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Application of *Mycobacterium smegmatis* as a surrogate to evaluate drug leads against *Mycobacterium tuberculosis*

Nada Lelovic[†], **Katsuhiko Mitachi**[†], **Junshu Yang**[‡], **Maddie R. Lemieux**[†], **Yinduo Ji**[‡], **Michio Kurosu**[†]

[†] Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, 881 Madison Avenue, Memphis, TN 38163, United States

[‡] Department of Veterinary and Biomedical Sciences, University of Minnesota, 205 VSB, 1971 Commonwealth Avenue, St. Paul, MN 55108, United States

Abstract

Discovery of new anti-tuberculosis (TB) drugs is a time-consuming process due to the slow-growing nature of *Mycobacterium tuberculosis* (Mtb). A requirement of biosafety level 3 (BSL-3) facility for performing research associated with Mtb is another limitation for the development of TB drug discovery. In our screening of BSL-1 *Mycobacterium* spp. against a battery of TB drugs, *M. smegmatis* (ATCC607) exhibits good agreement with its drug susceptibility against the TB drugs under a low-nutrient culture medium (0.5% Tween80 in Middlebrook 7H9 broth). *M. smegmatis* (ATCC607) enters its dormant form in 14 days under a nutrient-deficient condition (a PBS buffer), and shows resistance to a majority of TB drugs, but shows susceptibility to amikacin, capreomycin, ethambutol, and rifampicin (with high concentrations) whose activities against non-replicating (or dormant) Mtb were previously validated.

Keywords

Mycobacterium tuberculosis surrogate; *Mycobacterium smegmatis*; TB drugs; non-replicating *Mycobacterium smegmatis*

INTRODUCTION

Since the emergence of multidrug-resistant *Mycobacterium tuberculosis* (Mtb), the importance of discovery of new tuberculosis (TB) drugs has been documented in a number of scientific publications [1–5]. Public sector research agencies and nonprofit organizations play an important role in the development of TB drugs due to the fact that pharmaceutical companies have shied away from drug discoveries of neglected diseases including TB [6, 7]. A limited number of academic institutions have supported biosafety level 3 (BSL-3) laboratory and biocontainment facilities to perform TB researches in the U.S. In addition to

Address correspondence to Michio Kurosu, mkurosu@uthsc.edu.

This article is dedicated to the memory of Dr. Isao Kitagawa, Professor Emeritus of pharmaceutical sciences at Osaka University, an inspirational scientist.

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inconvenient accessibility for other researchers to the required facility, the pathogenicity, and a slow-growing nature of Mtb might discourage scientists from TB drug discovery. Particularly, a whole cell-based assay frequency of TB drug leads using Mtb strains is one of the factors that leads to a slow process of medicinal chemistry [1, 8–17]. Applying non-pathogenic *Mycobacterium smegmatis* strains to TB drug discovery has shown a limited number of successes in the past [18–21]. Under rich-nutrient culture media (or recommended culture conditions), a majority of *M. smegmatis* strains are not susceptible to the TB drugs (*e.g.*, rifampicin, INH, and ethambutol) [20]. In the screening of *M. smegmatis* strains susceptible to the TB drugs, it was found that *M. smegmatis* (ATCC 607) was effectively killed by the representative TB drugs (*e.g.*, rifampicin, INH, ethambutol, and amikacin) in 2–4 days under a low-nutrient culture condition (0.5% Tween 80 in Middlebrook 7H9 broth). To the best of our knowledge, it is the first observation that an *M. smegmatis* strain shows similar drug susceptibility to Mtb H37Rv strain. Herein, we report 1) drug susceptibility of *M. smegmatis* (ATCC607) against 20 antibacterial agents including the 1st and 2nd line, and investigational TB drugs, 2) mechanistic studies of antibacterial effects of the representative TB drugs against *M. smegmatis* (ATCC607), and 3) responses of some TB drugs against non-replicating *M. smegmatis* (ATCC 607).

MATERIALS AND METHODS

General / Chemicals and Reagents

All chemicals and antibiotics were purchased from commercial sources and used without further purification unless otherwise noted. Difco Middlebrook 7H10 agar, Middlebrook 7H9 broth, 44 Brain Heart Infusion Agar/Broth, Tween 80, ADC and OADC enrichment were purchased from Fisher Scientific. Resazurin (Alamar blue) was purchased from Sigma-Aldrich. Note: Middlebrook 7H9 contains following ingredients ($g \cdot L^{-1}$): ammonium sulfate (0.50), disodium phosphate (2.50), monopotassium phosphate (KH_2PO_4, 1.00), sodium citrate (0.10), magnesium sulfate (0.05), calcium chloride (0.0005), zinc sulfate (0.001), copper sulfate (0.001), ferric ammonium citrate (0.04), L-glutamic acid (0.50), pyridoxine (0.001), biotin (0.0005). Middlebrook 7H10 contains following ingredients ($g \cdot L^{-1}$): ammonium sulfate (0.50), disodium phosphate (1.50), monopotassium phosphate (KH_2PO_4, 1.50), sodium citrate (0.40), magnesium sulfate (0.025), calcium chloride (0.0005), zinc sulfate (0.001), copper sulfate (0.001), ferric ammonium citrate (0.04), L-glutamic acid (0.50), pyridoxine hydrochloride (0.001), biotin (0.0005), malachite green (0.00025), agar (15.00).

