Reduced Drug Uptake in Phenotypically Resistant Nutrient-Starved Nonreplicating Mycobacterium tuberculosis

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During active tuberculosis a spectrum of physiologically different Mycobacterium tuberculosis bacilli reside in human tissues. Subpopulations of the pathogen survive antibiotic treatment for a prolonged time in a dormant state of phenotypic drug resistance, a phenomenon independent of genetic mutations. Here, we used an established culture model of nutrient deprivation to shift down M. tuberculosis from growth to nonreplicating survival, which is characterized by a drastic loss of drug susceptibility. Liquid chromatography coupled with mass spectrometry techniques were employed to quantify drug penetration in replicating and nutrient-starved nonreplicating bacilli. We found that intracellular concentrations of fluoroquinolones, rifamycins, and linezolid were lower in nonreplicating M. tuberculosis. Studies with pump inhibitors suggest that the observed differences were independent of efflux processes. We conclude that decreased drug permeability contributes to phenotypic drug resistance of dormant M. tuberculosis.

Tuberculosis (TB), one of the most devastating diseases of mankind, is caused by Mycobacterium tuberculosis. Despite the availability of potent antibiotics, eradication of the pathogen in TB patients requires 6 to 9 months of combination chemotherapy. An attractive hypothesis suggests that prolonged treatment is related to minor populations of drug-resistant bacilli that survive in a state of dormancy. Indeed, nonreplicating bacilli generated by nutrient deprivation, oxygen starvation, or a combination of nitric oxide exposure and oxygen limitation in vitro are tolerant of antibiotics (1–4). However, when growth resumes, full susceptibility to drugs is reestablished. Display of such behavior, which is not genetically predetermined, has been termed phenotypic drug resistance (5).

Healthy individuals can usually control M. tuberculosis upon infection. Alveolar macrophages phagocytose the pathogen upon inhalation and finally form a well-structured macroscopic tissue reaction, a granuloma. It has been proposed that unfavorable microenvironments, such as nutrient shortage or low oxygen tension, inside the granuloma might trigger metabolic downshift of the pathogen to dormancy (5). This hypothesis is supported by physiological studies of culture models using nutrient starvation or oxygen depletion to generate nonreplicating M. tuberculosis (1, 2). Recent data have demonstrated that subpopulations of drug-tolerant persisters are present even in growing and stationary-phase cultures of M. tuberculosis (6). Growing evidence suggests that tubercle bacilli use metabolic shutdown at all stages of growth to ensure survival upon sudden environmental change, a capacity that might have been preserved from their soil-dwelling ancestors (7).

Numerous changes occur during the shift to the quiescent state. Transcriptome studies of nutrient-starved M. tuberculosis have demonstrated that most key functions, including carbohydrate/energy metabolism and replication machinery, are down-regulated (8). Accordingly, fluoroquinolones, a class of potent DNA gyrase inhibitors, were found to be inactive on nutrient-starved bacilli (1, 3). Oxygen deprivation or oxygen limitation/nitric oxide exposure, on the other hand, renders the pathogen only partially tolerant of fluoroquinolones (1, 2, 4). Some fluoroquinolones were reported to be nonlethal in the absence of protein biosynthesis (9). The drop in activity observed could therefore be related to insufficient formation of toxic inhibitor-gyrase-DNA complexes during dormancy, a condition associated with down-regulated transcription of ribosomal subunits (8). Another explanation could involve interference of the dormant state with accumulation of reactive oxygen species, which was identified as a general killing mechanism in bacteria upon antibiotic treatment (10). However, a change in cell wall permeability accompanying metabolic shutdown might contribute, as well. Variations in cell envelope permeability among different Mycobacterium species have been demonstrated (11, 12). Ziehl-Neelsen staining, the most common diagnostic method for M. tuberculosis, is known to be cell wall composition dependent. A loss in staining properties has been linked to persistence of the pathogen in host tissues of mice and humans (13). Similar observations have been made in nutrient-starved M. tuberculosis cultures, suggesting that, at least to some extent, the nutrient deprivation model reflects features of the pathogen in vivo (14). Moreover, significant cell wall thickening was reported for anaerobic M. tuberculosis cultures and aerobic genetically drug-resistant bacilli compared to the exponentially growing bacillus (15, 16). Altogether, growing evidence suggests that remodelling of the mycobacterial cell envelope occurs during downshift to dormancy.

