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Rifampicin Reduces Susceptibility to Ofloxacin in Rifampicin-resistant *Mycobacterium tuberculosis* through Efflux

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Abstract

Rationale—Central dogma suggests that rifampicin resistance in *Mycobacterium tuberculosis* develops solely through *rpoB* gene mutations.

Objective—To determine whether rifampicin induces efflux pumps activation in rifampicin resistant *M. tuberculosis* strains thereby defining rifampicin resistance levels and reducing ofloxacin susceptibility.

Methods—Rifampicin and/or ofloxacin minimum inhibitory concentrations (MICs) were determined in rifampicin resistant strains by culture in BACTEC 12B medium. Verapamil and reserpine were included to determine their effect on rifampicin and ofloxacin susceptibility. RT-qPCR was applied to assess expression of efflux pump/transporter genes after rifampicin exposure. To determine whether verapamil could restore susceptibility to first-line drugs, BALB/c mice were infected with a MDR-TB strain and treated with first-line drugs with/without verapamil.

Measurements and Main Findings—Rifampicin MICs varied independently of *rpoB* mutation and genetic background. Addition reserpine and verapamil significantly restored rifampicin susceptibility ($p = 0.0000$). RT-qPCR demonstrated that rifampicin induced differential expression of efflux/transporter genes in MDR-TB isolates. Incubation of rifampicin mono-resistant strains in rifampicin (2 µg/ml) for 7 days induced ofloxacin resistance (MIC > 2 µg/ml) in strains with an *rpoB531* mutation. Ofloxacin susceptibility was restored by exposure to efflux pump inhibitors. Studies in BALB/c mice showed that verapamil in combination with first-line drugs significantly reduced pulmonary CFUs after 1 and 2 months treatment ($p < 0.05$).

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Conclusion—Exposure of rifampicin resistant *M. tuberculosis* strains to rifampicin can potentially compromise the efficacy of the second-line treatment regimens containing ofloxacin, thereby emphasising the need for rapid diagnostics to guide treatment. Efflux pump inhibitors have the potential to improve the efficacy of anti-tuberculosis drug treatment.

Keywords

Mycobacterium tuberculosis; drug resistance; rifampicin; efflux pumps; cross resistance

Rifampicin is one of the most important anti-tuberculosis (anti-TB) antibiotics; it exerts its bactericidal activity by inhibiting the early steps of gene transcription by binding to the β -subunit of RNA polymerase (RpoB) encoded by the *rpoB* gene (1). This activity is responsible for shortening the treatment period and reducing the proportion of recurrent TB cases. The currently accepted paradigm suggests that resistance to rifampicin develops through a process of spontaneous mutation (nonsynonymous single nucleotide polymorphisms [nsSNPs]) in the *rpoB* gene (2), followed by antibiotic selection during periods of poor adherence or monotherapy. Luria-Delbrück fluctuation tests show that rifampicin resistance appears spontaneously at a rate of 10^{-9} to 10^{-8} mutations per cell division (3, 4). These nsSNPs largely occur in an 81-bp region in the *rpoB* gene known as the rifampicin resistance-determining region (RRDR) (2). Each nsSNP results in a novel amino acid substitution, thereby altering the structure of the corresponding RpoB protein, which in turn is believed to alter the binding constant between rifampicin and the RpoB protein (1, 5). Currently it is believed that all known RRDR mutations cause high-level rifampicin resistance (greater than the critical concentration of 2 $\mu\text{g/ml}$) leading to clinical resistance (6-8).

Resistance to rifampicin threatens TB control, as patients with rifampicin resistance need to be treated with second-line anti-TB drugs, which are less effective, more toxic, and more expensive than first-line agents. In the absence of routine drug susceptibility testing (DST), patients with undetected drug-resistant TB are exposed to first-line treatment regimens (including rifampicin) for long periods while awaiting DST results. The consequence of exposing the *M. tuberculosis* bacillus to an antibiotic to which it is already resistant is largely unknown, with the exception that quantitative real-time polymerase chain reaction has demonstrated that rifampicin induced expression of the Tap-like efflux pump in certain drug-resistant strains of *M. tuberculosis* (9). However, the authors acknowledge that the relationship between rifampicin resistance and active efflux remains to be demonstrated. Furthermore, these findings do not address the question of whether induction of such genes through exposure to a specific antibiotic may influence the efficacy of an unrelated antibiotic (i.e., decrease in susceptibility).

In this study we demonstrate that the level of rifampicin resistance is defined by efflux, which regulates the intracellular concentration of rifampicin. We show that the activation of efflux pump and/or transporter genes by rifampicin leads to decrease in susceptibility to ofloxacin. In addition, we show that the inhibition of efflux significantly restores susceptibility to both rifampicin and ofloxacin. This is further supported by our observation that treating with verapamil in combination with first-line anti-TB drugs significantly reduced lung bacilli loads in BALB/c mice infected with a multidrug-resistant (MDR) strain. These findings have important implications for our understanding of the mechanisms of mycobacterial drug resistance. Furthermore, they emphasize the importance of rapid diagnostics and question the value of treatment guidelines in the absence of routine DST, and recommend further investigation into the use and development of efflux pump inhibitors for the treatment of MDR-TB.

