**Abstract**

Current tuberculosis (TB) treatment requires 6 months of combination therapy with isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol for active TB and 9 months of INH or 3 months of rifapentine (RFP) + INH for latent TB. The lungs of patients with active and latent TB contain heterogeneous mixtures of cellular and caseous granulomas harboring *Mycobacterium tuberculosis* bacilli ranging from actively replicating (AR) to nonreplicating (NR), phenotypically drug-resistant stages. Several *in vitro* models to obtain NR cells were reported, including exposure to hypoxia, nutrient starvation, acid + nitric oxide, and stationary phase. Overall, these models showed that RIF, RFP, PA-824 (PA), metronidazole (MZ), bedaquiline (BQ), and fluoroquinolones were the most active drugs against NR *M. tuberculosis*. In hypoxia at pH 5.8, some combinations killed AR plus NR cells, as shown by lack of regrowth in liquid media, whereas in hypoxia at pH 7.3 (the pH of the caseum), only RIF and RFP efficiently killed NR bacilli while several other drugs showed little effect. In conventional mouse models, combinations containing RFP, BQ, PA, PZA, moxifloxacin, sutezolid, linezolid, and clofazimine sterilized animals in ≤2 months, as shown by lack of viable bacilli in lung homogenates after 3 months without therapy. Drugs were less effective in C3HeB/FeJ mice forming caseous granulomas. Overall, *in vitro* observations and *in vivo* studies suggest that the search for new TB drugs could be addressed to low lipophilic molecules (e.g., new rpoB inhibitors with clogP < 3) killing NR *M. tuberculosis* in hypoxia at neutral pH and reaching high rates of unbound drug in the caseum.

**Keywords:** Dormant, drugs, *Mycobacterium tuberculosis*, nonreplicating, tuberculosis

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**Introduction**

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, a microorganism that usually attacks not only lungs but also other parts of the body such as spine, kidney, and brain. After more than one century from discovery of the tubercle bacillus by Robert Koch in 1882, the disease has not been eliminated, and the World Health Organization (WHO) estimates that in 2015, there were 10.4 million new TB cases worldwide, of which 56% involved men, 34% women, and 10% children.[1] In the last years, several efforts have been done by WHO in the commitment of high-income countries and of public, private, and philanthropic donors to increase investments in multidisciplinary TB research to accelerate TB elimination.[2]

The current antibiotic treatment of active TB consists in the administration of the first-line drugs isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for 2 months, followed by RIF and INH for 4 months. Poor adherence to long-term therapy increases the number of patients harboring multidrug-resistant (MDR) *M. tuberculosis* strains (i.e., resistant at least to INH and RIF) and extensively drug-resistant strains (i.e., MDR strains resistant to any fluoroquinolone (FQ) and to at least one injectable second-line drug, kanamycin, amikacin (AK), or capreomycin (CP).[3]

Besides active TB, an estimated 2 billion people in the world have latent TB infection (LTBI) i.e., they harbor *M. tuberculosis* in a nonreplicating (NR) (dormant) stage in their tissues, with 10% of persons reactivating to active TB lifetime.[3,4] As similar to active TB, the treatment of latent TB is also very long, including 9 months of INH or 3 months of RFP plus INH.[5-7]
The lungs of patients with active and latent TB contain heterogeneous mixtures of cellular and caseous granulomas harboring an array of tubercle bacilli ranging from actively replicating (AR) to NR drug refractory stages. While in cellular granulomas, AR bacilli are killed by current therapy, in low-vascularized caseous granulomas, the low oxygen tension stimulates aerobic/microaerophilic AR bacilli to transit into a dormant state in their hypoxic centers. Here, the NR bacilli survive extracellularly in a solid necrotic material (caseum) but are unable to multiply because of anoxic conditions and pH changes. Analysis of the lipid composition of human caseum revealed that it contains cholesterol, cholesteryl esters, triacylglycerols, and lactosylceramide, and its formation correlated with \textit{M. tuberculosis}-mediated dysregulation of host lipid metabolism. For unknown reasons, in about 10% of persons with LTBI, the solid caseous material softens and the granulomas expand to meet the bronchial tree. Expanding lesions fuse with the airway structure and form cavities, in which the caseum liquefies. In the liquefied material, the NR bacilli rapidly multiply on contact with air and are released into the Airways as a mixture of AR and NR cells in the sputum of highly contagious pulmonary TB patients.

