Diarylquinolines Are Bactericidal for Dormant Mycobacteria as a Result of Disturbed ATP Homeostasis*§

An estimated one-third of the world population is latently infected with *Mycobacterium tuberculosis*. These nonreplicating, dormant bacilli are tolerant to conventional anti-tuberculosis drugs, such as isoniazid. We recently identified diarylquinoline R207910 (also called TMC207) as an inhibitor of ATP synthase with a remarkable activity against replicating mycobacteria. In the present study, we show that R207910 kills dormant bacilli as effectively as aerobically grown bacilli with the same target specificity. Despite a transcriptional down-regulation of the ATP synthase operon and significantly lower cellular ATP levels, we show that dormant mycobacteria do possess residual ATP synthase enzymatic activity. This activity is indispensable for the survival of dormant mycobacteria, making it a promising drug target to tackle dormant infections. The unique dual bactericidal activity of diarylquinolines on dormant as well as replicating bacterial subpopulations distinguishes them entirely from the current anti-tuberculosis drugs and underlines the potential of R207910 to shorten tuberculosis treatment.

*Mycobacterium tuberculosis* infection results in more than 2 million deaths per year and is the leading cause of mortality in people infected with HIV² (1). The global epidemic of tuberculosis (TB) is fuelled by co-infection of HIV patients with TB and a rise in multidrug-resistant TB strains (2). Despite the fact that TB control programs have been in place for decades, approximately one-third of the world population is latently infected with *M. tuberculosis*. Reactivation of latent TB is a high risk factor for disease development, particularly in immunocompromised individuals, such as HIV-infected patients. For global control of the TB epidemic, there is an urgent medical need for new drugs active against dormant or latent bacilli. These so-called sterilizing drugs would be able to shorten the current 6-month treatment duration for drug-susceptible TB and also offer new treatment opportunities for latent TB.

Tubercle bacilli enter lungs of healthy individuals by inhalation, where they are phagocytosed by the alveolar macrophages that eliminate most of the invading mycobacteria (3). However, a small proportion of bacilli survive and exist in a nonreplicating hypometabolic state, and these bacilli are tolerant to killing by bactericidal anti-TB drugs, such as isoniazid (4). They can linger in these altered physiological environments for an individual’s lifetime and maintain the capability of causing active TB after reactivation. The pathophysiological conditions in human lesions, thought to lead to persistence, are reduced oxygen tension, nutrient limitation, and acidic pH (5, 6).

Recently, we identified a new chemical class, diarylquinolines (DARQs) that demonstrate potent anti-mycobacterial activity on replicating bacilli both *in vitro* and *in vivo* (7, and the lead compound, R207910 (or TMC207), is currently in Phase IIb clinical trials for the treatment of patients with multidrug-resistant TB. R207910 acts by specifically targeting the membrane-bound c-subunit of F₁F₀-ATP synthase, the ATP-synthesizing machinery of the cell (8). During synthesis of ATP, the energy stored in the electrochemical proton gradient across the membrane is utilized to drive protons from the periplasmic space into the cytoplasm through the F₀ subunit and supplying a torque to the F₁ unit to convert ADP into ATP (9, 10). Under nonrespiratory conditions, in several bacteria, ATP synthases can function in the reverse direction, hydrolyzing ATP to ADP through ATPase activity and as such pumping protons from the cytoplasm into the periplasmic space (11). In this way, ATP synthase generates the membrane potential required for the uptake of nutrients. In dormant mycobacteria, a considerable remodeling of the respiratory chain has been reported, including down-regulation of cytochrome aa₃, type oxidase and up-regulation of the cytochrome bd type menaquinone oxidase (12, 13). Several studies also suggest that hypometabolic, nonreplicating mycobacteria have decreased requirements for ATP synthase, since the genes encoding the components of the ATP synthase operon are down-regulated, both *in vitro* and *in vivo* (12–14). It is, however, an open question whether ATP syn-
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that plays an essential role in dormant bacilli and whether the enzyme is responsible for ATP synthesis and/or maintenance of the membrane potential. Furthermore, it is not known whether, despite ATP synthase down-regulation, this enzyme can still be utilized as a drug target in dormant bacilli and whether DARQs are still able to effectively kill dormant bacilli.