METHODS

Genotyping of Clinical Isolates

Drug-resistant and pan-susceptible *M. tuberculosis* isolates were cultured and genotypically characterized as described in the online supplement.

Rifampicin Minimum Inhibitory Concentration Determination

Drug-resistant isolates with different nsSNPs in the RRDR of the *rpoB* gene and representing three MDR-TB outbreaks (transmission) (n = 15) (Table 1), MDR-TB (n = 30), or mono-rifampicin (n = 15) (Table 2) resistant strains with different genetic backgrounds were selected. The minimum inhibitory concentrations (MICs) for rifampicin were determined by culture in enriched BACTEC 12B medium containing 2 to 200 µg/ml rifampicin (see online supplement).

Inhibition of Efflux Pumps

Rifampicin-resistant and pan-susceptible isolates were cultured at 37°C in enriched BACTEC 12B media containing reserpine (10–100 µg/ml) or verapamil (10–500 µg/ml). The growth index (GI₉₀) was retained at 80 µg/ml reserpine or 50 µg/ml verapamil for the rifampicin-resistant isolates, and 30 µg/ml reserpine and 10 µg/ml verapamil for pan-susceptible isolates.

Restoration of Rifampicin Susceptibility

Rifampicin-resistant isolates were cultured in enriched BACTEC 12B medium containing 2 µg/ml rifampicin and either 80 µg/ml reserpine or 50 µg/ml verapamil. Similarly, pan-susceptible isolates were cultured in enriched BACTEC 12B medium containing 0.002 µg/ml rifampicin and either 30 µg/ml reserpine or 10 µg/ml verapamil. The GI for the respective isolates and treatment conditions was measured for 9 consecutive days. See online supplement for calculation of percent rifampicin susceptibility restored.

Rifampicin-Induced Expression of Efflux Pump and Transporter Genes

Selected MDR-TB isolates were cultured (OD₆₀₀ of 0.6) in duplicate in enriched 7H9 medium. Thereafter they were incubated in the presence or absence of rifampicin (2 µg/ml) for 24 hours. Total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA). cDNA was synthesized and the level of expression of putative efflux and transporter genes (Table 3) was quantified by quantitative real-time polymerase chain reaction (see online supplement).

Measuring Rifampicin-Induced Susceptibility to Ofloxacin

The ofloxacin MIC for five rifampicin mono-resistant isolates was determined by culture in enriched BACTEC 12B media containing 0.005 to 5 µg/ml ofloxacin. The GI for each culture condition was assessed for 9 consecutive days. Thereafter, each mid log culture in enriched BACTEC 12B media was exposed to 2 µg/ml rifampicin for 0 hours, 24 hours, and 7 days, respectively. Thereafter, the MIC for ofloxacin was determined in enriched BACTEC 12B media containing 2 µg/ml rifampicin and varying concentrations of ofloxacin (0.005–5 µg/ml), as described above.

Restoration of Ofloxacin Susceptibility

Selected rifampicin mono-resistant isolates were cultured in enriched BACTEC 12B medium containing 2 µg/ml rifampicin for 7 days to activate efflux/transporter genes. Thereafter, rifampicin (2 µg/ml) and ofloxacin (0.2 or 2 µg/ml) were added to the respective

cultures for 24 hours. A subculture was prepared by inoculating enriched BACTEC 12B media containing ofloxacin (0.2 and 2 µg/ml) and rifampicin (2 µg/ml) with or without 80 µg/ml reserpine or 50 µg/ml verapamil. The GI of the different cultures was assessed consecutively for 9 days. *See online supplement for calculation of percent ofloxacin susceptibility restored.*

Statistical Analysis

STATISTICA version 7 or the Relative Expression Software Tool-384 (REST-384) was used (*see online supplement*).

In vivo Restoration of Susceptibility to First-Line Anti-TB Drugs

Balb/c mice were infected with a clinical MDR-TB strain, followed by subsequent treatment with either verapamil alone, three first-line anti-TB drugs (isoniazid, rifampicin, and pyrazinamide), or a combination of verapamil and the three first-line drugs (*see online supplement*).

RESULTS

As demonstrated in Tables 1 and 2, levels of resistance to rifampicin as measured by MICs varied among MDR-TB and rifampicin mono-resistant isolates, both among strains of different genetic lineage and among genetically clustered MDR outbreak strains. These data suggest that biological mechanisms in addition to the *rpoB* mutation and the genetic background of the strains are responsible for defining the respective rifampicin MIC values.