Bacterial and macrophage strains

Mycobacterium smegmatis (ATCC607) was purchased from ATCC. *Mycobacterium tuberculosis* $H_{37}Rv$ was acquired from BEI Resources (NIAID).

MIC assays

Log phase bacterial culture—All liquid bacterial culturing was performed with a conical flask with an air filter. A single colony of a bacterial strain (*M. tuberculosis*) was grown on a Difco Middlebrook 7H10 nutrient agar (enriched with 10% OADC and 0.4% glycerol). Seed cultures and larger cultures of *M. tuberculosis* were obtained using

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Middlebrook 7H9 broth enriched with 10% OADC and 0.4% glycerol. *M. smegmatis* (ATCC607) was cultured on a 0.5% Tween 80 Middlebrook 7H10 nutrient agar (0.4% glycerol). Seed cultures and larger cultures of *M. smegmatis* (ATCC607) were obtained using 0.5% Tween 80 in Middlebrook 7H9 (0.4% glycerol). The culture flasks were incubated for 3–4 days for *M. smegmatis* (ATCC607), and for 10–12 days for *M. tuberculosis* $H_{37}Rv$ in a shaking incubator at 37 °C with a shaking speed of 200 rpm and cultured to mid-log phase (optical density - 0.5). The optical density was monitored at 600 nm using a 96 well microplate reader.

Determination of minimum inhibitory concentration (MIC)—The antibiotics were dissolved in DMSO or water (a final concentration of 1 mg per 100 μL). This concentration was used as the stock solution for all studies. Bacterial cultures at 0.2 optical density, were treated with serial dilutions of inhibitors in aerobic conditions and incubated at 37 °C for 4 and 14 days for *M. smegmatis* and *M. tuberculosis*, respectively. Alamar blue (2%, 20 μL) was added and incubated in a static incubator at 37 °C for 4–12 h. The lowest concentration at which the color of Alamar blue was completely retained as blue was read as the MIC (Pink = Growth, Blue = No growth). The absorbance measurements were also performed using a Biotek Synergy XT, 96 well plate reader at 570 nm and 600 nm.

Generation of drug-resistant *M. smegmatis* (ATCC607) strains—Drug-resistant *M. smegmatis* (ATCC607) strains against rifampicin (RIF), ethambutol (EMB), isoniazid (INH), capreomycin (CAP), and amikacin (AMK) were generated according to the same procedure. *M. smegmatis* (ATCC607) (100 μL of 1×10^7 CFU•mL^{-1}) was plated on 0.5% Tween 80 Middlebrook 7H10 nutrient agar plate (55 cm^2) containing antibiotics (minimum bactericidal concentration (MBC)). The colonies grown on the antibiotic-containing agar plate were collected and suspended in a PBS buffer (~1×10^7 CFU•mL^{-1}), and 100 μL of the bacterial suspension was plated on the agar plate containing antibiotics (1.5xMBC). This process was repeated until the cells acquire >5 times higher MIC level than the wild type. The concentrations of antibiotic were gradually increased (2.0x, 2.5x, 3.0x, 3.5x, 4.0x, 5.0x, 7.0x, 8.0x, 9.0x, 10.0x, and 20xMBC). The isolated resistant cells were confirmed by the MIC assay with the generated resistant strain.

Genetic analyses of *M. smegmatis* (ATCC607) resistant strains—The chromosomal DNAs from the resistant mutants, including RIF^R-*M. smegmatis*, EMB^R-*M. smegmatis*, Cap^R-*M. smegmatis*, and AMK^R-*M. smegmatis*, INH^R-*M. smegmatis* and their parental control *M. smegmatis* (ATCC607), were isolated using a NucleoSpin Tissue kit (Macherey-Nagel). The target genes of these antibiotics, including *embB, rpoB, embB, rrs, inhA, katG*, and *ahpC*, were amplified using the purified genomic DNA as a template by PCR using high fidelity DNA polymerase (BioLabs) and gene-specific primers listed in Table S1 (supporting information). The PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) or ExoSAP-IT Express PCR Cleanup reagents (ThermoFisher Scientific) and sequenced. The DNA sequences of these target genes were blasted against their corresponding DNA sequences of *M. smegmatis* in the NIH Genome database.

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Formation of non-replicating *M. smegmatis* (ATCC607)—The starter culture of *M. smegmatis* (ATCC607) was obtained from a single colony by incubation in 0.5% tween 80-Middlebrook 7H9 medium at 37 °C for 4 days. The seed culture (1 mL) was inoculated into 0.5% Tween 80 Middlebrook 7H9 (50–75 mL). This culture was incubated in a shaking incubator (200 rpm, 37 °C) for 48–72 h. A stationary phase (OD ~1.0) culture was kept in a static incubator (37 °C) for 30, 60, or 150 days.

