Verapamil, and Its Metabolite Norverapamil, Inhibit Macrophage-induced, Bacterial Efflux Pump-mediated Tolerance to Multiple Anti-tubercular Drugs

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Drug tolerance likely represents an important barrier to tuberculosis treatment shortening. We previously implicated the Mycobacterium tuberculosis efflux pump Rv1258c as mediating macrophage-induced tolerance to rifampicin and intracellular growth. In this study, we infected the human macrophage-like cell line THP-1 with drug-sensitive and drug-resistant M. tuberculosis strains and found that tolerance developed to most antitubercular drugs, including the newer agents moxifloxacin, PA-824, linezolid, and bedaquiline. Multiple efflux pump inhibitors in clinical use for other indications reversed tolerance to isoniazid and rifampicin and slowed intracellular growth. Moreover, verapamil reduced tolerance to bedaquiline and moxifloxacin. Verapamil’s R isomer and its metabolite norverapamil have substantially less calcium channel blocking activity yet were similarly active as verapamil at inhibiting macrophage-induced drug tolerance. Our finding that verapamil inhibits intracellular M. tuberculosis growth and tolerance suggests its potential for treatment shortening. Norverapamil, R-verapamil, and potentially other derivatives present attractive alternatives that may have improved tolerability.

Keywords. tuberculosis; tolerance; persistence; efflux; verapamil; norverapamil; R-verapamil; efflux pump inhibitor.

The long duration of therapy required to reliably cure tuberculosis [1] presents a fundamental hurdle to its eradication. However, attempts to shorten the first-line tuberculosis drug regimen for smear-positive cases have met with unacceptably high relapse rates [2, 3]. It has been long recognized that when relapses occur, they typically involve genetically drug-susceptible organisms [1]. Consequently, relapse and the need for long treatment have been attributed to phenotypic drug resistance, also known as tolerance [3, 4]. Drug-tolerant organisms are killed poorly, yet their minimal inhibitory concentration (MIC) is unchanged. Existing tolerance models use indirect evidence to invoke a metabolically dormant bacterial population that is not easily killed by existing drugs [5].

Consequently, there has been considerable excitement about the recently developed compounds PA-824 and bedaquiline (TMC-207) [6, 7]. Bedaquiline has been shown to be active against both replicating and nonreplicating bacteria in culture [8]. It has recently been approved for multidrug resistant (MDR) tuberculosis based on increased culture conversion rates [9]. However, in murine models with drug-sensitive tuberculosis, bedaquiline shortens treatment only moderately [10]. One explanation for its failure to shorten treatment even further is that additional or alternative tolerance mechanism(s) may be present in vivo.

We recently uncovered a completely different mechanism for drug tolerance [11]. We found that Mycobacterium tuberculosis develops bacterial efflux
pump-mediated tolerance to isoniazid and rifampicin following macrophage residence [11]. Moreover, we observed that tolerant bacteria are enriched in the actively-dividing \textit{M. tuberculosis} population. This macrophage-induced rifampicin tolerance was inhibited by verapamil, a calcium channel antagonist recognized to inhibit bacterial efflux pumps in vitro [12]. Subsequent work in murine tuberculosis models has validated these findings. Verapamil has been shown to accelerate bacterial killing in mice infected with drug-resistant [13] or drug-sensitive tuberculosis [14] and decrease relapse rates with shortened treatment courses [14]. These data suggest the promise of strategies combining efflux inhibitors with existing tuberculosis drugs.

In this work, we have extended our prior findings by studying macrophage-induced tolerance and its inhibition for a diverse panel of drugs used to treat drug-sensitive and drug-resistant tuberculosis. We found that macrophage-induced tolerance developed broadly, including newer drugs such as moxifloxacin, linezolid, PA-824, and bedaquiline. Considering agents used for drug-resistant tuberculosis, verapamil inhibited tolerance to moxifloxacin and bedaquiline. Further investigation indicated that verapamil’s effect on macrophage-induced tolerance appears to be independent of its activity as a calcium channel blocker, an insight that may permit development of better-tolerated verapamil derivatives for clinical study in tuberculosis.

