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Chemical remodeling of the mycomembrane with chain-truncated lipids sensitizes mycobacteria to rifampicin

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The outer mycomembrane of *Mycobacterium tuberculosis* and related pathogens is a robust permeability barrier that protects against antibiotic treatment. Here, we demonstrate that synthetic analogues of the mycomembrane biosynthetic precursor trehalose monomycolate bearing truncated lipid chains increase permeability of *Mycobacterium smegmatis* cells and sensitize them to treatment with the first-line anti-tubercular drug rifampicin. The reported strategy may be useful for enhancing entry of drugs and other molecules to mycobacterial cells, and also represents a novel way to study mycomembrane structure and function.

The genus *Mycobacterium* contains several human pathogens that exhibit extraordinary drug tolerance, including *Mycobacterium tuberculosis*, which killed 1.6 million people in 2021.[1] The innate drug tolerance of these organisms, which necessitates long-duration combination chemotherapy, originates from their distinctive and highly protective cell envelope. The mycobacterial cell envelope consists of layers of plasma membrane, peptidoglycan, arabinogalactan, and an outer mycomembrane, the latter of which is composed mainly of extremely long-chain (up to 90 carbons) branched fatty acids called mycolic acids.[2] Mycolic acids, which are esterified to carbohydrates to form the major mycomembrane components arabinogalactan–mycolate (AGM) and trehalose dimycolate (TDM), impede the penetration of antibiotics to the interior of the bacilli and thus contribute to mycobacterial drug tolerance.[3-4] Impairment of mycomembrane integrity, for example through genetic manipulation, can enhance entry of other molecules, including drugs with intracellular targets.[5-6] Thus, strategies that weaken the mycomembrane and enhance permeability across this barrier can be useful tools for improving delivery of molecular cargo to mycobacteria and can potentially be leveraged in the development of novel adjunctive therapies.

The biosynthesis of the major mycolate glycolipids AGM and TDM, which is a conserved and essential process in mycobacteria, utilizes a common biosynthetic precursor, trehalose monomycolate (TMM) (Figure 1A).[7] TMM is synthesized in the cytoplasm from trehalose and mycolic acid,

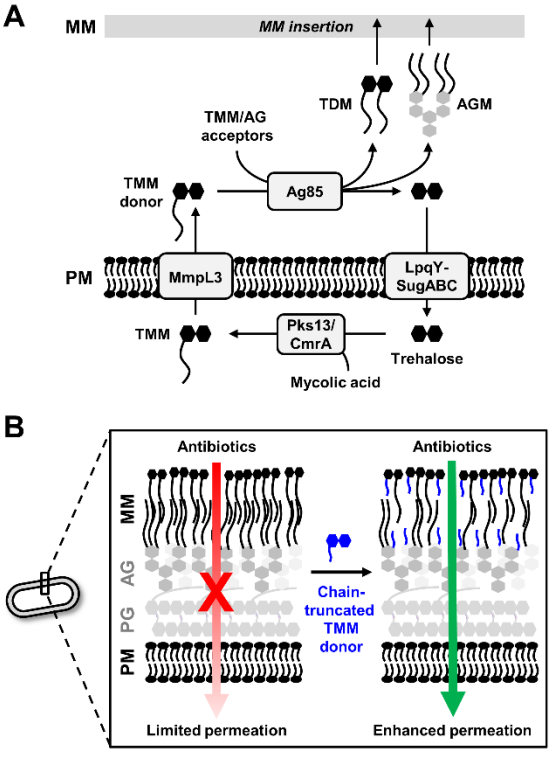

Figure 1. (A) Mycomembrane biosynthesis involves Ag85-mediated transfer of mycoloyl groups from the donor TMM onto glycan acceptor molecules to generate AGM and TDM. (B) Chain-truncated TMM analogues developed in this work are proposed to mimic TMM, installing short linear acyl chains in place of native mycoloyl groups to enhance cell envelope permeability toward antibiotics.

