MAJOR ARTICLE

Efflux-Pump–Derived Multiple Drug Resistance to Ethambutol Monotherapy in *Mycobacterium tuberculosis* and the Pharmacokinetics and Pharmacodynamics of Ethambutol

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Background. Ethambutol is used for the treatment of tuberculosis in cases where there is isoniazid resistance. We examined the emergence of drug resistance to ethambutol monotherapy in pharmacokinetic-pharmacodynamic studies of a hollow-fiber system.

Methods. Dose-effect and dose-scheduling studies were performed with ethambutol and log-phase growth *Mycobacterium tuberculosis* to identify exposures and schedules linked to optimal kill and resistance suppression. In one study, after 7 days of daily ethambutol, 300 mg isoniazid per day was administered to each system to determine its early bactericidal activity.

Results. Efflux-pump blockage reduced the mutation frequency to ethambutol 64-fold. In dose-effect studies, ethambutol had a maximal early bactericidal activity of 0.22 $\log_{10}$ colony-forming units/mL/day, as is encountered in patients. By day 7, resistance to both ethambutol and isoniazid had increased. Previous exposure to ethambutol halted isoniazid early bactericidal activity. Daily therapy, as opposed to more intermittent therapy, was associated with the least proportion of efflux-pump–driven resistance, consistent with a time-driven effect. Microbial kill was best explained by the ratio of area under the concentration-time curve to minimum inhibitory concentration ($r^2 = 0.90$).

Conclusion. The induction of an efflux pump that reduces the effect of multiple drugs provides an alternative pathway to sequential acquisition of mutations in the development of multiple drug resistance.

Ethambutol was the last drug added to the current 4-drug regimen used to treat tuberculosis. Ethambutol is added to isoniazid, rifampin, and pyrazinamide during the first few weeks of therapy to prevent rifampin resistance in cases where there is unrecognized isoniazid resistance. In addition, ethambutol is the primary drug for the treatment of *Mycobacterium avium* infection. Other mycobacteria that are susceptible to ethambutol include *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium gordonae*, *Mycobacterium marinum*, and *Mycobacterium scrofulaceum*. Although the pharmacokinetic-pharmacodynamic (PK/PD) properties of other antituberculosis drugs were recently determined, those of ethambutol are as yet unknown [1–9].

It is intriguing that when isoniazid-resistant *M. tuberculosis* isolates with mutations at Ser315 of the catalase-peroxidase gene (*katG* Ser315) were examined, these isolates often had concurrent resistance to ethambutol [10]. The *katG* Ser315 mutation is the most prevalent mutation in isoniazid-resistant clinical isolates, and it is believed that, as a result of good biofitness, this mutation increases the chance of acquiring high-level ethambutol resistance [10]. In the laboratory, exposure of *M. tuberculosis* to static isoniazid concentrations led to concurrent resistance to both isoniazid and

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ethambutol via induction of a multidrug resistance–like pump [11]. However, it is unknown whether exposure to ethambutol and to concentration-time profiles similar to those encountered in patients with tuberculosis also lead to simultaneous resistance to ethambutol and isoniazid.

In the standard antituberculosis regimen, early bactericidal activity (EBA) is derived mostly from isoniazid effect. The EBA of an antituberculosis drug is the average rate of sputum bacillary decline during the first 2 days of therapy, with that resulting from isoniazid being the highest at 0.6 $\log_{10}$ colony-forming units (CFU)/mL sputum per day [12]. Because pyrazinamide demonstrates no EBA and the standard 10 mg/kg dose of rifampin has limited bactericidal activity, ethambutol is expected to provide most of the EBA when there is preexisting isoniazid resistance [13]. Ethambutol EBA is dose dependent, with a maximum of 0.25 $\log_{10}$ CFU/mL sputum per day at doses of $\geqslant$25 mg/kg per day, followed by a sputum bacillary decline of 0.1 $\log_{10}$ CFU/mL per day from days 3 to 14 [13]. Although the importance and the basis for EBA is controversial [4, 14, 15], this clinical index of microbial kill can nevertheless still be used to benchmark preclinical PK/PD models, which can subsequently be used to evaluate pharmacologic events associated with antituberculosis drugs.