Here we report that, despite a substantial down-regulation of the ATP synthase operon and reduced cellular ATP levels, DARQs effectively kill nonreplicating *M. tuberculosis*. We find that dormant mycobacteria have active and functional ATP synthase that is capable of synthesizing ATP, a scarce energy resource in a nonreplicating cell. The depletion of ATP in dormant mycobacteria by the R207910-mediated chemical inhibition of ATP synthase leads to potent bactericidal activity. Thus, targeting processes that generate ATP and concurrently disturb the cellular ATP homeostasis is an effective strategy against dormancy. R207910 exhibits no significant effect on the membrane potential in dormant or replicating mycobacteria, suggesting that ATP synthase is not critical for maintaining the membrane potential but is primarily used for the production of ATP.

We also demonstrate an increased susceptibility of dormant mycobacteria toward R207910 as compared with actively growing bacteria. Using R207910-resistant mycobacterial strains, we show that the drug specifically targets ATP synthase during dormancy. The unique dual bactericidal action of R207910 on dormant as well as actively replicating bacteria probably contributes to its remarkable sterilizing efficacy in mice, where R207910 as a monotherapy was shown to be as effective as the triple combination of rifampin, isoniazid, and pyrazinamide (7, 15). This may also explain the ability of this drug to render lungs of infected mice culture-negative faster than first line anti-TB drugs.

In conclusion, despite a number of changes in the energy metabolism during dormancy, the ATP production by ATP synthase remains essential for mycobacterial survival. The current study demonstrates the critical importance of ATP synthase in mycobacterial dormancy and suggests that the ATP synthase is an excellent target to treat latent TB infections.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Medium—** *M. tuberculosis* H37Rv, *Mycobacterium smegmatis*, and *Mycobacterium bovis* BCG were obtained from the American Type Tissue Culture collection. Mycobacteria were cultured in Middlebrook 7H9 medium (Difco) with 0.05% Tween-80 (Sigma) in log phase for a period of 3–4 days before the start of the experiment. The incubation was done in a 250-ml sterile Duran bottle with stirring conditions 37°C (500 rpm).

**Chemical Synthesis of DARQ Analogs—** DARQ compounds are synthesized as described in a earlier patent (WO2004/011436) and previous reports (7).

**MIC Microdilution Assays and Determination of Minimum Bactericidal Concentration (MBCg90) and Wayne Cidal Concentration (WCCg90)—** Minimum inhibitory concentration (MIC, µg/ml) of *M. tuberculosis* against several DARQ compounds was determined by using 7H9 liquid broth and 7H10 agar dilution as described in earlier studies (16) with minor modifications using Alamar blue or resazurin for fluorometric measurement (7). MBCg90 (µg/ml) was determined as the concentration where 90% of the aerobic grown bacteria were killed after 5 days of treatment by colony-forming unit (CFU) counting using 7H10 agar dilution according to Clinical and Laboratory Standards Institute guidelines. WCCg90 (µg/ml) was determined as the concentration where 90% of the dormant bacteria (grown in the Wayne model) were killed after 5 days of treatment by CFU counting using 7H10 agar dilution.

**Dormancy Assays—** Drastic oxygen depletion (hypoxia model) of mycobacterial cultures was achieved by placing loosely capped tubes containing different drug concentrations inside an anaerobic jar (BBL) along with anaerobic gas generation envelopes (using palladium catalysts), as described previously (17). After 7 days of anaerobiosis, the dormant cultures were harvested by low speed centrifugation, washed twice with 7H9 medium to remove the drugs, and resuspended in drug-free medium. The CFUs of the treated and untreated cultures were determined by plating on 7H10 agar to evaluate the bactericidal activity. In the Wayne model, gradual O2 depletion induces dormancy in mycobacterial cultures, as previously described (18). Briefly, after induction of dormancy for 18 days by gradual O2 depletion, cultures without exposure to oxygen were treated further with various drug concentrations for a period ranging from 4 to 21 days, and CFUs were counted on 7H10 plates. In the NO model, dormancy was obtained by incubating cultures with 150 µM of a NO donor (diethylenetriamine/nitric oxide (DETA/NO)) for 2 h, followed by treatment with several compounds for 10 h as previously described (19).

