MmpL3 Is the Cellular Target of the Antitubercular Pyrrole Derivative BM212

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MmpL3 Is the Cellular Target of the Antitubercular Pyrrole Derivative BM212

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The 1,5-diarylpyrrole derivative BM212 was previously shown to be active against multidrug-resistant clinical isolates and Mycobacterium tuberculosis residing within macrophages as well as against Mycobacterium avium and other atypical mycobacteria. To determine its mechanism of action, we identified the cellular target. Spontaneous Mycobacterium smegmatis, Mycobacterium bovis BCG, and M. tuberculosis H37Rv mutants that were resistant to BM212 were isolated. By the screening of genomic libraries and by whole-genome sequencing, we found that all the characterized mutants showed mutations in the mmpL3 gene, allowing us to conclude that resistance to BM212 maps to the MmpL3 protein, a member of the MmpL (mycobacterial membrane protein, large) family. Susceptibility was unaffected by the efflux pump inhibitors reserpine, carbonylcyanide m-chlorophenylhydrazone, and verapamil. Uptake/efflux experiments with [14C]BM212 demonstrated that resistance is not driven by the efflux of BM212. Together, these data strongly suggest that the MmpL3 protein is the cellular target of BM212.

Having identified a group of related compounds with potent antimycobacterial activity, we proceeded to identify the cellular target of BM212 as the prototype compound in its class. Here, by complementary genetic approaches, we propose that the MmpL3 protein is the cellular target for BM212.

MATERIALS AND METHODS

Synthesis of BM212. All chemicals were used at a purity of ≥95%. A Discovery Microwave System apparatus (from CEM Corporation) was used for Stetter and Paal-Knorr reactions. Melting points were determined with open capillaries on a Gallenkamp apparatus and are uncorrected. Microanalyses were conducted with a Perkin-Elmer 240C or a Perkin-Elmer series II CHNS/O Analyzer 2400 instrument. Fluka silica gel 60 (230 to 400 mesh) was used for column chromatography. Fluka TLC plates (silica gel 60 F254) were used for thin-layer chromatography (TLC). Fluka aluminum oxide (activity II, according to the Brockmann scale) was used for chromatographic purifications. Fluka Stratacom aluminum oxide plates with a fluorescent indicator were used for TLC to check the purity of the compounds. High-performance liquid chromatography (HPLC) analyses were conducted with a Waters Alliance 2695 instrument, using a UV-visible light (Vis) Waters PDA 996 detector and working at 333 nm. All synthesized compounds were ≥95% pure. 13C nuclear magnetic resonance (NMR) and 1H NMR spectra were recorded with a Bruker AC 400 spectrometer in CDCl3 (with tetramethylsilane [TMS] as the internal standard).

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BM212 was synthesized as previously reported (3, 4). Briefly, 1-(4-chlorophenyl)pentane-1,4-dione was obtained by reacting the 4-Ch-benzaldehyde with methyl vinyl ketone. The resulting 1,4-diketone was cyclized in the presence of 4-Ch-aniline to yield 1,2-bis(4-chlorophenyl)-5-methyl-1H-pyrole. BM212 was finalized by reacting 1,5-diarylpyrrole with formaldehyde and N-methylpiperazine under Mannich reaction conditions.

**DNA cloning and amplification.** The shuttle cosmid pYUB18 (12 kb) was used to construct genomic libraries (10). Primers YUB3 and YUB4 (Table 1), recognizing short sequences upstream and downstream of the BamHI restriction site, were utilized for the partial sequencing of genomic inserts. Subcloning experiments with one of the cosmids conferring BM212 resistance were performed by cloning into the pGEM-T Easy vector (Promega) and then into the final vectors pSUM39 (1) and pMD31 (9).