To test the hypothesis that efflux pumps and/or transporter proteins were involved in modulating MICs, rifampicin-resistant strains were cultured in the presence of rifampicin (at the critical concentration of 2 µg/ml) together with the efflux inhibitors reserpine (80 µg/ml) or verapamil (50 µg/ml). Under these conditions rifampicin susceptibility was significantly restored in both MDR-TB isolates and rifampicin mono-resistant isolates ($P = 0.0000$; Figure 1). For the 15 MDR-TB isolates tested, the percentage susceptibility restored ranged from 53 to 81% and 61 to 86% for reserpine and verapamil, respectively (Table 1, Figure 1). Restoration of rifampicin susceptibility was independent of both the *rpoB* mutation and the genetic background. Analysis of the percent growth inhibition in relation to varying concentrations of rifampicin showed that growth inhibition was independent of the rifampicin concentration for all the strains tested (*see Figures E1A and E1B in the online supplement*). To investigate whether the absence of complete bacterial killing was due to bacteriostasis, the change in growth index over time was plotted (Figures E2A and E2B). These plots suggest nonreplicating growth in the presence of rifampicin and the efflux pump inhibitors, implying a bacteriostatic effect.

Interestingly, the level of intrinsic rifampicin resistance could not be modulated in the pan-susceptible isolates when exposed to rifampicin (0.002 µg/ml) and reserpine (30 µg/ml) or verapamil (10 µg/ml), thereby confirming a previous finding that suggested that efflux pumps targeted by reserpine are not active in pan-susceptible strains (10).

Table 3 shows that exposure of the four MDR-TB strains to rifampicin at 2 µg/ml resulted in the differential expression of genes putatively involved in efflux and transport. However, no clear correlation between rifampicin stimulation and gene expression was observed across the four strains analyzed. When isolates with similar genetic backgrounds were comparing it was evident that five genes (*Rv2994*, *Rv1877*, *Rv1258c*, *Rv2333c*, *Rv3239c*) were up-regulated in strains with the low copy clade genotype, whereas four genes (*Rv1747*, *Rv1634*, *Rv3239c*, *whiB7*) were up-regulated and one gene (*Rv1002c*) was down-regulated in strains with the Beijing genotype (Table 3). Only one of these genes (*Rv3239c*) was up-

regulated in both genetic backgrounds. The notion that rifampicin stimulated the expression of genes regulating the intracellular concentration of antibiotics was further supported by our observation that exposure of rifampicin mono-resistant isolates to 2 µg/ml rifampicin for 7 days induced a 10-fold or greater increase in the ofloxacin MIC of the five mono-rifampicin-resistant isolates tested (Figure 2). In the absence of rifampicin, the ofloxacin MICs for the respective isolates ranged from 0.02 to 0.2 µg/ml, whereas in the presence of rifampicin the ofloxacin MICs increased to between 0.2 and 2 µg/ml (Figure 2). This implies that in a portion of the mono-rifampicin-resistant isolates, exposure to rifampicin reduced the susceptibility to ofloxacin to above the critical concentration of 2 µg/ml used to define resistance to this second-line drug (11). Comparison of the genetic characteristics of the rifampicin mono-resistant strains showed a distinct relationship between the location of the *rpoB* mutation and the intrinsic level of resistance to ofloxacin. Strains with an *rpoB* mutation at codon 526 showed an ofloxacin MIC of 0.02 µg/ml, whereas strains with a mutation at codon 531 showed an ofloxacin MIC of 0.2 µg/ml. This in turn correlated with the level of rifampicin-induced ofloxacin resistance, as strains with the codon 526 mutation reached an ofloxacin MIC of 0.2 µg/ml after 7 days of rifampicin induction. In contrast, strains with the codon 531 mutation reached an ofloxacin MIC of greater than 2 µg/ml after 7 days of rifampicin induction (Figure 2).

To establish whether efflux pumps and/or transporter proteins were responsible for increasing the ofloxacin MIC, the influence of the efflux pump inhibitors reserpine or verapamil on the ofloxacin MICs were determined in rifampicin-exposed isolates. The overall percentage susceptibility restored ranged from 73 to 82% and from 69 to 87% for reserpine and verapamil, respectively (Table 4).

To determine whether efflux pump inhibitors could restore susceptibility toward first-line anti-TB drugs, verapamil was tested in a model of progressive pulmonary tuberculosis in Balb/c mice. Tuberculous mice infected with an MDR clinical isolate were treated with verapamil alone or verapamil plus first-line anti-TB drugs, and their efficiency was compared with animals treated with first-line anti-TB drugs alone and control animals treated with a saline solution. Figure 3 shows that animals receiving only verapamil or only first-line anti-TB drugs had similar bacilli loads at each time point, which were also similar to the control group. In contrast, the combination of first-line anti-TB drugs and verapamil significantly decreased cfu at every time point ($P < 0.05$), particularly after 60 days of treatment, where a 75% reduction in cfu was observed when compared with the control nontreated group (Figure 3). Together, these results suggest that the efflux pump-blocker verapamil is able to restore susceptibility to first-line anti-TB drugs in mice infected with an MDR strain.