**RNA Isolation and Real Time Quantitative PCR—** Dormant and aerobically grown bacterial pellets were resuspended in Trizol and disrupted with acid-washed glass beads in the Mixer Mill (MM 301). Total RNA was isolated as previously described (20) and cleaned up with the RNeasy Protect Bacteria minikit (Qiagen). Reverse transcription was carried out from 0.5 µg of total RNA using random hexamer primers and SuperScript II RT as described by the manufacturer (Invitrogen). The sequence of the TaqMan FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-N,N,N′,N′-tetr methylrhodamine) probes (Eurogentec) and primers for the mycobacterial genes analyzed in this study are available upon request. Real time quantitative PCR was carried out using the ABI PRISM 7900HT sequence detection system (Applied Biosystems) as described by the manufacturer. Amplification of endogenous 16 S ribosomal RNA was performed to standardize the amount of cDNA sample added to each reaction.

**Total Cellular ATP Measurement Assay—** ATP levels of log phase aerobic and dormant cultures of *M. tuberculosis* were measured using the ATP bioluminescence assay kit (Roche Applied Science), as previously described (8). ATP levels were followed during dormancy (Wayne model) from day 0 to day 21. Additionally, *M. tuberculosis* cultivated under Wayne dormancy conditions for 18 days were treated with R207910 (0.006–6 µg/ml), isoniazid (0.3 µg/ml), and dicyclohexylcarbodiimide (DCCD) (100 µg/ml) for 4 days, and ATP levels were measured.

**Measurement of ATP Synthesis Activity—** ATP synthesis of dormant *M. smegmatis* grown under Wayne conditions for 10
days was measured as described previously (8). Briefly, inverted membrane vesicles were prepared using a precooled French pressure cell at 20,000 p.s.i. The membrane vesicles were preincubated with R207910 or DCCD under stirring conditions at room temperature for 10 min. The ATP synthesis activity was determined by energizing the membranes with NADH and quantifying the amount of ATP produced using the luciferin/luciferase system (ATP Bioluminescence Assay Kit HS II; Roche Applied Science). Data are presented as averages ± S.E.

**Measurement of the Membrane Potential**—Membrane potential was measured using tetra-[3H]phenylphosphonium bromide (Amersham Biosciences) in aerobic and Wayne dormant (10 days) *M. smegmatis*, as previously described (21). Briefly, cells were energized with 20 mM glucose, tetra-[3H]phenylphosphonium bromide was added, and cells were centrifuged through silicon oil. Supernatant and cell pellet were dissolved in scintillation fluid and counted with a liquid scintillation analyzer (TRI-CARB 2100TR; Packard). Membrane potential (in mV) was calculated according to the Nernst relationship. Values represent averages ± S.E. of four independent experiments. Comparisons were performed using Student’s *t* test (StatXact). *p* values of <0.05 were considered as statistically significant.

**RESULTS**

**Bactericidal Activity of R207910 on Dormant M. tuberculosis**—The effect of DARQ lead compound, R207910, on the growth of nonreplicating *M. tuberculosis* was tested in three different *in vitro* dormancy models. As a control for target specificity during dormant conditions, we tested the efficacy of R207910 on the R207910-resistant strain of *M. tuberculosis* (BK12), carrying a point mutation in the α-helix of the c-subunit of the ATP synthase complex (7). Both wild type and mutant strains were subjected to dormancy using either gradual (Wayne) or drastic (hypoxia) O2 depletion or NO treatment in the presence of 0.1 and 10 μg/ml R207910 (Fig. 1).