Here, we aimed to investigate whether decreased drug penetration contributes to the phenotypic drug resistance of dormant M.
tuberculosis. A panel of antibiotics, including fluoroquinolones, rifamycins, ethambutol, linezolid, mefloquine, and thioridazine, with different physicochemical properties and mechanisms of action was selected to study intracellular drug concentrations. To allow broad and cost-effective application, we employed liquid chromatography coupled with mass spectrometry (LC-MS) instead of radiolabeling/scintillation counting methods to determine absolute concentrations of drugs in cell lysates.

MATERIALS AND METHODS

Antibiotics and chemicals. Ofloxacin, rifapentine, mefloquine, thioridazine, verapamil, and reserpine were obtained from Sigma; moxifloxacin, gentamycin, rifabutin, and linezolid from Sequoia Research Products; and levofloxacin and rifampin from Fluka. Fluoroquinolone stock solutions (10 mM) were prepared in water. Stock solutions of all other compounds were prepared in 90% dimethyl sulfoxide (DMSO). Phosphate-buffered saline (PBS) was provided by Invitrogen. Ultrapure methanol and acetonitrile were purchased from Fisher Scientific.

Growth conditions. M. tuberculosis H37Rv (ATCC 27294) was cultured at 37°C in Middlebrook 7H9 broth (Becton, Dickinson) supplemented with 0.5% albumin, 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.05% Tween 80 or on Middlebrook 7H11 agar (Becton, Dickinson) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton, Dickinson) and 0.5% glycerol. Agar plates were incubated for 3 to 4 weeks prior to determination of CFU. Nutrient-starved nonreplicating cultures were generated as described previously (1, 8). Briefly, exponentially growing bacilli were harvested at an optical density of 0.6 at 600 nm (OD600) at 0.3 to 0.4, washed twice with PBS-0.025% Tween 80, and resuspended in PBS-0.025% Tween 80. After 14 days of starvation at 37°C and constant rolling (2 rpm), the cultures were used for drug penetration studies.

Antibiotic susceptibility testing. The MBC90 was defined as the minimal drug concentration required to kill 90% of bacteria after 5 days of exposure. Similarly, the 90% Loebel-cidal concentration (LCC90) was defined as the minimal drug concentration required to kill 90% of bacteria after 5 days of exposure. Bactericidal activity against the nutrient-starved pathogen. These results indicated that nutrient-starved nonreplicating M. tuberculosis displayed a more than 100-fold loss of bactericidal activity against the nutrient-starved pathogen. These results confirm that nutrient-starved nonreplicating M. tuberculosis is

### TABLE 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>MBC90 (µM)</th>
<th>LCC90 (µM)</th>
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</thead>
<tbody>
<tr>
<td>Moxifloxacin</td>
<td>0.31–0.63</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.31–0.63</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>1.25–2.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.078</td>
<td>25</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>0.039</td>
<td>10</td>
</tr>
<tr>
<td>Rifapentine</td>
<td>0.078</td>
<td>10</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>2.5–5.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Thioridazine</td>
<td>40.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Linezolid</td>
<td>10.0</td>
<td>&gt;100</td>
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Impaired Drug Uptake in Dormant M. tuberculosis
highly resistant to a variety of antituberculosis agents. The data obtained with moxifloxacin, ofloxacin, rifampin, and ethambutol are in agreement with previous studies on nutrient-starved *M. tuberculosis* (1, 3). Interestingly, the bactericidal activities of mefloquine and thioridazine remained comparable in both growing and nutrient-starved nongrowing *M. tuberculosis*.

**Intracellular drug accumulations differ between replicating and nutrient-starved nonreplicating *M. tuberculosis***. Drug penetration assays in *M. tuberculosis* were performed with 10 antituberculosis candidates using a constant incubation concentration of 10 μM. Steady-state intracellular drug accumulations in replicating and nonreplicating cultures were determined after 30 min of exposure. The results were normalized to the number of CFU per sample and expressed as the total quantity of drug per bacterium (Fig. 1). Corresponding intracellular concentrations, IC/EC ratios, and fold differences in intracellular accumulation are collated in Table 2.

