Antimicrobial activity against *Mycobacterium tuberculosis* under *in vitro* lipid-rich dormancy conditions

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**Abstract**

Although tuberculosis treatment is dependent on drug-susceptibility testing (DST) and molecular drug-resistance detection, treatment failure and relapse remain a challenge. This could be partially due to the emergence of antibiotic-tolerant dormant mycobacteria, where host lipids have been shown to play an important role. This study evaluated the susceptibility of *Mycobacterium tuberculosis* to two antibiotic combinations – rifampicin, moxifloxacin, amikacin and metronidazole (RIF-MXF-AMK-MTZ), and rifampicin, moxifloxacin, amikacin and pretomanid (RIF-MXF-AMK-PA) – in a lipid-rich dormancy model. Although their effectiveness in *in vitro* cultures with dextrose as a carbon source has been proved, we observed that none of the antibiotic mixtures were bactericidal in the presence of lipids. The presence of lipids may confer tolerance to *M. tuberculosis* against the mixture of antibiotics tested and such tolerance could be even higher during the dormant stages. The implementation of lipids in DST on clinical isolates could potentially lead to a better treatment strategy.

Tuberculosis (TB) is now the leading cause of death worldwide among infectious diseases. This is due in part to the emergence of drug resistance in *Mycobacterium tuberculosis*, which results in treatment failures and relapses, especially in immunocompromised patients [1]. Although TB treatment is dependent on the implementation of drug-susceptibility testing (DST), together with molecular detection of drug resistance-associated gene mutations, drug tolerance is present in emerging dormant mycobacteria, making the complete sterilization of the bacterial population in the infected host more difficult. Dormant mycobacteria have low metabolic activity, are drug tolerant and originate in the granuloma, and some can be released in the sputum, although they are normally nonculturable by conventional laboratory techniques [2]. It has been shown that lipids have an important role during the survival and persistence of dormant mycobacteria [3, 4]. *M. tuberculosis* influences the host lipid homeostasis in order to create a lipid-rich environment inside the granuloma. This environment can be evidenced in sputum samples, which mainly contain cholesterol, palmitic, stearic and oleic acids [3]. It has been proposed that *M. tuberculosis* uses lipids as carbon and energy sources, rather than dextrose, during dormancy [5]. Nevertheless, the influence of host lipids in the transition of *M. tuberculosis* to its dormant drug-tolerant form has been poorly studied. Since *in vitro* models that mimic *in vivo* conditions better are needed, we studied the drug susceptibility of *M. tuberculosis* in a lipid-rich hypoxia dormancy model, first proposed by Wayne and Hayes and originally containing dextrose as the carbon source [6]. In this model, the authors described two populations of *M. tuberculosis* during its adaptation to dormancy, with these being termed the nonreplicating persistence 1 and 2 (NRP1 and NRP2) stages of dormancy. They can be distinguished from aerobic cultures of *M. tuberculosis* by their low metabolic activity and drug tolerance profile, and the length of the bacilli. Wayne and Hayes also reported that the shift from aerobic cultures into the NRP1 stage occurred when the declining level of dissolved O₂ approached 1% of the original saturation level, while for the NRP2 stage the dissolved O₂ content dropped below about 0.06% of the original saturation level. The length of the bacilli increased from a median length of 2.23 μm during active aerobic growth to 2.94 μm during both stages of dormancy. Moreover, the dormant population became resistant to isoniazid, ciprofloxacin and rifampicin while being susceptible to...
metronidazole [6]. Using this model, we evaluated the susceptibility of \textit{M. tuberculosis} to two anti-TB drug combinations: (1), rifampicin, moxifloxacin, amikacin and metronidazole, and (2), rifampicin, moxifloxacin, amikacin and pretomanid. Their sterilizing effect has previously been demonstrated among 12 anti-TB drugs, which were evaluated individually and in combination, using dextrose as the carbon source, against aerobic and hypoxic acidic cultures of \textit{M. tuberculosis} H37Rv [7, 8].