**METHODS**

**Bacterial Strains, Methods, and Chemicals**

The \textit{M. tuberculosis} strain CDC1551 was a gift from W. R. Bishai (Johns Hopkins University). H37Rv and an isogenic \textit{rpoB} mutant (H526Y) were from D. R. Sherman (Seattle BioMed). \textit{Mycobacterium marinum} strain M (BAA-535) was obtained from ATCC. \textit{M. tuberculosis} were grown to mid log phase in Middlebrook 7H9 medium (Becton Dickinson) with 0.05% Tween-80 and albumin, dextrose, catalase (Middlebrook ADC Enrichment, BBL Microbiology) prior to infection. Rifampicin, isoniazid, streptomycin, rifabutin, ethambutol, ethionamide, kanamycin, cycloserine, capreomycin, clofazimine, para-aminosalicylic acid (PAS), linezolid, verapamil, thioridazine, pipeline, and R- and S-verapamil were purchased from Sigma. Norverapamil and moxifloxacin was purchased from Santa Cruz Biotechnology. PA-824 was provided by David Sherman (Seattle BioMed) and bedaquiline was provided by Clifton Barry (NIAID).

**Macrophage Growth and Infection**

THP-1 macrophages were grown in RPMI, supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. THP-1 cells were differentiated with 100 nM phorbol 12-myristate 13-acetate for 48 hours and allowed to recover for 24 hours prior to infection. Subsequently, \(5 \times 10^5\) THP-1 macrophages were infected at a multiplicity of infection of 1 for 3 hours at 37°C. Cells were washed with media, and 6 \(\mu\)g/mL streptomycin was added to the media for the duration of the intracellular growth (Figure 1). Media was changed daily. To lyse macrophages and release bacteria, each well was washed once with \(1\times\) phosphate-buffered saline (PBS) and then with \(\text{dH}_2\text{O}\), with the latter being removed immediately. Then, 100 \(\mu\)L of \(\text{dH}_2\text{O}\) was added, and the cells were incubated at 37°C for 15 minutes. Finally, 900 \(\mu\)L of 7H9 medium with 0.05% Tween-80 was added and the wells scraped with a pipette tip.

![Figure 1. Schematic of protocols used to test effect of efflux pump inhibitors on macrophage-induced tolerance as well as intracellular Mycobacterium tuberculosis growth.](image-url)
Colony-forming units (CFU) were enumerated from triplicate wells on supplemented 7H10 agar. For determination of antibiotic killing, the percent survival was calculated by dividing the CFU for each well by the mean pretreatment CFU.

**Minimum Inhibitory Concentration Assays**

MICs were determined by adding approximately 10^6 CFU M. tuberculosis to round bottom 96-well plates containing 100 µL of drug-supplemented 7H9 ADC media lacking Tween-80. The plates were incubated at 37°C for 6–8 days, prior to incubation with Alamar Blue for 1 day. The MIC was defined as the lowest concentration that prevented growth (color change) [15]. In this study, we determined MICs for rifampicin, INH, linezolid, PA824, and bedaquiline (Supplementary Table 1).

**Drug and Efflux Inhibitor Treatment**

Infected macrophages or macrophage lysates were treated with anti-tuberculosis drugs at 3× the published MIC for H37Rv [16] except for bedaquiline, rifabutin, and linezolid. Bedaquiline was used at approximately 5× the MIC based on available drug stocks. Rifabutin concentration was chosen to be similar to rifampicin. We observed poor growth (data not shown), leading us to use 10 µg/mL subsequently (Supplementary Table 1). Verapamil and thioridazine were used at 1/5 the MIC, and piperine was used at 100 µg/mL (color change) [15].