For several strains of *Mycobacterium bovis* BCG and *M. tuberculosis* H37Rv, results from whole-genome sequencing were confirmed by the cloning of the *mmpL3* gene into pBluescript, followed by the targeted resequencing of *mmpL3*. All primers used for PCR amplification are listed in Table 1. Amplification reactions were performed with a final volume of 40 μl containing 200 μM each deoxynucleotide triphosphate (dNTP), 500 nM each primer, 2% dimethyl sulfoxide (DMSO), 1.6 mM MgCl2, 40 ng of plasmid DNA or 100 ng of genomic mycobacterial DNA, and 1 U of *Pfu* DNA polymerase (Promega). Cycling conditions were as follows: denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing for 30 s at a temperature dependent on the primers used, and elongation at 72°C for a time dependent on the product size, with a final elongation step at 72°C for 5 min.

PCR products were blunted into pBluescript at the EcoRV site, and the *mmpL3* gene was sequenced by using primers listed in Table 1.

**Bacterial strains, media, and growth conditions.** *Escherichia coli* HB101 was used as a host strain and for the construction of genomic libraries; the *E. coli* XL1-Blue MRF’ Tet strain (Stratagene) was used for cloning procedures. These strains were grown in Luria-Bertani (LB) broth or on LB agar (20). Plates were supplemented, when required, with tetra-cycline (12.5 μg/ml) (Sigma), ampicillin (50 μg/ml) (Sigma), kanamycin (50 μg/ml) (Sigma), and streptomycin (50 μg/ml) (Duchefa Biochimie B.V.). All strains were grown aerobically at 37°C with shaking (200 rpm). The plates were incubated at 37°C for 1 day.

*Mycobacterium smegmatis* wild-type strain mc2155 and mutant strains MSMEG-GR12.5 and MSMEG-BM1 to MSMEG-BM10 (Table 2) were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment broth (Difco) and 0.05% Tween 80 or on 7H11 agar (Difco) supplemented with 10% OADC and 0.5% glycerol. When required, kanamycin was added at a concentration of 25 μg/ml. All cultures were grown aerobically at 37°C with shaking (200 rpm). The plates were incubated at 37°C for at least 3 days.

*M. smegmatis* wild-type strain mc2155 and mutant strains SRBM212_20, SRBM212_21, SRBM212_24, and SRBM212_26 (Table 2) were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin-dextrose-catalase (OADC) enrichment broth (Difco) and 0.05% Tween 80 or on 7H11 agar (Difco) supplemented with 10% OADC and 0.5% glycerol. All cultures were grown aerobically at 37°C with shaking (160 rpm). The plates were incubated at 37°C for at least 5 days.

*Mycobacterium bovis* wild-type strain BCG and mutants resistant to BM212, BCG-BM40 to BCG-BM44, and BCG-BM49 to BCG-BM56 (Table 3), were grown as described above for *M. smegmatis* at 37°C for about

![FIG 1 Chemical structure of BM212. Me, methyl.](http://aac.asm.org/)