We examined the relationship between ethambutol exposure, bactericidal activity, and efflux-pump–related resistance to both ethambutol and isoniazid. The studies were performed by mimicking the serum concentration-time profiles of ethambutol encountered in patients with tuberculosis. With daily ethambutol dosing, there is a triphasic oscillation in concentration consisting of a 2 h time to peak concentration (t_{max}), followed by a biphasic decline at a periodicity of 24 h, which is less than the *M. tuberculosis* doubling time [16–18]. Because the ethambutol plasma-to–epithelial lining fluid ratio is 1 [19], the serum concentrations were assumed to adequately mirror those at the site of pulmonary tuberculosis. PK/PD parameters associated with *M. tuberculosis* microbial kill, EBA, and resistance emergence were then determined.

METHODS

Bacterial isolate. *M. tuberculosis* H37Rv (ATCC 27294) was used in all studies. This strain was chosen because it has a lower ethambutol minimum inhibitory concentration (MIC) than most clinical isolates and is highly susceptible to ethambutol. Stock cultures of the *M. tuberculosis* were stored at −80°C in Middlebrook 7H9 broth with 10% oleic acid-albumin-dextrose-catalase (OADC) (Remel Inc.) and 10% glycerol. For each study, the bacterial stock was thawed and incubated at 37°C for 4 days in Middlebrook 7H9 broth under shaking conditions and 5% carbon dioxide to achieve log-phase growth. All cultures and studies were performed under BSL-3 conditions, as sanctioned by the University of Texas Southwestern Medical Center Environmental Health and Safety Committee.

Drugs and supplies. All drugs were purchased from Sigma-Aldrich. Ethambutol and isoniazid were dissolved in sterile water to the desired drug concentrations. Reserpine was first dissolved in dimethylsulfoxide and then diluted to the desired concentration in Middlebrook 7H9 broth. Hollow-fiber cartridges and caps were purchased from FiberCell Systems, and tubing was purchased from Masterflex.

MIC and mutant frequency. The ethambutol MIC was determined using the agar dilution method, as well as the E-test (Biodisk). Mutation frequency studies of critical concentrations of 5 mg/L ethambutol [20] were performed on Middlebrook 7H10 agar, as well as on agar supplemented with the efflux-pump inhibitor reserpine at a final concentration of 10 mg/L, shown elsewhere not to kill log-phase growth *M. tuberculosis* [3, 4]. Ethambutol-resistant colonies were counted on 20 agar plates coated with 0.2 mL of nondiluted inoculum.

In vitro pharmacodynamic model of tuberculosis. Our pharmacodynamic model of tuberculosis, which utilizes hollow-fiber technology, has been described in detail elsewhere [1–5, 9]. In our current experiments, bacilli grew in log-phase growth in the peripheral compartment and were exposed to antibiotic concentration-time profiles that mimicked those given to patients with tuberculosis. Ethambutol was administered into the central compartment of these systems via computer-controlled syringe pumps to achieve a t_{max} of 2 h. Median flow rates were set to mimic an α half-life ($t_{1/2}$) of 3 h, from 0 to 12 h, and a β $t_{1/2}$ of 12 h, from 13 to 24 h, as encountered in patients [16–18]. Similarly, isoniazid was administered to mimic a t_{max} of 1 h and a $t_{1/2}$ of 3 h, as encountered in slow acetylators, as described elsewhere [4].

