Involvement of Holliday Junction Resolvase in Fluoroquinolone-Mediated Killing of Mycobacterium smegmatis

Quanxin Long, Qinglin Du, Tiwei Fu, Karl Drlica, Xilin Zhao, Jianping Xie

Institute of Modern Biopharmaceuticals, State Key Laboratory Breeding Base of Eco-Environment and Bio-Resource of the Three Gorges Area, Key Laboratory of Ministry of Education Eco-Environment of the Three Gorges Reservoir Region, School of Life Sciences, Southwest University, Beibei District, Chongqing, China; Second Affiliated Hospital and the Key Laboratory of Molecular Biology of Infectious Diseases of the Ministry of Education, Chongqing Medical University, Yuzhong District, Chongqing, China; Public Health Research Institute and Department of Microbiology, Biochemistry, and Molecular Genetics, New Jersey Medical School, Rutgers Biomedical and Health Sciences, Newark, New Jersey, USA; State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiang-An District, Xiamen, Fujian Province, China

The absence of the Holliday-junction Ruv resolvase of Mycobacterium smegmatis increased the bacteriostatic and bactericidal activities of the fluoroquinolone moxifloxacin, an important antituberculosis agent. The treatment of ruvAB-deficient cells with thiourea and 2,2'-bipyridyl lowered moxifloxacin lethality to wild-type levels, indicating that the absence of ruvAB stimulates a lethal pathway involving reactive oxygen species. A hexapeptide that traps the Holliday junction substrate of RuvAB potentiated moxifloxacin-mediated lethality, supporting the development of small-molecule enhancers for moxifloxacin activity against mycobacteria.

The fluoroquinolone moxifloxacin is becoming an important antituberculosis agent, as both a first-line therapeutic (1) and a second-line treatment for multidrug-resistant tuberculosis (2, 3). Compounds capable of potentiating moxifloxacin activity should accelerate bacterial death, shorten treatment time, and reduce the emergence of resistance. As a test with a model system, we constructed a transposon-mutagenized library of Mycobacterium smegmatis strains and screened for mutants whose growth was more readily blocked by moxifloxacin. One positive hit from the screening, M. smegmatis mutant M136, carried an insertion in ruvA, whose gene product participates in the resolution of Holliday junctions during DNA recombination (4). The existence of a hexapeptide that traps Holliday junctions and therefore inhibits RuvABC activity (5, 6) allowed us to test the idea that moxifloxacin lethality can be increased by a small molecule.

M. smegmatis strain mc²155 was grown in 7H9 liquid medium (supplemented with 0.5% glycerol and 0.05% Tween 80) and on 7H10 solid medium at 37°C, as described previously (7). Moxifloxacin was obtained from Bayer AG (Wuppertal, Germany). Gatifloxacin was obtained from Lummy Pharmaceuticals (Chongqing, China); norfloxacin, oxolinic acid, rifampin, isoniazid, chloramphenicol, and kanamycin were obtained from Sangon Biotech (Shanghai, China). Thiourea and 2,2'-bipyridyl were obtained from Sigma-Aldrich (St. Louis, MO). Transposon mutagenesis was performed by infecting M. smegmatis with the temperature-sensitive mycobacterial phasmid φMycMarT7 (8), obtained from C. Sassetti (University of Massachusetts Medical School, Worcester, MA). Individual colonies grown on 7H10 agar containing 20 μg/ml kanamycin at 37°C (the nonpermissive temperature for plasmid replication) were tested for failure to form colonies on agar containing 0.025 μg/ml moxifloxacin. The transposon insertion site was identified by DNA sequence determination following the digestion of mutant chromosomal DNA with SacII restriction endonuclease, circularization of the digested fragments with T4 DNA ligase, the transformation of Escherichia coli strain DH5α-λpir, and the recovery of transposon-derived kanamycin resistance-expressing plasmid. The DNA sequence determination used an outward primer from the transposon (5′-GCT TTC TTG ACG AGT TCT TCT GAG-3′). Wild-type ruvAB from Mycobacterium tuberculosis was cloned into pALACE (9), and the recombinant plasmid (pALACE-ruvAB) was introduced into strain M136 by electroporation to generate the complemented strain M. smegmatis M136C. Antimicrobial susceptibility (MIC) was determined by broth dilution, as per the CLSI protocol (10). The hexapeptide WRWYCR (5, 6) was synthesized by GL Biochem (Shanghai, China) and dissolved in 10% acetic acid.

