Time–kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of Mycobacterium tuberculosis

Jurriaan E. M. de Steenwinkel1*, Gerjo J. de Knegt1, Marian T. ten Kate1, Alex van Belkum1, Henri A. Verbrugh1, Kristin Kremer2, Dick van Soolingen2 and Irma A. J. M. Bakker-Woudenberg1

1Department of Medical Microbiology and Infectious Diseases, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands; 2National Tuberculosis Reference Laboratory, National Institute of Public Health and the Environment Centre for Infectious Disease Control (CIb/LIS), Bilthoven, The Netherlands

*Corresponding author. Tel: +31-10-7032174; Fax: +31-10-7033875; E-mail: j.desteenwinkel@erasmusmc.nl

Received 1 March 2010; returned 17 June 2010; revised 23 August 2010; accepted 8 September 2010

Objectives: The pharmacodynamics of tuberculosis (TB) treatment should be further explored, to prevent emergence of resistance, treatment failure and relapse of infection. The diagnostic drug susceptibility tests guiding TB therapy investigate metabolically active Mycobacterium tuberculosis (Mtb) isolates under static conditions and as such are not informative with respect to the time–kill kinetics of anti-TB drugs and the emergence of resistance in metabolically lowly active or even dormant mycobacterial cells.

Methods: In vitro, the killing capacity of rifampicin, isoniazid, ethambutol and amikacin regarding the degree of killing, killing rate and selection of resistant mutants was investigated in metabolically highly active versus metabolically lowly active Mtb cells.

Results: Isoniazid showed rapid and high killing capacity towards highly active mycobacteria, but due to the emergence of resistance could not eliminate the Mtb. Efflux pump-mediated isoniazid resistance was predominant. Rifampicin revealed a relatively slow and time-dependent killing capacity, but achieved elimination of all mycobacteria. Ethambutol was not bactericidal. Amikacin showed a high and extremely rapid killing activity that was not time dependent and could eliminate all mycobacteria. Exposure of lowly active Mtb populations to isoniazid, rifampicin or amikacin led to the emergence of resistant mutants. Compared with the highly active mycobacteria, elimination of the susceptible lowly active mycobacteria required a 64-fold increased isoniazid concentration and a 4-fold increased rifampicin concentration, whereas amikacin was equally effective irrespective of the metabolic state of the mycobacteria.

Conclusions: The anti-TB drugs differ significantly regarding their time–kill kinetics. In addition, the metabolic state of Mtb significantly affects its susceptibility to antimicrobials, with the exception of amikacin. Optimization of dosage of anti-TB drugs is required to achieve maximum drug concentrations at the site of infection in order to maximize reduction in Mtb load and to minimize the emergence and selection of resistance.

Keywords: isoniazid, rifampicin, ethambutol, amikacin, TB, mutant selection window, antimicrobial activity

Introduction

Tuberculosis (TB) remains one of the most important preventable infectious diseases worldwide. With over two billion persons latently infected with Mycobacterium tuberculosis (Mtb) worldwide, nine million patients with active TB diagnosed each year and almost two million deaths due to this disease annually, TB is a major cause of mortality and morbidity.1 Although the incidence of TB is stabilizing, from a worldwide perspective the prevalence is still rising. Improvement in TB treatment is urgently needed, since the global spread of drug-resistant TB is being observed.2,3 Optimal dosing and treatment duration, which is of the utmost importance to obtain maximum efficacy and to prevent resistance, has not been fully established. Although the usefulness of new drugs is evaluated,3 more efficient use of the available drugs could be a more realistic short-term goal. This may also serve the development of combined regimens of conventional and new drugs. For optimization of current treatment regimens, regarding dose and duration, insight into the dynamics of the activity of the anti-TB drugs is of critical importance. The...
in vitro drug susceptibility tests currently used are not informative in this way, as they provide only endpoint data obtained after a continuous 2–4 week drug exposure of the Mtb isolate. In this respect in vitro tests do not simulate the in vivo situation, being a static equilibrium, where the pharmacokinetic curve reflects fluctuating drug concentrations.\(^6\) Moreover, besides the degree of killing the rate of killing is also highly clinically significant, but this is not measured in current susceptibility assays.