Ethambutol dose-effect studies. *M. tuberculosis* cultures were grown to log-phase as described above. On day 4 of log-phase, 20 mL of the 10^6 CFU/mL cultures were inoculated into the peripheral compartment of the in vitro pharmacodynamic models, and the systems were incubated at 37°C under 5% carbon dioxide. Starting 24 h after inoculation, ethambutol was administered daily for 7 days to mimic the serum 0–24 area under the concentration time curve (AUC_{0-24}) and peak concentrations (C_{max}) achieved by human doses of 0, 6.25, 12.5, 25, 50, 75, and 100 mg/kg per day. On days 8 and 9, isoniazid was administered in all systems every 24 h to mimic AUC_{0-24} and C_{max} achieved by a daily dose of 300 mg administered to slow acetylator patients with tuberculosis. Starting from the first ethambutol infusion, the central compartment of each system was sampled 10 times over 48 h to establish that intended ethambutol concentration-time profiles were achieved. The *M. tuberculosis* cultures were sampled on days 0, 2, 5, 7, and 10. Each sample was washed twice in saline to prevent drug carryover, as described elsewhere [1–3, 5, 9]. The cultures

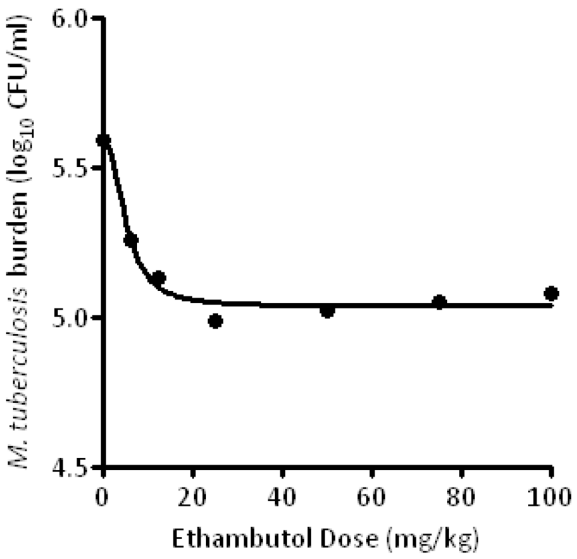

Figure 1. Effect of human equivalent ethambutol doses on *Mycobacterium tuberculosis*.

were then serially diluted, and the size of the *M. tuberculosis* population was determined by plating on Middlebrook 7H10 agar, supplemented with 10% OADC. To determine the sizes of the ethambutol-resistant and isoniazid-resistant populations, the same cultures were also plated on Middlebrook 7H10 agar, supplemented with either 5.0 mg/L ethambutol or the critical concentration of 0.2 mg/L isoniazid [20]. Colonies were counted after 3 weeks of incubation at 37°C under 5% carbon dioxide.

Ethambutol dose-scheduling studies. On the basis of the results of the dose-effect study, 2 sets of dose-scheduling studies were performed. In the first study, we wanted to explore further the potential for efflux-pump induction with paired regimens of either daily or once-a-week ethambutol monotherapy dosing for up to 21 days. An inoculum of 4.5 $\log_{10}$ CFU/mL *M. tuberculosis*, less than the inverse of the mutation frequency, was used for this study to minimize chances of preexisting resistance resulting from chromosomal mutations. Three paired regimens were evaluated: regimen A, associated with 20% of maximal kill (20% effective dose [ED_{20}]); regimen B, associated with 50% of maximal kill (median effective dose [ED_{50}]); and regimen C, associated with 80% of maximal kill (80% effective dose [ED_{80}]). The relationships between dosing schedule, microbial kill, and the emergence of efflux-pump–related drug resistance were evaluated by inspection. Efflux-pump–related resistance to ethambutol was defined as resistance that could be halted by 10 mg/L reserpine on Middlebrook agar and was expressed as a percentage of the total resistant subpopulation at the end of the study (day 21).

