PNAS COMMENTARY

The driving force for mycolic acid export by mycobacterial MmpL3 is proton translocation

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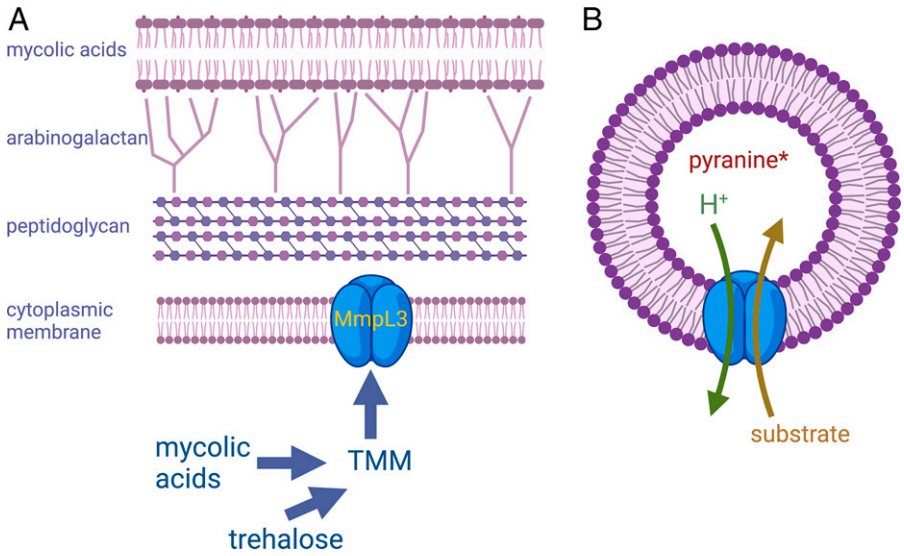

Fig. 1. (*A*) Schematic of the mycobacterial cell wall showing the outer mycolic acid bilayer and location of MmpL3 in the cytoplasmic membrane. Mycolic acids are synthesized in the cell and linked to trehalose to form TMM, which is exported by MmpL3 trimers. (*B*) Outline of the proteoliposome assay. MmpL3 is incorporated into liposomes and proton translocation monitored by measuring the internal pH using the reporter pyranine. * fluorescent probe. Figure created with BioRender.com.

Mycobacterium tuberculosis, the causative agent of tuberculosis, remains a major global human pathogen (1, 2). The COVID-19 pandemic has worsened the situation for treatment of tuberculosis, with millions of cases undiagnosed and an increase in the number of deaths to about 1.5 million per year. There remains an urgent need for new drugs to treat tuberculosis as well as for an increased understanding of the physiology of the bacterium.

One of the defining features of mycobacteria is the cell wall, an unusually thick and waxy structure which provides a formidable barrier for the penetration of antibiotics (3). This cell wall contains a second lipid bilayer, analogous to the outer membrane of gram-negative bacteria, and often referred to as the outer mycomembrane. The main components of this lipid bilayer are the mycolic acids; these are long-chain fatty acids (C60 to C90) comprising a beta hydroxy fatty acid and an alkyl side chain (Fig. 1). Mycolic acids are esterified to the arabinogalactan layer which, in turn, is linked to the peptidoglycan to form a covalently linked mAGP complex. In addition, mycolic acids can be linked to trehalose to form "cord factor" (trehalose dimycolate) in the cell wall. The synthesis of mycolic acids has been well studied, and the biosynthetic pathways are largely established in mycobacteria. Once produced inside the bacterium, mycolic acids need to be exported through the cytoplasmic membrane (Fig. 1). This process is mediated by the MmpL3 protein (MmpL denotes "mycobacterial membrane protein Large") which exports mycolates in the form of trehalose monomycolate (TMM) (4). Stevens et al. (5) provide mechanistic insight into how mycolic acids are transported across the cytoplasmic membrane by MmpL3.

