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A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs

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Abstract

The current understanding of the mechanism of anti-tuberculosis drug resistance has been shaped by the history of development of anti-tuberculosis drugs in the past 60 years and was arrived at as part of inductive generalization. Recently, these standard beliefs have been tested in controlled hollow fiber systems experiments. Drug resistance in *Mycobacterium tuberculosis* was shown to be related to pharmacokinetic-pharmacodynamic (PK/PD) factors, and factors such as pharmacokinetic variability. Poor PK/PD exposures due to our current non-optimized dosing regimens initiate a chain of evolution driven events, starting with induction of multi-drug efflux pumps, followed by the development of chromosomal mutations in time, which together lead to high level resistance multi-drug resistant tuberculosis and extremely drug resistant tuberculosis.

Introduction

Global efforts to eliminate tuberculosis (TB) by 2050 have been threatened by recent worldwide emergence of multidrug-resistant TB (MDR-TB) and extensively drug resistant-TB (XDR-TB), estimated at 440 000 and 25 000, respectively, in 2008 [1]. This is despite injection of enormous resources to support the World Health Organization (WHO) recommended directly observed treatment strategy (DOTS) programs which are meant to prevent TB drug resistance. MDR-TB refers to simultaneous resistance to rifampin and isoniazid, while XDR-TB refers to MDR-TB plus additional resistance to at least one injectable drug plus a quinolone. Both forms of TB are difficult to treat, are expensive, long, more likely to fail and more likely to result in death. The need for extended therapy using combinations of drugs remains a practical obstacle to effective TB control. We review the clinical, laboratory and pharmacokinetic/pharmacodynamics (PK/PD) factors associated with development of drug resistant TB and propose a new scenario based on evolution and PK/PD science. There has been a general paucity of data in this area; however the few pivotal studies in the past two years point towards a departure from standard beliefs on how *Mycobacterium tuberculosis* resistance arises.

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Current beliefs of how *M. tuberculosis* resistance emerges

The understanding of the mechanism of anti-TB drug resistance has been shaped by the history of development of anti-TB drugs in the past 60 years, and was arrived at as part of inductive generalization. Unfortunately, this approach is prone to bias. Based on observations in regimens tested between 1952 and 1980, each drug in the regimen was assigned special roles in treatment of *M. tuberculosis*. Pyrazinamide, isoniazid, ethambutol, rifampin and streptomycin are each thought to target certain specific populations of the *M. tuberculosis* such as bacilli under acidic, aerobic and/or hypoxic conditions within caseous foci, at the edge of pulmonary cavities and inside macrophages, respectively [2;3]. Resistance suppression is defined as one drug preventing resistance to another, not one drug preventing resistance to itself. The resulting belief, almost universally accepted, is that if patients take these multi-drug regimens without defaulting, then MDR-TB and XDR-TB emergence would be ameliorated. Accordingly, it is believed that missing drug doses leads to 'effective monotherapy' for some bacillary populations because of different drug half-life's and differential drug penetrations into effective compartments. It has been theorized that resistance evolves independently for each drug one at any one time through 'unlinked processes', leading to the standard step-wise pick up of mutations that leads to sequential acquisition of resistance [2;4]. Finally, the belief has been that resistance arises from replicating bacilli, so that non-replicating persistent bacilli (NRP) do not mutate and cause drug resistance. Recently, each of these staple beliefs has been challenged in well designed experiments that applied both PK/PD and none PK/PD methodology.

Just what do you mean by "resistant"?

The term "drug resistance" is ambiguously defined in many situations. What is drug resistance, especially in the context of *M. tuberculosis*? The WHO defines drug resistance as "the ability of certain microorganisms to withstand attack by antimicrobials." In the context of *M. tuberculosis*, this is defined as the ability of >1% proportion of a bacilli to grow in the presence of critical concentration of drug [5]. The critical concentrations themselves are defined as the concentration of antibiotic that inhibit growth in 95% of wild type strains that have hitherto not been exposed to drug. Thus, these are essentially epidermiologic cut-off values. The current critical concentrations that define resistance are shown in table 1.

