Mutations in MmpL3 alter membrane potential, hydrophobicity and antibiotic susceptibility in Mycobacterium smegmatis

Matthew B. McNeil, Devon Dennison and Tanya Parish*

Abstract
MmpL3 is a promising target for novel anti-tubercular agents, with numerous compound series identified as MmpL3 inhibitors. Despite this, there is an incomplete understanding of MmpL3 function. Here we show that Mycobacterium smegmatis MmpL3 mutant strains had an altered cell wall hydrophobicity, disrupted membrane potential and growth defects in liquid media. Compensatory mutations that restored normal growth also returned membrane potential to wild-type. Mycobacterium smegmatis MmpL3 mutant strains were resistant to two anti-tubercular agents, SQ109 and AU1235, but were more sensitive to rifampicin, erythromycin and ampicillin. Exposure of Mycobacterium smegmatis to AU1235 affected the cell wall composition and increased the potency of rifampicin. However, MmpL3 mutants did not prevent the dissipation of membrane potential following exposure to SQ109. These results demonstrate that in Mycobacterium smegmatis, MmpL3 contributes to a number of important phenotypes such as membrane potential, cell wall composition, antibiotic susceptibility and fitness.

The mycobacterial membrane protein MmpL3 transports trehalose monomycolates (TMM) across the mycobacterial inner membrane as a precursor to the synthesis of mycolic acids [1]. MmpL3 is essential in Mycobacterium smegmatis and Mycobacterium tuberculosis and is required for M. tuberculosis growth during the acute and chronic phases of infection in mice [1–3]. Despite an incomplete understanding of MmpL3 function, whole cell screening strategies have identified several small molecule inhibitors of MmpL3 [2–8]. The aim of this current study was to improve the understanding of mycobacterial MmpL3.

Four Mycobacterium smegmatis strains with independent single nucleotide polymorphisms (SNPs) in MmpL3 (MSMEG_0250) were isolated as resistant mutants to a compound under development in our laboratory derived from a known class of MmpL3 inhibitors [7]. The frequency of resistance was $6.8 \times 10^{-8}$. MmpL3 is an integral membrane protein with 12 transmembrane helices [9]. The MmpL3 mutations are located in transmembrane helices 3 or 4 (Table 1). These residues are conserved in M. tuberculosis and mutations S293T and Y257C are equivalent to S288T and Y252C in M. tuberculosis, which are associated with resistance to MmpL3 inhibitors in M. tuberculosis [7, 10].

MmpL3 mutant strains were tested for resistance to the M. tuberculosis MmpL3 inhibitors, AU1235 and SQ109. Minimum inhibitory concentrations (MICs) were determined on Middlebrook 7H10 plus 10 % v/v OADC (oleic acid, albumen, d-glucose, catalase; Becton Dickinson) supplement (7H10-OADC) solid medium in 24-well plates; compounds were prepared as two fold serial dilutions in DMSO starting at 100 µM. All MmpL3 mutants were greater than four fold resistant to AU1235 (Table 1). In contrast, only two of the four mutants, RM3 (S293T) and RM6 (S293A), showed greater than four fold resistance to SQ109, strain RM5 (Y257C) showed a small shift (two fold), and strain RM2 (I297F) was not resistant (Table 1). These data confirm that MmpL3 appears to be the target or mode of resistance in Mycobacterium smegmatis as in M. tuberculosis.