We treated Wayne dormant *M. tuberculosis* for 7 days with R207910 to determine its killing efficacy. As can be seen in Fig. 1A, R207910 at 10 μg/ml leads to a 1.8-log10 reduction in CFU counts, whereas no apparent effect was observed at 0.1 μg/ml. The viability of dormant bacilli was not affected by isoniazid treatment, an inhibitor of biosynthesis of cell wall mycolic acids. In contrast, metronidazole at 100 μg/ml led to a 1.9-log10 reduction in CFUs as compared with untreated control. Metronidazole is a prodrug that is effective only under anaerobic conditions. However, it has been shown to be ineffective against latent mycobacteria in a Cornell mouse infection model (22). DCCD, a nonspecific ATP synthase inhibitor, showed a drastic killing effect at 100 μg/ml (Fig. 1A).

In the hypoxia model, *M. tuberculosis* cultures were subjected to drastic O2 depletion in an anaerobic chamber. R207910 reduced the mycobacterial viability by 2.1-log10 CFUs at 10 μg/ml but had no apparent effect at 0.1 μg/ml (Fig. 1B). Metronidazole tends to be slightly more potent than in the Wayne model, leading to a 2.5-log10 CFU reduction at 100 μg/ml. This can be explained by the extreme hypoxic conditions created in the anaerobic chamber, and as such, the effects of metronidazole, which works best in a severely O2-depleted environment, tend to be enhanced.

NO has been shown to inhibit aerobic respiration in several bacterial systems, and it induces a dormancy response in mycobacteria similar to oxygen depletion (19). Treatment of midlog

![FIGURE 1. Effect of R207910 on wild type and resistant BK12 M. tuberculosis grown under oxygen depletion or NO stress.](image)
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TABLE 1
Comparative killing activity of TB compounds (rifampicin, isoniazid, and R207910) for actively replicating and dormant M. tuberculosis

Killing was represented as MBC\_90 for actively replicating and WCC\_90 for dormant bacilli. The WCC\_90/MBC\_90 ratio reflects the comparative killing efficiency. R207910 is slightly more potent toward dormant bacteria with a low WCC\_90/MBC\_90 ratio as compared with rifampicin and isoniazid. Isoniazid has no detectable activity on dormant bacillus, and rifampicin is significantly less active on dormant as compared to actively replicating bacilli.

| Compound  | MBC\_90 (µg/ml) | WCC\_90 (µg/ml) | WCC\_90/MBC\_90 |
|-----------|-----------------|-----------------|-----------------|
| Rifampicin| 0.03            | 0.5             | 17              |
| Isoniazid | 0.25            | >64             | >256            |
| R207910   | 2               | 1               | 0.5             |

Figure 2. Comparative R207910 killing kinetics for aerobically grown and dormant M. tuberculosis. R207910 was tested against dormant (Wayne) and aerobically grown cultures of M. tuberculosis. The dormant and aerobic cultures, with equal start A\_600 values or comparable starting CFUs (10^8 to 10^9 CFU/ml), were treated with 10 µg/ml R207910 or 10 µg/ml isoniazid. Killing was monitored by measuring CFU/ml after 1, 4, 7, and 14 days on 7H10 plates and plotted as log_{10} CFU reduction, calculated against nontreated controls. Note that for dormant mycobacteria treated with R207910 (10 µg/ml), the CFU counts were below the detection limit at 14 days (10^2 CFU/ml).

To further evaluate this result, we measured killing kinetics of R207910 and isoniazid on Wayne dormant and aerobically grown bacilli (Fig. 2). For this purpose, both cultures were diluted to equal A\_600 values or ~10^8 to 10^9 CFU/ml and treated with R207910 (10 µg/ml). For aerobic cultures, we observed an initial 2.2-log_{10} drop in CFUs by day 14 and no further reduction by day 21 (Fig. S1). As expected, isoniazid had no effect on dormant bacterial cultures. However, in aerobic cultures, isoniazid killed about 2.2 log_{10} during the first week, with no additional killing during the second week.

R207910-mediated killing of dormant bacilli at a 10 µg/ml concentration was highly potent, since no bacteria could be counted by day 14 (Fig. 2). In contrast, the killing kinetics of R207910 at 1 µg/ml on Wayne dormant M. tuberculosis suggested a characteristic biphasic killing with a 2.9-log_{10} drop in CFUs by day 14 and no further reduction by day 21 (Fig. S1).