Clinical experience is that inadequate exposure to anti-TB drugs may result in the emergence of resistant mycobacteria.\(^5,6\) However, the mechanism by which resistance is induced is not always known. The term ‘phenotypic drug resistance’ is used to indicate drug-tolerant mycobacteria that exhibit reduced susceptibility to anti-TB drugs without known genetic mutations. Phenotypic drug resistance might, for instance, be due to increased efflux pump activity, porin loss, permeability decrease or drug-modifying enzymes, resulting in reduced intracellular drug concentrations.\(^7–9\) ‘Genotypic drug resistance’ indicates resistance associated with known mutations in the genome of Mtb and relates to known levels of resistance.

In this study we aimed to visualize the concentration-dependent and time-dependent killing capacity of relevant, currently used anti-TB drugs towards Mtb and the emergence of resistance in relation to the metabolic state (growth phase) of the Mtb population. In the present study, the drug concentrations are also static; however, by using a wide range of concentrations and assessment of mycobacterial killing at different timepoints during drug exposure (instead of an endpoint assay), insight is provided into the dynamic killing capacity of fluctuating drug concentrations. This knowledge will contribute to a better understanding of the time–kill kinetics of anti-TB drugs and the emergence of resistance, and as such may help to optimize the use of these drugs.

Materials and methods

**Mtb cultures**

The Mtb strain used was H37Rv, a clinical isolate and reference strain nowadays commonly used in vitro as well as in animal TB models. The MICs determined as described by the CLSI (formerly the NCCLS) document M24-A\(^{10}\) were 0.125 mg/L for rifampicin, 0.125 mg/L for isoniazid, 8 mg/L for ethambutol and 2 mg/L for amikacin. Mtb suspensions were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid–albumin–dextrose–catalase enrichment (OADC; Baltimore Biological Laboratories, Baltimore, MD, USA), 5% glycerol (Scharlau Chemie SA, Sentmenat, Spain) and 0.05% Tween 20 (Sigma Chemical Co., St Louis, MO, USA), under shaking conditions at 96 rpm at 37°C. Vials with Mtb suspensions were stored at −80°C. Cultures on solid medium were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC for 21 days at 37°C with 5% CO\(_2\).

**Metabolic activity of highly active/low-density and lowly active/high-density Mtb cultures**

The metabolic activity of Mtb in cultures was assessed by measuring the adenosine-5’-triphosphate (ATP) level using the firefly luciferase bioluminescence assay.\(^11\) With a luminometer, light emission, which is proportional to the ATP concentration in Mtb, was quantified. The microbial ATP kit HS (BioThema AB, Dalarö, Sweden) was used according to the recommendations of the manufacturer.\(^11\)

**Anti-TB drugs**

Rifampicin, isoniazid, ethambutol and amikacin were purchased from Sigma Chemical Co. (I-3377, R-3501, E-4630 and A-3650). Dilutions of anti-TB drugs were prepared according to the recommendations of the manufacturers.

**Concentration- and time-dependent bactericidal activity of anti-TB drugs**

Mtb vials were defrosted and used to generate Mtb cultures in the early logarithmic phase of growth and at a density of 5 × 10\(^8\) cfu/mL (range 4–6 × 10\(^8\)), as confirmed by quantitative plate counts. These cultures were exposed to each of the individual anti-TB drugs at concentrations ranging from 0.0005 mg/L to 256 mg/L in 125 mL Erlenmeyer flasks (Corning Costar, Cambridge, MA, USA) for 6 days at 37°C, under shaking conditions at 96 rpm. On days 1, 2, 3 and 6, samples of 500 μL were taken for cfu counting, providing the mycobacterial suspensions did not show visible agglomeration, as from aggregated Mtb cultures accurate cfu counts could not be established. Log cfu values were plotted against time (in days) to obtain time–kill curves. In order to prevent drug carry-over, samples were washed once by centrifugation at 14,000 ×g for 10 min and resuspended in medium without anti-TB drugs. The washed suspensions were serially diluted in PBS and plated onto antibiotic-free 7H10 agar supplemented with OADC. After 21 days of incubation at 37°C and 5% CO\(_2\), cfu values were determined.