DISCUSSION

This study challenges the dogma that an nsSNP in the *rpoB* gene of *M. tuberculosis* is the sole cause of clinical resistance to rifampicin (6). We have demonstrated that the level of rifampicin resistance varies independently of the mutation in the *rpoB* gene and the genetic background of clinical isolates of *M. tuberculosis*. This finding was consistent with a recent study that reported varying levels of rifampicin resistance in isogenic *in vitro*-generated mutants with identical *rpoB* gene mutations (4). It implies that the level of rifampicin resistance in both clinical isolates and *in vitro*-generated mutants is determined by both an altered RpoB structure and other biological mechanisms. The restoration of rifampicin susceptibility by the synergistic activity of the newly developed anti-TB drug SQ109 supports the notion (12). However, the mechanism of action of SQ109 remains unknown. Our observation that inhibition of efflux pumps and/or transporter proteins significantly restored rifampicin susceptibility in rifampicin-resistant strains suggests the importance of

these proteins in defining the intracellular concentration of anti-TB drugs. This finding is concordant with a previous study, which also showed that efflux pump inhibitors increased susceptibility to rifampicin (13). The authors concluded that active efflux may explain rifampicin resistance in *M. tuberculosis* strains without any *rpoB* resistance causing mutations. In contrast to previous understanding, our results suggest that *rpoB* gene mutations confer low-level rifampicin resistance (≥ 2 mg/ml) and that clinical resistance is achieved through activation of efflux pumps and/or transporter proteins. According to this hypothesis, we assume that rifampicin diffuses passively into the cell (10) and that the intracellular concentration of rifampicin is rapidly reduced by actively pumping rifampicin out of the cell. Consequently, the concentration of rifampicin required to ensure binding between the mutant RpoB and rifampicin is well in excess of the critical concentration used to define clinical resistance. We hypothesize that inhibition of efflux/transporter proteins by efflux pump inhibitors will allow for the intracellular concentration of rifampicin to increase, leading to inhibition of bacterial growth. In this study, the MIC for the rifampicin/efflux pump inhibitor combination could not be established, suggesting that this drug combination was bacteriostatic (14).

The *in vivo* activation of both drug-specific and broadly active efflux pumps has been demonstrated for several anti-TB drugs, including isoniazid and ethambutol (15). In this study, the activation of efflux and/or transporter genes by rifampicin appears to be a phenotype uniquely associated with rifampicin-resistant isolates, as efflux pumps and/or transporter proteins inhibited by reserpine and verapamil are seemingly not expressed in pan-susceptible isolates (10). This may be explained by the fact that concentration used to treat pan-susceptible isolates was too low to stimulate activation of the efflux and/or transporter genes. We suggest that a threshold concentration of rifampicin is required for gene activation, which may be above the MIC of pan-susceptible isolates leading to bacterial killing before gene activation.

Our analysis of gene expression of putative efflux pump and transporter genes in this study clearly demonstrated that rifampicin induced differential expression of some of these genes. Interestingly, only one of these genes was consistently up-regulated (*Rv3239c*) in response to rifampicin among the different strains analyzed. This is a probable transmembrane protein that contains an N-terminal approximately 500 amino acid domain that is similar to various antibiotic resistance and efflux proteins (16-18). No further information on this protein has been reported. Within the low copy clade strains analyzed, an additional four genes were consistently up-regulated (*Rv1258c*, *Rv1877*, *Rv2333c*, and *Rv2994*). *Rv1258c* is a probable conserved integral membrane transport (efflux) protein that is similar to TAP protein multidrug-resistance efflux pump from *Mycobacterium fortuitum* (18). This protein has been previously shown to be up-regulated in response to isoniazid, ethambutol, ofloxacin, and rifampicin (9, 13, 16, 19). *Rv2333c*, *Rv1877*, and *Rv2994* are probable conserved integral membrane proteins, similar to many antibiotic and drug efflux proteins (16-18, 20, 21).

Analysis of the gene expression of putative efflux and transporter genes in strains with a Beijing genotype after exposure to rifampicin for 24 hours showed up-regulation of three genes (*Rv1634*, *Rv1747*, and *whiB7*) and down-regulation of one gene (*Rv1002c*). *Rv1634* is a possible drug efflux membrane protein similar to many antibiotic resistance (efflux) proteins, including DTDP-glucose dehydratase (GRAE) from *Streptomyces violaceoruber* (16, 17, 20, 21). *Rv1747* is a probable conserved transmembrane ATP-binding protein ABC transporter similar to other *M. tuberculosis* ABC-type transporters and contains an ATP/GTP-binding site motif A (P-loop) and an ABC transporter family signature (22). This ABC transporter has been implicated in drug resistance, specifically to isoniazid (16, 23). *WhiB7* is a *WhiB*-like and MDR effector gene that has been reported to regulate the expression of

the efflux gene, *Rv1258c*, which is involved in rifampicin efflux (9, 24). *Rv1002c* is a conserved membrane protein predicted to be in the GT-C superfamily of glycosyltransferases, which is also believed to extrude undetermined substrates (18).