In summary, by measuring the bactericidal concentrations as well as killing kinetics, we demonstrated that R207910 kills dormant M. tuberculosis at least as effectively as actively replicating bacilli. This makes R207910 the only TB drug with equal bactericidal activities on different bacterial subpopulations.

Specificity of DARQ Activity in Dormant Mycobacteria—Target specificity of DARQs was assessed by introducing chemical changes in the DARQ scaffold. During dormancy, bacteria might have alternative means of energy or ATP production (e.g., substrate level phosphorylation), and as such, ATP synthase might be redundant. Hence, it is postulated that R207910 might affect other cellular targets as well (23). At first, we studied the correlation between various structural and chemical changes in R207910 and its analogs versus their effect on biological activity. This leads to the determination of a structure-activity relationship. Over 400 DARQ derivatives were prepared and tested on actively replicating and aerobically grown M. tuberculosis cultures in in vitro MIC determination assays (data not shown). Five compounds (Fig. S2A) with MICs between 0.09 and 20 µg/ml were selected for evaluating their bactericidal effect on Wayne dormant M. tuberculosis. R207910 at 1 and 10 µg/ml led to 1.3- and 2.1-log_{10} CFU reduction, respectively, as compared with untreated dormant control cultures (Table 2). As can be seen in Table 2, an analogue with a methyl substituent on the first aryl moiety of R207910 (named 18040971-AAA) retained
explored the effect of dormancy on the ATP synthase operon in M. tuberculosis (12–14). We investigated transcriptional up-regulation of the dormancy regulon in the three metabolic pathways that lead to the transcriptional reprogramming of dormant mycobacteria. This increased susceptibility is observed despite the transcriptional down-regulation of the ATP synthase operon in dormant mycobacteria. In order to show that ATP synthase still has the residual functional activity during dormancy, we measured synthesis of ATP by the ATP synthase enzyme in membrane vesicles isolated from dormant mycobacteria. This demonstrates high susceptibility of dormant mycobacteria to lower concentrations of R207910.

### Table 2

| Compound ID | MIC$_{90}$/µg/ml | Log$_{10}$ reduction (dormant cultures) |
|-------------|-----------------|----------------------------------------|
|             |                 | 10 µg/ml | 1 µg/ml |
| R207910     | 0.09            | 2.1     | 1.35    |
| 18040971-AAA| 0.16            | 2.13    | 1.55    |
| 17996134-AAA| 1.74            | 1.14    | 0.87    |
| 25756770-AAA| 8.19            | 0.98    | 0.29    |
| 18086601-AAA| 20.21           | 0.23    | 0.11    |

Different DARQ analogs were tested on M. tuberculosis cultures grown in aerobic conditions or in the Wayne oxygen depletion model. MIC$_{90}$ values on aerobically grown bacilli were compared with the bactericidal activity (log$_{10}$ reduction as measured against untreated control) on dormant bacteria in 7H10 agar. The bactericidal activity was evaluated at concentrations of 1 and 10 µg/ml. Each value is the mean of a minimum of two different experimental values, and each experiment was repeated at least two times. A strong correlation between structural variations of DARQs and their biological effects against replicating and dormant bacilli is observed.

its bactericidal activity on dormant bacteria with 1.55-log$_{10}$ killing at 1 µg/ml. However, replacement of the dimethylamino group by azole groups (25756770-AAA) led to a decrease in the activity on both aerobic (MIC 8.19 µg/ml and dormant bacilli (0.29-log$_{10}$ CFU reduction). Interestingly, changing the chain length in R207910 between the hydroxyl and the terminal dimethylamino group (18086601-AAA) also decreased activity on both aerobic (MIC 20.21 µg/ml) as well as dormant bacilli (0.11-log$_{10}$ CFU reduction). These data suggest a strong correlation between structural variations of the DARQ scaffold and the subsequent effect on their activities against replicating and dormant bacilli (Fig. S2A). The sharp overlap between the bactericidal activities of DARQ analogs on dormant and replicating bacilli implies that their effects are highly target-specific, irrespective of mycobacterial growth conditions and physiological states.