**Statistical Analyses**

Statistical analyses were performed using Prism 5.01 (GraphPad). Means were compared via ANOVA, with post-test evaluation using the Dunnett or Bonferroni test. P values are abbreviated as follows: *P < .05; **P < .01; ***P < .001.

**RESULTS**

**Drugs Known to Inhibit Bacterial Efflux Pumps Reverse Macrophage-induced Tolerance to Isoniazid and Rifampicin**

We used 2 functional assays to test candidate efflux inhibitors: (1) inhibition of intracellular growth in the absence of antitubercular drugs (intracellular growth inhibition) and (2) potentiation of antitubercular drug activity when added to infected macrophages (intracellular tolerance inhibition) or to macrophage lysates after the bacteria have been allowed to grow intracellularly (lysate tolerance inhibition; Figure 1). The lysate tolerance inhibition assay allows the drug’s effects on bacterial efflux pumps to be tested independently of its intracellular penetration or its effects on macrophage transporters [20]. As in our previous work [11], we operationally defined macrophage-induced antimicrobial tolerance as the occurrence of reduced antimicrobial killing following M. tuberculosis macrophage residence.

We previously showed that 40 µg/mL verapamil reversed rifampicin but not isoniazid tolerance in M. tuberculosis. In conjunction with our finding that Rv1258c mediates rifampicin but not isoniazid tolerance [11], this observation suggested that macrophage residence induces distinct M. tuberculosis efflux pumps with reduced sensitivity to verapamil. Indeed, we found that increasing the concentration of verapamil (80 µg/mL, which is still below the MIC) reduced M. tuberculosis isoniazid tolerance (Figure 2A).

Along with verapamil, we evaluated other drugs that have been shown to inhibit bacterial efflux pumps in vitro [12]. These included thioridazine and piperine, a derivative of black pepper used to enhance the bioavailability of coadministered drugs [21]. Like verapamil, thioridazine reduced both rifampicin and isoniazid tolerance; however, piperine only inhibited rifampicin tolerance (Figure 2A–F). Piperine was poorly soluble above 100 µg/mL, precluding testing of higher concentrations. The finding that the M. tuberculosis Rv1258c efflux pump mutant had both reduced drug tolerance and intracellular growth [11,22] suggested that the bacterial efflux pump inhibitors should also have these dual effects [23]. Prior work had demonstrated that verapamil inhibits intracellular growth in both M. marinum and M. tuberculosis [11,24], as does thioridazine in M. tuberculosis [25]. We confirmed these findings and found that piperine likewise inhibits intracellular M. tuberculosis growth (Figure 2G and 2H).

Of these efflux-blocking drugs, verapamil appeared the most promising for further study. It has a long track record of broad and relatively safe clinical use [26,27], and animal studies show it to be 40- to 80-fold concentrated in the lungs [28,29]. In contrast, the other inhibitors were less attractive because (1) non-toxic plasma levels in humans are far below the levels needed to reverse tolerance in vitro and pulmonary concentration is doubtful (piperine [30]) or (2) potential for significant adverse reactions (thioridazine). So we pursued verapamil further, while including thioridazine in key experiments given the existing literature advocating its use in drug-resistant tuberculosis [31].

**Macrophage-induced Tolerance Develops to Other Drugs Used to Treat Drug-sensitive Tuberculosis**

We next asked if macrophage-induced tolerance develops to other drugs used for drug-sensitive tuberculosis and, if so, whether verapamil reduces tolerance to them. For ethambutol, macrophage-induced tolerance developed but was not inhibited by verapamil even at 80 µg/mL (Figure 3B). We did not test pyrazinamide given its poor efficacy in most in vitro assays [32]. We
also evaluated rifabutin, an alternative rifamycin that may be substituted for rifampicin given its reduced induction of CYP3A4. As with rifampicin, macrophage-induced tolerance to rifabutin developed and was inhibited by verapamil (Figure 3C and 3D). Finally, we tested streptomycin because it is still used in some first-line regimens [33]. Macrophage-induced streptomycin tolerance developed but was not inhibited by verapamil (Figure 3E). In fact, coadministration with either verapamil or thioridazine was associated with a small inhibition in killing by streptomycin. This finding was surprising because verapamil has been shown to reduce the MIC to streptomycin in some clinical isolates [34]. It raised the question of whether verapamil might impede the efficacy of regimens that include streptomycin. However, we found that the addition of verapamil to isoniazid plus streptomycin did not impede M. tuberculosis killing (Figure 3F).