PK/PD dose selection and clinical application to prevent drug resistance

When a drug dose is administered to patients it becomes part of the non-deterministic process of pharmacokinetic variability. In other words, a particular dose does not lead to a specific concentration-time profile in all patients, but rather a distribution determined in part by alleles of genes encoding enzymes involved in xenobiotic metabolism, the particular physique of each patient as is the case of pyrazinamide [6], or even dietary considerations. This means that in some patients, despite patients taking all their drug doses low drug concentrations could still be encountered, which could lead to emergence of drug resistance. Thus, resistance emergence could occur in part due to non-deterministic causes that have nothing to do with DOTS or default.

The response of the pathogen to a particular drug concentration-time profile is itself related to several PK/PD factors. For *M. tuberculosis*, PK/PD factors have been derived in monotherapy studies in the hollow fiber system (HFS) [7–12]. First, the shape of the concentration-time curve has been related to resistance emergence for each of the first line anti-TB drugs. Studies with isoniazid and pyrazinamide revealed that the relationship between drug exposure and population of drug-resistant *M. tuberculosis* was a series of curves that changed with time, starting with a "U" shaped curve, which then evolved over time to an inverted "U" curve (figure 1). In other words, the relationship is defined by a

quadratic function, with time as part of the defining characteristics of the leading coefficient (see reference 11). Therefore, in interpreting indices at which resistance can be suppressed, the duration of therapy should be taken into consideration. Rifampin resistance emergence and suppression are linked to the peak concentration (C_{max}) to MIC, with optimal suppression of resistance at a free drug C_{max}/MIC of 175 [10]. Isoniazid resistance emergence was demonstrated to be closely linked to both C_{max}/MIC and AUC/MIC [13]. On the other hand, both pyrazinamide and ethambutol resistance emergence were associated with the % time concentration persisted above MIC ($\%T_{MIC}$) [11;14]. The lessons are obvious, resistance emergence to a drug depends on the drug exposure achieved, and in many situations the actual shape of the concentration-time curve, which often differ from the PK/PD parameter linked to microbial kill.

These PK/PD results, as well as the exposures associated with optimal kill, can be used for several purposes. The first is to refine susceptibility breakpoints. Setting susceptibility breakpoints using the PK/PD approach does not just rely on the MIC distribution in wild type isolates, but also on the doses and the drug exposures achieved by the doses in patients, given pharmacokinetic variability. An isolate is defined as being drug-resistant if it has an MIC that precludes it from being effectively killed by antibiotic concentrations achieved in at least 90% of patients given a particular dose. Put simply, if an isolate cannot be effectively killed at site of infection in most patients by a drug after taking the maximum tolerated dose, then it is resistant to that drug. Using this approach, new critical concentrations for each of the first line anti-TB drugs, as well as moxifloxacin, were recently proposed, as shown in table 1. The most dramatic changes are proposed for isoniazid and rifampin, and thus the definition of MDR-TB itself. These are the two drugs in which PK/PD studies have been performed using at least two independent models (mice, hollow fibers and guinea pigs) [10, 13, 15–17] and population pharmacokinetic studies are available from at least 3 independent groups [18–22]; utilizing any of these studies leads to the same conclusion on breakpoints, so that it is unlikely that bias from any one PK/PD model can be invoked. Nevertheless, further work is needed to confirm these proposed susceptibility breakpoints. The breakpoints we have proposed, as well as the currently accepted breakpoints, need to be examined in large datasets of combination therapy clinical studies, and each breakpoint examined for whether it can predict microbiologic failure.

The second use of PK/PD exposures that could suppress resistance is to design doses and dosing schedules that could suppress drug resistance emergence. This is because it has been demonstrated for virtually each anti-TB agent examined; that the dose associated with maximal kill is not necessarily the one that prevents resistance emergence. Indeed, for moxifloxacin and ciprofloxacin such exposures associated with maximal bactericidal effect were also the ones associated with maximal amplification of the resistant sub-populations [7;8]. Thus, computer aided clinical trial simulations have been utilized to determine doses that best suppress resistance suppression for moxifloxacin, pyrazinamide, and rifampin. In HFS studies, a 24h AUC/MIC greater than 53 was associated with suppression of drug resistance. Monte Carlo simulations revealed that this target could be achieved by 59% of patients treated with 400 mg of drug and by 93% in patients treated with 800mg of moxifloxacin daily [7]. Similarly, Goutelle et al examined if rifampin doses of 600 mg and 1200 mg could adequately achieve the C_{max}/MIC of 175 needed to suppress drug resistance [18]. The standard dose of 600 mg a day fared badly, while 1,200 mg performed better. We performed similar studies for pyrazinamide's ability to achieve % time above MIC of ≥67% needed to suppress resistance emergence [11]. Doses ≥3G a day administered daily achieved the target in >90% of patients. All such doses advocated by this approach still need to be shown to be safe for patients. In the case of pyrazinamide, however, toxicodynamic analysis and meta-analysis recently suggested low rates of hepatotoxicity even at these high doses, provided they are administered no more than 2 months [12].