**Selection of drug-resistant Mtb**

In order to detect drug-resistant Mtb, in the highly active/low-density culture, the samples taken after 6 days of exposure to each of the individual anti-TB drugs at concentrations ranging from 0.0005 mg/L to 256 mg/L were cultured onto anti-TB drug-containing 7H10 agar plates. The concentrations of the drugs in the subculture plates were 4-fold the ‘critical’ concentrations of the agents;\(^10\) i.e. 4 mg/L rifampicin, 0.8 mg/L isoniazid, 20 mg/L ethambutol and 20 mg/L amikacin. Only the drug-resistant Mtb were able to grow on these drug-containing subculture plates, whereas both susceptible and drug-resistant Mtb showed growth on the subculture plates without anti-TB drugs.

Detection of drug-resistant Mtb in the lowly active/high-density culture after 6 days of drug exposure was performed using the same selection method as described earlier. The high-density/late-log-phase culture containing 0.9 × 10\(^8\) cfu/mL (range 0.3–1.3 × 10\(^8\) cfu/mL) was obtained after 4 days of incubation of the early-log-phase culture in the absence of anti-TB drugs at 37°C.

**Characterization of drug-resistant mutants**

Occurrence of phenotypic drug resistance caused by induction of efflux pumps was investigated by plating the samples from the anti-TB drug-exposed cultures not only onto agar plates containing anti-TB drugs but also onto agar plates containing a combination of anti-TB drugs and 20 mg/L reserpine (RO875-16; Sigma Chemical Co.), a known efflux pump inhibitor.\(^16\) In our assay we confirmed that the presence of 20 mg/L reserpine alone in the agar plates did not affect the growth of H37Rv (data not shown). From plates containing anti-TB drug and 20 mg/L reserpine, 10 colonies were picked randomly and investigated for known drug resistance-associated mutations (genotypic resistance).

From the isoniazid-resistant mycobacteria obtained from the highly active/low-density culture exposed to 64 mg/L isoniazid, the stability of resistance was also explored by repeated culture in isoniazid-free medium. To this aim the BACTEC MGIT-960\(^{18\text{a}}\) system (Becton Dickinson Co., Franklin Lakes, NJ, USA) was used. Two colonies picked from the
isoniazid/reserpine-containing agar plates were each resuspended in 600 μL of Middlebrook 7H9 medium and cultured in an MGIT™ control tube (isoniazid-free medium), in a 0.2 mg/L isoniazid-containing MGIT™ tube and in a 1.0 mg/L isoniazid-containing MGIT™ tube. The time to positivity of each of these three tubes was registered. The mycobacteria from the positive control tube (without isoniazid) subsequently underwent further stability control, by inoculating a new set of MGIT™ tubes containing isoniazid-free medium, medium containing 0.2 mg/L isoniazid and medium containing 1.0 mg/L isoniazid. This procedure was repeated for a total of five times. The percentage of isoniazid-resistant organisms in the total (stable) Mtb population was assessed and it was investigated whether isoniazid genotypic resistance could be detected.

From the drug-resistant Mtb isolates obtained, 10 colonies were randomly picked from the reserpine-containing plates and investigated for the presence of genotypic resistance. The GenoType® MTBDRplus assay (Hain Lifescience GmbH, Nehren, Germany) was used to detect the most common mutations in rpoB conferring rifampicin resistance and those in katG and inhA leading to isoniazid resistance. The GenoType® MTBDRsl assay (Hain Lifescience GmbH) was used to detect mutations in the rrs gene related to amikacin resistance. Both assays were performed according to the manufacturer’s recommendations.

In addition to the GenoType® MTBDRplus assay to detect mutations in the katG gene, deletion of the katG gene (so-called △katG) was also determined, as described by Bergval et al. In short, 10 colonies grown on 7H10 agar containing 0.4 mg/L isoniazid were selected and DNA was prepared. Primer sets specific for katG codon 315 and for katG codon 463 were used in a PCR assay using a GeneAmp® PCR system 9700 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Isolates were scored △katG if one or both of the PCR products were absent.