**Fluoroquinolones**. The three fluoroquinolones tested accumulated in the intracellular compartment of *M. tuberculosis*. In replicating bacteria, moxifloxacin, ofloxacin, and levofloxacin accumulated to intracellular concentrations of approximately 125 μM, i.e., 12-fold. Of the different drug classes tested, the fluoroquinolones showed the greatest decrease in intracellular accumulation as a result of nutrient starvation (*P* < 0.05). Ofloxacin accumulation decreased 12-fold and was most affected by nutrient deprivation of the bacillus. Ofloxacin did not accumulate above the incubation concentration in nutrient-starved nonreplicating bacteria. The effect of varying the incubation concentration of ofloxacin was tested. At 1, 5, and 20 μM, the ratios of intracellular ofloxacin accumulation between the two physiological states remained unchanged (data not shown).

**Rifamycins**. Intracellular accumulations of rifampin and rifapentine in replicating *M. tuberculosis* were moderate and similar (19.6 μM and 13.2 μM, respectively). In contrast, rifabutin accumulated to drastically higher intracellular concentrations of up to 89 μM in replicating bacteria. Nutrient starvation caused significant decreases in intracellular accumulation of all three rifamycins (*P* < 0.05). The fold decrease in intracellular accumulation upon nutrient starvation ranged between 1.5 and 2.7. At varying incubation concentrations of 1, 5, and 20 μM, rifampin accumulated to similar levels in growing and nongrowing bacilli (data not shown).

**Other antituberculosis agents**. The intracellular concentration of linezolid reached 68.5 μM in replicating *M. tuberculosis*. This decreased by half in nonreplicating bacteria (*P* < 0.05). In contrast, the intracellular accumulation of ethambutol, mefloquine, and thioridazine did not decrease in the nonreplicative state. Intracellular concentrations of ethambutol reached 23 μM and 21 μM in replicating and nonreplicating bacteria, respectively. Of all the antibiotics tested, mefloquine accumulated the most in replicating *M. tuberculosis*, to approximately 400 μM. Mefloquine accumulation did not decrease significantly in nonreplicating bacteria. Only thioridazine did not accumulate within the intracellular compartment of *M. tuberculosis*. Intracellular accumulations of rifampin and rifapentine in replicating *M. tuberculosis* were moderate and similar (19.6 μM and 13.2 μM, respectively). In contrast, rifabutin accumulated to drastically higher intracellular concentrations of up to 89 μM in replicating bacteria. Nutrient starvation caused significant decreases in intracellular accumulation of all three rifamycins (*P* < 0.05). The fold decrease in intracellular accumulation upon nutrient starvation ranged between 1.5 and 2.7. At varying incubation concentrations of 1, 5, and 20 μM, rifampin accumulated to similar levels in growing and nongrowing bacilli (data not shown).

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The uptake of fluoroquinolones in replicating and nonreplicating bacteria was examined over a 30-min exposure in nongrowing bacteria. The intracellular concentrations of fluoroquinolones at 30 min of drug incubation were due to delayed equilibrium. Next, we determined whether the strongly reduced accumulation was mediated by the physiological downshift to dormancy. Several efflux pumps were shown to actively transport fluoroquinolones and rifamycins out of M. tuberculosis cells. Several efflux pumps were shown to actively transport fluoroquinolones and rifamycins out of M. tuberculosis cells. To test the hypothesis that increased efflux could be responsible for the reduced IC/EC ratios for these drugs observed in the nonreplicating state, we performed drug accumulation assays in the presence of two efflux pump inhibitors, verapamil and reserpine. There was no detectable change in ofloxacin or rifampin accumulation levels upon preincubation of M. tuberculosis in either growth state in the presence of either efflux inhibitor.