On the basis of the results of this study and the dose-effect study above, a more intensive dose-scheduling study was performed to better characterize the PK/PD parameter (AUC/MIC, C_{max}/MIC, or $\%T_{MIC}$) associated with effect. In this study, the cumulative weekly doses of 1% effective dose, 5% effective dose, ED_{20}, ED_{50}, and ED_{80} were administered once a week, or as 7 doses administered daily. One system was a nontreated control. Each hollow-fiber system was sampled at 0, 1, 4, 8, 12, 18, 23.5, 25, 28, 32, 36, 48, and 72 h for ethambutol concentrations achieved. Cultures in each hollow-fiber system were sampled on days 0, 3, 7, 10, and 14, and processed as described above to determine the total bacterial population. The cultures were also plated on Middlebrook 7H10 agar that had been supplemented with either one of the following: 5 mg/L ethambutol or 5 mg/L ethambutol combined with 10 mg/L reserpine.

Measurement of ethambutol concentration. Middlebrook 7H9 broth samples with drug were diluted 1:10 with deionized water, and a 20-μL volume sample was injected directly without further processing. The liquid chromatography–mass spectrometry–mass spectrometry, or LC-MS/MS, method was used to analyze samples on a Shimadzu high-performance liquid chromatography, or HPLC, system with an ODS-3 Inertsil Varian column 50 × 2.1 mm (5 μm) at 40°C. The isocratic mobile phase (0.2 mL/h) consisted of 50% of 0.1% formic acid in deionized water and 50% of 0.1% formic acid in methanol (vol/vol). Detection was accomplished by using an API 3000 mass spectrometer that was programmed in the multiple reaction-monitoring, or MRM, mode, monitoring the transition of the mass charge 205.10 *m*/*z* (mass to change ratio) precursor ion to the 116.10 *m*/*z* product ion for ethambutol. The method was linear from 1 to 1000 ng/mL ($r = 0.999$); accuracy was within ±5%.

PK/PD modeling. Ethambutol pharmacokinetic parameters were determined with ADAPT II software [21]. The relationship between ethambutol exposure and the total *M. tu-*

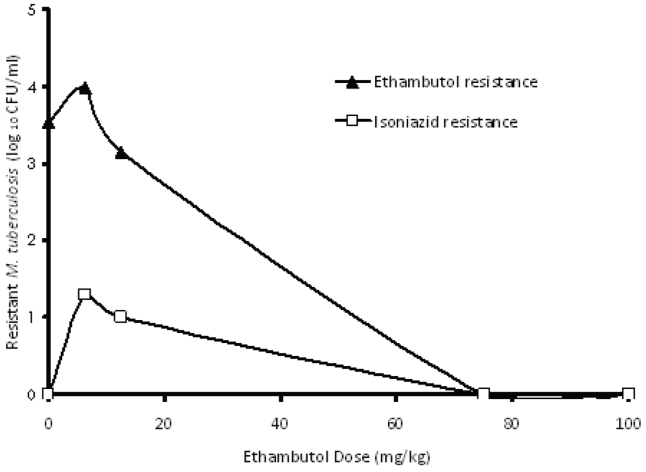

Figure 2. Emergence of isoniazid and ethambutol resistance after ethambutol monotherapy.

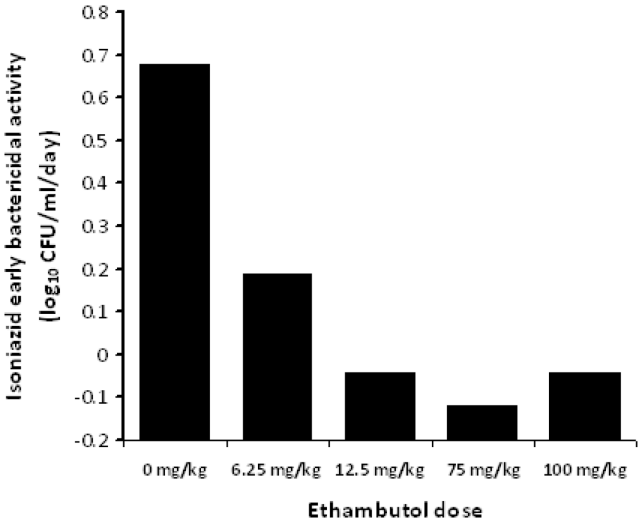

Figure 3. Early bactericidal activity of isoniazid after exposure to several ethambutol doses.

berculosis population was modeled with the inhibitory sigmoid E_{max} model, as described elsewhere [1–9]. All PK/PD modeling was performed using GraphPad Prism software (version 5.0; GraphPad Software).