Formation of non-replicating *M. smegmatis* (ATCC607) under a nutrient-deficient condition—The starter culture of *M. smegmatis* (ATCC607) was obtained from a single colony by incubation in 0.5% tween 80-Middlebrook 7H9 medium (35 mL) at 37 °C for 4 days. The culture was centrifuged (4,500 rpm for 10 min.) and suspended in phosphate-buffered saline (PBS, pH 7.2) for 14 days (37 °C)

Antimycobacterial activity of TB drugs against non-replicating *M. smegmatis* (ATCC607)—Non-replicating *M. smegmatis* (ATCC607) generated via the procedure described above was kept in the presence of each drug (5xMBC or 20xMBC) (total volume 200 μL) for 5–15 days at 37 °C. The cultures were diluted 4,000 times (10×20×20), and 100 μL of the diluted cell culture was plated on 0.5% Tween 80 Middlebrook 7H10 nutrient agar plate. Colony-forming units (CFUs) of survival *M. smegmatis* cells on agar plates were counted after incubation at 37 °C for 4 days.

RESULT

Susceptibility of *M. smegmatis* (ATCC607) against representative TB drugs

The MIC values of representative TB drugs, and positive- and negative-controls against *M. smegmatis* (ATCC607) were examined under a nutrient-deficient condition (0.5% Tween 80-Middlebrook 7H9 broth). These data are summarized in Table 1. Table 1 also includes their MIC values (reported in literature/databases or obtained in our lab as noted) [22–31], drug target(s), and drug-resistant mechanism(s) against *M. tuberculosis* (Mtb). A majority of FDA-approved TB drugs (isoniazid, rifampicin, ethambutol, bedaqline, aminoglycosides, streptomycin, capreomycin, ethionamide, clofazimine, and cycloserine) showed good correlation in the MIC values between *M. smegmatis* (ATCC607) strain and Mtb $H_{37}Rv$. Drug susceptibility of rifampicin (RIF) against *M. smegmatis* was 16–19 times higher MIC value than that against Mtb. On the other hand, the fluoroquinolones (moxifloxacin and ciprofloxacin) exhibited 5–10 times lower MIC values against *M. smegmatis* than those against Mtb. Pyrazinamide is known to show pH-dependent susceptibility against Mtb *in vitro* [32, 33]. At pH 6.6 it killed *M. smegmatis* efficiently with much lower MIC value (0.097–1.6 μg/mL) than that against Mtb. The MIC value of tunicamycin, a MraY/WecA inhibitor [34, 35], against *M. smegmatis* exhibited equal to that against Mtb. Glycopeptide antibiotics, vancomycin, and ristocetin A [8], had 3–10 times better susceptibility to *M. smegmatis* than Mtb. The negative-controls showed a good agreement in their susceptibility: colistin did not show antibacterial activity against *M. smegmatis*. Under the aerobic conditions, metronidazole was not effective in inhibiting the growth of *M. smegmatis* [36]. In these susceptibility tests, all TB drugs showed susceptibility against *M. smegmatis* under

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a slow growth condition, and a majority of TB drugs showed good or meaningful correlations for their MIC values except pyrazinamide.

Determination of *M. smegmatis'* mechanisms of resistance against representative TB drugs

We have generated drug-resistant strains of *M. smegmatis* (ATCC607) against rifampicin (RIF), ethambutol (EMB), isoniazid (INH), capreomycin (CAP), and amikacin (AMK). Their MIC values were 5-times or higher than those of the wild-type strain. It has been studied that RIF, EMB, CAP and AMK exhibit antibacterial activity by targeting RpoB (bacterial RNA polymerase) [37, 38], EmbB (*embB* gene encoding arabinosyltransferase activity) [39–41] and 16S ribosome RNA (encoded by the *rrs* gene) [42, 43], respectively. INH targets InhA, and drug-resistant Mtb strains have mutations in the *katG* (Mtb catalase-peroxidase) and/or *ahpC* (alkyl hydroperoxide reductase C) gene [38, 44–46]. To understand whether the resistance of *M. smegmatis* to these antibiotics has resulted from the mutations of their target genes identified in the mutant Mtb cells, we isolated the chromosomal DNAs from the resistant mutants, including RIF^R-*M. smegmatis*, EMB^R-*M. smegmatis*, CAP^R-*M. smegmatis, and* AMK^R-*M. smegmatis* (intermediate resistant), and their parental control *M. smegmatis* (ATCC607). Except for *embB,* the other target genes, including *rpoB*, *rrs*, *inhA*, *katG*, and *ahpC*, were obtained by PCR and they were sequenced. The DNA sequences of these target genes were blasted against their corresponding DNA sequences in the NIH Genome database [47]. The DNA sequence alignment revealed E401Q, E462Q, A638G, A653G, and G656S mutations in the protein sequence of RpoB in the RIF^R-*M. smegmatis* mutant, compared with their parental control (Figure 2A). One of important mechanisms in *M. smegmatis* against RIF is its ADP-ribosylation by mono ADP-ribosyltransferase encoded by *arr* (MSMEG_1221). In this experiment, we cannot rule out the possibility of mutation in the *arr* gene in RIF^R-*M. smegmatis* mutant [48]. A544G, A562T, E591Q, and R598P mutations were identified in the protein sequence of KatG, whereas no mutation of *inhA* occurred in the INH^R-*M. smegmatis* mutant compared to its parental control (Figure 2B). Three nucleotide mutations of A564G, C818T, and A869G were identified in 16S rRNA gene (*rrs*) of the Cap^R-*M. smegmatis* strain (Figure 3A). Five nucleotide mutations of G710A, G809A, C818T, A869G, and G997A were revealed in the *rrs* of the AMK^R-mutant (Figure 3B). These results observed in RIF-, INH-, CAP-, and AMK-resistant *M. smegmatis* strains suggested that the mutations in the same target genes identified in the Mtb mutants contribute to resistance to the corresponding antibiotics.