Results

Metabolic activity of Mtb by growth phase

We differentiated between ‘metabolically highly active’ and ‘metabolically lowly active’ Mtb as assessed by determination of the amount of ATP per viable Mtb. The highly active/low-density Mtb population increased from the start of the experiment with 5.0 × 10^5 cfu/mL to 0.9 × 10^8 cfu/mL on day 3. The metabolic activation during the 3 day period was reflected by a 6-fold increasing ATP content per cfu, being 1.5 × 10^-18 mol ATP/cfu (range 1.4 – 1.6 × 10^-18) at the beginning of the experiment and 0.9 × 10^-17 mol ATP/cfu (range 0.7 – 1.2 × 10^-17) on day 3. From day 4 onwards the ATP content per cfu was lower and remained at a constant level of 4.7 × 10^-18 mol ATP/cfu (range 2.8 – 7.4 × 10^-18), reflecting metabolically lowly active mycobacteria showing a constant but reduced metabolism.

Time–kill kinetics of anti-TB drugs towards highly active/low-density Mtb

In the absence of anti-TB drugs, the density of the Mtb H37Rv culture increased from 5.0 × 10^5 cfu/mL at the start of the experiment to 5.7 × 10^6 cfu/mL on day 1, 3.6 × 10^7 cfu/mL on day 2 and 1.1 × 10^8 cfu/mL on day 3. On day 6 the Mtb suspension was aggregated due to increased density of mycobacteria, and as a result appropriate quantification of the non-drug-exposed Mtb on day 6 could not be performed.

The time–kill kinetics of rifampicin, isoniazid, ethambutol and amikacin on the highly active Mtb cultures are shown in Figures 1 and 2. The data shown in the figures ranged from the concentrations that proved ineffective (identical growth compared with the control) up to 256 mg/L.

Rifampicin showed clear concentration-dependent killing activity, which was also strongly time dependent. At a high concentration of 128 mg/L >99% killing was achieved within 1 day of exposure. A moderate concentration of 8 mg/L was needed to achieve >99% killing after 3 days of exposure (Table 1) and this concentration eliminated all Mtb after 6 days of exposure (Figure 1).

Isoniazid also showed concentration-dependent killing activity, which was more rapid compared with rifampicin (Figure 1). At a low concentration of 0.125 mg/L >99% of the Mtb was killed within only 1 day of exposure (Table 1). Within 2 days of exposure a 2-fold lower concentration killed >99%; however, from that time onwards the number of cfu in the Mtb cultures increased again. This regrowth of Mtb was observed over a wide range of isoniazid concentrations, and was caused by the development of an isoniazid-resistant Mtb subpopulation. After 6 days of drug exposure, a concentration of 32 mg/L was needed to achieve >99% Mtb killing (Table 1). Only at a concentration of >128 mg/L was the expansion of isoniazid-resistant mycobacteria completely prevented (Table 2).

Ethambutol showed moderate killing activity that was almost independent of its concentration, and not time dependent (Figure 2). Even at the highest concentration of 256 mg/L, Mtb could not be fully eliminated. The concentrations of ethambutol needed to achieve >99% killing were >256 mg/L on day 1, >256 mg/L on day 2, 64 mg/L on day 3 and 2 mg/L on day 6 (Table 1).

Amikacin showed strong concentration-dependent killing capacity and a high killing rate (Figure 2). Killing of >99% after 1 day was achieved at a concentration of only 2 mg/L. To achieve >99% killing after 6 days of exposure, a modest decrease in concentration of only 4-fold was required (Table 1), indicating that the killing activity of amikacin was largely independent of time of exposure to this agent. The amikacin concentration needed for 100% elimination of Mtb after 6 days of exposure was only 16 mg/L (Figure 2 and Table 2).