In the last years, unprecedented efforts have been done in TB drug discovery in the attempt to eradicate \textit{M. tuberculosis} from humans, but the battle is still long because drugs do not penetrate well both the complex lung TB lesions and the cell wall of NR bacilli living in low-vascularized necrotic granulomas. Understanding more in depth the biology of NR bacilli and set up new \textit{in vitro} and \textit{in vivo} models to measure NR killing by drugs/drug combinations may be important to find new, shorter, anti-TB therapies.

**The Nonreplicating \textit{Mycobacterium tuberculosis}**

\textit{M. tuberculosis} slows down its metabolism when unfavorable conditions in the tissues of TB patients occur, including low oxygen tension and nutrient shortage or a combination of these and other stresses. This is supported by physiological studies of culture models by exposing bacilli to various adverse conditions. Several models to obtain NR cells were reported together with description of their metabolic and molecular characteristics. The most commonly used stressing conditions were hypoxia, nutrient starvation, acids and/or nitric oxide stationary phase, an NR model using the streptomycin-starved strain 18b (SS18b) was also described. Other models and reviews on NR cells were also published.

Given the multiplicity of models, different NR subpopulations were generated, implying some uncertainty in the nomenclature and the need to define the phenotypic/molecular characteristics of different NR bacilli. In general, the term “dormant” was associated with NR cells generated in the hypoxic model of Wayne with early and late gene cascades being reported, such as the DosR Regulon and the enduring hypoxic response, respectively. In the last years, a number of investigators used the term “persisters” to indicate phenotypically drug-resistant (drug-tolerant) NR bacilli. Low numbers of drug-tolerant persisters were shown to be present in the early exponential phase of \textit{M. tuberculosis}, followed by an increase up to ~1% of stationary phase cells. Persister transcriptome analysis of cells obtained by several \textit{in vitro} dormancy models showed overexpression of toxin-antitoxin systems and identified a small number of genes upregulated in all cases, likely representing a core dormancy response. The dormant state of persisters was believed to be a stochastic phenomenon independent of genetic mutations, but a recent study showed that after exposure of \textit{M. tuberculosis} to RIF, persisters cells with high levels of hydroxyl radical generated genetically resistant mutants. Overall, these observations may shed a new light on the interplay between genotypic (chromosomal mutations) and phenotypic (dormancy) drug resistance.

**In vitro Activity of Drugs Against Nonreplicating \textit{Mycobacterium tuberculosis}**

It is commonly thought that TB lesions contain coexisting \textit{M. tuberculosis} bacilli at different physiological stages. Several \textit{in vitro} models have been proposed to study activity of drugs against NR bacilli. Although none of them is truly representative of microenvironments met by \textit{M. tuberculosis} in the tissues of LTBI- and TB-patients, the models have great value to understand differences in the activity of drugs against NR bacilli.

In this study, to compare killing activity (log$_{10}$CFU reduction) of drugs against \textit{M. tuberculosis}, we performed a literature search using the terms “persistent, \textit{Mycobacterium tuberculosis}, drug,” “dormant, \textit{Mycobacterium tuberculosis}, drug,” and “nonreplicating, \textit{Mycobacterium tuberculosis}, drug” in the PubMed database. After reviewing abstracts and articles, we decided to restrict the study to marketed drugs and new drugs in phases 1–3 of clinical development (www.newtbdrugs.org). CFU reductions were determined after subtracting drug-treated CFUs from drug-untreated CFUs in tables and graphs of selected papers. Activity against NR \textit{M. tuberculosis} of molecules recently discovered or under preclinical development was reviewed elsewhere.

