Activity of Drug Combinations against Dormant Mycobacterium tuberculosis

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Received 9 December 2009/Returned for modification 28 February 2010/Accepted 21 March 2010

Aerobic (5-day-old cultures) and nonreplicating (dormant) Mycobacterium tuberculosis (5-, 12-, and 19-day-old cultures) bacteria were treated with rifampin (R), moxifloxacin (MX), metronidazole (MZ), amikacin (AK), or capreomycin (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 regrowth in solid and liquid media. R-MX-MZ-AK and R-MX-MZ-CP also caused a strong decrease of nonreplicating bacilli in 7 days in a cell-based dormancy model.

In people with latent tuberculosis (TB), a group estimated to be one-third of the world’s population, Mycobacterium tuberculosis 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 M. tuberculosis 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 M. tuberculosis is insensitive to isoniazid (3, 10, 13), but some inhibition is induced by rifampin (R) (3, 10, 13), moxifloxacin (MX) (3), amikacin (AK) (3), capreomycin (CP) (3, 5), and metronidazole (MZ) (3, 13). Few studies have investigated the activity of drug combinations against nonreplicating M. tuberculosis (8, 12). Here, we report the effects of R, MX, AK, CP, and MZ, alone and in combination against dormant bacilli in the Wayne model and inside adipocytes.

M. tuberculosis strain H37Rv was grown in tubes containing Dubos Tween-albumin (DTA) broth inoculated with about 1 × 10^6 CFU/ml (8, 13). Aerobic (A), replicating populations were obtained by incubation of 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, and 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, and CP (8, 4, 8, 8, and 20 µg/ml, respectively, for their maximum drug concentrations in serum [C_{max}]) were added to A5 cultures ([2 ± 1] × 10^7 CFU/ml) and, by syringe, to H5, H12, and H19 cultures ([4.2 ± 2] × 10^7 CFU/ml, [2.6 ± 1.3] × 10^7 CFU/ml, [2.1 ± 0.4] × 10^7 CFU/ml, respectively). After 7, 14, and 21 days, 1 ml of aerobic or hypoxic cultures was washed and resuspended in 1 ml of DTA broth, and 0.2 ml was 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 number of days to reach a growth unit of ≥ 75 (days to positivity [DTP]). M. tuberculosis killing was defined as lack of regrowth in MGIT tubes after > 100 days (DTP > 100 days).

Among single drugs, MX and R were particularly active in decreasing the number of CFU, with the highest activities being shown by MX against A5 and H5 bacilli and by R against H12 and H19 bacilli (P value of < 0.05 in comparison to the control; Student’s t test) after 14 and 21 days (Fig. 1). The combination R-MX was active against A5, H5, H12, and H19 bacilli, with < 2 log_{10} CFU/ml remaining after ≥ 14 days. After 21 days of exposure to R-MX, R-MX-MZ, R-MX-AK, R-MX-MZ-AK, R-MX-MZ-CP, and R-MX-MZ-CP, no CFU of A5, H5, H12, and H19 populations were observed.

Since dormant M. tuberculosis 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 L). No single drug killed A5, H5, H12, and H19 bacilli after 7, 14, and 21 days, as shown by regrowth in MGIT tubes (DTP, ≤ 19 days). Again, MX and R were the most effective, with the highest activities being shown by MX against A5 and H5 populations (P value of < 0.05 in comparison to the control) (Fig. 2A to C) and by R against H12 and H19 populations (P value of < 0.05 in comparison to the control). 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 value of < 0.05 in comparison to the control). Lower levels of activity were shown by AK and CP (Fig. 2G to L).

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 to 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 bacilli after 14 and 21 days of exposure (P < 0.05).

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† Published ahead of print on 29 March 2010.
and H5 bacilli (Fig. 2I). Noticeably, after 21 days all four populations examined (A5, H5, H12, and 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. 2L).

The correlation between CFU and DTP 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 DTP can be used instead of the CFU to evaluate the drug activity against aerobic and hypoxic $M. tuberculosis$ cultures.

To explore whether these drugs can be effective in a cell-based assay of nonreplicating persistence (10), survival of $M. tuberculosis$ in adipocytes was determined. Preadipocyte cells (3T3F442A cell line; European Collection of Cell Cultures, Salisbury, United Kingdom) were differentiated into mature adipocytes by using bovine insulin (1 μg/ml; Sigma-Aldrich) for 12 days. Adipocytes were infected at a multiplicity of infection of 1 CFU per adipocyte for 4 h at 37°C, washed with phosphate-buffered saline (PBS), and incubated with complete medium (Dulbecco’s modified Eagle’s medium [DMEM]–8% heat-inactivated fetal calf serum, 20 mM HEPES). Three days after infection, drugs were added. After 7 days of exposure, infected adipocytes were washed with PBS and lysed in distilled water containing 0.01% Triton X-100, and the number of CFU was 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 by about 3.5 log$_{10}$.

In the Wayne model, the susceptibility to drugs varied with the physiologic stage of $M. tuberculosis$. However, R-MX-MZ-AK and R-MX-MZ-CP killed all four stages examined, ranging from aerobic (A5) to microaerophilic-anaerobic (H5, H12, and H19) stages.
FIG. 2. Survival of *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]) by using the BACTEC MGIT 960 system. Five-day-old aerobic (A5), replicating cultures were incubated aerobically with drugs. Five-, 12-, and 19-day-old hypoxic (H5, H12, and H19, respectively), nonreplicating cultures were incubated anaerobically with drugs. R, rifampin; MX, moxifloxacin; MZ, metronidazole; AK, amikacin; CP, capreomycin. In panels G to L, DTP of R-MX and R-MX-MZ are shown (dashed lines) for a better comparison of overall results. Means and standard deviations from three experiments are shown.
H12, and H19 bacilli. These observations show that it is possible to kill all cells of heterogeneous populations presumably living in active and latent TB (10). Longer incubation time affected adipose tissues.

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 *M. tuberculosis* inside adipocytes, which represent a cell type 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 CFU could not be determined.

In conclusion, by using regrowth in broth as a test much more sensitive than CFU measurement to ascertain *M. tuberculosis* death, we found that R-MX-MZ-ACK and R-MX-MZ-CP killed both aerobic and dormant (microaerophilic/anaerobic and drug-tolerant bacteria) *M. tuberculosis* in vitro in 3 weeks and showed strong activity against dormant, intra-adipocytic *M. tuberculosis* in 1 week. Given the abundance of adipocytes throughout the body, our observations can be important in combating nonreplicating *M. tuberculosis* also inside the adipose tissues.

The finding that tubercle bacilli surviving bacterial agents such as R and MX (9, 11) can be killed by the 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 bacterial 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 *M. tuberculosis*.

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 *M. tuberculosis* 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.

This work was supported by the European Project StopLATENT-TB grant agreement 200999. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**REFERENCES**