**TABLE 1 Oligonucleotides used in this work**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM1</td>
<td>GATGCGGCACATCTGACCTTCG</td>
<td>Sequencing of the <em>M. smegmatis</em> 5.2-kb Scal/EcoRV fragment containing the <em>mmpL3</em> gene</td>
</tr>
<tr>
<td>BM2</td>
<td>AACGCGCCCAAGGAGACTCG</td>
<td></td>
</tr>
<tr>
<td>BM3</td>
<td>GCCCTGCACATCAGGGCTTCA</td>
<td></td>
</tr>
<tr>
<td>BM4</td>
<td>GAATTCTTCTGACGTGCGCG</td>
<td>pYUB18 cosmid sequencing</td>
</tr>
<tr>
<td>BM5</td>
<td>AGTGGCAGCGGGGTTGAACTCGG</td>
<td></td>
</tr>
<tr>
<td>BM6</td>
<td>CACAGGCGACACCTAGGCTC</td>
<td></td>
</tr>
<tr>
<td>BM7</td>
<td>TTTCCTCAGCAGCGCGCAGACACCCT</td>
<td></td>
</tr>
<tr>
<td>YUB3</td>
<td>GCAATGCAGAAGCCAGTTTCA</td>
<td></td>
</tr>
<tr>
<td>YUB4</td>
<td>TGGCCGCGGCGACGTTTCA</td>
<td></td>
</tr>
<tr>
<td>MMPLB1</td>
<td>GACGTGTTTGAACACAAAA</td>
<td>Sequencing of the <em>M. bovis</em> BCG <em>mmpL3</em> gene</td>
</tr>
<tr>
<td>MMPLB3</td>
<td>AACGGCGAATGGAAAGTCT</td>
<td></td>
</tr>
<tr>
<td>MMPLB5</td>
<td>ATCGTGTCTTATACCCGTGTTG</td>
<td></td>
</tr>
<tr>
<td>MMPLB7</td>
<td>ACCCGCGACAAAAGAATCAC</td>
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</tr>
<tr>
<td>MMPLB9</td>
<td>TGGTGTGTAAGAAGCTGCTG</td>
<td></td>
</tr>
<tr>
<td>Rv0206_seq1</td>
<td>GACTGTGTTGCAACACAAAA</td>
<td>Subcloning and sequencing of the <em>M. tuberculosis</em> <em>mmpL3</em> gene</td>
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<tr>
<td>Rv0206_seq2</td>
<td>GAATGGAGATGCTGCGGC</td>
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<tr>
<td>Rv0206_seq3</td>
<td>TCAGGCGAGTGATCTTCGC</td>
<td></td>
</tr>
<tr>
<td>Rv0206_seq4</td>
<td>CATCATCAGGCGGGGCGG</td>
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<tr>
<td>Rv0206_seq5</td>
<td>ATCAGTGTCGTCGCGGG</td>
<td></td>
</tr>
<tr>
<td>Rv0206_PCRf</td>
<td>AGAGATCGCGTGTTTTCGCG</td>
<td></td>
</tr>
<tr>
<td>Rv0206_PCRr</td>
<td>GCTTTCTTCAACATGCGGTCA</td>
<td></td>
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</tbody>
</table>
3 to 4 weeks under static conditions, while plates were incubated at 37°C for at least 3 weeks.

Wild-type *M. bovis* BCG and *M. bovis* mutant strains BCGRBM212_1 and BCGRBM212_2 (Table 3) were also grown in Middlebrook 7H9 medium (Difco) supplemented with 10% ADC, 0.05% Tween 80, and 0.2% glycerol or on 7H10 agar (Difco) supplemented with 10% ADC and 0.5% glycerol at 37°C for about 10 days with shaking (160 rpm). The plates were incubated at 37°C for at least 4 weeks.

*M. tuberculosis* wild-type strain H37Rv and mutant strain TBRBM212_1 (Table 3) were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% OADC, 0.05% Tween 80, and 0.2% glycerol at 37°C for approximately 3 weeks with shaking (160 rpm). The plates were incubated at 37°C for at least 8 weeks.

### TABLE 2 MICs of BM212 and amino acid substitutions in the *mmpL3* gene of *M. smegmatis* resistant to BM212

<table>
<thead>
<tr>
<th><em>M. smegmatis</em> strain(s)</th>
<th>MIC (µg/ml)</th>
<th>Amino acid change (position)</th>
<th>Mutation(s) in another <em>M. smegmatis</em> ORF(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc2155 (wt)</td>
<td>3.125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSMEG-GR12.5</td>
<td>12.5</td>
<td>A254V, I296L</td>
<td></td>
</tr>
<tr>
<td>MSMEG-BM1, MSMEG-BM2, MSMEG-BM7, MSMEG-BM8</td>
<td>12.5</td>
<td>I249T</td>
<td></td>
</tr>
<tr>
<td>MSMEG-BM3, MSMEG-BM4</td>
<td>12.5</td>
<td>F240L, L196P</td>
<td></td>
</tr>
<tr>
<td>MSMEG-BM6</td>
<td>12.5</td>
<td>A326T, A347V</td>
<td></td>
</tr>
<tr>
<td>MSMEG-BM9</td>
<td>12.5</td>
<td>V689G</td>
<td></td>
</tr>
<tr>
<td>MSMEG-BM10</td>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRBM212_20</td>
<td>25</td>
<td>A326T</td>
<td>MSMEG_2883, T130T</td>
</tr>
<tr>
<td>SRBM212_21</td>
<td>25</td>
<td>A326T</td>
<td>MSMEG_0931c, +C: 3682514, T&gt;C (noncoding); deletion of MSMEG_1716–MSMEG_1726</td>
</tr>
<tr>
<td>SRBM212_24</td>
<td>25</td>
<td>V689G</td>
<td></td>
</tr>
<tr>
<td>SRBM212_26</td>
<td>25</td>
<td>V197M</td>
<td></td>
</tr>
</tbody>
</table>

* The different nomenclatures of strains refer to mutants isolated during different experiments (see Materials and Methods). wt, wild type.