A rapid comparison of NR CFU reductions generated by 25 drugs tested at a total of 53 concentrations is represented as bars in Figure 1. The bars in the first three columns represent CFU reductions after 7 days of drug exposure in Wayne hypoxic models (18–21-day-old cells) at pH 5.8 (mimicking the environment of cellular granulomas), pH 6.6 (the pH of most mycobacterial media), and pH 7.3 (mimicking the environment of caseous granulomas). The bars in the other five columns of Figure 1 represent CFU reductions after 6 ± 1 days of drug exposure under the following conditions: pH 5.5 + NO, 7 days; 42-day nutrient starvation; 28-day stationary phase; ≥60-day stationary phase; SS18b. Overall, the most active drugs against \textit{M. tuberculosis} were the rifamycins, RIF and RFP. Indeed, 1–10 µg/ml of RIF reduced CFUs by 4.5–6.3-log$_{10}$ in the pH 5.8, 6.6, and 7.3 Wayne models (19-day-old cells) and...
by 3.3–6.0-log_{10} in the 42-day starvation,[49] 28-day stationary phase,[50] and SS18b model.[27,55-57] Furthermore, 10 µg/ml of RFP reduced CFUs by 3.3–4.9-log_{10} in the pH 5.8 and 7.3 Wayne models (19-day-old cells).[41,43]

Other effective agents were the new anti-TB drugs PA-824 (PA) (pretomanid), FQs, and metronidazole (MZ), as shown by ≥2.0-log_{10} CFU reduction in at least one model. Indeed, PA-824 reduced CFUs by 2.1-log_{10} in the pH 6.6 Wayne model (21-day-old cells)[47] and by 2.3–4.2-log_{10} in the pH 5 plus NO model,[48] ≥60-day stationary phase,[53] and SS18b model.[27] As to the FQ, 5 µg/ml of moxifloxacin (MX) and 4 µg/ml of gatifloxacin (GF) reduced CFUs by 3-log_{10} in the 28-day stationary phase[50] and in the SS18b model.[27] MZ (8 µg/ml) reduced CFUs by 2.2-log_{10} in the pH 6.6 Wayne model (19-day-old cells).[46]

The drugs with <2 log_{10} CFU reduction were bedaquiline (BQ) (1.8–1.9-log_{10} reduction in the pH 6.6 Wayne model [18-day-old-cells][45] and in the SS18b model[27]), the protein synthesis inhibitors AK, sutezolid (SZ), linezolid (LZ), CP (1–1.8-log_{10} reduction in the pH 6.6 Wayne model[45-46] and the SS18b model[50]), thioridazine (TH), nitazoxanide (NZ), clofazimine (CL), and meropenem + clavulanic acid (0.9–1.4-log_{10} reduction in the pH 5.8 Wayne model[41,42] or the SS18b model[55-57]). In all models examined, the least active drugs were INH, EMB, PZA, nicosamide (NC), PBTZ169, and SQ109.

However, results obtained by different 7-day-drug exposure models need some analysis. For instance, in the Wayne model at pH 5.8 (19-day-old cells), when exposure was prolonged from 7 to 14 or 21 days, RFP, PA, BQ, MZ, CL, NC, NZ increased their activity by >100 times.[41,42] Furthermore, in this model, PZA showed a time-dependent killing, reaching a 1.4-log_{10} CFU reduction on day 21.[41] In contrast, CFU reduction by MX, AK, and LZ was similar on day 7, 14, or 21.[41,42] The activity of RIF, BQ, and PA in the Wayne model at pH 6.6 also increased by >100 times when incubation was extended from 7 to 14 or 21 days.[45-47] In general, in the same model, a dose–response increase in CFU reduction of NR M. tuberculosis was observed. In the pH 5 + NO model, dose–response CFU assays showed that the rank of activity
at 10 µg/ml was PA > RIF > BQ > MX. Dose–response studies in 42-day nutrient starvation and/or ≥60-day stationary phase models showed that 10 µg/ml RIF and PA were very active while FQ showed low activity. High killing by 1 µg/ml of RIF and 4 µg/ml of GF in the 28-day stationary phase model was possibly due to the presence of semidormant cells in the cultures, in comparison with ≥60-day stationary phase models. The SS18b model showed that RIF, PA, and BQ were active against NR *M. tuberculosis*; however, MX, SZ, and LZ efficiently reduced CFUs (from 1.8 to 3-log). After comparison of all these models, the most evident conclusion was that RIF, RFP, and PA were the most active drugs *in vitro* against NR *M. tuberculosis*. However, in the Wayne model at pH 7.3 mimicking environment of caseum, only RIF and RFP efficiently killed NR bacilli, while other drugs (PZA, PA, BQ, CL, NC, NZ, TH, INH, AK, MX) had no or little effect. This novel model may be important for testing activity of drugs against NR *M. tuberculosis*.