Response of TB drugs against non-replicating *M. smegmatis* (ATCC607)

Mtb can persist many years within host tissues [49, 50]. Subpopulations of Mtb to enter a dormant phase lead to the long-term treatment adherence for TB and recurrence of TB. *In vitro* studies using the dormant forms of Mtb have demonstrated that non-replicating Mtb cells show resistance to a majority of TB drugs [1, 51]. We have cultured *M. smegmatis* (ATCC607) strain for up to 150 days in medium containing 0.5% Tween 80 as the source of the primary carbons [52]. Bactericidal activity of amikacin (AMK), capreomycin (CAP), rifampicin (RIF), isoniazid (INH), and ethambutol (EMB) were examined against *M. smegmatis* (ATCC607) cultured for 30 days, 60 days, and 150 days. AMK killed non-replicating forms (incubation periods of 30, 60, and 150 days) of *M. smegmatis* in 5 days at

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5-times the minimum bactericidal concentration (5xMBC) (Table 2) [53]. Similarly, susceptibility of the other TB drugs (CAP, RIF, INH, and EMB) showed no apparent difference against non-replicating *M. smegmatis* cultured for 30, 60, and 150 days. CAP was also determined to be an effective agent for killing non-replicating *M. smegmatis* at 5xMBC; over 2-log (99%) reductions of colony-forming unit (CFU) were observed in 5 days [54]. No CFU was counted for non-replicating *M. smegmatis* treated with CAP (5xMBC) for 15 days. RIF showed efficacy against non-replicating *M. smegmatis* in a concentration-dependent manner: a 25–30% reduction of the CFU with the drug concentration at 5xMBC for 5 days, and over 90% reduction at 20xMIC for 5 days [55, 56]. 15 Days of treatment of RIF against non-replicating *M. smegmatis* did not significantly show the CFU reduction. INH, a negative control, was not effective in killing non-replicating *M. smegmatis* at 20xMBC for 5 and 15 days, respectively [57, 58]. EMB was not efficacious in killing non-replicating *M. smegmatis* at 20xMBC for 5 days. However, it showed bactericidal activity against non-replicating *M. smegmatis* in a time-dependent manner: 15 Days treatment of EMB showed no countable colony [59]. The same susceptibility testing was performed with non-replicating *M. smegmatis* generated under a nutrient-deficient condition (stored in a PBS buffer for 14 days). AMK, CAP, RIF, and EMB displayed an equal or very similar trend of antimycobacterial activities to those observed against non-replicating *M. smegmatis* formed via 30–150 days incubation in the growth medium.

DISCUSSION

M. smegmatis is a useful research surrogate for pathogenic *Mycobacterial* species in laboratory experiments. For example, it is an excellent expression host for the production of recombinant proteins from various mycobacterial species [60]. However, under the recommended growth conditions (nutrient-rich conditions), *M. smegmatis* strains are resistant to many TB drugs (*e.g.*, rifampicin and isoniazid); *M. smegmatis* strains displayed resistance (MIC >20 $\mu g \bullet mL^{-1}$) to the key drugs in the 1st-line anti-TB drugs, such as rifampicin (RIF) and isoniazid (INH) [20]. Although resistant mechanisms have not been studied thoroughly, a number of TB scientists agree that *M. smegmatis* has intrinsic resistance to these drugs [61, 62]. Nonetheless, low susceptibility of *M. smegmatis* to several TB drugs discourages many scientists to apply *M. smegmatis* as a surrogate to screening TB drug leads. Under a slow-growing culture condition (0.5% tween 80-Middlebrook 7H9 broth), *M. smegmatis* (ATCC607) displayed antimicrobial susceptibility against all TB drugs tested (Table 1). Importantly, a majority of the 1st- and 2nd-line TB drugs showed a good correlation in their MIC levels against *M. smegmatis* (ATCC607) to those against M. *tuberculosis* (Mtb) $H_{37}Rv$ (a laboratory strain. Pyrazinamide showed better susceptibility against *M. smegmatis* (ATCC607) at an initial pH of 6.6 than that against Mtb [63]. All positive- and negative-control agents showed a good correlation in the MIC values between *M. smegmatis* (ATCC607) and Mtb. The observed drug susceptibility agreement between *M. smegmatis* (ATCC607) and Mtb premises that *M. smegmatis* strains could be reliable surrogates for Mtb in a slow-growing culture medium. We have spent about half a year to isolate mutant strains against rifampicin (RIF), isoniazid (INH), capreomycin (CAP), amikacin (AMK), and ethambutol (EMB). These resistant strains showed 5-times or higher MIC than those against the wild-type strain. The target genes (*rpoB*, *katG, ahpC, inhA,* and

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rrs) for RIF, INH, Cap, and AMK were obtained successfully from their resistant strains. Analyses of the amino acid or gene sequence alignments between the wild-type strain and these drug-resistant mutants revealed that RIF, INH, CAP, and AMK-resistant *M. smegmatis* strains show their resistances by the mutations of the same target genes that are identified in the corresponding Mtb mutants. Although a limited number of the drug- resistant mechanistic studies have been performed, it could be concluded that *M. smegmatis* (ATCC607) acquires the predicted resistant mechanisms against four TB drugs (selected from two of 1st and 2nd line TB drugs). These genotypic data further support that *M. smegmatis* (ATCC607) is a reliable and convenient surrogate for identifying new anti-TB drug leads. However, we have not performed whole-genome sequencing of the mutants, thus, we cannot rule out the possibility that other factors or genes beside the known target genes contribute to the observed drug resistances.