Selection of drug-resistant mutants in highly active/low-density Mtb

In the highly active Mtb population, drug-resistant mutants were only observed after exposure to isoniazid and not after exposure to the other anti-TB drugs. Isoniazid concentrations of ≥4 mg/L resulted in equal numbers of mycobacteria on the subculture plates without anti-TB drugs and the drug-containing subculture plates, whereas >128 mg/L resulted in elimination of all mycobacteria, including the resistant Mtb. The isoniazid concentrations within which resistant mutants were selected ranged from 0.031 up to 64 mg/L, whereas in a concentration window between 4 and 64 mg/L isoniazid equal numbers of mycobacteria on the subculture plates without anti-TB drugs and the drug-containing subculture plates were cultured. Surprisingly, genotypic characterization revealed no mutations in inhA or katG, as assessed by the GenoType® MTBDRplus assay. However, using the katG gene PCR, according to Bergval et al., a deletion of the katG gene was demonstrated in two of the 10 isolates tested.

Additional differentiation between phenotypic and genotypic isoniazid-resistant isolates demonstrated that only 1.9% of the
isoniazid-resistant mycobacteria that were selected after 22 days of exposure to 64 mg/L were able to grow in the presence of the efflux pump inhibitor. This suggests that the majority of the isoniazid-resistant population represents efflux pump-mediated phenotypic drug resistance. Twenty colonies that were picked from the isoniazid/reserpine-containing plates for genetic characterization revealed no mutations in the \textit{inhA} or \textit{katG} gene using the GenoType\textsuperscript{w} MTBDRplus assay. However, deletion of (a part of) the \textit{katG} gene was demonstrated in two out of eight isolates tested.

The stability of the isoniazid-resistant mutants was demonstrated by subculture of two mutant isolates in MGIT\textsuperscript{TM} medium without isoniazid, with 0.2 mg/L isoniazid or with 1.0 mg/L isoniazid. Mutants showed similar growth rates in all media. This indicates that the isoniazid resistance was conserved even without isoniazid pressure, suggesting that genotypically resistant mutants were present. The genetic characterization of the isoniazid-resistant mutants that after subculturing consecutively five times remained stable revealed no mutations in the \textit{inhA} or \textit{katG} gene, as assessed by the GenoType\textsuperscript{w} MTBDRplus assay.

\textbf{Figure 1.} Concentration- and time-dependent bactericidal effect of anti-TB drugs on metabolically active Mtb strain H37Rv. Cultures of Mtb at low density in the early logarithmic phase of growth (5×10\textsuperscript{5} cfu/mL) were exposed to rifampicin or isoniazid at 2-fold increasing concentrations for 6 days at 37°C. After 1, 2, 3 and 6 days of exposure, quantitative cultures were performed on subculture plates without anti-TB drugs. Due to aggregation of the mycobacteria in the culture, at 6 days of exposure to some of the low concentrations, accurate cfu counts could not be performed.
However, in these stable genotypically resistant mutants, a deletion of part of the \( \text{katG} \) gene was observed in one out of two.

**Selection of drug-resistant mutants in lowly active/high-density \( \text{Mtb} \)**

In the lowly active \( \text{Mtb} \) population, drug-resistant mycobacteria were selected during exposure to rifampicin, isoniazid and amikacin, but not during exposure to ethambutol.

After rifampicin exposure, selection of resistant mutants was only observed in a rifampicin concentration window between 32 and 256 mg/L. Resistant mycobacteria did not emerge at a concentration of \( \geq 512 \) mg/L. Analysis of 10 rifampicin-resistant colonies showed an altered \( \text{rpoB} \) gene sequence. Eight colonies had a mutation at codon 526 of the \( \text{rpoB} \) gene; six of these had the \( \text{His526Tyr} \) mutation and two had an unknown mutation at this position. One colony exhibited the \( \text{Ser531Leu} \) mutation and the remaining colony had an unknown mutation at codon 531 or 533 (Table 2).

After isoniazid exposure resistant \( \text{Mtb} \) were also observed. Isoniazid concentrations of \( \geq 256 \) mg/L resulted in equal numbers of mycobacteria on the subculture plates without anti-TB drugs and the drug-containing subculture plates, whereas \( \geq 512 \) mg/L resulted in elimination of all mycobacteria.