The results of a battery of potency assays in microplates against NR *M. tuberculosis* are shown in Table 1, including the Wayne cidal concentrations (WCC<sub>90</sub>) and the Loebel cidal concentrations (LCC<sub>90</sub>). The minimum inhibitory concentrations (MIC<sub>90</sub>) obtained by the luminescence-based low oxygen recovery assay (LORA) and at pH 5 + NO showed that 10 µg/ml RIF, BQ, MX, and PA were active against NR *M. tuberculosis*; however, MX, SZ, and LZ efficiently reduced CFUs (from 0.31 to 1.25 µg/ml), followed by BQ, MX, PA, depending on the test. For instance, BQ and MX showed MIC<sub>90</sub> ranging from ≤0.2 to 4 µg/ml in three assays (WCC<sub>90</sub>, LORA MIC<sub>90</sub>, pH 5 + NO MIC<sub>90</sub>) while PA MIC<sub>90</sub> ranged from 0.4 to 4.3 µg/ml in two assays (LORA MIC<sub>90</sub> and pH 5 + NO MIC<sub>90</sub>). However, as to RIF, BQ, MX, PA, and several other drugs, the carryover effect, i.e., the percentage of drug removed by activated charcoal, was very high (99.9%) showing the limitations of liquid-based MICs for assessing drug activity under NR and AR conditions. Due to the good activity of RIF, BQ, MX, and PA, both *in vitro* (against NR and AR *M. tuberculosis*) and in animal models, these four molecules were recently named “dual active molecules.”

### Table 1: Inhibitory (MIC<sub>90</sub>) and bactericidal (WCC<sub>90</sub>, MBC ≥99) LCC<sub>90</sub>) concentrations of drugs (µg/ml) against nonreplicating *M. tuberculosis*<sup>*</sup>