RESULTS

The ethambutol MIC was 0.03 mg/L. The mutation frequency to ethambutol was 1.57×10^{-5}. However, in the presence of reserpine, the mutation frequency was 1.99×10^{-7}, a 64-fold reduction.

Population pharmacokinetic analysis of ethambutol concentrations in the hollow-fiber systems revealed a volume of 227 ± 47 L, a k_a of 31.340 ± 8.472, a k_e of 0.214 ± 0.076 per h during the α-phase and 0.035 ± 0.008 per h during the β-phase. The time to maximum concentration was 2 h in all the systems. Thus, the triphasic concentration-time profile of ethambutol was adequately mimicked. In terms of dose effect (as shown in Figure 1), the *M. tuberculosis* burden on day 2 was related to ethambutol exposure (equivalent human dose in mg/kg) by the following equation:

$$\text{effect } (\log_{10}\text{CFU/mL}) = 6.20 - \frac{(1.16 + \text{dose}^{1.89})}{(\text{dose}^{1.89} + 6.30^{1.89})} .$$

The r^2 for this regression was 0.97, and the P value was <.001. Examination of the relationship between exposure and EBA (as opposed to bacillary burden) revealed that the maximal EBA (E_{max}) was 0.22 (95% confidence interval, 0.14–0.29) $\log_{10}$ CFU/mL per day. Beyond the period corresponding to the EBA (days 2–7), the maximal rate of decrease in bacillary burden was 0.04–0.10 $\log_{10}$ CFU/mL per day. On day 7, the relationship between ethambutol exposure and resistance to ethambutol and isoniazid was as shown in Figure 2. Indeed, when the systems were treated on days 8 and 9 with an isoniazid exposure equivalent to 300 mg per day, the EBA demonstrated phenotypic tolerance in all systems with previous exposure to ethambutol monotherapy, as shown in Figure 3. On the other hand, the isoniazid EBA in the control cultures, as yet untreated with ethambutol, was 0.67 $\log_{10}$ CFU/mL per day.

When 3 ethambutol regimens were administered as either a daily or as a once-a-week regimen, in hollow-fiber systems with a low inoculum, results were conflicting (Figure 4), with the once-a-week regimen showing greater kill at the end of the first dosing interval for regimens A and C (which is consistent with the C_{max}/MIC-driven effect) but equivalent amounts of kill for regimen B regardless of dose schedule (which is consistent with the AUC/MIC driven effect). By day 14 all regimens had failed and looked alike as a result of resistance emergence. On day 21, the relationship between the resistant subpopulation resulting from efflux pumps versus dosing schedule was as shown in Table 1. Because once-a-week regimens were associated with a higher proportion of efflux-pump–related resistance, the emergence of this resistant subpopulation was $\%T_{MIC}$-linked. The next experiment evaluated a more extensive dose-scheduling dosing regimen, and on the basis of the inhibitory sigmoid E_{max} model, the r^2 was 0.25 for $\%T_{MIC}$, 0.56 for C_{max}/MIC, and 0.90 for AUC/MIC for microbial kill. The EC_{50} was an AUC_{0-168}/MIC of 552.9 or an AUC_{0-24}/MIC of 79.0.