In a second approach to characterize the target specificity of R207910 during dormancy, we tested the efficacy of R207910 on the resistant strain of M. tuberculosis (BK12) bearing a mutation in c-subunit of ATP synthase (7). At the highest concentration of 10 µg/ml, R207910 had no effect on this mutant strain in Wayne conditions (Fig. 1A), but NO (Fig. 1B) and hypoxia (Fig. 1C) conditions. Isoniazid had no effect under these conditions, suggesting that bacilli were indeed in a nonreplicating state. The ATP synthase inhibitor DCCD had a drastic killing effect and led to a 5.3-, 2.8-, and 4.3-log$_{10}$ drop in CFUs in Wayne (Fig. 1A, hypoxia (Fig. 1B), and NO (Fig. 1C), respectively. The lack of cross-resistance between these two compounds suggests that although DCCD binds to the same target as R207910, its binding pocket is different. In summary, our results demonstrate that killing of mycobacteria by DARQs critically depends on the structure of the DARQ compound as well as critical amino acid compositions in the target protein.

**ATP Synthesis in Nonreplicating Mycobacteria**—The dormancy physiological phase involves changes in several metabolic pathways that lead to the transcriptional reprogramming of different bacterial genes. In a first step, we confirmed transcriptional up-regulation of the dormancy regulon in the three dormancy models, as previously reported (12–14). We explored the effect of dormancy on the ATP synthase operon under O$_2$ depletion and NO stress conditions by real time quantitative PCR for atpA to atpH. In M. tuberculosis, the ATP synthase enzyme complex can have a bimodal function: synthesis of ATP and generation of a membrane potential.

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**R207910 or TMC207 and Dormant Mycobacteria**

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DISCUSSION

Treatment of active TB in humans by a regimen combining four drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) leads to rapid killing of the bacteria in the sputum during the first 2 months (24). However, relapses occur if the therapy is not continued further for 4 months to kill the remaining bacilli. These bacilli are likely to be located in caseous regions with limited supply of O2 and nutrients and exhibit a dormant phenotype. Penetration of drugs into these microenvironments tends to be restricted, and these bacteria seem to be tolerant to antimycobacterial drugs, such as isoniazid (25). Therefore, there is an urgent need for the development of new drugs that specifically target dormant bacilli.

Our study demonstrates that DARQ lead compound R207910 effectively killed dormant mycobacteria in all three in vitro dormancy model systems used that mimic the microenvironment encountered by mycobacteria within granulomas in humans. Surprisingly, we observed a higher susceptibility of dormant bacilli to DARQs as compared with aerobically grown cultures, a unique property that distinguishes R207910 from presently used first and second line anti-TB drugs, such as rifampicin (WCC90/MBC90 of 17). This dual action of DARQs might explain why the combination of R207910 with the standard of care regimen (rifampin, isoniazid, and pyrazinamide) led to a 50% faster reduction of CFU counts than the control regimen (7, 15). The sterilizing efficacy of R207910 in mice, involving measurement of relapse rates 3 months after cessation of treatment, demonstrated that R207910 reduces the number of relapses compared with the standard of care combination therapy (26). Moreover, treatment of Guinea pigs with R207910 resulted in an almost complete eradication of M. tuberculosis throughout both primary and secondary lesions in the lung granulomas after 6 weeks of treatment (27).

In correlation with transcriptional down-regulation of ATP synthase, the ATP levels are also significantly decreased in dormant mycobacteria, suggesting an energy adaptation for this new physiological state. It has already been suggested that mycobacterial isolates from human lesions use alternative energy pathways, since several genes responsible for the lipid metabolism are up-regulated (28). Considering the bactericidal action of R207910 on dormant M. tuberculosis, it is likely that nonreplicating mycobacteria still need minimal amounts of ATP to survive, provided by a residual activity of ATP synthase. This suggests that dormant mycobacteria can be more prone to alterations in cellular ATP pools due to inhibition of ATP synthase as compared with actively growing bacilli. Our data thus provide a nice example of a down-regulated enzyme acting as a highly efficient target, whereas generally enzymes that are up-regulated during dormancy were considered to be promising targets for latent TB.