| Drug (marketed or in trial) | Low O<sub>2</sub>; Wayne pH 6.6 (WCC<sub>90</sub> 20D+5d) | Low O<sub>2</sub>; LORA (MIC<sub>90</sub>) | pH 5 + NO (MIC<sub>90</sub>) | NARA MBC ≥99; CARA MBC ≥99 | Starvation (LCC<sub>90</sub>, 14D+5d) | Reference |
|-----------------------------|---------------------------------|---------------------------------|--------------------------|-------------------------------|-------------------------------|----------|
| Rifampin                    | 0.5; 0.82; 0.4 ≤0.4              | 0.31-1.25                       | 0.63-1.25 ; 5-10         | 8.23; 20.6; 20.6              | 45; 59; 60/61/48/48/59; 16; 60 | 59/59    |
| Rifapentine                 | 0.4                              |                                 |                          | 8.8; 8.8                      | 59/59; 62/48/48              | 59/59    |
| PA-824                      | 7.2                              | 4.3; 0.82                       | 0.4-0.78                 | 0.78 ; 12.5-25                | 45; 59; 60/48/48/59          | 59/59    |
| Bedaquiline                 | 1; 2.6; 1.4                      | 1.56-3.13                       | 1.56 ; 12.5-25 =11.1     | 45; 59; 60/48/48/59          | 59/59; 60/48/48/59          | 59/59    |
| Moxifloxacin                | 4; 2.4                           | ≤0.2                            | 0.78-3.13                | >100                          | 40.1                          | 59/59    |
| Ofloxacin                   | 8.7                              |                                 | 1.56-3.13; ≥100          |                               |                               | 59/59    |
| Ciprofloxacin               | 10.9                             |                                 |                          |                               |                               | 59/59    |
| Levofloxacin                | 18.1                             |                                 |                          |                               |                               | 59/59    |
| Gatifloxacin                | 18.7                             |                                 |                          |                               |                               | 59/59    |
| Metronidazole               | 8.6                              | 6.8                             |                          |                               |                               | 59/59    |
| Clofazimine                 | 23.7                             | 0.4; 0.67                       |                          | >47.3                         | 59/61; 62/59                 | 59/59    |
| Amikacin                    | 5.9                              | ≤0.3                            |                          | >58.7                         | 59/59; 56/59                 | 59/59    |
| Linezolid                   | >33.7                            | >43; 9.6                        | 16.5                     | >33                           | 59/61; 62/48/48              | 59/59    |
| Capreomycin                 | 6.7                              | 1.1; 3.9                        |                          | >66.9                         | 59/61; 62/59                 | 59/59    |
| Meropenem                   | 38.3                             |                                 |                          | >38.3                         | 59/59; 62/48/48              | 59/59    |
| Clavulanic acid             | >19.9                            |                                 |                          | >19.9                         | 59/59; 62/48/48              | 59/59    |
| Niacinamide                 | 0.3                              |                                 |                          |                               |                               | 59/59    |
| Nitrozoxamide               | 3.13                             | 6.25 ; 6.25                     |                          | 18.5; 18.5                    | 59/61; 59/59                 | 59/59    |
| Thioridazine                | 7.4; 18.5                        | 4.34                            |                          | 18.5; 18.5                    | 59/61; 59/59                 | 59/61    |
| Pyrazinamide                | >12.3                            | 11.1                            |                          | >12.3                         | 59/61; 59/59                 | 59/59    |
| Ethambutol                  | >20.4                            | >26.5                           | 25-50                    | 50-100; >100                  | >20.4                         | 59/61/48/48/59 |
| Isoniazid                   | >64; >13.7                       | >17.5; >13.7                    | 12.5                     | 12.5 ; >100                   | >13.7                         | 45; 59/61; 62/48/48 |

*MIC<sub>90</sub>: Minimum Inhibitory Concentration 90, WCC<sub>90</sub>: Wayne Cidal Concentration 90, MBC ≥99: Minimum Bactericidal Concentration ≥99, LCC<sub>90</sub>: Loebel Cidal Concentration 90, "D: days of stress exposure, d: days of drugs exposure.
**In vitro Activity of Drug Combinations Against Nonreplicating Mycobacterium tuberculosis**

The *in vitro* activity of drug combinations against NR *M. tuberculosis* is depicted in Figure 2. Shown are log$_{10}$ CFU reductions after 7 ± 1 days of drug exposure in the Wayne model (19-day-old cells) at pH 5.8 [41] (panel A) and at pH 6.6 [46] (panel B), in the 28-day stationary phase [50,63,64] (panels C and D), and in the SS18b model [58] (panel E). CFUs were reduced by 0.5 log$_{10}$ at 0.1 µg/ml of RIF or by >5 log$_{10}$ at 1 or 8 µg/ml of RIF, and addition of various agents in 2-, 3- or 4-drug combinations usually increased RIF activity, but CFUs were often below the limit of detection. In the stationary phase, RIF activity was increased by MZ [63]. In the Wayne model, to overcome the limit of CFU detection, the killing of NR *M. tuberculosis* was demonstrated as lack of regrowth of drug-exposed cells in MGIT 960 tubes after >100 days of incubation. This parameter (day-to-positivity after >100 days, DTP >100) was much more sensitive than CFUs and demonstrated that RIF + MX + AK + PA sterilized both 19-day-old NR and 5-day-old AR bacilli in 14 days and that several RIF + MX-containing combinations sterilized 19-day-old NR bacilli in 21 days [41,46]. This suggests that the stringent DTP >100 assay may be used in place of CFU counts when studying the sterilizing activity of new drugs or combinations. In the SS18b model, the cell wall inhibitor in phase 1 trial PBTZ169 improved the NR killing of BQ and CF [58]. Again, these observations are in keeping with the knowledge that RIF, BQ, MX, and PA are pivotal drugs in the killing of NR *M. tuberculosis*.