Together these results indicate that different mechanisms may be activated in response to rifampicin in rifampicin-resistant strains. We suggest that differential expression of a variety of efflux pumps and/or transporter genes may define the level of rifampicin resistance. However, we acknowledge that we were unable to demonstrate a direct relationship between extrusion of rifampicin and activation of specific genes. The up-regulated efflux pumps and/or transporter proteins appear to be promiscuous in their activity as we observed the modulation of the intracellular concentration of ofloxacin in the presence of rifampicin. Both of these anti-TB drugs are cyclic and probably recognized by the same efflux pumps and/or transporter proteins or combinations thereof. This is supported by previous observations that reserpine was able to modulate the ofloxacin MIC (25, 26).

Both the level of intrinsic ofloxacin resistance and rifampicin-induced ofloxacin resistance appear to be dependent on the location of the mutation in the *rpoB* gene. However, the mechanism underlying these different phenotypes remains to be determined. It is tempting to speculate that different *rpoB* mutations may change gene expression or that such mutations may alter the interaction with transcriptional regulators enabling differential expression of certain genes. This observation needs further investigation.

The ability of the cell to regulate the intracellular concentration of ofloxacin by efflux to a level equivalent to the critical concentration (2 µg/ml) suggests that rifampicin can induce clinical resistance to ofloxacin in rifampicin-resistant clinical isolates harboring the *rpoB* ser531leu mutation. These findings have important clinical and therapeutic implications. Current World Health Organization treatment guidelines promote the use of first-line anti-TB drugs until the drug-resistance profile of a patient's sputum culture is known (27). During such diagnostic delay periods, exposure of a rifampicin-resistant strain to rifampicin is likely to stimulate expression of efflux pumps and/or transporter proteins, thereby inadvertently inducing resistance to ofloxacin, one of the most effective second-line anti-TB drugs. This drug-induced "conditioning" of the bacilli may compromise the efficacy of the second-line treatment regimen, in part explaining the long duration of therapy and slow conversion rates observed.

This study accentuates the need to introduce rapid drug sensitivity screening methods, including MIC testing, to ensure optimal efficiency of the treatment regimen administered to treat MDR-TB. Furthermore, we highlight the fact that the physiology of drug-resistant strains may be very different from that of drug-susceptible strains and that the definition of the critical concentration used to define resistance needs to be cognizant of the fact that drug-pathogen interactions may modulate susceptibility toward another drug (antagonism), thereby compromising the usefulness of that drug. Last, the restoration of susceptibility to perhaps the most important anti-TB drug (rifampicin) would greatly benefit the treatment of MDR- and extensively drug-resistant TB cases. This is supported our observation that the combination of verapamil and three first-line anti-TB drugs significantly reduced pulmonary bacilli burdens in mice infected with an MDR-TB strain. Accordingly, we suggest that the development of anti-TB drugs that target mechanisms that define the intracellular concentration of anti-TB drugs may synergistically improve treatment outcome as well as enable the shortening of the duration of treatment. SQ109 is an example of such a drug (12).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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AT A GLANCE COMMENTARY**Scientific Knowledge on the Subject**

This study challenges the dogma that a nonsynonymous single nucleotide polymorphism in the *rpoB* gene of *Mycobacterium tuberculosis* is the sole cause of clinical resistance to rifampicin. In contrast to previous perceptions, we show that the level of rifampicin resistance is determined by the activation of efflux and transporter genes. The activation of these genes by rifampicin leads to a decrease in susceptibility to ofloxacin, which may compromise the efficacy of second-line anti-tuberculosis treatment.

What This Study Adds to the Field

This emphasizes the need for rapid diagnostics to guide treatment and the development of *M. tuberculosis*-specific efflux pump inhibitors to enhance the activity of current and newly developed anti-tuberculosis drugs.

Figure 1.

Normalized growth curves of multidrug-resistant tuberculosis (MDR-TB) isolates cultured in the presence or absence of rifampicin and/or reserpine or verapamil. MDR-TB isolates ($n = 15$) were cultured in enriched BACTEC 12B medium in the absence rifampicin (*open circles*), in the presence of rifampicin ($2 \mu\text{g/ml}$) (*open squares*), in the presence of verapamil ($50 \mu\text{g/ml}$) (*open diamonds*), in the presence of reserpine ($80 \mu\text{g/ml}$) (*solid circles*), in the presence of rifampicin ($2 \mu\text{g/ml}$) and verapamil ($50 \mu\text{g/ml}$) (*solid squares*), and in the presence of rifampicin ($2 \mu\text{g/ml}$) and reserpine ($80 \mu\text{g/ml}$) (*open triangles*). Growth curves were normalized using STATISTICA software and the percent susceptibility restored was calculated using the formula:

$$\frac{((GI_{\text{RIF}} - GI_{\text{EPI+RIF}}) - (GI_{\text{CONTROL}} - GI_{\text{EPI}}))}{GI_{\text{RIF}}},$$

where GI_{RIF} = growth index in the presence of $2 \mu\text{g/ml}$ rifampicin, GI_{EPI} = growth index in the presence of either $50 \mu\text{g/ml}$ verapamil or $80 \mu\text{g/ml}$ reserpine, $GI_{\text{EPI+RIF}}$ = growth index in the presence of $2 \mu\text{g/ml}$ rifampicin and either $50 \mu\text{g/ml}$ verapamil or $80 \mu\text{g/ml}$ reserpine, and GI_{CONTROL} = growth index in the absence of any drug and diluted 1:100. The percent susceptibility restored ranged from 55 to 81% for reserpine and from 61 to 86% for verapamil (Table 1) irrespective of the genetic background. Restoration of rifampicin susceptibility was statistically significantly ($P = 0.0000$; 95% CI) when compared with treatment in the absence of the efflux pump inhibitor.