The screening of about 3,000 colonies led to the isolation of 3 transposon-insertion mutants that failed to grow when spotted on moxifloxacin-containing agar that allowed wild-type M. smegmatis to grow. Strain M136 showed the largest drop in moxifloxacin MIC and was chosen for further characterization. An insertion site analysis revealed a transposon insertion between nucleotides 14 and 15 in the ruvA coding sequence (Fig. 1A). Using a pair of primers directed inward toward the insertion site, amplification by PCR generated a DNA fragment from mutant DNA that was about 2.1 kbp larger than that from wild-type DNA, supporting the conclusion that an insertion had occurred at the identified site. The introduction of a plasmid containing wild-type ruvAB (from M. tuberculosis strain H37Rv) into the mutant strain M136 restored wild-type susceptibility to moxifloxacin in agar plates (Fig. 2A).
FIG 1 Transposon insertion in ruvA associated with increased activity of moxifloxacin. (A) Results of nucleotide sequence analysis for insertion site. The transposon insertion in strain M136 occurred in codon 5 (between nucleotides 14 and 15) of the ruvA open reading frame. The asterisk indicates the location of the transposon insertion. (B) Growth on moxifloxacin-containing agar. Culture aliquots of 10 μl were applied to agar plates containing 0.0125 μg/ml moxifloxacin and 1% acetamide; the inoculated plates were then incubated at 37°C for 36 h. Strains containing an intact ruvAB transcription unit grew (wild-type [Wt] mc²155, wild type with vector [pALACE] alone, M136 with ruvAB cloned into pALACE), while those lacking a functional ruvAB operon did not (M136 and M136 with the pALACE vector). Three replicate experiments gave similar results.

1B), while that of a plasmid containing no insert (M136 pALACE) or ruvA alone did not. These data indicate that the polar effect of transposon insertion inactivated both ruvA and ruvB. Collectively, these data demonstrate that the inactivation of ruvAB increases bacteriostatic susceptibility of M. smegmatis to moxifloxacin.

We also assessed the effect of the ruvA insertion on the bacteriostatic activities of other quinolones and antituberculosis agents (Table 1). Strain M136 exhibited an 8-fold decrease in MIC for moxifloxacin, a 4-fold decrease for gatifloxacin, and a 2-fold decrease for norfloxacin and oxolinic acid. Thus, with M. smegmatis, the effect of RuvAB depends on quinolone structure. The introduction of a plasmid containing wild-type ruvAB into strain M136 restored wild-type quinolone susceptibility (Table 1). The ruvA insertion had no effect on susceptibility for the antituberculosis agents isoniazid, rifampin, and capreomycin (Table 1).

Since growth inhibition and killing are mechanistically distinct for quinolones (11), we determined whether the absence of RuvAB also affects lethal activity. In these experiments, we compared moxifloxacin lethality against wild-type and ruvAB mutant strains at the same fold MIC rather than at the same absolute drug concentration. Such drug concentration normalization is expected to distinguish factors that specifically affect killing from those that affect growth inhibition, such as drug uptake, efflux, and target interactions. The rate of killing was about 1,000 times higher with the insertion mutant than that with the wild type when the cells were treated with 8× the MIC of moxifloxacin. These conditions resulted in about 10,000-fold more cells being killed with the mutant than with the wild-type cells after a 12-h treatment (Fig. 2A). The complemented strain, M136C, behaved like wild-type cells (Fig. 2A). Thus, the absence of RuvAB enhances killing by moxifloxacin.