Mechanisms of resistance emergence

It is believed that during non-compliance, one of several mechanisms may lead to emergence of drug resistance. According to the pharmacokinetic mismatch hypothesis, during non-compliance the drug with the short half-life disappears quickly, leaving *M. tuberculosis* exposed to the drug with the longer half-life as monotherapy. In some scenarios, even without non-compliance, if the half-life of two drugs are very mismatched (e.g., rifapentine and isoniazid), then the same situation can arise especially during intermittent phases of therapy. We recently expressed this as a falsifiable hypothesis and tested it in HFS for rifampin and isoniazid with and without pre-existed resistant sub-populations (Srivastava et al. In revision). The drugs were administered as well matched regimens, or isoniazid administered 6h, 12hr, or 24 hr after rifampin. Essentially the more mismatched regimens performed better and the pharmacokinetic mismatch hypothesis was rejected. Another theory on resistance emergence has been the time in mutant suppression window hypothesis. In our work on rifampin, isoniazid, pyrazinamide, and ethambutol in the HFS this hypothesis also failed to explain emergence of resistance to these agents [10;11;13;14]. However, one mouse study confirmed this theory for moxifloxacin [23].

New evolution based understanding

In *M. tuberculosis*, as in all bacteria, DNA replication allows a narrow baseline rate of chromosomal mutations, a balance between the ability to adapt to the changing environment via mutations and safeguarding genetic information from collapse if mutation rates are too high. DNA repair enzymes are central to this process. Mutations in genes that encode DNA repair enzymes would lead to hyper-mutable strains. As an example, deficiencies in *M. tuberculosis* MutT1 results in a 16-fold increase in spontaneous mutation frequency that leads to increased rifampin resistance in the laboratory; mutants in which two DNA repair genes *ada/alkA* and *ogt,* involved in alkylation damage repair, are inactivated increases mutation frequencies to rifampin 100 fold [24; 25]. It has been argued, convincingly that indeed non-stable mutators formed in the inflammatory lesions in patients may be even be more common [26]. Such families of hyper-mutators could lead to higher mutation rates in genes associated with anti-TB drug resistance than predicted from a stable genome approach. Epidemiologic evidence has been presented that suggests that this might be why there is a greater propensity to drug resistance in the Beijing strain [27].

One dogma has been that in latent TB, non-replicating bacilli (which have minimal DNA replication), do not generate resistant mutants. Thus, it has been assumed that most resistance arises from “luxuriantly” replicating bacilli but not in those bacteria not growing [28; 29], and that isoniazid monotherapy is appropriate for treating latent TB. Recently, Fortune and her team infected macaques with *M. tuberculosis*, and then examined bacteria recovered from lesions with either active TB, or latent TB, or reactivation TB [03]. Whole genome sequencing revealed that mutation rates were as high in bacteria in granulomas during latent TB as in log phase growth cultures. The mutations were a result of oxidative damage, either cytosine deamination (GC>AT) or the formation of 8-oxoguanine (GC>TA) [30]. This seminal observation suggests that drug resistant mutations arise more commonly during latency than assumed, and that treatment of latent TB may contribute to the emergence of drug resistant TB. Indeed, the highest risk factor for isoniazid monoresistance is prior treatment for latent TB [31].