Mutations associated with drug resistance often have a fitness cost [11, 12]. We determined if MmpL3 SNPs affected the aerobic growth of Mycobacterium smegmatis in liquid medium. Strains were grown in Middlebrook 7H9 medium supplemented with 10 % v/v OADC and 0.05 % w/v Tween 80 or LB broth plus 0.05 % Tween 80 in 125 ml conical flasks at 37°C with shaking at 125 r.p.m. RM3 (S293T) grew as well as the wild-type (WT) under both conditions (Fig. 1a, b). RM2 (I297F) had a growth impairment in LB-Tw but not 7H9-OADC-Tw (Fig. 1a, b). RM5 (Y257C) and RM6 (S293A) had a growth impairment in both media (Fig. 1a, b). Furthermore, RM5 (Y257C) and RM6 (S293A) mutants formed cellular aggregates in both media after reaching an OD$_{590}$ of ~0.1, whilst RM2 (I297F) formed cellular aggregations in LB-Tw only (data not shown). Strains RM5...
hypothesized that the growth defects of M. smegmatis mutant strains could be caused by disruptions in PMF. We measured the $\Delta \psi$ of each strain using the fluorescent dye DiOC2(3) (3,3'-diethyloxacarbocyanine iodide) [13, 14]. Briefly, strains were grown to OD$_{590}$=0.8, washed and resuspended in 7H9-Tw, and incubated with 15 $\mu$M DiOC2 (3) for 20 min. Excess DiOC2(3) was removed by washing; an equal volume of loaded culture (50 µl) was added to 7H9-Tw in a black, clear bottomed, 96-well plate and read in a Synergy-4 plate reader. Data were expressed as the ratio between the red and green wavelengths (excitation at 488 nm; emission at 610 and 530 nm, respectively). All the mutant strains had a higher red/green ratio than WT, indicating a change in $\Delta \psi$ (Fig. 2a). Furthermore, the change in $\Delta \psi$ was correlated with the severity of the growth impairment, i.e. RM3, which grew as well as the WT, had the smallest change, whilst RM5 and RM6 had the largest increase and significant growth impairments (Fig. 2a). The ratio of SRM1 (S293A, G750D) shifted from the parental mutant towards WT levels, suggesting that the compensatory mutation rescued the growth defect by partially restoring the membrane potential (Fig. 2a). To the best of our knowledge, this is the first demonstration that mutations in MmpL3 directly affect $\Delta \psi$ and show that MmpL3 contributes to an optimal $\Delta \psi$ in M. smegmatis.

MmpL3 inhibitors fall into two classes, those that inhibit $\Delta \psi$ and $\Delta \mathrm{pH}$ (such as SQ109) [15]. We hypothesized that if SQ109 activity against M. smegmatis was mediated through the dissipation of $\Delta \psi$, then the resistant mutant strains would not have changes in $\Delta \psi$ when exposed to SQ109. We measured the $\Delta \psi$ after 45 min exposure to SQ109. All MmpL3 mutant strains showed the same disruption in $\Delta \psi$ as the WT strain (Fig. 2b). Thus, the mechanism of action of SQ109 against M. smegmatis does not involve dissipation of the $\Delta \psi$.

Disruption of the mycolic acid layer of the mycobacterial cell wall could result in changes in hydrophobicity [16, 17]. To determine if mutations in MmpL3 affected cell wall

### Table 1. Resistance profiles of M. smegmatis mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MmpL3 SNP</th>
<th>M. tuberculosis equivalent</th>
<th>Solid agar MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AU1235</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>MmpL3-RM2</td>
<td>I297F</td>
<td>I291</td>
<td>12.5</td>
</tr>
<tr>
<td>MmpL3-RM3</td>
<td>S293T</td>
<td>S288</td>
<td>25</td>
</tr>
<tr>
<td>MmpL3-RM5</td>
<td>Y257C</td>
<td>Y252</td>
<td>12.5</td>
</tr>
<tr>
<td>MmpL3-RM6</td>
<td>S293A</td>
<td>S288</td>
<td>100</td>
</tr>
<tr>
<td>MmpL3-SRM1</td>
<td>S293A, G750D</td>
<td>S288, G745</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MmpL3-SRM2</td>
<td>S293A, G750D</td>
<td>S288, G745</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MmpL3-SRM3</td>
<td>S293A, G750D</td>
<td>S288, G745</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MmpL3-SRM4</td>
<td>S293A, G750D</td>
<td>S288, G745</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

MICs were determined on solid medium in 24-well plates and are defined as the lowest concentration which prevented growth. Amp, ampicillin; Cm, chloramphenicol; Em, erythromycin; EtBr, ethidium bromide; Km, kanamycin; Rif, rifampicin; nd, not determined.
composition, we measured hydrophobicity using a hydrocarbon adhesion assay [17]. Strains were grown to $OD_{590}=0.8$ in 7H9-OADC-Tw, harvested, washed and resuspended in phosphate-urea-magnesium buffer (17.4 g l$^{-1}$ $K_2HPO_4$, 7.26 g l$^{-1}$ $KH_2PO_4$, 1.8 g l$^{-1}$ urea, 0.2 g l$^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 7.1) to a final $OD_{590}=0.8$ [16, 17] in