Rifampicin and Isoniazid-resistant M. tuberculosis Strains Develop Macrophage-induced Drug Tolerance and Use Efflux Pumps for Intracellular Growth

Although resistance in M. tuberculosis has been traditionally ascribed to chromosomal mutations in drug targets or activators of pro-drugs, a growing body of data suggests that efflux may also contribute [35]. Efflux pump inhibitors have been shown to increase M. tuberculosis in vitro drug susceptibility [34, 36–38]. Moreover, a recent report found that verapamil administered alongside isoniazid, rifampicin, and pyrazinamide to mice infected with a MDR M. tuberculosis strain reduced pulmonary

Figure 2. Effects of efflux inhibitors on macrophage-induced tolerance to isoniazid and rifampicin and intracellular Mycobacterium tuberculosis growth. A–F, THP-1 macrophages were infected with CDC 1551 and lysed at 2 hours (gray bars) or 96 hours (black bars) postinfection. The released bacteria were left untreated or were treated for an additional 48 hours with 0.6 µg/ml isoniazid (INH) or 1 µg/ml rifampicin (RIF) in the presence or absence of 80 µg/ml verapamil (VER), 100 µg/ml piperine (PIP), or 2 µg/ml thioridazine (TRZ) prior to enumeration of colony-forming units (CFU). G and H, THP-1 macrophages were infected with CDC 1551 for 48 hours (G) and 96 hours (H) and subsequently left untreated (UNT) or treated for an additional 48 hours with 40 µg/ml VER, 100 µg/ml piperine, or 2 µg/ml TRZ prior to enumeration of CFU. Results (A–H) are representative of at least 3 independent experiments. Error bars represent SEM. Significance testing was performed using 1-way ANOVA with Dunnett’s post-test. Abbreviations: ANOVA, analysis of variance; SEM, standard error of the mean.
bacillary loads [13]. We asked 2 questions in the context of drug-resistant tuberculosis: (1) does macrophage-induced tolerance manifest in the presence of genetic drug resistance and (2) does the addition of an efflux pump inhibitor potentiate killing of M. tuberculosis by the drug to which it has become resistant?

To test these possibilities, we used a rifampicin-monoresistant strain bearing a commonly reported rpoB mutation (H526Y) [39]. We used the intracellular tolerance assay previously described to take advantage of the additional intracellular drug concentration that may result from blocking macrophage transporters [20]. We found that genetic rifampicin resistance is further complicated by macrophage-induced tolerance; the partial killing by rifampicin observed when treatment is started shortly after infection is lost if treatment is delayed 96 hours (Figure 4A and 4B). Verapamil and thioridazine impaired growth of intracellular M. tuberculosis in both wild-type and rifampicin-resistant strains. However, no additional M. tuberculosis killing was seen in the rifampicin-resistant isolates when rifampicin combined with either inhibitor. We observed similar results in a katG isoniazid-resistant mutant (Figure 4C and 4D).

Together, these results suggest that macrophage-induced tolerance may further limit the efficacy of rifampicin and isoniazid for drug-resistant strains. Although efflux pump inhibitors retain the ability to kill intracellular drug-resistant M. tuberculosis, they fail to restore drug susceptibility.