M. smegmatis (ATCC607) enters its non-replicating state within 30 days in 0.5% tween 80-Middlebrook 7H9 medium. Noticeable loss of viability of non-replicating *M. smegmatis* was observed during extended culturing from 30 to 150 days (see viable cells vs time curve in SI). Conveniently, *M. smegmatis* generated under a nutrient-deficient condition (stored in a PBS for 14 days) could predict the efficacy of TB drugs against non-replicating (or dormant) Mtb. Antimycobacterial activity of AMK, CAP, RIF, and EMB (positive-controls) was demonstrated with non-replicating *M. smegmatis*. INH, a negative-control, was not effective in killing non-replicating *M. smegmatis*. *In vitro* evaluation of dormant form of Mtb has not provided useful information on the effect of drugs on clinical tests. Drug susceptibility of non-replicating *M. smegmatis* (ATCC607) showed some correlations with *in vitro* data obtained with non-replicating Mtb; for example, bactericidal activity of capreomycin (CAP) against non-replicating tubercle bacilli was previously reported [64–66].

In summary, the application of a non-pathogenic *M. smegmatis* to a preliminary screening of library molecules and iterative medicinal chemistry should facilitate TB drug discovery programs. Under nutrient limiting conditions, certain *M. smegmatis* strains would display similar drug susceptibility observed against Mtb. In our screening of libraries of antibacterial molecules, we have not observed disagreement in drug susceptibility profiles between *M. smegmatis* (ATCC607) and Mtb H37Rv. However, the MIC values obtained with *M. smegmatis* should not represent absolute data but are relative data to evaluate the antibacterial activity of molecules against Mtb. We recommend evaluating new molecules with the MIC values of <6.25 mg•mL^{-1} against *M. smegmatis* (ATCC607) in bacterial growth inhibitory activity (MIC) assays against Mtb strain(s). Assay results of drug susceptibility of new antimycobacterial agents against *M. smegmatis* (ATCC607) and its drug-resistant strains and their MIC correlations to Mtb strains will be reported elsewhere.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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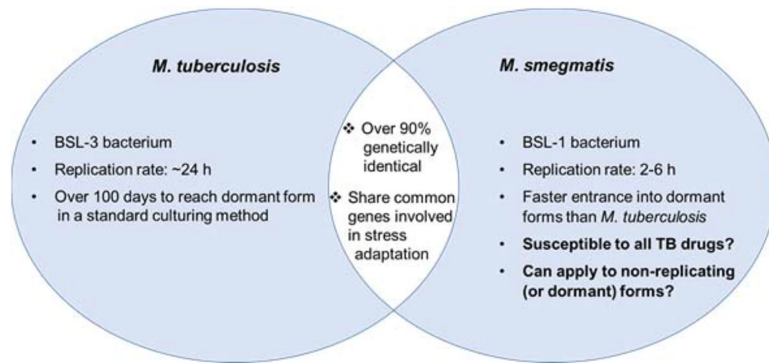

Figure 1.

Identification of *M. smegmatis* that is susceptible to TB drugs and beneficial of its application to TB drug discovery.

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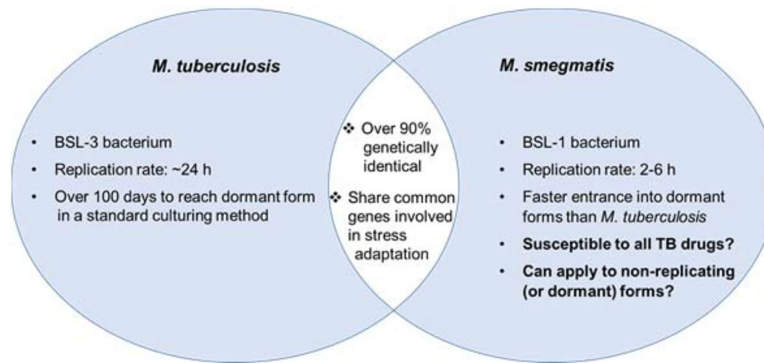

Figure 2.

The amino acid alignments of the bacterial RNA polymerase subunit protein B (RpoB) between Rifampicin^{R} (Rif^{R}) mutant and parental control *M. smegmatis* (**A**) and KatG protein between Isoniazid^{R} (INH^{R}) mutant and parental control *M. smegmatis* (**B**). The red arrow represents the site mutation in RpoB or KatG.