Figure 2. Induction of ofloxacin (OF) resistance (increased minimum inhibitory concentration [MIC]) by exposure of rifampicin mono-resistant isolates with different genetic backgrounds and *rpoB* mutations to rifampicin (2 μ g/ml) for varying time intervals. *Medium shaded bars* = OFL MIC without exposure to rifampicin; *darkly shaded bars* = OFL MIC after exposure to rifampicin for 24 hours; and *lightly shaded bars* = OFL MIC after exposure to rifampicin for 7 days.

Figure 3.

Pulmonary bacillary loads (cfu) in mice infected with multidrug-resistant tuberculosis (MDR-TB) strain. Mice infected with the MDR-TB strain were treated with verapamil alone (*bars 3 and 4*), first-line anti-TB drugs alone (*bars 5 and 6*), a combination of verapamil and first-line anti-TB drugs (*bars 1 and 2*), or a saline solution (*bars 7 and 8*). Verapamil in combination with first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide, and verapamil) significantly reduced the cfu at 30 (*open bars*) and 60 (*solid bars*) days after initiation of treatment. Data are expressed as means $\pm$ SD, five mice per time point. * Represents statistical significance ($P < 0.05$).

TABLE 1
VARIATION IN THE LEVEL OF RIFAMPICIN RESISTANCE AND RESTORATION
OF RIFAMPICIN SUSCEPTIBILITY IN MULTIDRUG-RESISTANT
TUBERCULOSIS OUTBREAK ISOLATES

	Evolutionary Lineage (28–30)*	Spoligotype	IS6110 Cluster Type	<i>rpoB</i> Mutation	Other Resistance- Causing Mutations	Rifampicin MIC ($\mu\text{g/ml}$) [†]	% Susceptibility Restored at 2 $\mu\text{g/ml}$ Rifampicin [†]	
							80 $\mu\text{g/ml}$ Reserpine	50 $\mu\text{g/ml}$ Verapamil
MDR-TB outbreak isolates	Beijing (n = 5)	2	220	TTG (Ser531Leu)	<i>inhA</i> -15prom <i>embB</i> 306(Met306Val) <i>pncA</i> 103(Tyr103Ter)	30–150 (90)	55–81 (70)	70–85 (78)
	Low copy clade (n = 3)	115	DRF150	TTG (Ser531Leu)	<i>katG</i> 315(Ser315Thr) <i>embB</i> 306(Met306Ile) <i>pncA</i> Del58	60–170 (60)	53–68 (67)	61–71 (70)
	Atypical Beijing (n = 7)	2	464	GTC (Asp516Val)	<i>katG</i> 315(Ser315Asn) <i>inhA</i> -17prom	10–80 (20)	68–75 (71)	72–86 (77)

Definition of abbreviations: MDR-TB = multidrug-resistant tuberculosis; MIC = minimum inhibitory concentration.

*
n = number of isolates.

[†]
Data presented as range (median).

TABLE 2
VARIATION IN THE LEVEL OF RIFAMPICIN RESISTANCE IN
MYCOBACTERIUM TUBERCULOSIS ISOLATES WITH DIFFERENT GENETIC
BACKGROUNDS

	Evolutionary Lineage (28, 30, 31)	Spoligotype	IS6110 Cluster Type	rpoB Mutation	RIF MIC (mg/ml)
RIF mono-resistant	Various (n = 15)	Various	Various	Various	10–150 (90)
Drug-resistant isolates	Beijing (n = 7)	2	220	Ser531Leu	5–90 (10)
	F11 (n = 4)	77	1027	Ser531Leu	70–120 (110)
	F28 (n = 4)	82/81	11061	Ser531Leu	30–100 (95)
	Low copy clade (n = 15)	115	338	Ser531Leu	30–120 (70)

Definition of abbreviations: MIC = minimum inhibitory concentration; RIF = rifampicin.

*
n = number of isolates.

†
 Data presented as range (median).

