Activity of drug combinations against dormant *Mycobacterium tuberculosis*

Running title: Drug combinations against dormant *M. tuberculosis*

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Abstract

Aerobic (5-day-old cultures) and nonreplicating (dormant) *Mycobacterium tuberculosis* (5-, 12-, 19-day-old cultures) were treated with rifampin (R), moxifloxacin (MX), metronidazole (MZ), amikacin (AK), capreomicin (CP) for 7, 14, and 21 days. R-MX-MZ-AK and R-MX-MZ-CP killed both aerobic and dormant bacilli in 21 days, as shown by lack of re-growth in solid and liquid media. R-MX-MZ-AK and R-MX-MZ-CP caused also a strong decrease of nonreplicating bacilli in 7 days in a cell-based dormancy model.
In people with latent tuberculosis (TB), which are estimated to be one third of the world population, *Mycobacterium tuberculosis* (Mtb) is presumed to lie in a nonreplicating (dormant) state in caseous lesions of the lungs with little access to oxygen (2, 13), or in extrapulmonary sites containing adipose tissue (10). Nonreplicating Mtb may be obtained by adaptation of replicating cultures to hypoxia through the self-generated formation of an oxygen gradient (Wayne model) (13-14), or inside adipocytes (10). Dormant Mtb is insensitive to isoniazid (3, 10, 13), but some inhibition is exerted by rifampin (R) (3, 10, 13), moxifloxacin (MX) (3), amikacin (AK) (3), capreomycin (CP) (3, 5), metronidazole (MZ) (3, 13). Few studies investigated the activity of drug combinations against nonreplicating Mtb (8, 12). Here, we report the effect of R, MX, AK, CP and MZ, alone and in combination, against dormant bacilli in the Wayne model and inside adipocytes.

Mtb strain H37Rv was grown in tubes containing Dubos Tween-Albumin (DTA) broth inoculated with about $1 \times 10^6$ CFU/ml (8, 13). Aerobic (A), replicating, populations were obtained by incubating the tubes at 37°C with loosened screw-caps for 5 days (A5). For preparation of hypoxic (H), nonreplicating bacilli, tight-fitting rubber caps were put under the screw-caps, and the tubes were incubated for 5, 12 and 19 days (H5, H12, H19, respectively). Control tubes with 1.5 μg/ml methylene blue as an indicator of oxygen depletion were added in each experiment (8, 13). To determine drug activity, R, MX, MZ, AK, CP (8, 4, 8, 8, 20 μg/ml, respectively, corresponding to their $C_{\text{max}}$) were added to A5 cultures ($2\pm1 \times 10^7$ CFU/ml) and, by syringe, to H5, H12, H19 cultures ($4.2\pm2$, 2.6±1.3 and 2.1±0.4, x $10^7$ CFU/ml, respectively). After 7, 14 and 21 days, 1 ml of A or H cultures were washed and resuspended in 1 ml of DTA broth, and 0.2 ml were inoculated in Middlebrook 7H10 agar (Difco) plates for CFU determination, and in liquid medium
(BACTEC MGIT 960 system; Becton Dickinson, Sparks, MD) for determination of the days to reach a growth unit of \( \geq 75 \) (days to positivity, DTP). Mtb killing was defined as lack of re-growth in MGIT tubes after \( >100 \) days (DTP \( >100 \) days).

Among single drugs, MX and R were particularly active to decrease A5 and H19 CFUs, with the highest activity being shown by MX against A5 and H5 bacilli and by R against H12 and H19 bacilli \((P<0.05, \text{versus control, Student } t \text{ test})\), respectively, after 7, 14 and 21 days (Fig. 1). Their combination R-MX was active against A5, H5, H12 and H19 bacilli, with \(<2 \log_{10} \text{CFU/ml} \) remaining after \( \geq 14 \) days. After 21 days of exposure to R-MX, R-MX-MZ, R-MX-AK, R-MX-MZ-AK, R-MX-CP and R-MX-MZ-CP, no CFUs of A5, H5, H12 and H19 populations were observed.