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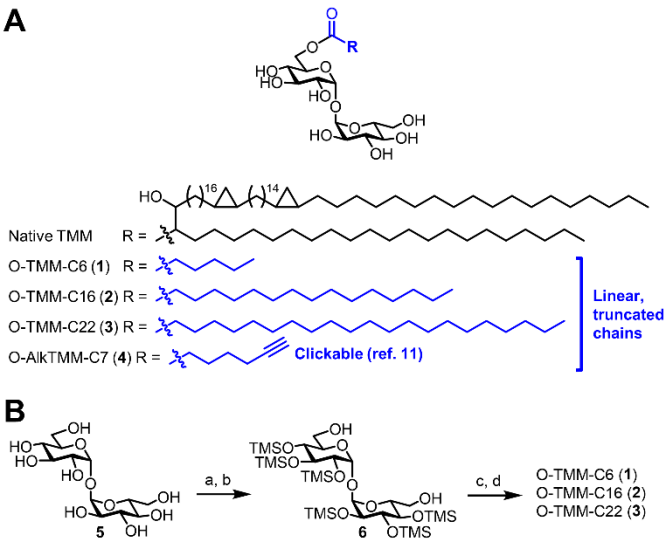
then it is translocated into the periplasm, where it serves as the universal mycoloyl donor that is used to build the mycomembrane.[8] Mycoloyltransferase enzymes (e.g., the antigen 85 complex (Ag85)) transfer the mycoloyl groups from TMM onto acceptor molecules, including arabinogalactan to form AGM, and TMM to form TDM.[9-10] Previously, our group demonstrated that, when administered to live mycobacterial cells, synthetic TMM analogues containing modified acyl chains (e.g., modified with click chemistry, fluorophore, or photocrosslinking groups) undergo mycoloyltransferase-mediated incorporation of their acyl chains into AGM and TDM, thus remodeling the mycomembrane with unnatural chemical moieties for various applications.[11-16] On the basis that this platform allows remodeling of the mycomembrane with unnatural acyl groups and there is an inverse correlation between lipid size and membrane permeability,[17] we hypothesized that TMM analogues with truncated acyl chains would replace a sufficient amount of native mycolates in mycobacterial cells to permeabilize the mycomembrane toward antibiotics (Figure 1B).

We designed and synthesized three target TMM derivatives (Scheme 1A). Native TMM has an α-branched, β-hydroxylated mycolate chain containing a total of 60–90 carbons in mycobacteria.[4] We designed TMM analogues **1**–**3**, which have acyl chains with lengths of 6, 16, and 22 carbons, and thus are linearized and severely shortened relative to native mycolates, so as to enhance permeability upon incorporation. Despite this significant simplification of the acyl chain, analysis of the literature strongly suggested that these structures would be incorporated into the mycomembrane by mycoloyltransferase activity. Published *in vitro* enzyme activity assay data show that purified Ag85 mycoloyltransferases utilize substrate analogues with very short acyl chains ranging from 4 to 16 carbons.[9, 18-19] Moreover, in our prior studies, we found that TMM analogues with alkyne-containing acyl chains ranging from 5 to 15 carbons were efficiently metabolically incorporated into the mycomembrane in live mycobacterial cells, and the labeled glycolipids could be imaged using click chemistry.[11, 13-14] As shown in Scheme 1B, we synthesized the target chain-truncated TMM analogues **1**–**3** starting from trehalose (**5**). Using an established method, we produced TMS-protected diol **6**,[20] which was esterified with the appropriate carboxylic acid using DCC/DMAP and desilylated under acidic conditions.

With TMM analogues **1**–**3** in hand, we evaluated their effect on growth using *Mycobacterium smegmatis* (*Msmeg*). *Msmeg* is an avirulent species that has the same cell envelope architecture, major mycomembrane components, and biosynthetic pathways as pathogenic mycobacteria, and is thus commonly used as a model mycobacterial organism. First, we tested whether the TMM analogues alone impacted growth. *Msmeg* was cultured in 7H9 liquid medium in the presence of 0–1,000 μM of truncated TMM analogues and optical density at 600 nm (OD_{600}) was monitored. None of the compounds exhibited a significant impact on *Msmeg* growth, whereas the positive control INH prevented growth (Figure S1, Electronic Supporting Information, ESI). Thus, whereas TMM analogues with small linear acyl groups metabolically incorporate into the



Scheme 1. (A) Structures of native TMM and chain-truncated TMM analogues (**1**–**3**). (B) Synthesis of TMM analogues **1**–**3**: (a) TMSCl, Et_3N; (b) K_2CO_3, CH_3OH; (c) carboxylic acid, DCC, DMAP, CH_2Cl_2; (d) Dowex H^+ resin, CH_3OH.

mycomembrane as noted above,[11, 13-14] they do not substantially inhibit growth on their own.