TABLE 3
SELECTION OF EFFLUX AND TRANSPORTER GENES PUTATIVELY INVOLVED
IN MAINTAINING THE HOMEOSTASIS OF THE INTRACELLULAR
ENVIRONMENT

Gene	Transporter Type	Function	Primer Sequence	Efflux Pump Gene Expression (Fold Changes)			
				LCC Low RIF MIC	LCC High RIF MIC	Beijing Low RIF MIC	Beijing High RIF MIC
<i>PstB</i>	ABC	Active import of inorganic phosphate and export of drugs	Forward:5' GTT CCC GAT GTC AAT CAT GG 3' Reverse:5' ACC ACC AGA GAG TCG AAA CG 3'	2.14 *	-2.00 †	-2.27 †	11.43 *
<i>Rv2686c</i>	ABC	Active transport of drugs	Forward:5' ACG ACA TTC GAG GAC CCT AC 3' Reverse:5' ACG ATG ATG CTG GTC AAC AA 3'	1.73	3.28 *	-1.40	1.10
<i>Rv2687c</i>	ABC	Export of highly hydrophobic drugs	Forward:5' CTA CAG GTG CGG CAG AAG TT 3' Reverse:5' GAC GAA GAA GAA CCC GAT GA 3'	1.24	-1.35	-2.15 †	-1.14
<i>Rv2688c</i>	ABC	Export of toxic compounds	Forward:5' ACA GTC CCA CCG AAC TGA AG 3' Reverse:5' ATG AAT GGT CTC GAC GTG GT 3'	1.42	3.39 *	2.29 *	1.32
<i>Rv1747</i>	ABC	Transport of drug across the membrane	Forward:5' TCT GGA GCT GTT CGT TGA TG 3' Reverse:5' ACC CAG GAC ATC TGG TCA AG 3'	-1.90	-2.53 †	2.15 *	7.92 *
<i>drrA</i>	ABC	Export of antibiotic in the cell wall	Forward:5' ACG ACC ATG GTG GAC ATC TT 3' Reverse:5' AAC ACC AGG TTC TGC TCA CC 3'	-1.18	1.17	-1.83	-1.64
<i>drrB</i>	ABC	Export of antibiotic in the cell wall	Forward:5' CTG AGC TTG CCC ATT TTG AT 3' Reverse:5' TCA CCT GTG AGG CTG TCT TG 3'	-1.62	-1.95	1.21	3.48 *
<i>drrC</i>	ABC	Export of antibiotic in the cell wall	Forward:5' AAC CGG TTG CTA ACT CGA TG 3' Reverse:5' CAG CGG AAC AAT GCT GTA GA 3'	-2.05 †	-1.54	-1.39	1.29
<i>Rv1348</i>	ABC	Active export/translocation of drugs across the membrane	Forward:5' GTT CTT GGG TAC CAC GTT CG 3' Reverse:5' GTG GTC GAA CAC CAC AGT TG 3'	-1.95	-1.35	-1.38	-1.10
<i>Rv1456c</i>	ABC	Active export of antibiotic across the membrane	Forward:5' ATA TGC ATT CGT CGC TGT TG 3' Reverse:5' GGG TAC CCC GGT GAA GTA TT 3'	5.28 *	1.93	1.01	-4.08 †
<i>Rv1463</i>	ABC	Active transport and energy coupling across the membrane	Forward:5' AGA ACT GCT CAA GCC CAA GA 3' Reverse:5' ACG TAT TCC GGG TGG ATG TA 3'	1.72	1.26	-1.39	-1.81
<i>Rv2994</i>	MFS	Efflux of drugs	Forward:5' CTA TCT CAC GCG GGT CTG TT 3' Reverse:5' ACA GGA AGA CAC CGA TCC AC 3'	2.60 *	3.38 *	1.90	-2.45 †
<i>Rv1877</i>	MFS	Efflux of drugs	Forward:5' AAT CGC TGT ACC TGG TCG TC 3'	2.24 *	6.94 *	-1.29	-1.36