Nutrient starvation does not delay equilibration of drug accumulation. Nutrient starvation does not delay equilibration of drug accumulation. The plots of intracellular ofloxacin accumulation over time at both physiological stages are presented in Fig. 3. In replicating bacteria, intracellular ofloxacin accumulation proceeded rapidly within the first 3 min of incubation and continued to gradually increase until the end of the experiment. Likewise, steady-state accumulation was reached within the first 3 min in nonreplicating M. tuberculosis, but intracellular concentrations remained consistently lower than in replicating cells over the entire incubation period. The results demonstrate that nutrient starvation brings about a drastic reduction in steady-state ofloxacin accumulation without delaying the equilibrated state.

Growing and nutrient-starved nonreplicating M. tuberculosis bacilli have comparable cell dimensions. The length distribution of bacteria could have an impact on total drug accumulation studies. In order to show that differences in drug uptake observed in our study were independent of cell size, we examined the length and shape of replicating and nonreplicating M. tuberculosis bacilli. Samples of bacilli taken directly before drug treatment were inactivated and underwent acid-fast staining prior to bright-field microscopy analysis at ×1,000 magnification. The average cell lengths of growing and nutrient-starved bacilli were 1.8 ± 0.4 μm (n = 500) and 1.7 ± 0.3 μm (n = 500), respectively. The difference in average dimensions was not statistically significant. This suggests that 14 days of nutrient starvation does not influence the dimensions or size of the pathogen under the experimental conditions adopted in this study.

DISCUSSION

Antituberculosis drugs have to cross various physical barriers before reaching their targets: (i) the granuloma as a host-derived containment for the pathogen, (ii) the infected host cell, and (iii) the pathogen’s envelope. In this study, we focused on the M. tuberculosis cell wall and membrane barrier in replicating versus nonreplicating bacilli. Unlike genetic drug resistance, found in all microbes and conferred by DNA mutations, the phenotypic drug resistance observed in M. tuberculosis is mediated by the physiological downshift to dormancy. Accordingly, full drug susceptibility is restored once growth resumes. While the cell envelope of exponentially growing M. tuberculosis is notably impermeable to small molecules, we speculated that nonreplicating bacilli are even more impermeable and that this contributes to the phenomenon of phenotypic drug tolerance.
To test our hypothesis, we selected a panel of drugs and drug classes active against *M. tuberculosis* and compared their intracellular accumulations in replicating versus nutrient-starved nonreplicating bacilli. Nutrient starvation, first studied by Loebel et al. (28, 29), causes the pathogen to arrest growth, minimize respiration, and become resistant to drugs while maintaining viability, thereby mimicking some of the features of *M. tuberculosis* persistence (8, 30). Loebel’s starvation model was selected, since it allows repeated sampling without perturbing the culture conditions and physiological state of the bacilli. Mass spectrometric analysis was chosen to measure drug concentrations in cell lysates. Such analytical methods provide the required sensitivity but are limited to detecting only parent compounds and known stable metabolites. Isoniazid, which is rapidly converted to a range of transient free radicals and adduct derivatives (31), was therefore excluded. The general limitations of our study are 2-fold. (i) We investigated only one out of several *in vitro* dormancy models. Which model is most relevant in respect to the dormant state of *M. tuberculosis* in *vivo* remains a point of discussion. (ii) All data presented were obtained from the well-characterized *M. tuberculosis* laboratory strain H37Rv. Determining whether our results translate to other genetic lineages of the pathogen or drug-resistant clinical isolates will be part of our future work.

The majority of the drugs tested here showed significantly decreased accumulation in nutrient-starved *M. tuberculosis*. Accumulation of the fluoroquinolones was most affected by the nonreplicating state, consistent with the marked loss of bactericidal activity in starved versus replicating cultures. Intracellular accumulation of the rifamycins was impaired to a lesser extent under starvation conditions, also consistent with the observation that they retain measurable bactericidal activity against persisters, though much reduced compared to that achieved in growing cultures. However, the decrease in rifamycin and fluoroquinolone uptake alone was not able to account for the loss of drug susceptibility exhibited by nonreplicating cultures. For instance, ofloxacin accumulation decreased 12-fold, while its bactericidal activity was 300-fold lower in nonreplicating bacilli. These results indicate that several factors, including altered cellular permeability, contribute collectively to the drug tolerance phenotype of persistent tubercle bacilli. Interestingly, the IC/EC ratios of the fluoroquinolones and rifamycins in nonreplicating bacilli approached 1 for most compounds and were independent of the extracellular concentration used in the assay, suggesting mostly passive uptake.