**In vivo Sterilizing Activity of Drug Combinations Against Mycobacterium tuberculosis**

Besides *in vitro* tests, the sterilizing activity of a drug combination can be determined in animal models reaching different stages of granuloma formation, ranging from conventional mouse models, which do not develop caseating granuloma and cavitary lesions (e.g., the BALB/c mice), to guinea pig, rabbit, and macaque models, all showing necrosis, caseation, liquefaction, and cavity formation [13]. In the low-cost mouse models, drug efficacy is assessed by CFU counts in organs at selected time points during treatment (a measure of bactericidal activity) and by the proportion of mice with culture-positive relapse 3 months after discontinuation of treatment (a measure of sterilizing activity) [65]. Relapse was defined as the presence of *M. tuberculosis* colonies upon plating of entire undiluted lung homogenate [66].

The number of months of treatment necessary to sterilize mice by various regimens (lack of colonies after completion of treatment plus 3 months without treatment) is shown in Table 2. In BALB/c mice, the RIF-containing combinations RIF$_{10}$ + INH$_{10}$ + PZA$_{150}$ and RIF$_{40}$ + INH$_{10}$ + PZA$_{150}$ sterilized animals in 6 and 3 months, respectively [65,67]. Furthermore, the RFP-containing combinations RFP$_{10}$ + INH$_{10}$ + PZA$_{150}$...
and RFP_{20} + INH_{10} + PZA_{150} sterilized BALB/c mice in 3 and 2.5 months, respectively.\textsuperscript{67} This indicated that increased doses of RIF and of the long-lasting rifamycin RFP (recently suggested for short treatment of LTBI)\textsuperscript{5-7} consistently shortened the duration of sterilizing therapy in BALB/c mice. In the combination RIF_{10} + INH_{10} + EMB_{100} + PZA_{150}, substitution of MX for INH reduced the sterilizing time from 4 to 3 months.\textsuperscript{68} BALB/c mice were sterilized in 2 months by BQ_{25} + PZA_{150}\textsuperscript{69} and BQ_{25} + CL_{20} + PZA_{150},\textsuperscript{69} BQ_{25} + PZA_{150}, BQ_{25} + TBA-354_{50} + PZA_{150} and SZ_{50} + PA_{10} \textsuperscript{70} and in 1.5 months by BQ_{25} + RFP_{10} + CL_{20} + PZA_{150},\textsuperscript{69} SZ_{20} + TB-354_{50} and SZ_{50} + TB-354_{30}.\textsuperscript{70} and by 1 month of BQ_{25} + PA_{100} + LZ_{100} + PZA_{150} followed by 1 month of BQ_{25} + PA_{100} + PZA_{150}.\textsuperscript{71} These observations also show that besides RIF and RFP, other drugs such as BQ, PA, SZ, LZ, CL, MX, TBA-354, and PZA are promising to shorten anti-TB therapy in the future, and all but TBA-354 are presently tested in combination in human trials (www.newtbdrugs.org). Indeed, because of side effects in phase 1 studies, clinical development of TBA-354 was discontinued in 2016 (https://www.tballiance.org/news/phase-1-clinical-trial-tb-drug-candidate-tba-354 discontinued).