A

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M. semgmatis  1081  IDHFGNRRLRTVGELIQNQIRVGLSRMERVVRERMTTQDVQAITPQTLINIRPVVAAIKE  1260
                    IDHFGNRRLRTVGELIQNQIRVGLSRMERVVRERMTTQDV+AITPQTLINIRPVVAAIKE
RifR mutant   361   IDHFGNRRLRTVGELIQNQIRVGLSRMERVVRERMTTQDVEAITPQTLINIRPVVAAIKE  420

M. semgmatis  1261  FFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGLSRERAGLQVRDVHPSHYGRMCPIETP  1440
                    FFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGLSRERAGL+VRDVHPSHYGRMCPIETP
RifR mutant   421   FFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGLSRERAGLEVRDVHPSHYGRMCPIETP  480

M. semgmatis  1441  EGPNIGLIGSLSVYARVNPFGFIETPYRKVENGVVTDQIDYLTADEEDRHVVAQANSPTD  1620
                    EGPNIGLIGSLSVYARVNPFGFIETPYRKVENGVVTDQIDYLTADEEDRHVVAQANSPTD
RifR mutant   481   EGPNIGLIGSLSVYARVNPFGFIETPYRKVENGVVTDQIDYLTADEEDRHVVAQANSPTD  540

M. semgmatis  1621  ENGRFTEDRVMVRKKGGEVEFVSADQVDYMDVSPRQMVSVATAMIPFLEHDDANRALMGA  1800
                    ENGRFTEDRVMVRKKGGEVEFVSADQVDYMDVSPRQMVSVATAMIPFLEHDDANRALMGA
RifR mutant   541   ENGRFTEDRVMVRKKGGEVEFVSADQVDYMDVSPRQMVSVATAMIPFLEHDDANRALMGA  600

M. semgmatis  1801  NMQRQAVPLVRSEAPLVGTGMELRaaidagdvvvadKNGVIEEVSADYITVMGDDSTRQS  1980
                    NMQRQAVPLVRSEAPLVGTGMELRAAIDAGDVVVADK GVIEEVSADYITVM DD TRQS
RifR mutant   601   NMQRQAVPLVRSEAPLVGTGMELRAAIDAGDVVVADKTGVIEEVSADYITVMADDGTRQS  660
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B