Mycobacterial species can be identified on the basis of the types of mycolic acids they synthesize; *M. tuberculosis* produces three types of mycolates—the alpha-, methoxy-, and keto- mycolic acids. Shorter-chain mycolic acids are also produced by other actinobacteria, notably, *Corynebacterium*, *Nocardia*, and Rhodococcus. Mycolic acids are essential for the structural integrity of the cell wall in mycobacteria but are not essential for the viability of corynebacteria, demonstrating differences between the bacterial species (4). Export of mycolate is mediated by MmpL3 in mycobacteria and CmpL1/4 in corynebacteria. MmpL3/CmpLs are members of the RND (resistance, nodulation, and division) family of transporters. Most interest has focused on

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MmpL3, since it is essential for the viability of *M. tuberculosis* both in vitro and in vivo, demonstrating its pivotal role in the synthesis of the cell wall. MmpL3 is a good drug target, since it is both essential and vulnerable to inhibition (6). A number of chemical scaffolds have been identified which appear to inhibit MmpL3 function, and these are the focus of intensive drug discovery and development efforts (7).

A major barrier to the rational development of new drugs targeting MmpL3 has been the lack of a biochemical or functional assay. Hypomorph (underexpressing) and resistant strains as well as biophysical interaction studies have been used to support the identification of MmpL3 as the target of novel antitubercular agents but have not proved successful or useful in designing improved analogs (8, 9). Stevens et al. (5) develop new assays to explore the function of MmpL3/CmpL1 and confirm the on-target activity of current MmpL3 inhibitors.

Stevens et al. provide mechanistic insight into how mycolic acids are transported across the cytoplasmic membrane by MmpL3.

MmpL3 is an integral membrane protein, with 12 transmembrane helices and two periplasmic loops. Its natural state is most likely to be a trimer similar to other members of the RND family, with the C-terminal domain mediating oligomerization (expression of a truncated protein lacking this domain results in a monomeric protein). A major barrier to the design of a functional MmpL3 assay has been the difficulty of working with integral membrane proteins. *M. tuberculosis* proteins often are not expressed well in *Escherichia coli*, so Stevens et al. (5) used a mycobacterial expression host, the fast-growing *Mycobacterium smegmatis,* to purify $MmpL3_{MTB}$. Using a strain in which the native *M. smegmatis* MmpL3 was replaced by the *M. tuberculosis* allele, they were able to purify the full-length *M. tuberculosis* MmpL3 protein. In addition, they expressed and purified CmpL1 from *C. glutamicum*.

Previous work has suggested that MmpL3 is a proton translocator, based on its homology to other RND family proteins and evidence that its function is dependent on the proton motive force. This suggests an antiporter mechanism with the substrate TMM. Stevens et al. (5) demonstrate that proton translocation does occur, using proteoliposomes containing full-length MmpL3 or CmpL1 protein. The fluorescent pH probe pyranine was used to monitor pH inside vesicles containing the various purified MmpL3 proteins (Fig. 1). Using this technique, they are able to demonstrate that MmpL3-mediated proton translocation occurs, an important step forward in understanding the role of this protein. They also demonstrate that proton flux is dependent on a proton gradient, since it only occurs when the extravesicular pH is higher or lower than the intravesicular pH. In the presence of a substrate mimic containing trehalose linked to a C16 acyl chain, proton translocation is increased. The rate of translocation is dependent on the length of the acyl chain, with shorter chains (C4 to C14) inhibiting proton translocation. The C-terminal truncation of MmpL3 demonstrates proton translocation activity but is not affected by substrate mimics. Interestingly, while CmpL1 also has proton translocation activity, it is not stimulated by substrate mimics, and is seen only when the extravesicular pH is neutral or acidic. These data demonstrate striking differences between the corynebacterial and mycobacterial transporters, both in terms of proton translocation and in terms of the substrates they bind.