Resistance has been hitherto assumed to arise from chromosomal mutations of genes encoding proteins related to either conversion of prodrug to active moiety or target proteins (table 1). The role of efflux pumps in drug resistance has been more recently examined. First, microbial kill by isoniazid in log-phase growth bacilli in the HFS was terminated by

efflux pump related mechanisms [9]. This finding led to considerable controversy [28;29], especially around the meaning of “tolerance” and “resistance”. The rapid emergence of resistance to isoniazid was confirmed in a subsequent PK/PD study, and was linked to both C_{max}/MIC and AUC/MIC [13]. Further insight was gained in a study in which ethambutol monotherapy led to “tolerance”, essentially low level resistance to the ethambutol that was linked to % time above the MIC [14]. Even more remarkable was the finding that isoniazid resistance could also be demonstrated after the ethambutol monotherapy, with the same “U” shaped curve encountered as if isoniazid had been administered. Administration of isoniazid after 7 days of daily ethambutol resulted in reduction in isoniazid effect in a rank order proportional to the original isoniazid dose [14]. This meant that multiple drug resistance can arise from administration of a single drug, and the resistance was proportional to drug concentrations used against the bacteria. This led us to propose a new scenario for the emergence of multi-drug resistant TB; one based on both evolution and PK/PD exposures (figure 2). In this scenario, efflux pumps are induced first, and lead to drug resistance or tolerance to more than one drug. This occurs in the face of PK/PD drug exposures that have not been optimized to suppress resistance. The bacteria continue to replicate under protection of the efflux pumps, sometimes in with a background of stable and non-stable mutator genes, and enable the bacteria to generate the canonical chromosomal mutations associated with high level resistance. We predicted that this is a common scenario, and that efflux pumps such as those encoded by Rv1258c would lead to multiple drug resistance to rifampin, quinolones and streptomycin [14]. Recently, Louw et al sequenced MDR-TB clinical isolates from Cape Town, and demonstrated these efflux pump derived clusters of resistance to rifampin and quinolones [32]. In our proposed paradigm, poor compliance and pharmacokinetic mismatching play a less significant role, while reduced exposures due to poor dosing (our current non-optimized dosing) and pharmacokinetic variability start a chain of evolution driven events rather quickly, which leads to MDR-TB and XDR-in the long run. The traditional role of combination therapy in reducing acquired drug resistance via one drug protecting another is still maintained in this scenario, provided the drugs are not expelled from bacterial cells by the same efflux pump.

The role of the immune system in the evolutionary scenario we have proposed for *M. tuberculosis* drug resistance is yet to be determined. There are some hints, especially in the case of rifamycin drug resistance, that the immune system may play an important role in suppressing drug resistance [33;34]. It seems that rifamycin monoresistance arises more commonly when there is a background of advanced immunodeficiency. However, unlike microbial kill of other bacteria by granulocytes in which the contribution of the extent to which phagocytes contribute to microbial kill have been well characterized [35; 36], no similar studies have been performed for emergence of drug resistance. Studies examining *M. tuberculosis* are even more scant. Studies to establish this are on-going.

Acknowledgments

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Review highlights

- *Mycobacterium tuberculosis* resistance to pyrazinamide and ethambutol are linked to % time concentration persists above MIC while resistance to rifampin is linked to C_{max}/MIC ratio and isoniazid to both C_{max}/MIC and AUC/MIC.
- Mutation rates are high and drug resistance emerges in Mycobacterium tuberculosis even during latency
- In our current poorly optimized regimens, induction of efflux pumps might lead to multiple drug tolerance/resistance and emergence of MDR-TB
- We propose a new evolutionary scenario for MDR-TB emergence that integrates PK/PD, efflux pumps, mutator genes, and standard chromosomal mutations