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M. semgmatis  1621  ISAGDLIMLGGCAAVEHAAAETGHPIEVPCRLGRTDAPQEWTDIEWFSALQPTADAFPNY  1800
                    ISA DLIMLGGCAAVEHAAAE GHPIEVPCRLGRTDAPQEWTDIEWFSAL+PTADAF NY
INHR mutant   541   ISAADLIMLGGCAAVEHAAAEAGHPIEVPCRLGRTDAPQEWTDIEWFSALEPTADAFRNY  600
```

Figure 3.

16S rRNA gene rrs sequence alignment between: (**A**) Capreomycin^R (CAP^R) mutant and its parental control *M. smegmatis* (ATCC607), and (**B**) Amikacin^R (AMK^R) mutant and its parental control *M. smegmatis* (ATCC607).

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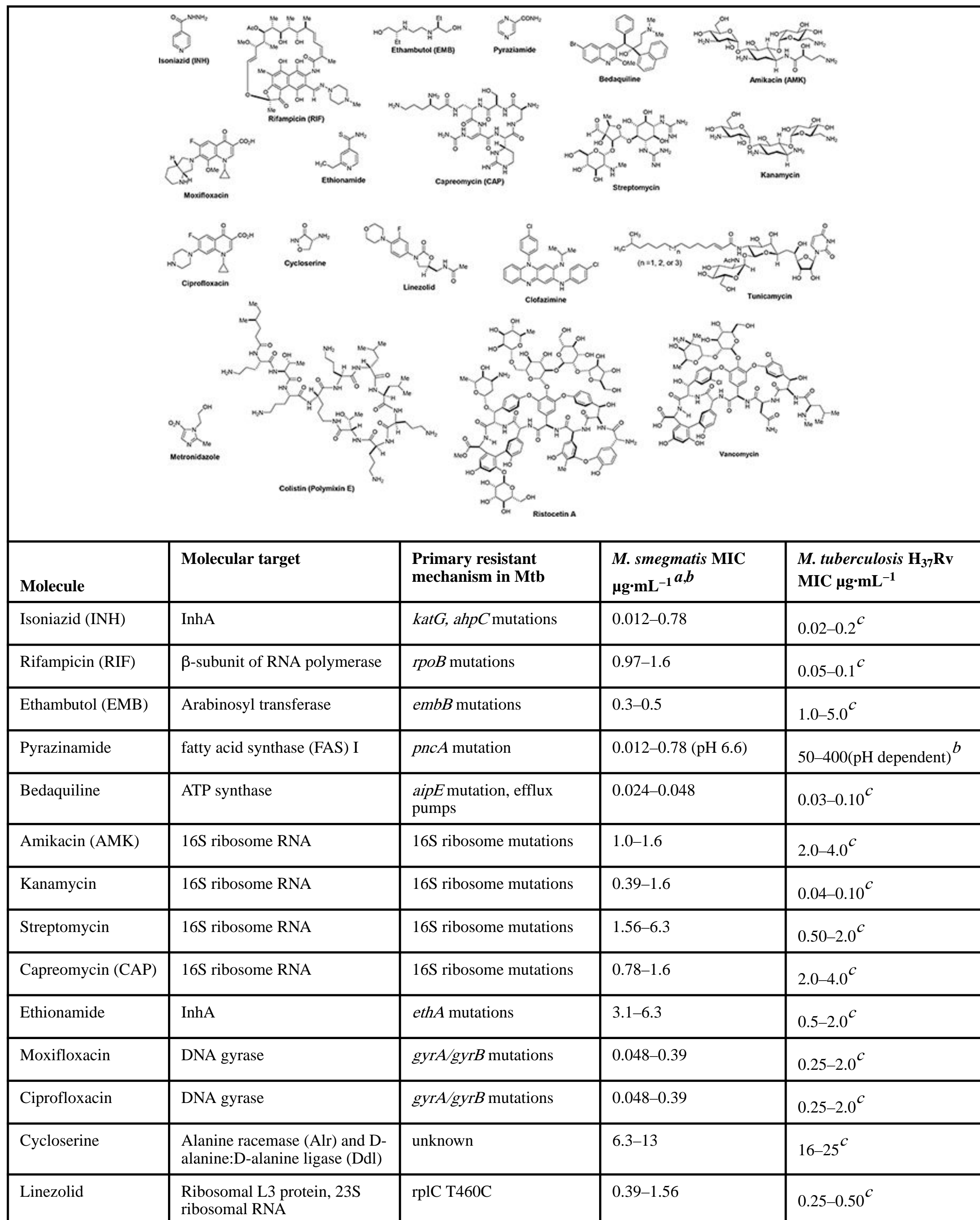
Table 1

Comparison of MIC values of representative TB drugs, positive- and negative-controls against *M. smegmatis* (ATCC607) and *M. tuberculosis* $H_{37}Rv$.



Molecule	Molecular target	Primary resistant mechanism in Mtb	*M. smegmatis* MIC μg·mL^{-1} [a,b]	*M. tuberculosis* $H_{37}Rv$ MIC μg·mL^{-1}
Isoniazid (INH)	InhA	*katG, ahpC* mutations	0.012–0.78	0.02–0.2[c]
Rifampicin (RIF)	β-subunit of RNA polymerase	*rpoB* mutations	0.97–1.6	0.05–0.1[c]
Ethambutol (EMB)	Arabinosyl transferase	*embB* mutations	0.3–0.5	1.0–5.0[c]
Pyrazinamide	fatty acid synthase (FAS) I	*pncA* mutation	0.012–0.78 (pH 6.6)	50–400(pH dependent)[b]
Bedaquiline	ATP synthase	*aipE* mutation, efflux pumps	0.024–0.048	0.03–0.10[c]
Amikacin (AMK)	16S ribosome RNA	16S ribosome mutations	1.0–1.6	2.0–4.0[c]
Kanamycin	16S ribosome RNA	16S ribosome mutations	0.39–1.6	0.04–0.10[c]
Streptomycin	16S ribosome RNA	16S ribosome mutations	1.56–6.3	0.50–2.0[c]
Capreomycin (CAP)	16S ribosome RNA	16S ribosome mutations	0.78–1.6	2.0–4.0[c]
Ethionamide	InhA	*ethA* mutations	3.1–6.3	0.5–2.0[c]
Moxifloxacin	DNA gyrase	*gyrA/gyrB* mutations	0.048–0.39	0.25–2.0[c]
Ciprofloxacin	DNA gyrase	*gyrA/gyrB* mutations	0.048–0.39	0.25–2.0[c]
Cycloserine	Alanine racemase (Alr) and D-alanine:D-alanine ligase (Ddl)	unknown	6.3–13	16–25[c]
Linezolid	Ribosomal L3 protein, 23S ribosomal RNA	rplC T460C	0.39–1.56	0.25–0.50[c]

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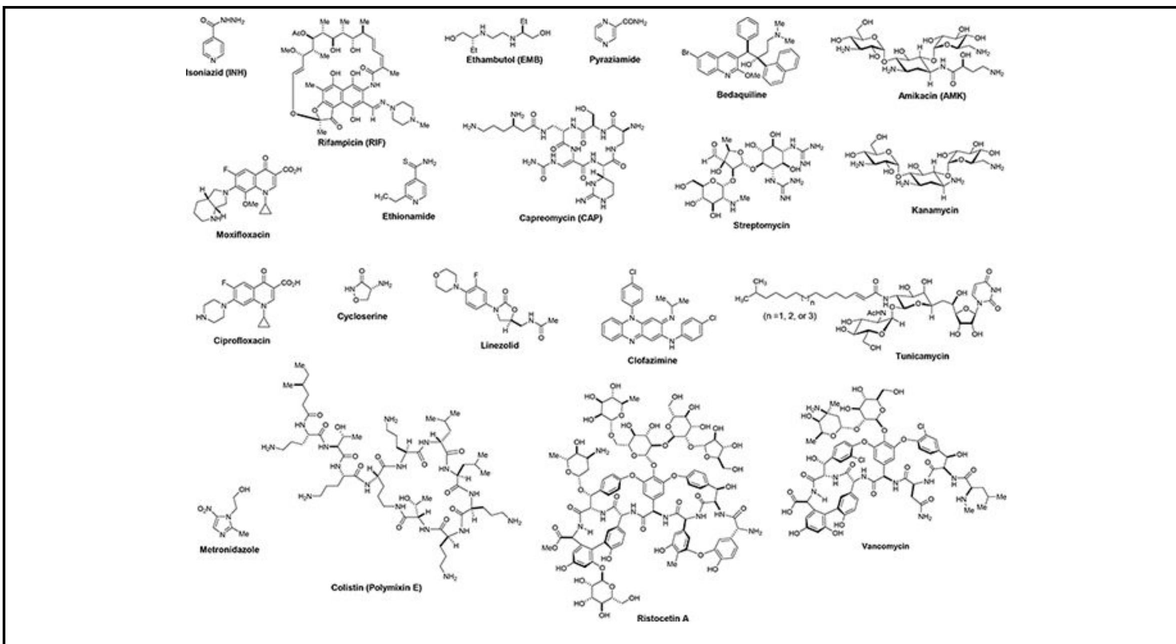

Molecule	Molecular target	Primary resistant mechanism in Mtb	*M. smegmatis* MIC μg·mL^{-1} [a,b]	*M. tuberculosis* $H_{37}Rv$ MIC μg·mL^{-1}
Clofazimine	Bacterial membrane	unknown	0.048–0.19	0.13–0.20[c]
Tunicamycin	MraY, WecA	unknown	6.25–12.5	6.3–12.5[a,c]
Vancomycin	Cell wall biosynthesis	unknown	3.1–12.5	6.25–25.0[a,c]
Ristocetin A	Cell wall biosynthesis	unknown	0.19–0.39	0.5–3.9[c]
Colistin	Bacterial membrane	unknown	>50	>50[a,c]
Metronidazole	A prodrug that is activated by a nitroreductase enzyme to reactive species (effective only hypoxic conditions)	unknown	>50	>50[a,c]

[a] All MIC data were generated in this studies. Microplate Alamar (Risazurin) blue assays were applied (see Experimental). All experiments were triplicated.