Gene	Transporter Type	Function	Primer Sequence	Efflux Pump Gene Expression (Fold Changes)			
				LCC Low RIF MIC	LCC High RIF MIC	Beijing Low RIF MIC	Beijing High RIF MIC
<i>Rv1258c</i>	MFS	Export of drugs	Reverse:5' CGG TCC AGG AAG TTT ACG AA 3' Forward:5' GGT ATG CCG TGT TGG CTA TC 3' Reverse:5' CCG CGT CTG TAT CAC GTA GTT 3'	2.16 *	4.47 *	2.01 *	1.10
<i>Rv1634</i>	MFS	Efflux of sugars and drugs	Forward:5' CCA CCA ACG AGT TTC TGA CA 3' Reverse:5' ACC CCA TCA GAT ACG ACG AG 3'	6.59 *	-2.48 †	89.26 *	11.47 *
<i>efpA</i>	MFS	Export of drugs	Forward:5' TAG GTT TCA TCC CGT TCG TG 3' Reverse:5' TGA CCA GGT TGG GGA AGT AG 3'	1.80	1.94 *	1.38	1.38
<i>Rv2333c</i>	MFS	Efflux of drugs	Forward:5' TGA TCT TTC TCG ACG CAC TG 3' Reverse:5' CAG CGT GAA CAA CGA AAC AC 3'	4.08 *	5.64 *	-1.25	-2.22 †
<i>Rv2459c</i>	MFS	Transport of substrates	Forward:5' TGG ACG TCA ACA TCG TCA AT 3' Reverse:5' GTG ACC CCG AAC ACA AAA CT 3'	1.26	-1.10	2.63 *	-2.91 †
<i>Rv3239c</i>	MFS	Could be involved in efflux	Forward:5' CGG ACG CTG ACC CTA TTA GA 3' Reverse:5' ACA TGC AGT CGA CCG TTG TA 3'	17.33 *	20.32 *	20.11 *	4.32 *
<i>Rv3728</i>	MFS	Involved in efflux	Forward:5' GAT GGC ATC GGA AAA AGT GT 3' Reverse:5' CAC CAG CTC CAT GAT TTG TG 3'	11.51 *	-3.19 †	1.90	-22.86 †
<i>emrB</i>	MFS	Export of multiple drugs	Forward:5' TTC GAC TAC ATG GGC CTC TT 3' Reverse:5' TAT GAG CGG ATG TTC TGT GC 3'	1.17	-2.06 †	1.85	10.09 *
<i>mmpL7</i>	RND	Export of antibiotic	Forward:5' TGA AAT ACG GAA GCC TGG TC 3' Reverse:5' GAG GTA AGA GGC CAG CAC AC 3'	-1.06	1.89	2.93 *	-1.13
<i>whiB7</i>	Regulatory protein	Transcriptional regulation	Forward:5' CAG ACA AAG ATT GCC GGT TT 3' Reverse:5' TCG AGC CTT GGT CGA ATA TC 3'	-1.06	-2.57 †	5.72 *	6.80 *
<i>Rv2989</i>	Transcriptional regulator	Transcriptional mechanism	Forward:5' GAA AGC GTG CAG GTA TAT CG 3' Reverse:5' ACA CCG CCT TTG GCA ATA C 3'	1.85	2.53 *	1.55	-2.01 †
<i>iniA</i>	Membrane protein	Drug transport	Forward:5' AAG ATG ATC CAG CGT CTG CT 3' Reverse:5' TTG ACC TGG CTC AGG ATA CC 3'	1.42	-1.14	1.92	-1.55
<i>iniB</i>	Membrane protein	Drug transport	Forward:5' GCT AGC CAG ATC GGT GTC TC 3' Reverse:5' CGA CAG ATG AGG CAT AGC AG 3'	2.17 *	-1.11	8.94 *	-10.90 †
<i>iniC</i>	Membrane protein	Transcriptional mechanism	Forward:5' CAA CGA CAT TGA ACG ACG AC 3'	2.14 *	1.17	-62.69 †	1.21

Gene	Transporter Type	Function	Primer Sequence	Efflux Pump Gene Expression (Fold Changes)			
				LCC Low RIF MIC	LCC High RIF MIC	Beijing Low RIF MIC	Beijing High RIF MIC
<i>Rv1002c</i>	Membrane protein	Unknown function	Reverse:5' GAA CGG ATC GTT GAG TGG AT 3' Forward:5' CAT TTC TGG TGA TGG GCA TT 3' Reverse:5' CCA GGT TCC AGG TCT GTT GT 3'	-1.54	-14.88 [†]	-2.76 [†]	-8.43 [†]
<i>Rv3806c</i>	Membrane protein	Unknown Function	Forward:5' GTG TCG TCG GCG TAT TTG AT 3' Reverse:5' CGC AGA TAG GTG CTG GTG TA 3'	1.73	16.34 [*]	-1.71	-1.79
<i>Rv3679</i>	ATPase	Extrusion of anions	Forward:5' AAG AAC AAG CTG CCG GTC TA 3' Reverse:5' GGC AGC GCT TCT AAC AGA GT 3'	1.25	3.51 [*]	1.04	3.93 [*]

Definition of abbreviations. ABC = ATP-binding cassette; LCC = low copy clade; MIC = minimum inhibitory concentration; MFS = major facilitator superfamily; RIF = rifampicin; RND = resistance nodulation cell division family; SMR = small multidrug resistance family.

Identified through bioinformatical analysis and literature searches (26).

^{*} Significantly up-regulated.

[†] Significantly down-regulated.

TABLE 4
RESTORATION OF OFLOXACIN SUSCEPTIBILITY IN RIFAMPICIN MONO-RESISTANT ISOLATES AFTER EXPOSURE TO RIFAMPICIN (2 µg/ml) FOR 7 DAYS TO INDUCE OFLOXACIN RESISTANCE TO EITHER 0.2 µg/ml OR 2 µg/ml FOLLOWED BY THE ADDITION OF OFLOXACIN (0.2 µg/ml OR 2 µg/ml) AND EITHER VERAPAMIL (50 µg/ml) OR RESERPINE (80 µg/ml)

Evolutionary Lineage (28, 30, 31)	% Ofloxacin Susceptibility Restored	
	Verapamil, 50 µg/ml	Reserpine, 80 µg/ml
Beijing	76.68	82.08
LCC	82.83	78.97
F11	73.77	73.18
EAI	87.3	78.6
F13	69.00	81.70

Definition of abbreviation: LCC = low copy clade; EAI = East-African Indian.