### Drug susceptibility tests

MICs of compounds (BM212, isoniazid [INH], and pump inhibitors) for *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv strains were determined with both solid and liquid media.

For assays performed with solid media, 7H11 agar medium supplemented with 10% OADC and 0.5% glycerol was used to prepare plates containing 2-fold serial dilutions of BM212 dissolved in DMSO at a concentration ranging from 0.17 to 100 µg/ml. Cell cultures were grown to an optical density at 600 nm (OD_{600}) of 0.8 and diluted to a final concentration of 2 x 10^6 CFU/ml. One microliter of the diluted culture was then streaked onto each plate and incubated at 37°C for 3 to 4 days, 4 to 5 weeks, or 8 weeks for *M. smegmatis*, *M. bovis* BCG, or *M. tuberculosis* H37Rv, respectively. The MIC was defined as the lowest concentration of drug that prevented the formation of colonies.

For assays performed with liquid media, the MIC was defined as the lowest concentration of drug that yielded an absence of visual turbidity. BM212 and INH, prepared in ethanol and sterile water, respectively, were dissolved in 3 ml of Middlebrook 7H9 medium, to a final concentration ranging from 0.125 to 100 µg/ml, in 2-fold serial dilutions. The medium was then inoculated with mycobacteria to an OD_{600} of 0.003 of *M. smegmatis*, *M. bovis* BCG, or *M. tuberculosis* H37Rv and incubated at 37°C for 24 h, 10 days, or 12 days, respectively. A control tube or inkwell without any drug was included with each experiment, and INH was used as a control.

### Isolation of *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* mutants resistant to BM212

*M. smegmatis* mutant strains MSMEMG-BM1 to MSMEG-BM10 (Table 2), resistant to BM212, were isolated as follows. For *M. smegmatis* mc2155, 5 x 10^8 CFU/ml was plated onto 7H10 solid medium containing concentrations of BM212 ranging from 3.125 to 100 µg/ml, in 2-fold serial dilutions. The medium was then inoculated with mycobacteria to an OD_{600} of 0.003 of *M. smegmatis*, *M. bovis* BCG, or *M. tuberculosis* H37Rv and incubated at 37°C for 24 h, 10 days, or 12 days, respectively. A control tube or inkwell without any drug was included with each experiment, and INH was used as a control.

### Isolation of *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* mutants resistant to BM212

*M. smegmatis* mutant strains MSMEMG-BM1 to MSMEG-BM10 (Table 2), resistant to BM212, were isolated by plating about 10^10 cells onto 7H10 solid medium with concentrations of BM212 ranging from 3.125 to 100 µg/ml. Cell cultures were grown to an optical density at 600 nm (OD_{600}) of 0.8 and diluted to a final concentration of 2 x 10^6 CFU/ml. One microliter of the diluted culture was then streaked onto each plate and incubated at 37°C for 3 to 4 days, 4 to 5 weeks, or 8 weeks for *M. smegmatis*, *M. bovis* BCG, or *M. tuberculosis* H37Rv, respectively. The MIC was defined as the lowest concentration of drug that prevented the formation of colonies.