Quinolones kill bacteria in two ways (11). One way involves ongoing protein synthesis and a cascade of reactive oxygen species (ROS) (12); the second, observed only with the newer and more potent fluoroquinolones, kills cells even when protein synthesis and the ROS cascade are blocked (12, 13). To determine whether RuvAB acts on one or both killing mechanisms, we treated exponentially growing cultures of M. smegmatis with a fixed moxifloxacin concentration (8× the MIC) for various times in the presence or absence of chloramphenicol, an inhibitor of protein synthesis. Chloramphenicol had no effect on the killing of wild-type cells by moxifloxacin (Fig. 2A), as reported previously (14, 15). However, chloramphenicol reduced moxifloxacin-mediated killing of the ruvAB mutant to wild-type levels (Fig. 2A). We also supplemented moxifloxacin treatment with subinhibitory concentrations of 2,2′-bipyridyl plus thiourea, agents that inhibit hydroxyl radical accumulation (12, 16, 17). Thiourea plus 2,2′-bipyridyl had no effect on killing by moxifloxacin with wild-type cells, but the combination reduced the killing of the mutant to wild-type levels (Fig. 2B). Thus, the wild-type activity of RuvABC obscures the effects of moxifloxacin on the protein-synthesis-dependent ROS-mediated lethal pathway, which otherwise would make M. smegmatis be more readily killed by moxifloxacin.

To determine whether agents that trap Holliday junctions and therefore inhibit RuvABC action also potentiate moxifloxacin-mediated lethality, we combined moxifloxacin with a hexapeptide (WRWYCR) previously shown to inhibit branch migration and interfere with the resolution of Holliday junctions by RuvABC (5,

### Table 1: Antimicrobial Susceptibility of a ruvAB-Deficient Mutant of M. smegmatis

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant genotype</th>
<th>MIC (μg/ml) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mox</td>
</tr>
<tr>
<td>mc²155</td>
<td>Wild type</td>
<td>0.063</td>
</tr>
<tr>
<td>M136</td>
<td>Lacking ruvA</td>
<td>0.0078</td>
</tr>
<tr>
<td>M136C</td>
<td>Lacking ruvA complemented</td>
<td>0.031</td>
</tr>
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</table>

*a The compounds used were moxifloxacin (Mox), gatifloxacin (Gat), norfloxacin (Nor), oxolinic acid (Oxo), isoniazid (INH), rifampin (RIF), and capreomycin (Cap).

*b M136 transformed with a pALACE-ruvAB plasmid. ruvA rather than ruvA alone was cloned, because the transposon insertion has a polar effect on ruvB due to the overlapping and cotranscription nature of the two genes.
The dependent Holliday junction resolution appears to participate in the -bipyridyl. Thus, RuvAB-deletion in the absence of RuvAB is eliminated by chloramphenicol and by a more chromosome fragmentation and subsequent cell death (13, 20). Growth, as reflected in a reduced moxifloxacin MIC. The second thus improve the ability of moxifloxacin to inhibit bacterial growth, as reflected in a reduced moxifloxacin MIC. The second level arises from elevated fluoroquinolone concentrations causing chromosome fragmentation and subsequent cell death (13, 20). The increased moxifloxacin-mediated lethality associated with the absence of RuvAB is eliminated by chloramphenicol and by a combination of thiourea plus 2,2′-bipyridyl. Thus, RuvAB-dependent Holliday junction resolution appears to participate in the repair of the DNA fragmentation that initiates the protein synthe-
sis- and ROS-dependent killing pathway. Chromosome fragment-
at ion arising from a second lethal pathway, which we attribute to fluoroquinolone-mediated destabilization of gyrase-DNA com-
plexes (12, 13), does not appear to be repaired by RuvAB-medi-
ated recombination.