Macrophage-induced Tolerance and Efflux Pump Inhibitor Effects in the Context of Drugs Used for MDR and XDR Tuberculosis

Though the worse treatment outcomes in MDR and XDR tuberculosis are often attributed to the reduced potency of the drugs used in these cases, we wondered whether drug...
tolerance could be an additional factor. We therefore assessed a comprehensive panel of these drugs for development of macrophage-induced tolerance and its inhibition by verapamil and thioridazine. Macrophage-induced tolerance developed to all except bedaquiline (Figure 5, and see below). However, neither verapamil nor thioridazine significantly inhibited tolerance to any drug except for moxifloxacin (Figure 5 and Supplementary 1).

We did observe trends toward improved bacterial killing when PAS, ethionamide, and clofazimine were paired with verapamil (Figure 5).

Macrophage-induced Bedaquiline Tolerance Occurs Within Two Hours of Macrophage Infection and Is Verapamil-sensitive

When we examined bedaquiline in our tolerance assay, the results were initially puzzling. There was no evidence of M. tuberculosis killing at either time point in the presence of 0.3 µg/mL bedaquiline, approximately 5-fold the MIC (Figure 5). Nevertheless, addition of verapamil to the 96-hour lysate caused a 34% reduction in CFU in contrast to bedaquiline alone, which allowed 27% growth (Figure 5). The addition of thioridazine similarly enabled modest killing by bedaquiline (Supplementary Figure 1E). This finding could be explained if macrophage-induced tolerance to bedaquiline develops within 2 hours of macrophage infection. If so, verapamil should enhance bedaquiline killing in the 2 hour macrophage lysates. We found this to be the case; the verapamil-bedaquiline combination produced a 51% reduction in CFU (Figure 6A). In contrast, verapamil only enhanced isoniazid and rifampicin killing of 96-hour but not 2-hour macrophage lysates (Figure 6B and 6C).

Alternatively, the benefit of verapamil at the 2-hour time point could be explained by the presence of constitutive verapamil-sensitive tolerance. To examine this possibility, we compared bedaquiline activity on broth-grown, logarithmic phase vs macrophage-grown M. tuberculosis. In broth cultures, bedaquiline suppressed M. tuberculosis growth in the 7-day observation period, consistent with previous reports of its bacteriostatic effect (Figure 6D) [40]. In contrast, M. tuberculosis that had been macrophage-resident for either 2 hours or 96 hours continued to grow in the presence of bedaquiline (Figure 6E and 6F). Whereas the addition of verapamil led to initial modest killing in macrophage lysates, similar killing was not observed in broth-grown bacteria. These results confirmed that bedaquiline tolerance is not constitutive but instead develops rapidly following macrophage residence. Moreover, a 4-fold lower concentration of

Figure 4. Verapamil promotes killing of intracellular drug-resistant Mycobacterium tuberculosis. A and B, THP-1 macrophages were infected with H37Rv (WT) or an isogenic rpoB H526Y mutant. Starting 2 hours (gray bars) or 96 hours (black bars) postinfection, the cells were either left untreated or were treated with 1 µg/mL rifampicin for an additional 48 hours prior to lysis and enumeration of CFU. At the 96-hour time point, they were additionally treated with no efflux inhibitor, 40 µg/mL VER, or 2 µg/mL TRZ for 48 hours. C and D, THP-1 macrophages were infected with CDC1551 (WT) or an isogenic katG mutant. Starting 2 hours (gray bars) or 96 hours (black bars) postinfection, the cells were either left untreated or were treated with 0.6 µg/mL INH for an additional 48 hours prior to lysis and enumeration of CFU. At the 96-hour time point, they were additionally treated with no efflux inhibitor, 40 µg/mL VER, or 2 µg/mL TRZ for 48 hours. Results are representative of 3 independent experiments for panels A and B. For panels C and D, the results of the only experiment performed are shown. Error bars represent SEM. Significance testing was performed using 1-way ANOVA with Dunnett’s post-test. Abbreviations: ANOVA, analysis of variance; CFU, colony-forming units; INH, isoniazid; SEM, standard error of the mean; TRZ, thioridazine; VER, verapamil.
Verapamil had comparable efficacy in macrophage lysates, suggesting that the *M. tuberculosis* pumps mediating bedaquiline tolerance are distinct from those mediating rifampicin or INH tolerance, which are less verapamil-sensitive (Figure 6E–F).