For assays performed with liquid media, the MIC was defined as the lowest concentration of drug that yielded an absence of visual turbidity. BM212 and INH, prepared in ethanol and sterile water, respectively, were dissolved in 3 ml of Middlebrook 7H9 medium, to a final concentration ranging from 0.125 to 100 µg/ml, in 2-fold serial dilutions. The medium was then inoculated with mycobacteria to an OD_{600} of 0.003 of *M. smegmatis*, *M. bovis* BCG, or *M. tuberculosis* H37Rv and incubated at 37°C for 24 h, 10 days, or 12 days, respectively. A control tube or inkwell without any drug was included with each experiment, and INH was used as a control.
Control 7H10 plates containing 30 μg/ml of INH inoculated with either 5 × 10³ CFU/ml of M. smegmatis mc²155, 5 × 10⁴ CFU/ml of M. bovis BCG, or 5 × 10⁴ CFU/ml of M. tuberculosis H37Rv were used. The plates were incubated at 37°C for 7 days in the case of M. smegmatis, 4 weeks for M. bovis BCG, and 8 weeks for M. tuberculosis H37Rv.

MIC determinations in the presence of efflux inhibitors. The MICs of the efflux pump inhibitors reserpine (RES) (a plant alkaloid known to inhibit the transport of drugs extruded by ATP-energized pumps), carbonyl cyanide m-chlorophenylhydrazide (CCCP) (a proton uncoupler), and verapamil (VER) (a calcium channel blocker) were determined for M. smegmatis mc²155. The susceptibilities of M. smegmatis strains mc²155, MSMEG-GR12.5, and SRB1202_20 to BM212 were then tested in the presence of subinhibitory concentrations of CCCP (7.5 μg/ml), RES (12 μg/ml), and VER (40 μg/ml). The MICs of the pump inhibitors alone or in the presence of BM212 were determined in liquid medium, as reported above.

Whole-genome sequencing. Resistant mutants selected for whole-genome sequencing were sequenced with an Illumina Genome Analyzer II instrument in the paired-end mode with 51-bp reads. Genomic DNA was extracted from log-phase cultures by using a MasterPure purification kit (Illumina) according to the manufacturer’s instructions. A total of 2 to 3 μg of DNA from each sample was sheared by using a Covaris sonicator, and the standard Illumina sample preparation procedure was followed, including blunt-end repair, adapter ligation, PCR amplification, and size selection of 350- to 450-bp fragments on a 2% agarose gel, as described by the manufacturer (Multiplexing SamplePrep guide 1005361_D; Illumina). Samples were loaded onto a flow cell, clusters were generated by the manufacturer (Multiplexing SamplePrep guide 1005361_D; Illumina). Samples were then washed twice at 5,000 rpm for 20 min with 10 ml 0.1 M phosphate buffer containing 0.1 M LiCl, and filtered through 0.45-μm-pore-size filters (Millipore). The filters were washed twice with 5 ml of the same buffer, and the radioactivity retained on the filter was determined with a Perkin-Elmer TriLux Microbeta 1450 Counter by using an Ecolomue scintillation cocktail (ICN Biomedicals). The efflux experiment was repeated three times.

RESULTS
Isolation of M. smegmatis, M. bovis BCG, and M. tuberculosis H37Rv mutant strains resistant to BM212. The MIC of BM212 against different mycobacterial species was evaluated as described in Materials and Methods. BM212 showed good potency against all mycobacterial species tested, as previously described (3, 4, 7) (Tables 2 and 3).

We isolated and characterized mycobacterial strains resistant to BM212 in order to identify the cellular target of BM212. The plating of M. smegmatis onto solid media containing various concentrations of BM212 yielded multiple isolates that exhibited higher MICs of BM212 than the parent strain (Table 2). The MIC of BM212 for wild-type M. smegmatis strain mc²155 was 3.12 μg/ml (Table 2). Mutants arose at a frequency of 3.7 × 10⁻⁵. Fourteen out of 61 resistant isolates were characterized and showed MIC values ranging from 12.5 to 25 μg/ml, 4- to 8-fold higher than those for the parent strain (Table 2). Isolates of the vaccine mycobacterial strain M. bovis BCG and M. tuberculosis H37Rv that were resistant to BM212 were selected by using a similar strategy. The MICs of BM212 for wild-type M. bovis BCG and M. tuberculosis H37Rv were 0.78 and 1.5 μg/ml, respectively (Table 3). Fifteen out of 18 resistant isolates of M. bovis BCG were characterized and showed MIC values ranging from 3.12 to 20 μg/ml, 4- to 25-fold higher than those for the parent strain (Table 3). The MIC of BM212 for the only resistant M. tuberculosis strain was 20 μg/ml, 10-fold the MIC for the parent strain (Table 3). Mutants were isolated at frequencies of 1.7 × 10⁻⁸ for M. bovis and 2 × 10⁻⁹ for M. tuberculosis.