**Figure 2.** Concentration- and time-dependent bactericidal effect of anti-TB drugs on metabolically active \( \text{Mtb} \) strain H37Rv. Cultures of \( \text{Mtb} \) at low density in the early logarithmic phase of growth \( \left( 5 \times 10^5 \text{ cfu/mL} \right) \) were exposed to ethambutol or amikacin at 2-fold increasing concentrations for 6 days at 37°C. After 1, 2, 3 and 6 days of exposure, quantitative cultures were performed on subculture plates without anti-TB drugs. Due to aggregation of the mycobacteria in the culture, at 6 days of exposure to some of the low concentrations, accurate cfu counts could not be performed.
including the resistant Mtb. The analysis of these isoniazid-resistant mycobacteria revealed no mutations in \textit{inhA} or \textit{katG}, as assessed by the GenoType MTBDRplus assay (Table 2). Using katG gene analysis, in 3 of the 10 isolates, (partial) deletion of the \textit{katG} gene was observed.

Exposure to ethambutol did not result in selection of resistant Mtb. At a concentration of 4 mg/L multiplication of Mtb up to high levels resulting in aggregation of the Mtb suspension was prevented, and \geq 99.9\% killing of Mtb was obtained, whereas full elimination of Mtb was not achieved (Table 2). Continuation of ethambutol exposure up to 10 days did not result in a further decrease in Mtb numbers (data not shown).

Exposure to amikacin did result in resistant Mtb. Selection of only resistant mutants was found within an amikacin concentration window between 8 and 256 mg/L. Amikacin concentrations of \geq 8 mg/L resulted in equal numbers of mycobacteria on the subculture plates without anti-TB drugs and the drug-containing subculture plates, whereas \geq 512 mg/L resulted in elimination of all mycobacteria, including the resistant Mtb. Ten amikacin-resistant mutants were investigated and revealed a mutation in the \textit{rrs} gene at codon 1401 (Ala1401Gly) (Table 2).

### Discussion

The ideal anti-TB drug should exhibit a high killing rate, resulting in a rapid decrease in mycobacterial load and, hence, a reduced risk of spreading the disease and development of resistance. Also, the bactericidal activity of an anti-TB drug against mycobacteria at low growth rate is important, as deep-seated Mtb in tissues during the dormant state probably exhibit low metabolic activity, and may be more difficult to eliminate.\textsuperscript{16} Moreover, attainable tissue concentrations of anti-TB drugs at the infectious foci are important; they may be too low with the current dosing regimens. It is expected that suboptimal concentrations of anti-TB drugs facilitate the emergence of resistance by allowing enrichment and amplification of resistant mycobacterial subpopulations.\textsuperscript{5,6}

All first-line drugs currently used to treat TB have a long history of clinical use. However, their pharmacodynamics have yet to be fully elucidated and there are indications that improvement of dosage could be of great value. The results of the present study provide additional insight into the \textit{in vitro} killing dynamics of the anti-TB drugs, with respect to the degree of killing (concentration dependence), the rate of killing (time dependence) and the selection of phenotypically and/or genotypically resistant mutants. Furthermore, the metabolic activity (growth phase) of the mycobacterial population was taken into account. To our knowledge this is the first study in which the cornerstone anti-TB drugs isoniazid and rifampicin, as well as ethambutol and amikacin, have been investigated in this way.

Isoniazid and rifampicin differed strongly with respect to their killing rate of metabolically active mycobacteria. Noticeably, isoniazid showed extremely rapid and completely concentration-dependent killing. In contrast, rifampicin revealed relatively slow and strongly time-dependent killing. This evident difference in the dynamics of bactericidal activity between isoniazid and rifampicin is in concordance with therapeutic results obtained in human studies.

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**Table 1.** Concentration-dependent bactericidal effect (\geq 99\% killing) over time of anti-TB drugs towards highly active/low-density Mtb strain H37Rv

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lowest concentration (mg/L) resulting in \geq 99% killing at various timepoints during exposure to anti-TB drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>128</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.125</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>\geq 256</td>
</tr>
<tr>
<td>Amikacin</td>
<td>2</td>
</tr>
</tbody>
</table>

Cultures of Mtb strain H37Rv (5 \times 10^5 cfu/mL) were exposed to rifampicin, isoniazid, ethambutol or amikacin at 2-fold increasing concentrations for 6 days at 37°C. After 1, 2, 3 and 6 days of exposure, quantitative cultures were performed on plates without anti-TB drugs.