**Verapamil Inhibits Tolerance Independent of Its Activity as a Calcium Channel Antagonist**

We pursued additional studies to better characterize verapamil’s mechanism of action as an efflux inhibitor. Prior studies had found that verapamil derivatives without calcium channel blocking activity remain active P-glycoprotein inhibitors [41]. We wondered whether this was also true for verapamil’s inhibition of drug tolerance. As a simple first test, we asked whether supplemental calcium abrogated verapamil’s effect on drug tolerance. Using *M. marinum*, we found that supplemental CaCl₂ concentrations as high as 1 mM did not abrogate the inhibition of rifampicin tolerance by verapamil (data not shown).

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462  •  JID 2014:210 (1 August)  •  Adams et al
Next, we tested verapamil derivatives known to have reduced calcium channel blocking activity. Whereas verapamil’s major metabolite norverapamil achieves serum levels comparable to those of verapamil, it has only 20 percent of the cardiac activity [42]. We found that norverapamil was similarly effective as verapamil at inhibiting isoniazid and rifampicin tolerance and killing of intracellular *M. tuberculosis* in the absence of other drugs (Figure 7B and 7C). Furthermore, although verapamil is currently administered as a racemic mixture, its R-enantiomer has been shown to have reduced effects on cardiac conduction [43]. The result with norverapamil decoupling verapamil’s efflux pump inhibition from its calcium channel antagonism was corroborated by finding that R-verapamil was comparably effective as racemic verapamil at reducing macrophage-induced isoniazid tolerance (Figure 7D).

**DISCUSSION**

Our finding that macrophage residence induces *M. tuberculosis* tolerance to virtually all anti-tuberculosis drugs may have broad therapeutic implications. It is unclear whether any of the more recently-developed anti-tuberculosis drugs or compounds in late stages of clinical testing will permit tuberculosis treatment shortening. Indeed, we found that moxifloxacin, PA-824, linezolid, and bedaquiline are subject to macrophage-induced tolerance. Strategies specifically targeting *M. tuberculosis* efflux may provide new tools to increase the antitubercular drug efficacy. Moreover, because multiple *M. tuberculosis* efflux pumps have been shown to be essential for intracellular growth [22], they represent promising drug targets themselves.

Efflux pump inhibitors have been previously shown to reduce the in vitro drug susceptibility (MIC) to multiple drugs including streptomycin, INH, and ofloxacin; a similar effect has recently been reported for clofazimine and bedaquiline [34, 36–38]. Our findings represent a different phenomenon whereby drug concentrations above the MIC are not effective against a substantial population of macrophage-grown bacteria. Gupta et al’s study was designed to examine the effect of verapamil on growth inhibition but not killing in vitro. In our study assessing bacterial killing, we find that bedaquiline is growth inhibitory to broth-grown bacteria, an effect that is lost within 2 hours of macrophage culture. The addition of verapamil
restores growth inhibition to the macrophage-grown bacteria, providing an important impetus for studying bedaquiline in combination with an efflux inhibitor. We note that verapamil has been shown to enhance rifampicin intracellular accumulation [14], but direct evidence for bedaquiline efflux remains to be demonstrated. Moreover, although we used verapamil at sub-MIC levels, we cannot exclude the possibility that its enhancement of bedaquiline activity, and other drugs tested in these experiments aside from rifampicin, may be due at least in part to direct antimicrobial effects of verapamil.

Our observations that bedaquiline tolerance is induced earlier than isoniazid or rifampicin tolerance and that a higher verapamil concentration is required to inhibit isoniazid tolerance speak to the functional specificity of macrophage-induced drug efflux mechanisms. A better understanding of the most relevant *M. tuberculosis* efflux pumps for macrophage-induced anti-tuberculosis drug tolerance and their regulation may facilitate more targeted approaches to inhibit macrophage-induced tolerance.