Screening of cosmid libraries derived from BM212-resistant M. smegmatis isolates identified mutations in the mmpL3 gene as mediating resistance to BM212. Genomic DNAs from two BM212-resistant isolates of M. smegmatis, MSMEG-GR12.5 and MSMEG-BM10, were used to construct two cosmid libraries by using vector pYUB18 (10) and were transformed into E. coli cells. The isolated cosmid DNA was transformed into wild-type M. smegmatis mc²155 cells by electroporation, and the transformants were plated onto selective medium containing BM212 at concentrations ranging from 3.12 μg/ml to 50 μg/ml. Resistant colonies were first validated to ensure that they were truly resistant to BM212. The MIC of BM212 for isolated colonies was 12.5 μg/ml, i.e., 4-fold the MIC for the same strain transformed with vector pYUB18. Cosmid DNA was extracted from the resistant clones and retransformed into M. smegmatis mc²155 to confirm that resistance was due to the cosmid and not to the generation of spontaneous resistance. As expected, all the cosmids conferred resistance to BM212.

The cosmids responsible for the resistance phenotype were partially sequenced, and sequences were compared with those of M. smegmatis mc²155 by using BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All cosmids that mediated resistance contained a partially overlapping fragment. The cosmid derived from the screening of the genomic library from the resistant isolate MSMEG-GR12.5 contained a 22-kb genomic DNA insert with 2 partial genes and 16 intact genes. This fragment was digested with restriction enzymes and further subcloned into vectors pMD31 and pGem39. Transformants containing these plasmids were again screened for resistance to BM212. A 5.2-kb Scal/EcoRV fragment was found to confer resistance to BM212 and contained three genes, MSMEG_0249 (1,140 bp), MSMEG_0250 (3,042 bp), and MSMEG_0251 (699 bp), which code for an integral membrane protein, the MmpL3 protein, and a hypothetical protein, respectively. The cloned DNA was sequenced, and the sequence was compared with the reference M. smegmatis genome. While the sequences for MSMEG_0249 and MSMEG_0251 were identical to the wild-type sequence, the mmpL3 sequence contained 2 nonsynonymous mutations compared with the reference sequence. These encoded amino acid substitutions from alanine to valine at position 254 (A254V) and from isoleucine to leucine at position 296 (I296L) (Table 2).

For the cosmid derived from the screening of the genomic library from the resistant isolate MSMEG-BM10, a single muta-
tion rendering an amino acid substitution from valine to glycine at position 689 (V689G) in the mmpL3 gene was found (Table 2).

Given the results achieved with the screening of cosmid libraries, the mmpL3 genes from the other nine resistant M. smegmatis mutants (MSMEG-BM1 to MSMEG-BM9) were amplified and sequenced. The analysis of these sequences revealed point mutations which caused an amino acid change for each mutant tested (Table 2). Altogether, these results suggest that mutations in the mmpL3 gene are responsible for BM212 resistance. As shown in Fig. 2A, the majority of these amino acid changes map within the first six transmembrane segments of the predicted MmpL3 topology.

**Whole-genome sequencing of resistant isolates to identify mutations responsible for BM212 resistance.** Among isogenic mutants resistant to BM212, the isolates with the highest MICs, SRBM212_20, SRBM212_21, SRBM212_24, and SRBM212_26 (Table 2), were selected for whole-genome sequencing to identify
polymorphisms linked with resistance. We used an Illumina GenomeAnalyzer instrument with 51-bp reads in the paired-end mode, yielding a mean depth of coverage of 50× to 78× (number of reads covering each nucleotide) and a completion of >99.9% (fraction of sites covered by at least one read). Compared to the genome sequence of the parental strain \textit{M. smegmatis} mc²155, each strain was observed to have a nonsynonymous substitution in the \textit{mmpL3} gene (MSMEG_0250), including the mutations A326T, V689G, and V197M (Table 2). As shown in Fig. 2A, the first and the second of these amino acid changes map to transmembrane segments, while the last one lies within a cytoplasmic domain of the predicted MmpL3 topology.