To test our hypothesis that treatment with chain-truncated TMM analogues would sensitize *Msmeg* to antibiotics, we carried out co-treatment growth assays with the front-line antitubercular compound rifampicin (RIF). RIF has an intracellular target, RNA polymerase, and is a relatively lipophilic and large molecule (cLogP 3.710; MW 823 g/mol) that is hypothesized to accumulate in mycobacteria mainly through passive diffusion through the cell envelope.[21] Therefore, we predicted that modulating the lipid chains of the mycomembrane would impact RIF efficacy. First, we established in our *Msmeg* growth assay that the minimum inhibitory concentration (MIC) of RIF was 12.5 μg/mL (Figure S2A, ESI). Next, to determine whether TMM analogues affected RIF efficacy, we co-treated *Msmeg* with varying concentrations of TMM analogues **1**–**3** (0–1,000 μM) and sub-MIC-range concentrations of RIF (0.39–12.5 μg/mL) for 24 h, then measured OD_{600}. The TMM analogues sensitized *Msmeg* to RIF in a chain length- and dose-dependent manner (Figure 2). Whereas the longer-chain analogue O-TMM-C22 (**3**) had no discernible effect on RIF sensitivity and the intermediate-length analogue O-TMM-C16 (**2**) had at most a modest effect, the short-chain analogue O-TMM-C6 (**1**) had a pronounced effect. When administered at 1 mM, O-TMM-C6 decreased the RIF MIC by approximately 8-fold (MIC 1.56 μg/mL) despite having only a minor impact on growth when administered alone. This result indicates that O-TMM-C6 significantly sensitizes *Msmeg* cells to treatment with the lipophilic anti-tubercular drug RIF.

We also evaluated whether TMM analogue treatment affected the efficacy of INH in *Msmeg*. Like RIF, INH has an intracellular target, enoyl acyl carrier protein reductase (InhA), but contrary to RIF, INH is a relatively hydrophilic and small molecule (cLogP –0.668; MW 137 g/mol).[20] Similar to the RIF co-treatment experiments, we first established the MIC of INH in *Msmeg* as 6.25 μg/mL (Figure S2B, ESI), then we tested whether