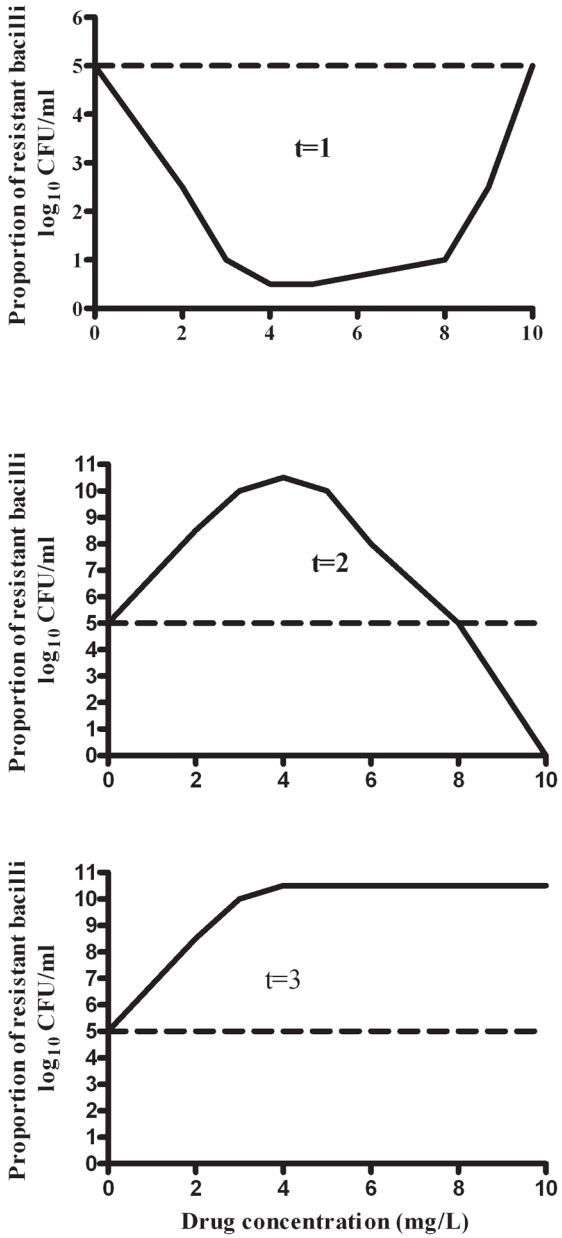

Figure 1. Change in size of drug-resistant *M. tuberculosis* population with exposure and time
Figure shows an upright "U" at the beginning of therapy (t=1), which evolves to an inverted "U" curve at t=2, and eventually reaches a time when no concentration of the antibiotic in question can suppress drug resistance at t=3.

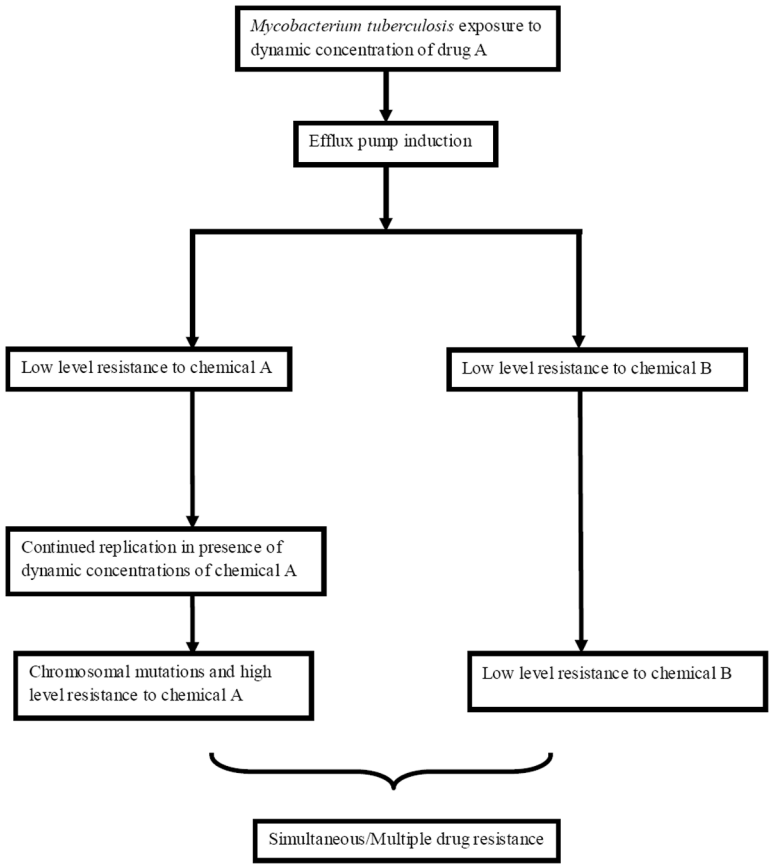

Figure 2. Proposed evolution of simultaneous drug resistance to antituberculosis drugs

Table 1

Susceptibility breakpoints and chromosomal mutations associated with drug resistance

Drug	Current breakpoint (mg/L)	Proposed breakpoint (mg/L)	Chromosomal mutations associated with resistance
Rifampin	1	0.0625	*rpoB*
Isoniazid	0.2/1.0	0.03/0.125	*katC, inhA, oxyR, ahpC, furA*
Streptomycin	5	-	*rrs, rpsL*
Pyrazinamide	100	50	*pncA*
Ethambutol	5.0/7.5	4	*embB*
Moxifloxacin	1	1	*gyrB*