Recently, M. tuberculosis-infected C3HeB/FeJ mice were reported to develop necrotic lung granulomas with abundant extracellular bacilli, so they are becoming a candidate to supplement, or even replace, conventional mouse strains for evaluation of TB drugs.\textsuperscript{72-74} As expected, drug treatments were less effective than in BALB/c mice; however, 2 months of RIF_{10} + INH_{10} + EMB_{100} + PZA_{150} followed by 4 months of RIF_{10} + INH_{10} + PZA_{150} sterilized C3HeB/FeJ mice in 4.5 months [Table 2].\textsuperscript{75}

## TARGETING NONREPLICATING MYCOBACTERIUM TUBERCULOSIS IN THE CASEUM

In conventional mouse models, M. tuberculosis bacilli were found primarily intracellularly, whereas in guinea pigs, the majority of bacteria in the necrotic lesions of the lungs were extracellular.\textsuperscript{76} Following drug treatment, a homogenous bacillary reduction

### Table 2: Sterilizing activity of drug combinations against M. tuberculosis-infected BALB/c and C3HeB/FeJ mice

| Drug doses, mg/kg | N° of months reported to have sterilized\* | Reference |
|------------------|----------------------------------------|-----------|
|                  | BALB/c mice | C3HeB/FeJ mice |
| RIF 10 + INH 10 + PZA 150 | 6 | 65 |
| BQ 25 + PZA 150 | 3 | |
| BQ 25 + PA 50 + PZA 150 | 4 | |
| BQ 25 + MX 100 + PZA 150 | 4 | |
| BQ 25 + LZ 100 + PZA 150 | 3 | |
| BQ 25 + RIF 10 + PZA 150 | 3 | |
| BQ 25 + RFP 10 + PZA 150 | 2 | |
| BQ 25 + CL 20 + PZA 150 | 2 | 69 |
| BQ 25 + RFP 10 + CL 20 + PZA 150 | 1.5 | |
| BQ 25 + SZ 50 + PA 50 | 3 | 70 |
| BQ 25 + PZA 150 | 2 | |
| BQ 25 + TBA-354 50 + PZA 150 | 2 | |
| SZ 50 + PA 50 | 0.5 | 70 |
| SZ 50 + TBA-354 25 | 1.5 | |
| SZ 50 + TBA-354 50 | 1.5 | |
| BQ 25 + PA 50 + LZ 100 | 3 | 71 |
| 1 BQ 25 + PA 100 + LZ 100/BQ 25 + PA 100 | 3 | |
| 2 BQ 25 + PA 100 + LZ 100/BQ 25 + PA 100 | 3 | |
| 2 BQ 25 + PA 100 + LZ 100/BQ 25 + PA 100 + LZ 50 | 3 | |
| BQ 25 + PA 100 + LZ 50 | 3 | |
| BQ 25 + PA 100 + PA 100 | 3 | |
| 1 BQ 25 + PA 100 + LZ 100/1 BQ 25 + PA 100 + PZA 150 | 1.5 | |
| RIF 10 + INH 10 + EMB 100 + PZA 150 | 4 | 68 |
| RIF 10 + MX 100 + EMB 100 + PZA 150 | 3 | |
| 2 RIF 10 + INH 10 + EMB 100 + PZA 150/4 RIF 10 + INH 10 | 4.5 | 75 |
| RIF 40 + INH 10 + PZA 150 | 3 | 67 |
| RFP 10 + INH 10 + PZA 150 | 3 | |
| RFP 20 + INH 10 + PZA 150 | 2.5 | |
| RFP 10 + INH 10 + EMB 100 + PZA 150 | 3 | |
| 2 RIF 10 + INH 10 + EMB 100 + PZA 150/4 RIF 10 + INH 10 + PZA 150 | 4.5 | 75 |

\*Sterilization was defined as lack of M. tuberculosis colonies upon plating of entire undiluted lung homogenates obtained from mice killed three additional months after completion of the indicated months of treatment. The acronyms of individual drugs in the combinations are indicated in the text
across granulomas was observed in mice, whereas in guinea pigs, the NR extracellular bacilli persisted in lesions with residual necrosis.[74] These observations indicated that drug development should be designed to target NR populations and that drug regimens should be evaluated in appropriate animal models.