\textit{M. tuberculosis} H37Rv was cultured in Dubos broth (Difco), without glycerol, containing 0.5% albumin (Sigma Fraction V) supplemented with a lipid mixture (oleic acid, palmitic acid and stearic acid, at a final concentration of 0.001% each, plus 0.01% cholesterol), as previously described [5]. Briefly, \textit{M. tuberculosis} was grown to the exponential and stationary stages of aerobic growth (containing \(2.4 \times 10^6 \pm 9 \times 10^5 \) c.f.u. ml\(^{-1}\) at day 7 of incubation and \(2.9 \times 10^5 \pm 2 \times 10^5 \) c.f.u. ml\(^{-1}\) at day 15 of incubation, respectively), as well as the NRPy and NRPr stages of dormancy (containing \(2.7 \times 10^6 \pm 8 \times 10^5 \) c.f.u. ml\(^{-1}\) at day 8 of hypoxia and \(8.1 \times 10^5 \pm 2 \times 10^5 \) c.f.u. ml\(^{-1}\) at day 29 of hypoxia, respectively). Hypoxic cultures were prepared from pre-cultures at the exponential stage of aerobic growth, and parallel cultures, supplemented with methylene blue (1.5 mg ml\(^{-1}\)), were used as an indicator of oxygen depletion. Once \textit{M. tuberculosis} reached each experimental stage, the antibiotic mixture rifampicin/moxifloxacin/amikacin/metronidazole or rifampicin/moxifloxacin/amikacin/pretomanid, was added. Rifampicin (Sigma Chemicals, USA), moxifloxacin (Bayer, Mexico), amikacin (Sigma Chemicals, USA), metronidazole (Sigma Chemicals, USA) and pretomanid (AdooQ BioScience, USA) were used at their maximum serum concentration of 8, 4, 8, 8 and 2 \(\mu\)g ml\(^{-1}\), respectively [8]. The antibiotic activity was evaluated at 7, 14 and 21 days by estimating survival and mortality by counting c.f.u.s in Middlebrook 7H10 agar with ADC enrichment and the most probable number (MPN) in Middlebrook 7H9 broth supplemented with ADC as described by the US Food and Drug Administration (FDA) [9]. Two biological replicates for every mycobacterial stage and three technical replicates for survival measurements were performed. Standard culture conditions, using Dubos broth (Difco), without glycerol, containing 0.5% albumin (Sigma Fraction V), supplemented with 0.2% dextrose, were used as a control to validate the reported efficiency of the drug combinations.

Fig. 1 shows the rate of killing of \textit{M. tuberculosis} for both antibiotic mixtures over 21 days in solid media. As demonstrated before, panel (a) shows that \textit{M. tuberculosis} was killed by day 21 in the presence of dextrose. By contrast, the upper charts of panel (b) show remarkable \textit{M. tuberculosis} survival in the presence of lipids. However, the killing curves for lipid aerobic cultures shown in the lower charts of panel (b) (exponential and stationary stages) show that the \textit{M. tuberculosis} population declined rapidly after 7 days, with a rate of killing of 84 to 96%, with this finally reaching 100% at day 21. Interestingly, the same mixtures of antibiotics were less effective at the hypoxic stages, with higher \textit{M. tuberculosis} survival and a rate of killing of only 49 to 65% at day 7 for the NRPy stage and one of 59 to 98% for the NRPr stage. Nevertheless, both hypoxic stages also showed a rate of killing of 99 to 100% by day 21. Moreover, the results in liquid media proved that the \textit{M. tuberculosis} population did not decrease over 21 days when survival was measured under lipid conditions for any drug combination. Instead, the counting reached up to \(>1100\) MPN ml\(^{-1}\) over 21 days of drug addition for every stage of growth with both drug combinations (data not shown). These results indicate that while \textit{M. tuberculosis} growth was arrested in the presence of lipids with the mixture of drugs, and it stopped growing on solid media by day 21, it was able to regrow in aerobic cultures in liquid media. This is probably explained by the availability of nutrients in the solid media. Despite the documented effectiveness of the selected drug combinations against \textit{M. tuberculosis} in \textit{in vitro} cultures with dextrose as the carbon source, we observed that none of the antibiotic mixtures were bactericidal in the presence of lipids.