Since dormant Mtb may not form colonies on agar \((2, 8)\), the samples for which results are shown in Fig. 1 were also inoculated in liquid medium (MGIT 960 tubes) in order to provide a more sensitive viability test (Fig. 2A to N). No single drug killed A5, H5, H12 and H19 bacilli after 7, 14 and 21 days, as shown by re-growth in MGIT tubes (DTP: \( \leq 19 \) days). Again, MX and R were the most effective, with the highest activity being shown by MX against A5 and H5 populations \((P<0.05, \text{versus control})\) (Fig. 2A to C) and by R against H12 and H19 populations \((P<0.05, \text{versus control})\), respectively. As expected, MZ was ineffective against A5 bacilli but its activity increased from H5 to H19 cells after 14 and 21 days of exposure \((P<0.05, \text{versus control})\). A lower activity was shown by AK and CP (Fig 2G to N).

Among two-drug combinations, R-MX was more active than R or MX alone against A5, H5, H12 and H19 bacilli but did not kill them (DTP: 15-27 days) (Fig. 2D to E). Among two-drug combinations, R-MX was more active than R against A5 and H5 bacilli after 14 and 21 days of exposure \((P<0.05)\), and than MX against H12 and
H19 bacilli after 7, 14 and 21 days ($P<0.05$), but did not kill them (DTP: 15-27 days) (Fig. 2D to F). However, R-MZ killed H19 populations in 14 days. After 21 days of exposure, R-MX-MZ killed H12 and H19 bacilli (DTP >100 days), and R-MX-AK killed A5 and H5 bacilli (Fig. 2I). Noticeably, after 21 days all four populations examined (A5, H5, H12, H19) were killed by R-MX-MZ-AK (Fig. 2I). In a similar way, after 21 days R-MX-CP killed A5 and H5 bacilli and R-MX-MZ-CP killed A5, H5, H12 and H19 bacilli (Fig. 2N).

The correlation between CFUs and DTPs was investigated by linear regression analysis. The correlation coefficients $R^2$ for A5, H5, H12 and H19 cells were 0.95, 0.95, 0.94 and 0.95, respectively (data not shown), indicating that the DTPs can be used instead of the CFUs to evaluate the drug activity against aerobic and hypoxic Mtb cultures.

To explore whether these drugs can be effective in a cell-based assay of nonreplicating persistence (10), survival of Mtb in adipocytes, was determined. Preadipocyte cells (3T3F442A cell line, European Collection of Cell cultures, Salisbury, UK) were differentiated into mature adipocytes using bovine insulin (1 $\mu$g/ml, Sigma-Aldrich) for 12 days. Adipocytes were infected at a multiplicity of infection of 1 bacterium/adipocyte for 4 h at 37°C, washed with PBS and incubated with complete medium (DMEM-8% heat-inactivated foetal calf serum, 20 mM Hepes). Three days after infection, drugs were added. After 7 days of exposure, infected adipocytes were washed with PBS, lysed in distilled water containing 0.01% TritonX-100 and the number of CFUs determined in Middlebrook 7H11 agar (Difco) plates in triplicate. R, MX and CP caused about 1 log$_{10}$ CFU reduction, in comparison with untreated adipocytes (Fig. 3). Among combinations, R-MX-MZ-AK, R-MX-MZ-CP and R-MX-AK, reduced the CFU numbers of about 3.5 log$_{10}$. 

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In the Wayne model the susceptibility to drugs varied with the physiologic stage of Mtb. However, R-MX-MZ-AK and R-MX-MZ-CP killed all four stages examined, ranging from aerobic (A5) to microaerophilic-anaerobic (H15, H12, H19) bacilli. These observations show that it is possible to kill all cells of heterogeneous populations presumably living in active and latent TB lesions (2, 13). After R-MX treatment, which inhibits transcription (R) and DNA gyrase (MX) (9, 11), re-growth was seen in MGIT tubes; these observations suggest that R-MX induced the survival of drug-tolerant Mtb (persisters) in A5, H5, H12 and H19 populations. However, H12- and H19-tolerants were killed by addition of MZ, a drug for anaerobes (1, 4), and A5- and H5-tolerants were killed by addition of the protein synthesis inhibitors AK and CP (9, 11), suggesting that protein synthesis occurs under stationary and/or microaerophilic conditions. Some support to this hypothesis comes from the knowledge that 2% protein synthesis was seen in 50-day old microaerophilic Mtb (6), and that mRNA transcripts were found in microaerophilic stationary-phase Mtb induced by R (7). Noticeably, A5-, H5-, H12-, and H19-tolerants were killed in 3 weeks by addition of MZ plus AK, or MZ plus CP.