Despite the low ATP levels and a reduced expression of ATP synthase, dormant bacteria still need to maintain a membrane potential for cellular viability. We tested the effect of R207910 on membrane potential of Wayne dormant M. smegmatis. It was seen that dormant bacilli are able to maintain an energized membrane or membrane potential (−135 mV), albeit at slightly lower levels in comparison with aerobically grown bacilli (−180 mV) (p < 0.001) (Fig. 4B). The presence of R207910 had no significant effect on this membrane potential both in aerobic and in dormant M. smegmatis (data not shown). This suggests that ATP synthase is not required for the generation of the membrane potential by pumping protons in the periplasm.

Taken together, dormant mycobacteria have functional ATP synthase: low but critically needed ATP levels and an energized membrane. Hence, dormant bacteria are not metabolically or physiologically inert but still operate some key cellular processes.

FIGURE 3. Decreased requirement of ATP synthesis in nonreplicating mycobacteria. A, real time quantitative PCR (RT-qPCR) was performed on cDNA generated from total RNA extracted from M. tuberculosis exposed to various dormancy conditions. The change in gene expression was calculated by normalizing Ct values of individual gene transcripts against Ct values of 16S rRNA. Normalized values were compared with control values from M. tuberculosis cultures grown under aerobic conditions, and fold change was calculated. Each value is the mean of two different experiments with S.D. of less than 10%. B, intracellular ATP levels and atpE mRNA expression in M. tuberculosis at different time points (up to 21 days) in Wayne Og depletion conditions. The inset represents the differences in ATP levels (nmol/mg) of dormant M. tuberculosis compared with aerobic culture. C, effect of treatment of R207910, isoniazid, and DCCD (4 days) on ATP levels in M. tuberculosis cultivated under Wayne dormancy conditions for 18 days.

FIGURE 4. ATP synthase is functional in dormant M. smegmatis. A, ATP synthesis activity in inverted membrane vesicles (50 μg/ml) from dormant M. smegmatis cultured for 10 days under Wayne O2 depletion conditions. Positive control was membrane vesicles with buffer containing ADP, P, and NADH, whereas negative control excluded NADH and P, from the assay buffer. B, effect of R207910 on membrane potential in dormant M. smegmatis cultured for 10 days under Wayne O2 depletion conditions. The membrane potential was measured using tetra-H[3H]phenylphosphonium bromide.
For dormant mycobacteria, a role for ATP synthase in synthesizing ATP and/or maintaining the membrane potential (by hydrolyzing ATP and pumping protons across the membrane) has been proposed (29). DARQs potently inhibited ATP synthesis activity but even at high concentrations had no significant effect on the membrane potential. This is further supported by the previous finding that the purified ATP synthase of Mycobacterium phlei appears to have only very low or latent ATP hydrolysis activity (30). It has been suggested that for restoring the redox balance and proton motive force during dormant conditions, the nitrate reductase complex, an alternative respiratory enzyme, can generate a proton motive force during dormant growth conditions (31). Furthermore, under low O₂ conditions, there are several changes in the respiratory chain complexes in mycobacteria, including up-regulation of cytochrome _bd_-type menaquinol oxidase that has a higher oxygen affinity and a non-proton-translocating NADH dehydrogenase (type II) that is required for oxidation of NADH within the cell (28).

In conclusion, our study shows that despite its down-regulation during dormancy, ATP synthase plays an essential role for mycobacterial survival and thus can serve as an ideal drug target for killing dormant as well as actively replicating bacteria. Unraveling the principles of energy metabolism during dormancy and understanding the mechanisms of antibiotic action can reveal critical weaknesses of dormant and persistent bacterial species. In _streptococci_, ATP synthase was also found to be differentially regulated in biofilm formation (32). The down-regulation of _F₁F₀-ATPase_ might make these bacteria more susceptible to changes in cellular ATP pools. Thus, validating ATP synthase as a target might also be useful to treat other persistent bacterial infections.

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