containing 20 μM *cis*-11,14-eicosadienoic acid.

ACCESSION NUMBERS

The structure of CmaA2 in complex with dioctylamine has been deposited in Protein Data Bank with accession code 3HEM.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00114-8.

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