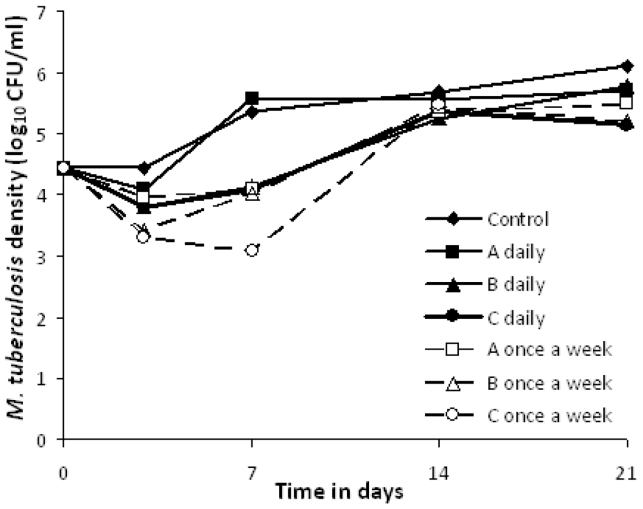

Figure 4. Dose-scheduling study with 3 pairs of doses administered either once a week or 7 equally divided daily doses. Dose scheduling demonstrated concentration-dependent relationship for total bacillary burden on day 1, but resistance had emerged in all regimens by day 14. Two nonpaired regimens were omitted from the graph for purposes of clarity.

Table 1. Reserpine-Inhibited Ethambutol-Resistant Subpopulation by Dosing Schedule

Regimen	Daily therapy, %	Once a week, %
Control (AUC_{0-168}/MIC, 0)	0.5	…
AUC_{0-168}/MIC, 46.46	28.7	60.5
AUC_{0-168}/MIC, 134.21	8.0	88.7
AUC_{0-168}/MIC, 269.42		74.8
AUC_{0-168}/MIC, 516.19	7.1	

NOTE. AUC, area under the concentration time curve; MIC, minimum inhibitory concentration.

DISCUSSION

The experimental system we applied has been used in the past for the bactericidal effect of antituberculosis drugs [1–5]. In the current study, this system was able to recreate the maximal ethambutol EBA of 0.25 $\log_{10}$ CFU/mL sputum per day and an extended bactericidal rate of 0.1 $\log_{10}$ CFU/mL per day, as in patients with tuberculosis [12, 13]. Although mutation frequency rates of 5 mg/L ethambutol were established 4 decades ago as 5×10^{-5} [22], the pattern and time to emergence of ethambutol resistance in patients with tuberculosis have not been adequately defined. This is because ethambutol was the last of the 4 first-line drugs introduced and came at a time when the advantage of combination therapy was already apparent. Our experimental system was able to provide such data, enabling us to better study the evolution of resistance to this drug.

It is believed that when there is concurrent drug resistance in isolates, the acquisition of the resistance is a sequential process, with the bacilli picking mutations resistant to one drug followed later by another drug [23]. This is based on the understanding of stable mutation frequencies, leading to the conclusion that the chance of simultaneous mutations developing resistance to 2 drugs in pulmonary cavities is incredibly small. Mutation frequency studies typically use static concentrations of drugs in growth media to establish mutation frequencies. However, it is a general principle of adaptive evolution that oscillations in the intensity of environmental stressors results in higher mutation rates and a greater need to adapt when compared with constant stressor pressure [24, 25]. Therefore, resistance emergence may be even more likely with dynamic concentrations of drugs, as opposed to when the bacillus is exposed to static concentrations of drugs. In our experimental pharmacokinetic system, resistance emerged quickly even when a low inoculum with minimal chance of having preexisting resistant isolates was used. Moreover, in dose-scheduling studies, once-a-week therapy regimens, which are associated with even more abrupt changes in drug concentrations than those found with regular daily dosing, were associated with greater proportions of efflux-pump–related resistance. Similarly, with pharmacokinetic changes in vitro and in patients, *M. tuberculosis* resistance to rifampin, moxifloxacin, ciprofloxacin, isoniazid, and pyrazinamide also emerged within a few days, well before the preexisting chromosomal mediated resistant subpopulation could grow enough to account for the total size of the resistant population [1–5, 9, 26, 27]. Furthermore, efflux pumps are increasingly a cause of clinically relevant *M. tuberculosis* drug resistance [28–31].