[b] Selected MIC data performed in an enriched medium were summarized in Supporting Information (for a comparison).

[c] The MIC data were cited from databases and/or literatures.

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Table 2

Antimycobacterial activity of representative TB drugs against non-replicating *M. smegmatis* (ATCC607) generated via long-term culturing and a nutrient deficient condition[a]

TB drugs	MBC ($\mu g \cdot mL^{-1}$)[b]	Drug conc. ($\mu g \cdot mL^{-1}$)[c]	Treatment time (days)	CFU·mL^{-1} [d]			
				***M. smegmatis* (30 days)**[e]	***M. smegmatis* (60 days)**[e]	***M. smegmatis* (150 days)**[e]	***M. smegmatis* (14 days, nutrient def.)**[f]
Amikacin (AMK)	1.6	7.5 (5 × MBC)	**5**	**0**	**0**	**0**	**0**
Amikacin (AMK)	1.6	7.5 (5 × MBC)	**15**	**0**	**0**	**0**	**0**
Capreomycin (CAP)	2.5	12.5 (5 × MBC)	**5**	**2.0×10^5**	**8.0×10^4**	**4.0×10^4**	**2.0×10^5**
Capreomycin (CAP)	2.5	12.5 (5 × MBC)	**15**	**0**	**0**	**0**	**0**
Rifampicin (RIF)	1.6	7.5 (5 × MBC)	**5**	**1.2×10^7**	**1.1×10^7**	**1.0×10^7**	**1.9×10^7**
Rifampicin (RIF)	1.6	32.0 (20 × MBC)	**5**	**6.0×10^5**	**4.0×10^5**	**3.9×10^5**	**6.8×10^6**
Rifampicin (RIF)	1.6	32.0 (20 × MBC)	**15**	**5.5×10^5**	**3.9×10^5**	**3.2×10^5**	**5.7×10^6**
Isoniazid (INH)	1.0	20.0 (20 × MBC)	5	2.8×10^7	2.5×10^7	2.4×10^7	2.8×10^7
Isoniazid (INH)	1.0	20.0 (20 × MBC)	15	2.6×10^7	2.3×10^7	2.2×10^7	2.5×10^7
Ethambutol (EMB)	0.5	10.0 (20 × MBC)	5	3.8×10^7	3.2×10^7	3.3×10^7	3.2×10^7
Ethambutol (EMB)	0.5	5.0 (5 × MBC)	**15**	**0**	**0**	**0**	**0**
No drug (control)	-	-	5	4.0×10^7	3.9×10^7	3.8×10^7	3.6×10^7

[a] Non-replicating *M. smegmatis* (ATCC607) was kept in the presence of each drug (5×MBC or 20×MBC) (total volume 200 μL) for 5 days at 37 °C. All experiments were triplicated.

[b] The minimum bactericidal concentration (MBC) was determined by colony-forming unit (CFU) of *M. smegmatis* (ATCC607) cultured on agar plates contacting TB drug.

[c] Drug concentrations used are 5- or 20-times the MBC of each drug.

[d] *M. smegmatis* colony-forming unit (CFU) grown on the agar plate (55 cm^2, 0.5 % tween 80-Middlebrook 7H11 agar base) was counted after 4 days at 37 °C.

[e] The culture was produced by inoculation of single colony of *M. smegmatis* (ATCC607) into 25 mL 0.5% tween 80-Middlebrook 7H9 medium (0.4% glycerol), followed by incubation under stationary conditions at 37 °C for 30, 60, and 150 days, respectively.

[f] The starter culture of *M. smegmatis* (ATCC607) was obtained from a single colony by incubation in 0.5% tween 80-Middlebrook 7H9 medium (0.4% glycerol) at 37 °C for 4 days. The culture was centrifuged (4,500 rpm for 10 min.) and suspended in PBS (pH 7.2) for 14 days (37 °C)