For two of the strains, these were the only polymorphisms detected in the entire genome. The other two strains had additional polymorphisms, although one was synonymous (MSMEG_2883, T130T), one was in a noncoding region, and one was a frameshift mutation in a hypothetical protein (MSMEG_0931c) (Table 2). Strain SRBM212-24 also had an 8,566-bp deletion of MSMEG_1716 to MSMEG_1726 (6 open reading frames [ORFs] of which are annotated as transposases and 3 of which are annotated as hypothetical proteins) (Table 2). These results strongly suggest that mutations in \textit{mmpL3} confer resistance to BM212.

\textbf{BM212-resistant isolates of \textit{M. bovis} BCG and \textit{M. tuberculosis} H37Rv contain mutations in \textit{mmpL3}.} Given the identification of mutations in \textit{mmpL3} as likely to be responsible for resistance to BM212 in \textit{M. smegmatis}, we determined the sequence of \textit{mmpL3} from the BM212-resistant \textit{M. bovis} BCG and \textit{M. tuberculosis} isolates. Of note, all the resistant \textit{M. bovis} BCG isolates had a common mutation in \textit{mmpL3} that resulted in a change from leucine to proline at position 320 (L320P) (Table 3). Some of the BCG isolates also had a second nonsynonymous mutation within the \textit{mmpL3} gene (Table 3). As shown in Fig. 2B, the majority of these amino acid changes map within the first six transmembrane segments of the predicted topology. The mutation identified in the \textit{mmpL3} gene of the resistant \textit{M. tuberculosis} strain results in a change from leucine to serine at position 215 (L215S) (Table 3) in the third transmembrane segment of the predicted MmpL3 topology (Fig. 2B).

\textbf{Mutations in \textit{mmpL3} do not mediate resistance to BM212 via drug efflux.} MmpL3 encodes one of the MmpL ( \textit{mycobacterial membrane protein, large} ) proteins, a family that has primary structure homology to the resistance-nodulation-cell division (RND) protein family, a group of proteins that are mainly involved in drug resistance in Gram-negative bacteria (16). Since energy-dependent bacterial efflux pumps can confer a multidrug resistance phenotype to clinically important pathogens by extruding the drug out of the cell before it can reach its target, we felt that it was important to exclude the possibility that resistance to BM212 mediated by mutations in the \textit{mmpL3} gene is due to the efflux of the compound.

We investigated whether the resistance phenotype could be modified by broadly acting efflux pump inhibitors. We tested three well-characterized MDR pump inhibitors; none of them changed the susceptibility of resistant \textit{M. smegmatis} isolates to BM212 (Table 4), suggesting that mutations in the \textit{mmpL3} gene did not cause resistance via drug efflux.

We also excluded the possibility that resistance to BM212 might be due to the overexpression of the \textit{mmpL3} gene. Quantitative PCR of \textit{mmpL3} transcripts from both wild-type and BM212-resistant \textit{M. smegmatis} strains, in both the presence and the absence of BM212, revealed no difference in \textit{mmpL3} expression (data not shown).

To further determine whether resistant \textit{M. smegmatis} strains extruded BM212 out of the cell, we formally tested the accumulation and efflux of [\textsuperscript{14}C]BM212 in both wild-type and BM212-resistant (MSMEG-GR12.5) \textit{M. smegmatis} strains (Fig. 3). [\textsuperscript{14}C]BM212 accumulated in both strains rapidly within 5 min, at which time concentrations remained constant for the following 60 min (Fig. 3). There was no difference in the rate or quantity of BM212 accumulation between wild-type and BM212-resistant strains. Taken together, these data strongly suggest that the mechanism of resistance to BM212 mediated by \textit{mmpL3} mutations is not via drug efflux.