**Clinical Insights and Therapeutic Opportunities**

Our results also provide insights into the results of ongoing clinical tuberculosis investigations. The development of macrophage-induced tolerance may explain why the substitution of moxifloxacin into current regimens has failed to consistently improve 2-month sputum culture conversion rates [44, 45]. If macrophage-induced tolerance is an important barrier in vivo to further tuberculosis treatment shortening, then it may be mitigated by concomitant treatment with an efflux inhibitor. Of the tested inhibitors, verapamil is the most appealing for further study: (1) it inhibits tolerance to multiple drugs; (2) is generally well-tolerated; (3) has well-characterized pharmacokinetics; (4) in animal models, it is significantly concentrated in the lungs [28, 29]; and (5) its metabolite, norverapamil, also inhibits...
macrophage-induced tolerance and achieves similar serum levels to verapamil [46]. Although verapamil’s calcium channel blocking activity is important for its use in treatment of cardiovascular diseases, this property is undesirable in tuberculosis. Our investigations suggest that verapamil’s inhibitory effects on M. tuberculosis tolerance are independent of its activity as a calcium channel antagonist. These results raise possible strategies that might improve verapamil safety and tolerability in tuberculosis, such as calcium supplementation [47] or use of derivatives having reduced calcium channel activity such as R-verapamil or norverapamil.

There are important practical concerns to studying verapamil as an adjunctive tuberculosis therapy. First, it is unclear whether the concentrations used in vitro are attainable in tuberculosis patients. However, efflux inhibition would be augmented by contributions from norverapamil and animal studies also suggest that verapamil is highly concentrated in the lung. At the same time, verapamil levels are known to be markedly lowered by rifampicin, due to CYP3A4 induction [48]. It may be possible to administer higher, compensatory doses of verapamil [14], though this will require careful study. Alternatively, rifabutin could be substituted for rifampicin given its reduced induction of CYP3A4.

Such rifamycin-related challenges would be avoided in MDR or XDR tuberculosis. Intriguingly, verapamil was able to restore the efficacy of isoniazid, rifampicin, and pyrazinamide in a murine MDR model [13]. When we sought to replicate these findings in an infected macrophage system using a rifampicin-monoressist strain with the same rpoB mutation, we found that both verapamil and thioridazine decrease intracellular growth. However, the combination of either inhibitor with rifampicin did not lead to greater M. tuberculosis killing, suggesting that this approach did not restore rifampicin efficacy. Our assay may simply have been too short to see such an effect. However, the efficacy of efflux pump inhibitors could also be strain-dependent, with the greatest benefit seen in strains over-expressing efflux pumps [49] or with low-level drug resistance, such as inhA promoter mutants. However, our findings also suggest that verapamil could still have utility as an adjunctive agent in drug-resistant tuberculosis due to its inhibitory effects on intracellular growth.

On a cautionary note, we found that verapamil appeared to reduce killing by the ribosome-targeting drugs streptomycin, kanamycin, and capreomycin, as well as the cell wall agent cycloserine. The mechanistic basis for this phenomenon is uncertain but might reflect inhibition of drug uptake [50]. Further studies will be required before using verapamil with these drugs.

In summary, our investigations merit clinical studies to determine if adjunctive treatment with efflux inhibitors permits tuberculosis treatment shortening. Although verapamil presents practical challenges, it is a well-characterized, globally available, and inexpensive drug. Verapamil derivatives or newer efflux pump inhibitors may ultimately prove more attractive for use in tuberculosis and possibly even prophylaxis for drug-resistant tuberculosis contacts. Given the conservation of many M. tuberculosis efflux pumps [23], we expect that macrophage-induced tolerance is a ubiquitous phenomenon that may extend to other medically important mycobacteria.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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