

Bedaquiline Targets the ϵ Subunit of Mycobacterial F-ATP Synthase

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The tuberculosis drug bedaquiline inhibits mycobacterial F-ATP synthase by binding to its c subunit. Using the purified ϵ subunit of the synthase and spectroscopy, we previously demonstrated that the drug interacts with this protein near its unique tryptophan residue. Here, we show that replacement of ϵ 's tryptophan with alanine resulted in bedaquiline hypersusceptibility of the bacteria. Overexpression of the wild-type ϵ subunit caused resistance. These results suggest that the drug also targets the ϵ subunit.

The diarylquinoline bedaquiline (trade name Sirturo, code names TMC207 and R207910) is a new tuberculosis drug discovered by Koen Andries and colleagues (1, 2, 16). The drug was shown to inhibit F-ATP synthase of mycobacteria (1). Genetic, biochemical, and modeling studies demonstrated that the c subunit of the F-ATP synthase, forming the membrane-embedded rotary ring of the enzyme, contains the binding site of the drug (1–9). Recent structural studies suggest that bound bedaquiline prevents the rotor ring from acting as an ion shuttle and thus stalls the ATP synthase operation (9).

Using recombinant purified F-ATP synthase ϵ subunit, which plays a key role in coupling proton translocation across the membrane with the catalytic site events in the cytoplasm, we showed previously via tryptophan fluorescence and nuclear magnetic resonance (NMR) spectroscopy that the drug interacts with this subunit near its unique and *Mycobacterium*-specific N-terminal tryptophan residue 16 (10). Based on these *in vitro* data, we proposed that bedaquiline may target, in addition to the c subunit, the ϵ subunit of F-ATP synthase (10). Here, we carried out genetic studies to provide *in vivo* evidence for this second binding site of bedaquiline on the F-ATP synthase.

To test our hypothesis that bedaquiline binds to the ϵ subunit near its tryptophan 16 residue, we replaced this amino acid with alanine by mutating the corresponding codon in the genome of *Mycobacterium smegmatis* mc²155 (ATCC 700084). We predicted that this amino acid exchange would change the bedaquiline susceptibility of the bacteria, either to hyposensitivity, if the drug binds more weakly to the mutated form of the ϵ subunit, or to hypersensitivity, if the mutation allows stronger binding. Site-directed, oligonucleotide-based genome mutagenesis (“recombineering” [11]) was carried out as described previously (12). Briefly, electrocompetent *M. smegmatis* harboring plasmid pJV53 and thus expressing the mycobacterial phage Che9c recombineering genes gp60 and gp61 was transformed with the double-stranded DNA oligonucleotide GGTGTGGCTGATCTGAACGTCGAGATCGTCGCCGTCGAGCGTGAGCTCGCGTCCGGACCCGCTACGTTTCGTGTTCAACCCGCACCACCGCCGGTGAGATCG (Integrated DNA Technologies, USA) containing the desired GCG alanine codon (underlined) to replace the genomic TGG tryptophan codon in position 16 of the ϵ subunit-encoding *atpC* gene. Colonies were PCR screened with 2 different primer pairs both containing the forward primer GCGCTTCCTGAGCCAGAATGA. One pair contained the reverse primer CGAACGTAGCGGGTCCGGACCA, matching the wild-type codon for

tryptophan, and one pair contained the reverse primer CGAACGTAGCGGGTCCGGACCGC, matching the mutated codon for alanine. For colonies that showed positive PCRs with the pair containing the primer matching the mutated version and a negative PCR with the pair containing the wild-type version of the gene, the introduction of the desired mutation was verified by sequencing (AIT Biotech, Singapore). Figure 1A and B show that the resulting strain *M. smegmatis atpC^{W16A}* displayed a growth behavior on Middlebrook 7H10 agar and in 7H9 broth (Becton Dickinson, USA) that was indistinguishable from that of the parental wild-type strain. This shows that the tryptophan-to-alanine alteration in the ϵ subunit of the F-ATP synthase did not affect bacterial growth (13). To determine whether the amino acid replacement changed bedaquiline susceptibility of the bacterium, growth inhibition dose-response curves were determined using the broth dilution method as described earlier (14) and bedaquiline purchased from Genegobio (Los Angeles, CA). Figure 1C shows that whereas wild-type *M. smegmatis* showed a MIC₅₀ of 10 nM, *M. smegmatis atpC^{W16A}* showed a MIC₅₀ of 2 nM, a 5-fold-increased sensitivity to bedaquiline. The observed hypersusceptibility of the bacterium may suggest that the replacement of the bulky tryptophan with an alanine provides more space for a stronger binding of the drug to the ϵ subunit. The hypersensitivity phenotype was complemented by the introduction of a wild-type copy of *atpC* carried by the plasmid pMV262-*atpC* and expressed by the plasmid's *hsp60* promoter (Fig. 1C). Together, these results suggest that bedaquiline interacts with the ϵ subunit of the F-ATP synthase in intact bacilli. pMV262-*atpC* was constructed by inserting a PCR-amplified DNA fragment of the coding sequence of the *atpC* gene into plasmid pMV262 via its BamHI and PstI sites (15). As the *atpC* coding sequence contained an internal BamHI site, the cloning was carried out in a two-step PCR process to eliminate this restriction site by introduction of a silent mutation. First, two