\section*{DISCUSSION}

All antibiotics in current usage were identified through the traditional screening of small-molecule libraries for activity against bacteria. Only subsequently was the cellular target(s) of the drug identified, sometimes decades after the agent entered clinical use. In some cases, such as with the first-line antitubercular agent isoniazid, the identification of the cellular target proved difficult and controversial (2, 14, 19), and in others, such as with metronidazole, the target(s) remains elusive.

The alternative to whole-cell-based screens, target-based screening, has failed to live up to expectations, at least for antibiotic development (15). Nonetheless, knowing the target of a hit compound greatly facilitates the process of progressing from hit to lead compound to clinical agent.

In this study, by complementary genetic approaches, we identified mutations in the \textit{mmpL3} gene (MSMEG_0250, BCG0243c, and RV0206c) as being responsible for resistance to BM212, a novel candidate antitubercular agent. We also provided evidence that resistance to BM212 from mutations in the \textit{mmpL3} gene are not due to an enhanced efflux of the drug, making it likely that the \textit{mmpL3} gene product is the cellular target for BM212 activity.

One point mutation was identified in 11 out of 14 characterized \textit{M. smegmatis} mutants resistant to BM212, giving rise to similar levels of resistance (4- to 8-fold the MIC for the wild-type strain). These mutations are localized at different nucleotide positions, but the majority map to the first six transmembrane segments of the predicted MmpL3 topology. Two independent mutants (MSMEG-BM10 and SRBM212_26) contain the same amino acid change, V689G; the latter strain additionally contains an 8,566-bp deletion and two other mutations. As this deleted region contains genes coding for hypothetical proteins, trans-
posases, and pseudogenes, as annotated by SmegmaList (http://mycobrowser.epfl.ch/smegmalist.html), it seems unlikely that this deletion is responsible for resistance, although further experiments would be necessary to clarify the role of this large deletion in resistance to BM212.

Two SNPs were identified in the mmpL3 gene in 5 out of 15 characterized M. bovis isolates resistant to BM212. One mutation, encoding the L320P change, occurs individually in nine other M. bovis mutants with small differences in the BM212 MIC values. This finding suggests that the L320P SNP is important for conferring BM212 resistance and that the second SNP observed for other mutants (I250M, T286K, T277M, A316T, and A294T) does not contribute to BM212 resistance. Also, in M. bovis, like M. smegmatis, the majority of these amino acid changes map to the first six transmembrane segments of the predicted MmpL3 topology. The only M. tuberculosis mutant resistant to BM212 presented the SNP L215S in the third transmembrane segment.

Since no good structural model of a close homologue of MmpL3 with significant sequence identity (>40%) is available, it is difficult to determine how the mutations that mediate resistance could alter the association of the protein with BM212. However, the fact that the mutated amino acids all lie within predicted transmembrane segments is not surprising given the hydrophobicity of the compound. However, if these amino acids do interact directly with the compound, BM212 might well inhibit MmpL3’s ability to act as a transporter.

It is noteworthy that we found a higher frequency of resistance in M. smegmatis than in both M. bovis BCG and M. tuberculosis. One possible explanation for this finding is that mutations in M. smegmatis mmpL3 are better tolerated, as mmpL3 appears to be essential in M. tuberculosis (22).

MmpL3 is a putative membrane protein belonging to the RND protein family of multidrug resistance pumps that mediate the transport of a diverse array of ionic or neutral compounds as well as heavy metals and fatty acids (16). The M. tuberculosis genome encodes 13 members of the MmpL (mycobacterial membrane protein, large) family (6). Despite their annotation as multidrug transporters, they do not appear to play a role in antimycobacterial drug resistance (8). A recent report has ascribed a role for mmpL3 in heme acquisition by M. tuberculosis (22), although that role appears to be dispensable in vitro, suggesting that MmpL3 may have other functions as well.

The use of high-throughput sequencing technologies in addition to classical genetics and biochemistry provides new opportunities for the rapid identification of likely targets for antibacterial agents. Our data suggest that the mmpL3 gene product is likely the cellular target for BM212. This suggests that MmpL3 represents a new potential druggable target for the treatment of TB. Novel targets such as this are critical given the current epidemic of drug-resistant TB.

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