A hexapeptide that traps Holliday junctions also increases moxifloxacin-dependent killing of cultured M. smegmatis. To our knowledge, this peptide represents the first example of a small-
molecule enhancer of fluoroquinolone lethality. Since the lethal activities of quinolones are similar with M. smegmatis and M. tu-
berculosis (14, 15) and since RuvA and RuvB are conserved among many bacterial species (Table 2; see also Fig. S1 and S2 in the supplemental material [21]), we anticipate that the conclusions drawn with M. smegmatis will also apply to M. tuberculosis and many other bacterial species. The absence of RuvA and RuvB orthologues in humans (Table 2; see also Fig. S1) suggests that RuvA and RuvB may serve as potential targets for clinically useful small-
molecule enhancers of moxifloxacin.

![FIG 2 Enhancement of moxifloxacin-mediated killing by a ruvAB deficiency. (A) Effect of ruvAB deficiency and chloramphenicol (Cm) on killing by moxi-
floxacin (Moxi). Cultures of M. smegmatis were treated with moxifloxacin at 8× the MIC for the indicated times. The CFU were determined at the indicated times and expressed as the percent survival relative to the CFU value determined at the time of drug addition. Circle, wild-type (Wt) strain mc²155; square, ruvAB mutant, strain M136; triangle, ruvAB complemented, strain M136C in the absence (filled symbols) or presence (open symbols) of chlor-
amphenicol (20 µg/ml). (B) Effect of RuvAB deficiency and 2,2′-bipyridyl plus thiourea (BT) on killing by moxifloxacin. The experiments were as in panel A except that subinhibitory concentrations of 2,2′-bipyridyl (0.25 mM) plus thiourea (100 mM) were included as indicated. Three replicate experiments gave similar results. The error bars represent the standard deviation.](http://aac.asm.org/)

![FIG 3 Effect of hexapeptide on moxifloxacin (Moxi)-mediated killing. An exponentially growing culture of M. smegmatis was treated with hexapeptide (WRWYCR) at 25 µg/ml (1× the MIC) alone (square), moxifloxacin alone at 8× the MIC (circles), or both hexapeptide and moxifloxacin (triangle). The CFU were determined at the indicated times and expressed as the percent survival relative to the CFU at the time of drug addition. Three replicate ex-
periments gave similar results. The error bars represent the standard deviation.](http://aac.asm.org/)

<table>
<thead>
<tr>
<th>Species</th>
<th>BLAST score</th>
<th>E value</th>
<th>Identity (%)</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. smegmatis</td>
<td>288/627</td>
<td>1 × e⁻¹⁰⁹/⁰</td>
<td>78/99</td>
<td>99/99</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>109/376</td>
<td>3 × e⁻⁵⁴/⁶ × e⁻¹²⁰</td>
<td>33/55</td>
<td>98/95</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>103/343</td>
<td>6 × e⁻⁶⁰/⁶ × e⁻¹¹⁷</td>
<td>32/50</td>
<td>98/95</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>103/389</td>
<td>9 × e⁻⁵²/⁶ × e⁻¹³²</td>
<td>34/56</td>
<td>98/95</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>33/49</td>
<td>0.84/3 × e⁻³</td>
<td>40/33⁰</td>
<td>24/24⁰</td>
</tr>
</tbody>
</table>

* RuvA and RuvB amino acid sequences of M. tuberculosis strain H37Rv were used to run BLAST the genome sequences of the human and bacterial species listed. Graphic alignments of RuvA and RuvB among the species listed above are presented in Fig. S1 in the supplemental material. The ClustalW alignments of RuvA and RuvB among the bacterial species are presented in Fig. S2 in the supplemental material.

b Although the identify is high, the short amino acid coverage (24% with human versus >95% with other bacteria [see Fig. S1 in the supplemental material]) rules out the existence of bacterial RuvA and RuvB orthologues in humans.
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