Ethambutol monotherapy not only led to resistance to itself but also to tolerance to isoniazid. The size of this resistant subpopulation was drastically reduced by reserpine, a drug also known to reverse resistance to isoniazid [4; 11]. These results are consistent with a pump that effluxes both isoniazid and ethambutol. Bacterial ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters have the capacity to extrude a wide variety of structurally unrelated chemicals from bacteria. Thus, one antibiotic may induce a pump that also extrudes other antibiotics. Alternatively, one antibiotic may induce a particular single pathway, which then leads to induction of many different efflux pumps. As an example, when *M. tuberculosis* is exposed to tetracycline, there is induction of *whiB7*,

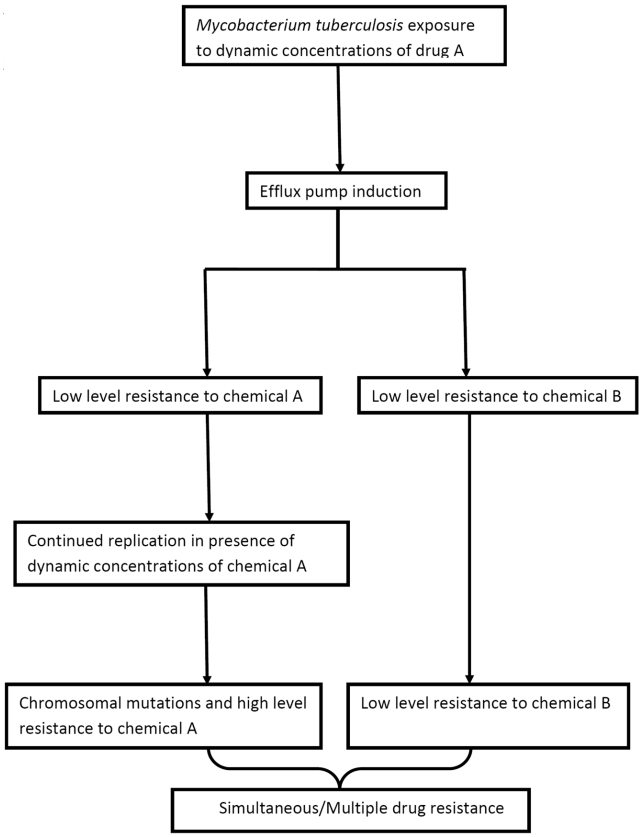

Figure 5. Proposed evolution of simultaneous drug resistance to antituberculosis drugs

a primary regulatory gene, whose expression leads to induction of at least 3 additional genes: Rv1258c, which encodes a *tap*-like efflux pump that confers low-level resistance to the tetracycline itself and aminoglycosides; Rv1473, which encodes an ABC transporter that effluxes macrolides; and *erm*, which confers resistance to lincosamide and streptogramin [32]. Interestingly, Rv1258c encodes for an MFS efflux pump that is inhibited by reserpine [33]. Moreover, Rv1258c is overexpressed in the presence of either isoniazid or rifampin in clinical isolates with chromosomal mutations in the *katG* and *rpoB* genes [30; 31]. Other transporters that extrude both ethambutol and isoniazid from *M. tuberculosis* include an ABC transporter encoded by *pstB*, which also extrudes rifampin, and the drug transporter encoded by *iniA* [11; 34]. Whatever the case, induction of multiple drug resistance by a single drug to structurally unrelated drugs is clearly a common survival strategy of the tubercle bacillus.