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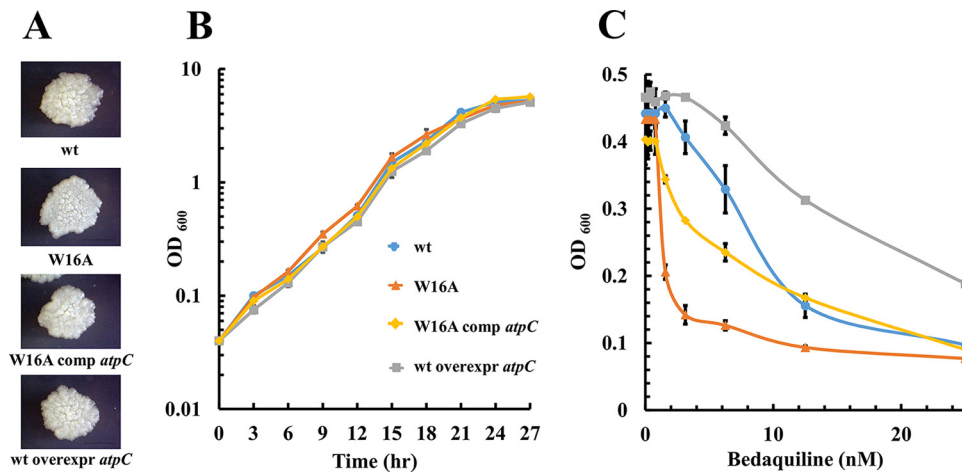


FIG 1 Growth and bedaquiline susceptibility of wild-type *M. smegmatis* and various genetic derivatives. (A) Growth on solid medium. Colonies are shown after 4 days of incubation. (B) Growth in liquid medium. (C) Bedaquiline growth inhibition dose-response curves. wt, wild-type *M. smegmatis*; W16A, *M. smegmatis atpC*^{W16A}; W16A comp *atpC*, *M. smegmatis atpC*^{W16A} complemented with a wild-type copy of the gene encoding the ϵ subunit via the introduction of plasmid pMV262-*atpC*; wt overexpr *atpC*, wild-type *M. smegmatis* overexpressing wild-type ϵ protein via introduction of pMV262-*atpC*; OD₆₀₀, optical density at 600 nm. The experiments were carried out three times independently. Mean values with standard deviations are shown.

overlapping *atpC* fragments, lacking the original BamHI site, were amplified using the primer pairs Forward-BamHI (GGTGTGGA TCCGCTGATCTGAACG)/Internal-reverse (CTCCACGAGGA TTCGGACGGTCTC) and Internal-forward (GAGACCGTCCG AATCCTCGTGGAG)/Reverse-PstI (ATGGACGCTGCAGTCG ATGTGACTAG). Then, using these two fragments as the template, the entire (BamHI site-mutated) *atpC* coding sequence was amplified with primers Forward-BamHI and Reverse-PstI.

To further confirm the binding of bedaquiline to the ϵ subunit *in vivo*, we carried out an overexpression experiment. If the drug indeed inhibits F-ATP synthase in intact bacteria by binding to the ϵ subunit of the enzyme, increasing the intracellular target concentration, i.e., overexpression of the wild-type ϵ subunit in wild-type bacteria, is expected to result in reduced sensitivity, i.e., increased resistance to bedaquiline. Figure 1C shows that this is the case. Wild-type *M. smegmatis* carrying wild-type ϵ subunit-overexpressing plasmid pMV262-*atpC* showed a 2.5-fold increase in MIC₅₀, from 10 nM to 25 nM, compared to wild-type bacteria.

Taken together, these genetic results support a model in which bedaquiline inhibits mycobacterial F-ATP synthase via a novel second mechanism involving the enzyme's ϵ subunit in addition to binding to its c subunit. Our data suggest that the drug interacts with the ϵ subunit and that this binding occurs close to tryptophan 16 of the protein. The results further suggest that the replacement of the bulky side chain of tryptophan 16 with the smaller side chain of alanine allows for a tighter binding of the drug. In conclusion, we provide *in vivo* evidence for a second binding site of the new tuberculosis drug bedaquiline involving subunit ϵ of the mycobacterial F-ATP synthase. This information may be useful for the discovery of the next generation of diarylquinolines. In this context, it is important to note that in our previous biophysical *in vitro* and modeling studies (10), we proposed a detailed binding epitope involving in addition to tryptophan 16, characterized here genetically, arginine 37 and threonine 19 in the ϵ subunit and phenylalanine 50 in the c subunit. To begin to dissect the details of the second binding epitope of bedaquiline on the F-ATP synthase genetically, we replaced arginine 37 (CGG) in the ϵ subunit with

glycine (GGG) by recombineering. We were able to generate viable bacteria carrying the desired mutation, and the mutated strain had the same generation time as that of wild-type bacteria, i.e., it showed wild-type-like growth behavior. Interestingly, bedaquiline MIC determinations showed no shift in drug susceptibility in the mutant *M. smegmatis* strain in which arginine 37 was replaced by glycine (data not shown). There are several possible explanations why changing arginine 37 in the ϵ subunit had no effect on bedaquiline MIC. First, the proposed binding model suggesting an interaction with arginine 37 is not correct and may require modification. Alternatively, the proposed binding model may be correct; however, the contribution of arginine 37 to bedaquiline binding may not be detectable by genetic means, i.e., the interaction might be too weak to cause phenotypic consequences when interrupted. In general terms, genetic confirmation of a biochemically determined binding epitope may have limitations. A comprehensive mutational analysis in which all proposed interacting amino acid residues are exchanged for multiple different amino acids is in progress.

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S.K. and G.B. carried out the experiments. G.G. and T.D. wrote the manuscript.

We declare that we have no conflicts of interest.

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