In line with in vitro results, 1-week exposure to R-MX-MZ-AK and R-MX-MZ-CP caused a strong decrease of nonreplicating Mtb inside adipocytes, a cell that may constitute a reservoir of dormant bacilli in humans with either active or latent TB (10); longer incubation time affected adipocyte viability and the CFUs could not be determined.

In conclusion, by using re-growth in broth as a test much more sensitive than CFUs to ascertain Mtb death, we found that R-MX-MZ-AK and R-MX-MZ-CP killed both aerobic and dormant (microaerophilic/anaerobic and drug-tolerant) Mtb in vitro in three weeks, and showed strong activity against dormant, intra-adipocytic Mtb, in
one week. Given the abundance of adipocytes throughout the body, our observations can be important to combat nonreplicating Mtb also inside the adipose tissues. The finding that tubercle bacilli surviving to bactericidal agents such as R and MX (9, 11) can be killed by addition of MZ plus a protein synthesis inhibitor such as AK or CP sheds a new light on how to design drug combinations effective against both active and latent TB. It is known that the bactericidal antibiotics nitroimidazoles, fluoroquinolones and aminoglycosides kill Gram-negative and Gram-positive bacteria by stimulating the production of reactive radicals (1-2, 4, 9); in this view, it will be important to study the killing mechanisms induced by these drugs in replicating and nonreplicating Mtb.

Overall, our observation that using plate counts can seriously underestimate nonreplicating populations after drug exposure is important to be considered when we assess the sterilizing effect of anti-TB agents in vitro and in vivo. To demonstrate that Mtb is dead, other than showing that it does not form colonies on solid media, we have to check that it does not grow after long-term incubation in broth.
REFERENCES


Fig 1. Survival of \textit{M. tuberculosis} in the Wayne dormancy culture model after 7, 14 and 21 days of exposure to drugs, as estimated by CFU counts. Five-day-old aerobic (A5), replicating, cultures were incubated \textit{aerobically} with drugs. Five-, 12- and 19-day-old, hypoxic (H5, H12 and H19, respectively), nonreplicating cultures were incubated \textit{anaerobically} with drugs. Dashed lines indicated the limit of $\log_{10}$ CFU per milliliter detected on Middlebrook 7H10 plates (5 CFUs/ml). R, rifampin; MX, moxifloxacin; MZ, metronidazole; AK, amikacin; CP, capreomycin. Error bars, standard deviations.

Fig 2. Survival of \textit{M. tuberculosis} in the Wayne dormancy culture model after 7, 14 and 21 days of exposure to drugs, as estimated by regrowth in liquid medium (day to positivity, DTP) using the BACTEC MGIT 960 system. Five-day-old aerobic (A5), replicating, cultures were incubated \textit{aerobically} with drugs. Five-, 12- and 19-day-old, hypoxic (H5, H12 and H19, respectively), nonreplicating cultures were incubated \textit{anaerobically} with drugs. R, rifampin; MX, moxifloxacin; MZ, metronidazole; AK, amikacin; CP, capreomycin. In the panels G to N, DTPs of R-MX and R-MX-MZ are shown again (dashed lines) for a better comparison of overall results. Means and standard deviations from three experiments are shown.

Fig 3. Survival of nonreplicating \textit{M. tuberculosis} inside adipocytes after 7 days of exposure to drugs, as estimated by CFU counts. R, rifampin; MX, moxifloxacin; MZ, metronidazole; AK, amikacin; CP, capreomycin. Means and standard deviations from triplicate plates of one representative experiment out of two are shown.
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