Fig. 1. Mutations in the MmpL3 result in growth impairments in $M. smegmatis$. Growth curves of $M. smegmatis$ strains were performed in (a) and (c) 7H9-OADC-Tw, as well as in (b) and (d) LB-Tw. Strains used were WT, MmpL3-RM2 (I297F), RM3 (S293T), RM5 (Y257C), RM6 (S293A) and SRM1-4 (S293A, G750D). Data are the average of three biological replicates with standard deviation. (e) Growth of MmpL3-RM3 and RM6 on 7H10-OADC after 3–6 days.
16 mm borosilicate tubes. Hexadecane was added to a final concentration of 0.5%, vortexed for 15 s and phases allowed to separate for 30 min [16]. The OD$_{590}$ of the lower phase was measured. Data are represented as the percentage of cells remaining in the lower phase; an increase in cellular hydrophobicity increases adherence to hexadecane and reduces the percentage of cells in the lower phase. RM2, RM3 and RM5 all demonstrated increased hydrophobicity compared to the WT strain with a significantly reduced fraction of cells remaining in the lower phase (Fig. 2c). The increased hydrophobicity of RM6 was not significantly different compared to WT (Fig. 2c). Despite this, the compensatory mutant, MmpL3-SRM1, derived from RM6 had significantly increased hydrophobicity (Fig. 2d). It is not obvious why SRM1, but not the parental strain, showed increased hydrophobicity, but it may be related to the slow growth rate. In conclusion, mutations in MmpL3 alter the cell wall composition of *M. smegmatis*.

Disruption of the mycobacterial cell wall resulting from mutations in MmpL3 might alter sensitivity to other antimycobacterial agents. We determined MICs for a range of
antibiotics. All mutant strains were more sensitive to ampicillin and the hydrophobic antibiotics, rifampicin and erythromycin (Table 1). There was no change in sensitivity to chloramphenicol or kanamycin (Table 1). MmpL3 mutants were equally sensitive to ethidium bromide, suggesting that altered resistance profiles were not due to changes in permeability. Numerous *M. tuberculosis* MmpL3 mutant strains have been identified, but there is only a single example of an MmpL3 mutation leading to enhanced sensitivity to rifampicin [10]. In addition, the expression of certain *M. tuberculosis* MmpL3 mutant alleles in *M. smegmatis* increased sensitivity to rifampicin, although rifampicin sensitivity was unchanged in *M. tuberculosis* [9]. These differences in resistance profiles could be attributable to species differences in the mycobacterial cell wall.

To determine if MmpL3 inhibitors also affected cell wall composition in *M. smegmatis*, we monitored hydrophobicity following exposure to AU1235. Cells were grown to OD$_{590}$=0.4, split into 20 ml volumes in 125 ml conical flasks with compound or DMSO (2% final concentration), exposed for 3h, harvested and hydrophobicity measured. Exposure to AU1235 increased cell wall hydrophobicity, with only 20% of cells remaining in the lower phase compared to 65% in untreated cells (Fig. 2e). Thus, inhibition of MmpL3 affects hydrophobicity of *M. smegmatis*.

Exposure to AU1235 and mutations in MmpL3 both increased the hydrophobicity of *M. smegmatis*. We hypothesized that this might be responsible for the increased sensitivity towards rifampicin, a hydrophobic compound, and that AU1235 could enhance the activity of rifampicin in combination. Synergy between AU1235 and rifampicin was tested using checkerboard assays on 7H10-OADC in 24-well plates. Fractional inhibitory concentrations (FICs) were determined for each compound. The potency of rifampicin increased 16-fold from 50 to 3.1 µM, FIC(A)=0.062 in the presence of 0.5× MIC of AU1235. In contrast sub-inhibitory concentrations of rifampicin did not alter the potency of AU1235, where FIC(B)=0.5. The two-drug combination was additive FIC=0.56, but borderline synergistic (FIC <0.5) [18]. This enhancement of the rifampicin in *M. smegmatis* is consistent with previous observations of AU1235 and other MmpL3 inhibitors showing exceptional antibacterial activity in an animal model of tuberculosis infection. *J Med Chem* 2016;59:6232–6247.


Li W, Upadhyay A, Fontes FL, North EJ, Wang Y et al. Novel insights into the mechanism of inhibition of MmpL3, a target of inhibitors are synergistic with rifampicin and ampicillin in *M. tuberculosis* [19, 20]. We hypothesize that the inhibition of or alterations in MmpL3 function alter the mycobacterial cell wall and sensitize it to other anti-mycobacterial agents.

### Funding information

This research was supported with funding from the Bill and Melinda Gates Foundation, under grant OPP1024038. The funder played no role in the study, the preparation of the article or the decision to publish.

### Conflicts of interest

The authors declare that there are no conflicts of interest.

### References


15. Li W, Upadhyay A, Fontes FL, North EJ, Wang Y et al. Novel insights into the mechanism of inhibition of MmpL3, a target of...


**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.