**Table 2.** Elimination (100\% killing) of susceptible or resistant Mtb strain H37Rv by anti-TB drugs, in relation to metabolic activity of the mycobacteria

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lowest concentration (mg/L) resulting in 100% killing after 6 days of exposure to anti-TB drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>susceptible mycobacteria</td>
</tr>
<tr>
<td></td>
<td>highly active/low-density Mtb</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>8</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>4</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>\geq 1024</td>
</tr>
<tr>
<td>Amikacin</td>
<td>16</td>
</tr>
</tbody>
</table>

Cultures of Mtb strain H37Rv at low density (5 \times 10^5 cfu/mL) or at high density (1 \times 10^6 cfu/mL) were exposed to rifampicin, isoniazid, ethambutol or amikacin at 2-fold increasing concentrations for 6 days at 37°C. After 6 days of exposure, quantitative cultures were performed on plates without anti-TB drugs and plates containing 4 mg/L rifampicin, 0.8 mg/L isoniazid, 20 mg/L ethambutol or 20 mg/L amikacin.

^a Detection of mutations in \textit{rpoB} for rifampicin, \textit{katG} and \textit{inhA} for isoniazid and \textit{rs} for amikacin.

^b \textit{\Delta katG} indicates that the \textit{katG} gene was (partly) deleted.
in studies in TB patients investigating the early bactericidal activity (EBA) of anti-TB drugs, assessed by the decline of viable Mtb in consecutive sputum samples during the initial days of therapy. Also, the EBA studies show the rapid killing of mycobacteria by isoniazid. Whereas in the present study a rapid and high killing capacity of isoniazid towards highly active mycobacteria was demonstrated, isoniazid could not eliminate all mycobacteria, due to the emergence of resistant mutants. This indicates that EBA studies in humans have a very limited predicting value relating to the sterilizing activity of anti-TB drugs.

Compared with isoniazid, the EBA of rifampicin was demonstrated to be substantially less. Based on this observation the essential therapeutic role of rifampicin is thought to be during the continuation phase of TB treatment, where rifampicin is responsible for sterilization. Also, in the present study, a slow and time-dependent killing activity of rifampicin was observed as well as elimination of the highly active mycobacterial population, provided adequate concentrations of rifampicin were available. We concluded that our in vitro data support the concept of a biphasic action of isoniazid and rifampicin against Mtb. Isoniazid played the most eminent role in the first phase of treatment, whereas rifampicin became important in the second phase of TB treatment. As a result, one could contemplate increasing dosing of isoniazid to accelerate the time to sputum-smear conversion. Increasing dosing of rifampicin to generate a faster sterilizing effect may result in the possibility of reduced total treatment duration.

The cessation of mycobacterial killing after 3 and 4 days of isoniazid exposure and resistance development were also observed in the studies of Gumbo et al. who investigated the bactericidal activity of isoniazid in an in vitro pharmacodynamic model in which Mtb strain H37Rv was exposed to isoniazid concentration–time profiles encountered in TB patients. Gumbo et al. demonstrated, as we now confirm, that the mycobacterial population remaining at the time bactericidal activity ceased represented an isoniazid-resistant subpopulation. We characterized the isoniazid-resistant mutants that were selected from the highly active Mtb population in the present study. Within a broad isoniazid concentration range only isoniazid-resistant mycobacteria were cultured. We concluded that the majority of the isoniazid-resistant mutants found are not due to genetic mutation in known drug resistance-associated genes. As such, they are not identified as genotypically resistant mycobacteria. We demonstrated that this resistance was efflux mediated, by the use of the efflux pump inhibitor reserpine. Efflux pump inhibitors were also applied in the studies of Viveiros et al. and Colangeli et al. who examined their role in isoniazid resistance in Mtb. Their studies suggested that efflux pump inhibitors can reverse mycobacterial tolerance to isoniazid and that even high-level resistance to isoniazid can be induced in isoniazid-susceptible Mtb strains by the induction of a reserpine-sensitive efflux mechanism. Gumbo et al. have also found in their pharmacodynamic model that isoniazid resistance is partly due to single point mutations in the katG gene and partly the result of an efflux pump mechanism. All these findings, including our data, indicate that there may be a role for efflux pump inhibitors in the treatment of TB, as they also have proven potency to treat multidrug-resistant TB.