The drugs used in this study, rifampicin, moxifloxacin, amikacin and pretomanid, are mainly used for drug-resistant TB, while rifampicin is also used as first-line drug against drug-susceptible isolates and metronidazole has been reported to have a potent \textit{in vitro} effect against dormant \textit{M. tuberculosis} under hypoxia, even higher than most commonly used drugs in TB regimens [6, 10]. Its implementation in clinical trials was nevertheless stopped due to the excessive toxicological side-effects that occur when it is used for long periods [11]. Furthermore, during the intensive search for new TB drugs or synergic drug combinations that better eliminate dormant \textit{M. tuberculosis}, Piccaro \textit{et al}. have reported on DST using 12 anti-TB drugs, individually and in combination, under aerobic and hypoxic conditions. They determined that the best synergism was given by the combinations rifampicin/moxifloxacin/amikacin/metronidazole and rifampicin/moxifloxacin/amikacin/pretomanid [7, 8]. Although these combinations are not used in current TB regimens, they are predicted to eliminate dormant \textit{M. tuberculosis} under \textit{in vitro} conditions. In our study, we confirmed this prediction, but only when dextrose was used as the carbon source, supporting the hypothesis that shifting the carbon source of a pathogen also changes its drug-susceptibility profile [12]. We propose that a population of drug-tolerant \textit{M. tuberculosis} emerged due to the presence of lipids, and that this drug tolerance was even higher under hypoxic conditions. These results support the hypothesis that treatment failure and relapses could potentially be associated with sub-optimal DST techniques (using different carbon sources), which in turn could lead to selection of incorrect or less efficient drug regimens. A recent study reinforced this hypothesis when implementing host-mimicking media in DST against clinical strains of drug-resistant \textit{Staphylococcus} and virulent Gram-negative pathogens – their results differed greatly from those obtained by conventional DST [13]. These reports, taken together with our results, indicate the importance of considering models that better mimic the microenvironment of \textit{M. tuberculosis} during DST. To better assess this,
we must understand the conditions that catalyze the transition from drug-susceptible to dormant and drug-tolerant mycobacteria. Dormancy is induced through several stress conditions [3], but what makes DST more complex for *M. tuberculosis* is the diversity of the dormant population. Not all dormant bacteria are drug tolerant [14] while there is a very heterogeneous set that survive drug treatment, namely the bacterial persisters [15–18]. In some cases, reactivated mycobacteria adopt a resistant phenotype without genetic modifications and mathematical models have predicted that antibiotic tolerance facilitates the appearance of drug resistance [19].

![Fig. 1. Survival of *M. tuberculosis* H37Rv in solid media after exposure to RIF/MXF/AMK/MTZ and RIF/MXF/AMK/PA combinations. (a) Drug-susceptibility testing (DST) in the presence of dextrose as a carbon source, showing the c.f.u. ml⁻¹ obtained from every stage of growth at four time-points (0, 7, 14 and 21 days) after the addition of the drug mixtures. (b) DST in the presence of lipids. The upper charts show the c.f.u. ml⁻¹ as described for (a). The lower charts in (b) show the percentage of survival of *M. tuberculosis* in the presence of lipids, with 100 % representing the total population per stage prior to the addition of antibiotics (day 0). Black bars and the blue lines indicate the exponential (Exp) stage of growth; grey bars and green lines indicate the stationary (Stat) stage; black-and-white striped bars and red lines indicate the NRP1 stage; white bars and yellow lines indicate the NRP2 stage of dormancy. The mean±SD of survival was charted.](image-url)
Drug tolerance, relapses and treatment failures are not restricted to TB, but could also be important in dealing with non-tuberculous mycobacteria and other persistent pathogens. There are several reports in which drug combinations are tested on persistent mycobacteria with variable results [20]. The implementation of better host-mimicking environments in DST could lead to more valuable and reliable results. The results of this study still need to be confirmed with clinical M. tuberculosis isolates; however, they offer guidance on possible variations of DST results that could influence the selection of more appropriate treatment regimens for TB. In summary, we have shown for the first time, to the best of our knowledge, that the presence of lipids, such as cholesterol and long-chain fatty acids, as carbon sources, may confer some tolerance against the tested antibiotics on M. tuberculosis, and that this tolerance could be even higher during the dormant stages. The implementation of DST in clinical strains that include lipids as a carbon source could potentially lead to better treatment strategies and better culture conditions for the development of new drugs.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