Phenotypic high-throughput screening has identified numerous compound series which appear to target MmpL3 (10). Assignment of MmpL3 as the target has largely been based on the isolation of resistant mutants carrying mutations in the gene. However, demonstration of functional inhibition of MmpL3 has been lacking due to the absence of suitable assays. Previous work has used binding or competition assays to support the premise that MmpL3 is the target (9). Using surface plasmon resonance Stevens et al. (5). demonstrate that the C16 substrate mimic binds to MmpL3 but not CmpL1, consistent with their data on its ability to stimulate proton translocation only for the *M. tuberculosis* protein. The shorter-chain substrate mimics did not bind to either protein, consistent with their inability to promote proton translocation. Binding of known MmpL3 inhibitors with whole-cell activity was also seen. These data support the use of biophysical methods to investigate the mechanism and/or function of RND transporters. However, using analogs of the indole carboxamide (ICA) series, there was no relationship between binding and whole-cell activity. Three ICA molecules had the same binding mechanism and bound both MmpL3 and CmpL1, which was consistent with whole-cell activity against both species. However, one analog demonstrated no binding but had significant whole-cell activity. Since there is no correlation between binding affinity and whole-cell activity, this suggests caution in using SPR data to inform structure–activity relationships or for rational drug design. Further work to understand the nature of this discrepancy is warranted.

The availability of a functional assay allows Stevens et al. (5) to connect the dots between whole-cell activity and inhibition of the target protein. Using their proteoliposomes, they are able to demonstrate that ICAs inhibit proton translocation in a pH-dependent fashion. Two molecules effected a concentration-dependent inhibition of proton translocation, but only at low pH, whereas another ICA inhibited proton flux only at pH8 and was not concentration dependent. The reasons for these differences are not known, but the authors propose that this may reflect differences in the molecules themselves at different pH, for example, if they have ionizable groups. Alternatively, it may reflect one of the limitations of the assay, which is that integration of the protein into the lipid layer can occur in both orientations, which can complicate the interpretation of the data if bidirectional flux is occurring. Further work to establish why inhibition is pH dependent is needed. However, the demonstration that MmpL3 proton translocation is affected by inhibitors provides an important mechanistic link between inhibitor binding and MmpL3. In addition, the availability of a simple assay opens the way for more rational drug design, as well as further mechanistic studies.

The link between whole-cell activity and on-target activity is an important part of drug discovery and basic research

and can both provide information for rational drug design and illuminate aspects of bacterial physiology. Since both mycobacteria and corynebacteria produce mycolic acids and have similar transporters, one might expect that MmpL3 inhibitors would also be effective against the *Corynebacterium glutamicum* homolog. Whether inhibition of corynebacterial transporters would inhibit growth is an open question, since mycolic acids are not essential in this organism. However, it is possible that a buildup of mycolates in the cell would be toxic and that inhibition of mycolate transport would reduce the growth rate, as was seen with CmpL1/4 double-deletion mutant strains.

Stevens et al. (5) tested this question by determining whether a range of scaffolds active against *M. tuberculosis* were also inhibitory to *C. glutamicum*. Since mycolate export is mediated by two redundant transporters, CmpL1 and CmpL4, they used strains in which either CmpL1 or CmpL4 was deleted. SQ109 and BM212 had similar low-level activity against all three corynebacterial strains, suggesting an off-target effect. Since these inhibitors are known to have other targets in *M. tuberculosis*, including disruption of the proton motive force, this may not be surprising. Surprisingly, three ICAs also had activity against a single *Corynebacterium* strain, suggesting inhibition of CmpL1. However, given the fact that mycolates are not essential for corynebacterial growth, it seems unlikely that inhibition of CmpL1 is the only effect of these molecules. Further work is needed to establish the mechanism of action in corynebacterium.

MmpL3 plays an important role in the physiology of the mycolate-containing bacteria and is the focus of a number of drug discovery efforts for tuberculosis; therefore, understanding mechanisms of resistance is important. Mutations in MmpL3 lead to resistance to several MmpL3 inhibitor classes; the majority of these mutations are located in the presumed proton translocation channel (7, 11). Now that Stevens et al. (5) have demonstrated proton translocation by the wild-type protein, it should be feasible to determine the effect of mutations on protein function and inhibitor action. This type of information would be invaluable in selecting the best molecule classes to pursue.

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