The emergence of resistant mutants during exposure to the anti-TB drugs in relation to the metabolic activity of the mycobacteria was also explored in the present study. Whereas in the highly active Mtb population resistant mutants were only observed after exposure to isoniazid, in the lowly active Mtb population selection of resistant mutants was found after exposure to isoniazid, rifampicin or amikacin. Selection of resistant Mtb is primarily expected from the high-density mycobacterial population in view of the mutation frequency. As the mutation frequency for isoniazid is known to be relatively high compared with other drugs, selection of isoniazid-resistant mutants even from the low-density population is not surprising. Again, the isoniazid-resistant genotypic mutants were difficult to characterize, but we conclude that a minority of genotypically resistant mutants was characterized by deletion of the katG gene, whereas the majority of isoniazid-resistant genotypic mutants remained unclassified. The rifampicin-resistant mutants, on the other hand, all exhibited mutations in their rpoB gene. Whereas, compared with highly active bacteria, for elimination of lowly active Mtb a 64-fold higher isoniazid concentration was needed, for rifampicin only a 4-fold increase in concentration was required. It is therefore concluded that rifampicin is superior to isoniazid with respect to killing of Mtb with low metabolic activity. This supports the existing concepts of the role of rifampicin in TB therapy. We also found that the killing of isoniazid-resistant or rifampicin-resistant mutants required extremely high drug concentrations. As it is expected to be impossible to achieve such high drug concentrations at the site of infection, it is important to avoid selection of resistant mutants. Our data underline the need to optimize the dosage schedule of an anti-TB drug in order to achieve the maximum-attainable drug concentration that results in maximum reduction in mycobacterial load, and in this way to minimize the selection of resistant mutants.

Ethambutol exhibited primarily bacteriostatic activity. Not even at 1024 mg/L for 6 days did ethambutol kill all mycobacteria. This confirms the limited efficacy of ethambutol against actively multiplying Mtb and its poor efficacy against the lowly active Mtb subpopulation, as also seen in other studies. It is clear that the primary role of ethambutol in the treatment of TB is to minimize the risk of emergence of resistance to the other anti-TB drugs administered.

Amikacin displayed concentration-dependent, rapid bactericidal activity. Interestingly, complete elimination of highly active Mtb compared with lowly active Mtb required similar amikacin concentrations, indicating that amikacin is effective irrespective of the growth phase and metabolic state of the Mtb cells. In this respect, amikacin was superior to the other agents. However, as with isoniazid resistance and rifampicin resistance, for killing of amikacin-resistant mutants extremely high drug concentrations are required.

In conclusion, the classes of anti-TB drugs differ substantially with respect to the dynamics of their mycobacterial effect. In addition, these drugs are significantly different in their capacity to kill metabolically lowly active Mtb. This might be an important observation as the deep-seated, lowly active or dormant mycobacteria in the infected tissues are probably responsible for relapse of TB. Also, the risk of selection of resistance is strongly dependent on the class of drug used and on the growth phase (metabolic activity) of the Mtb. With respect to isoniazid, phenotypic resistance is the dominant resistance type, and reserpine-inhibitable efflux pumps might play a role as a...
mechanism contributing to this phenotypic resistance. However, clinical relevance should be further evaluated. In genotypically isoniazid-resistant Mtb as yet unknown genetic mutations contribute to isoniazid resistance. The present study indicates that increasing rifampicin and/or amikacin dosing resulting in enhanced anti-TB drug concentration at the site of infection may significantly improve the therapeutic outcome of TB.

Acknowledgements
We would like to thank Miranda Kamst and Rina de Zwaan [National Tuberculosis Reference Laboratory, National Institute of Public Health and the Environment Centre for Infectious Disease Control (Ctb/LIS), Bilthoven, The Netherlands] for technical support.

Funding
This work was supported by the departments of the authors.

Transparency declarations
None to declare.

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