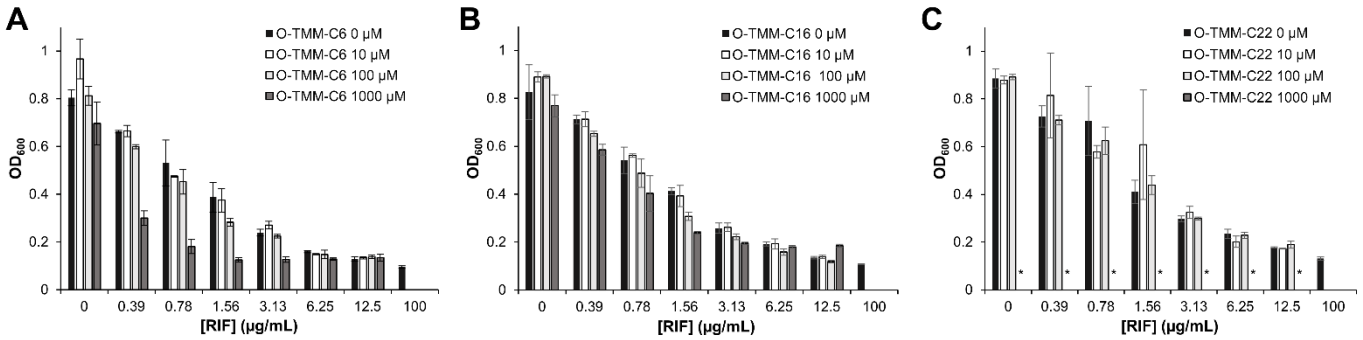


Figure 2. Co-treatment of *Msmeg* with TMM analogues and RIF. *Msmeg* was cultured in 96-well microplates in 7H9 liquid growth medium containing (A) O-TMM-C6 (**1**), (B) O-TMM-C16 (**2**), or O-TMM-C22 (**3**) or DMSO control at the indicated concentrations along with RIF at various sub-MIC-range concentrations, 0 μg/mL as the negative control, or 100 μg/mL as the positive control. After 24 h, growth was assessed by measuring OD_{600}. Data shown are average values of three technical replicates and are representative of three independent experiments. Error bars represent the standard deviation of three technical replicates. *O-TMM-C22 was insoluble in the assay at 1 mM concentration.

the TMM analogues modulated the MIC. In contrast to the results observed in the RIF co-treatment experiments, the TMM analogues did not noticeably sensitize *Msmeg* to INH treatment (Figure S3, ESI). Our results are consistent with prior work, which demonstrated that a *M. tuberculosis* Ag85C mutant with an impaired mycomembrane exhibited increased uptake of the lipophilic probe chenodeoxycholate compared to the wild-type strain, but uptake of hydrophilic INH was equivalent in the two strains.[5] Together, these results suggest that diffusion of INH through the mycomembrane is not the limiting step of cell entry, and that chain-truncated TMM analogues may selectively enhance uptake of molecules whose primary barrier to internalization is diffusion across the mycomembrane.

Given the promising activity of O-TMM-C6 (**1**) in RIF co-treatment, we next examined the compound in pre-treatment experiments. If TMM analogues exert their activity through chemical remodeling of the mycomembrane, as we hypothesize, then pre-treatment of *Msmeg* cells with O-TMM-C6 should also impact RIF inhibitory activity, potentially more potently than co-treatment and in a time-dependent manner. To test this idea, we cultured *Msmeg* in 1 mM O-TMM-C6 for varying durations, washed the cells to remove unincorporated O-TMM-C6, then incubated cells with a sub-MIC concentration of RIF (0.78 μg/mL) for 24 h and measured growth. As above, treatment with TMM analogue or RIF alone at these concentrations had minimal effect, whereas *Msmeg* cells pre-treated with O-TMM-C6 exhibited progressively higher sensitivity toward RIF as pre-treatment time increased, with activity escalating between 2–8 h of pre-treatment (Figure 3A). This time frame aligns with the doubling time of *Msmeg* (~4 h in a 96-well plate) and the previously observed metabolic labeling plateau for clickable TMM analogues in *Msmeg*.[11] Moreover, pre-treatment of *Msmeg* with O-TMM-C6 for 8 h prior to sub-MIC RIF exposure completely abolished growth, whereas co-treatment with TMM analogue and RIF over 24 h only partially decreased growth. Together, these data are consistent with the proposed mycomembrane remodeling mechanism of TMM analogue activity.

Finally, we investigated whether sensitization of *Msmeg* to RIF could be due to enhanced cellular permeability, as hypothesized. To evaluate *Msmeg* permeability, we used ethidium bromide, which is a fluorescent probe whose fluorescence intensity is enhanced upon intercalation into intracellular DNA and is thus sensitive to factors that modulate envelope permeability.[21-23] *Msmeg* was pre-treated with O-TMM-C6 (**1**) for 8 h, washed, re-suspended in phosphate-buffered saline (PBS) containing ethidium bromide, and then uptake kinetics were measured using a fluorescence plate