The C3HeB/FeJ mice showed heterogeneity in the size and degree of liquefaction of caseous lesions between mice and between lesions within the same mouse. Furthermore, unlike the common belief that the pH of caseum was acidic, the pH of liquefied caseous material from C3HeB/FeJ mice was found to be 7.4 (range 7.2–7.5).[73] This value was similar to that found in lesions of M. tuberculosis infected guinea pigs (pH 7.2, range 7.0–7.5), rabbits (pH 6.4–7.4), and humans (pH 6.1–7.4).[73] Overall, C3HeB/FeJ mice may be a good model for mimicking human TB. Due to the variety of granulomatous lesions in these mice, PZA had no or little activity in a subset of animals with large caseous lesions, where the pH approached 7.4. Indeed, sterilization occurred when PZA was administered in combination with the first-line drugs in mice with less extensive disease.[19] A similar dichotomous activity was seen in BQ-treated C3HeB/FeJ mice.[77]

A limited activity of CL, a poorly soluble, lipophilic drug, was reported in C3HeB/FeJ mice.[78] The lipophilic character of a drug is mostly expressed as hydrophobicity (calculated octanol/water partitioning coefficient, clogP).[19] Drugs with clogP < 0 are hydrophilic.[42] Compounds with moderate hydrophobicity (clogP between 0 and 3) are optimal for oral administration owing to a good balance of solubility and permeability. In the current TB therapy, only RIF is lipophilic (clogP of 3.85) while the other three drugs (INH, EMB, PZA: clogP of −0.71, −0.12, −0.71, respectively) (http://www.drugbank.ca) are hydrophilic.

The caseum is devoid of vascular supply, and in M. tuberculosis-infected rabbits, it was found that only the free drug fraction (fraction unbound, f<sub>u</sub>) can penetrate this matrix through passive diffusion.[19] Hydrophobic and aromatic ring count of a drug were shown to be proportional to caseum binding and compounds with clogP < 1 had a high chance of achieving f<sub>u</sub> >10%.[19] Lipophilic drugs nonspecifically bind to caseum macromolecules at the outer edge of the caseum core, preventing further passive diffusion toward the center of the necrotic core.[19]

Indeed, the f<sub>u</sub> of the highly lipophilic agents CL and BQ (clogP of 7.39 and 6.37, respectively) was <0.01%, while that of hydrophilic molecules such as INH and PZA was >99.9%. Drugs with intermediate clogP values such as RFP, Rif, PA, and SZ (clogP of 4.83, 3.85, 2.8, and 1.22, respectively) showed increasing free fraction (f<sub>u</sub> of 0.5, 5.1, 7.3, and 30.1%, respectively).[19,42] Other drugs including LZ and MX (clogP of 0.61 and 0.01, respectively) showed f<sub>u</sub> of 29.3 and 13.5%, respectively.

In summary, to combat the battle against TB, we should know more in depth the biology of the caseum and of NR bacilli[79,80] and find new tools for the search of novel sterilizing combinations. In the Wayne model at pH 5.8, it was reported that lipophilic drugs were more active than hydrophilic agents against NR M. tuberculosis.[42] However, in the Wayne model at pH 7.3, only RIF and RFP (inhibitors of the subunit beta of RNA polymerase, rpoB) efficiently killed NR bacilli, while several other drugs had no or little effect irrespective of being lipophilic or hydrophilic.[43] It is known that RIF accumulates and maintains therapeutic levels in the caseum, where dormant M. tuberculosis resides[19] and that high-dose treatments with RIF in TB patients reduced the time to culture conversion.[81] In addition, there is increasing evidence that RFP + INH regimen for the treatment of latent TB is as effective, better tolerated, and more likely to be completed compared to INH.[67,82] In this respect, novel combinations containing optimized rifamycins dosages might shorten treatments of active and latent TB in the future.

In summary, on the basis of in vitro observations on NR bacilli and recent studies on drug penetration in caseum,[19] we suggest that the search for new TB drugs could be addressed to molecules with low lipophilicity (e.g., new rpoB inhibitors with clogP < 3) killing NR M. tuberculosis in hypoxia at neutral pH (e.g., at around pH 7.3).

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Conflicts of interest
There are no conflicts of interest.

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