Intracellular penetration of ethambutol remained unchanged in replicating and nonreplicating *M. tuberculosis*, while its bactericidal activity decreased drastically in the latter. Ethambutol affects cell wall biosynthesis by specifically inhibiting the arabinosyltransferase, a capacity that is dispensable during nonreplicating survival. In addition, penetration of ethambutol in the cytosol is likely not required for its activity, given the transmembrane location of its target (32, 33). These two factors likely account for the “disconnect” between cytosol accumulation and the activity of ethambutol. Similarly, thioridazine is believed to inhibit type II NADH dehydrogenase, a key component of the respiratory chain within the *M. tuberculosis* cellular membrane (34). In addition, the highly hydrophobic phenothiazines are thought to be largely sequestered in the mycobacterial cell wall (35), in agreement with the very low intracellular concentrations of thioridazine observed in this study. It should be emphasized here that in our assay, the intracellular compartment where drug concentrations were measured included only the cytosolic fraction and excluded membranes and cell wall components, which are eliminated by centrifugation.

Mutations in or induction of efflux systems is thought to confer low levels of resistance to the fluoroquinolones and rifamycins, consistent with their MICs being affected by efflux pump inhibitors (verapamil and/or reserpine) in selected clinical isolates resistant to the two drug classes (36–38). Mycobacterial efflux pumps suspected to be associated with fluoroquinolone and rifampin resistance include members of the major facilitator superfamily and ATP binding cassette transporters (36, 39). These studies, however, did not report intracellular drug concentrations in the presence and absence of efflux inhibitors. To test the possibility that enhanced efflux might reduce intracellular drug concentrations in starved bacilli, we tested the effects of standard efflux inhibitors on fluoroquinolone and rifamycin accumulation in the cytosol of replicating and nutrient-starved *M. tuberculosis*. Neither reserpine nor verapamil affected the intracellular concentrations of ofloxacin or rifampin in the replicating or nonreplicating pathogen at steady state. It was previously discovered that the activation of efflux genes by rifampin is a phenotype uniquely associated with rifampin-resistant isolates, as efflux pumps and/or transporter proteins inhibited by reserpine and verapamil are not expressed in pansusceptible isolates (40). Failure to detect an effect of efflux pump inhibitors in this study is in agreement with those results, since we have exclusively worked with the fully susceptible wild-type *M. tuberculosis* laboratory strain H37Rv.

Collectively, the results of this study demonstrate that the intracellular penetration of different drugs and drug classes is affected to different extents in the nonreplicating state. Uptake of various drug classes is likely driven by a combination of multiple active and passive mechanisms, each of which is differently affected once *M. tuberculosis* ceases to grow and begins a dormant life style. Our results for fluoroquinolone and rifamycin uptake in replicating versus nonreplicating cells suggest that active transport is involved in intracellular accumulation of these molecules, while this is markedly reduced or even turned off in nutrient-starved bacilli. To date, however, outer membrane protein candidates that could mediate facilitated or active transport into the TB bacillus have not been unambiguously identified (41). Besides the modulation of active transport systems, cell wall alterations have been suggested to account for the loss of bactericidal activity of small molecules against nonreplicating bacilli. The biosynthesis of cell wall components is controlled by global regulators, such as sigma factors, serine threonine kinases, and two-component systems. Cell wall components implicated in virulence are induced under conditions that mimic the pathogen’s environment during infection (30). The precise remodelling mechanisms and structural changes brought about during dormancy remain to be elucidated in order to understand how they affect small-molecule permeation.

In conclusion, our findings indicate that new lead compounds aiming to shorten tuberculosis chemotherapy must not only target functions relevant in dormancy but also effectively penetrate the persistent bacillus. The inclusion of simple drug penetration assays, in addition to *in vitro* potency assays, for replicating and nonreplicating bacteria will overcome some of the issues associated with, and reduce attrition rates during, lead-finding and optimization campaigns.
ACKNOWLEDGMENTS
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REFERENCES