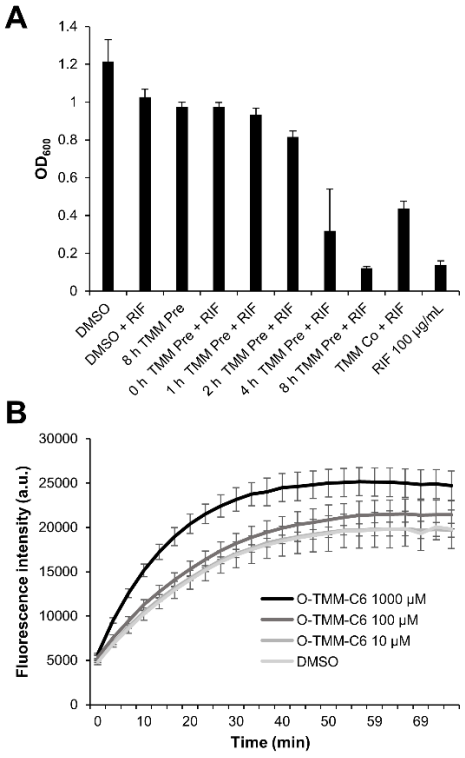


Figure 3. Pre-treatment of *Msmeg* with O-TMM-C6 sensitizes bacteria to RIF and enhances cellular permeability. (A) *Msmeg* was cultured in 96-well microplates in 7H9 medium containing 1 mM O-TMM-C6 (**1**) or left untreated (DMSO control) for the indicated durations. Cells were then washed, incubated for 24 h in RIF (0.78 μg/mL) or left alone as control, and OD_{600} was measured. Pre-treatment conditions ("Pre") were compared to co-treatment conditions ("Co"), in which *Msmeg* was co-treated with 1 mM O-TMM-C6 and RIF (0.78 μg/mL) for 24 h followed by OD_{600} reading as in Figure 2A. (B) *Msmeg* was pre-treated with O-TMM-C6 (**1**) for 8 h as in (A), then treated with ethidium bromide and fluorescence (Ex 535 nm/Em 595 nm) was monitored. Data shown are average values of three technical replicates and are representative of three independent experiments. Error bars represent the standard deviation of three technical replicates.

reader. Bacteria pre-treated with 1 mM O-TMM-C6 exhibited a significantly enhanced rate of ethidium bromide uptake, strongly suggesting that chain-truncated TMM analogues function through modulating cell envelope permeability.

We propose that O-TMM-C6 enhances envelope permeability through Ag85-mediated replacement of native mycolic acids with truncated acyl chains (Figure 1). Consistently, we found using an LC-MS enzyme assay that Ag85A converted O-TMM-C6 into a diacylated trehalose product resembling TDM (Figure S4, ESI), showing that Ag85 uses O-TMM-C6 as an acyl donor to generate glycolipids with shortened chains. Although it is difficult to directly confirm incorporation of O-TMM-C6 into the mycomembrane in cells, we previously demonstrated Ag85 incorporation of a similar compound with a trackable alkyne handle, O-AlkTMM-C7 (**4**, Scheme 1A).[11-12] With O-AlkTMM-C7 already known to incorporate into the mycomembrane, here we showed that it also enhances *Msmeg* permeability to EtBr and sensitivity to RIF similar to O-TMM-C6, although less potently (Figures S5 and S6, ESI). Together, these data support a mechanism whereby Ag85 uses TMM analogues as acyl donors to remodel the mycomembrane with chain-truncated lipids, which in turn modulates cell envelope permeability.

Novel strategies to modulate mycomembrane permeability are valuable tools for studying the mycomembrane and may lead to new adjunctive therapies for mycobacterial infections. Here, we developed compounds designed to chemically remodel the mycomembrane with chain-truncated lipids. We found that a water-soluble TMM analogue bearing a major lipid truncation, O-TMM-C6 (**1**), permeabilized *Msmeg* cells and sensitized them to RIF, which is used to treat tuberculosis. Prior evidence from TMM labeling probes (e.g., **4**) and the data herein are consistent with the hypothesis that O-TMM-C6 acts through metabolic replacement of native mycolic acids. Future research will focus on further mechanistic studies, expanding the types of drugs being tested for increased uptake, tuning the TMM analogues to enable structure–activity relationship studies, and testing in pathogens. Finally, we emphasize that the reported strategy for modulating mycomembrane permeability may be advantageous because: (i) it is tunable with respect to the TMM structure; (ii) it does not require genetic manipulation; (iii) it can be used to transiently modulate permeability. Along with previously reported trehalose-based metabolic inhibitors,[25-26] photosensitizers,[27] and antibody-recruiting molecules,[28] the present study also contributes to a growing toolbox of strategies that target mycobacterial trehalose metabolism[16] toward novel TB therapeutics.

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