Given the foregoing, as well as our experimental results, we would like to propose a general model for the emergence of resistance to multiple drugs by *M. tuberculosis*. The model, as illustrated in Figure 5, has a first step consisting of induction of efflux pumps. This allows the bacteria more time for multiple replications in the face of ongoing chemical pressure; more replications increase the chances of emergence of chromosomal mutations associated with drug resistance. Indeed, whereas induction of efflux pumps may impose energy costs, in general this ancient mechanism provides a more rapid response to toxins for an organism with a doubling time of $\geqslant$24 h, allowing for the development of a chromosomal mutation that least compromises biofitness. The polyspecific nature of the pumps mean that instead of sequential acquisition of resistance mutations to 2 unrelated compounds [10], the critical first event may often be induction of an efflux pump which transports the 2 or more drugs, enabling rapid emergence of high-level resistance to both. This would explain, for example, the important observation by Parsons et al [10] that high-level ethambutol resistance without concurrent isoniazid resistance is rarely encountered in the clinic. However, the proposed model will need to be validated using other tuberculosis disease models and, ultimately, in patients. The model that we propose likely does not operate in exclusivity. As an example, mutations in genes involved in DNA repair mechanisms may lead to hypermutable strains to many antibiotics (mutator phenotypes). Clinical isolates of the Beijing genotype have been demonstrated to have mutations in the repair genes *mut* and *ogt*, which lead to higher rates of simultaneous rifampin and isoniazid resistance [35, 36]. Less stable mutator phenotypes resulting from antituberculosis drugs, oxidative stressors, and other environmental stressors may be even more common than stable mutator phenotypes [37, 38]. Thus, efflux-pump induction, stable mutators, and inducible mutators likely work in various permutations and combinations, so that the concept of sequential acquisition of mutations that lead to 2 different drugs, based on stable baseline mutation frequencies, may be an oversimplification.

The clinical implications of our findings are as yet unclear. However, it is intriguing that, in 3 independent studies in India and in the United States that evaluated >4500 *M. tuberculosis* isolates from patients with tuberculosis, ethambutol resistance was almost always accompanied by isoniazid resistance [10, 39, 40]. However, a considerable proportion of isoniazid-resistant isolates did not have ethambutol resistance [40]. This pattern suggests a link between exposure to ethambutol and emergence of isoniazid resistance. A study examining the effect of efflux-pump inhibitors on the MICs of a large number of ethambutol-resistant clinical *M. tuberculosis* isolates, with and without known ethambutol and isoniazid associated chromosomal mutations, is the ideal next step. If the MICs in these isolates decrease in the presence of an efflux-pump inhibitor consistent with our proposed model (Figure 5), then the clinical strategy of using ethambutol as an insurance in case of isoniazid resistance compared with the potential of ethambutol to limit the effectiveness of isoniazid would need to be evaluated. It is also unclear whether the efflux-pump induction also limits the effectiveness of ethambutol and isoniazid analogs such as SQ 109 and ethionamide, respectively. Thus, additional studies are needed. On the other hand, if the role played by efflux-pump induction is confirmed, efflux-pump inhibitors could be developed as a therapeutic strategy to forestall the emergence of multiple drug resistance.

Finally, both microbial kill and resistance emergence were evaluated using classic PK/PD approaches. Less frequent dosing of the same cumulative dose was associated with higher proportions of reserpine inhibitable resistance, whereas daily therapy fared better, which strongly suggests that T_{MIC} was more important for suppressing efflux-pump–related drug resistance. However, concentration-related measures, especially AUC/MIC, were more important in terms of determining microbial kill. Such differences between the PK/PD parameter associated with microbial kill and resistance seem to be common when *M. tuberculosis* has been studied [5, 9]. This should not be a surprise, given that the mechanism of kill (inhibition of specific target) may vary from those associated with drug-resistance emergence (eg, efflux-pump induction). Nevertheless, the concentration-related kill strongly suggests that higher doses than those currently used may be more effective for ethambutol. However, the exact doses remain to be identified and the toxicity of such doses determined.

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