The Changing Basis of Anti-TB Therapy and Prospects of Shorter Duration of Therapy: The View of a Midget Sitting on the Shoulders of Giants

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Tawanda Gumbo, MD
Assistant Professor of Medicine
Division of Infectious Diseases
Department of Medicine
UT Southwestern Medical Center, Dallas, TX

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I heard last night, your song of death
the song of the cough in your chest
and of the dampness in the handkerchief
of the ruby stain showing flowers of blood

Evaristo Carriego's *Misas herejes* (1908) (trans. Vera Reber)

Introduction and History: The epic struggle between TB and humans.

Every medical student knows that our ancestors got infected with *Mycobacterium bovis* from domesticated animals, and then *Mycobacterium tuberculosis* evolved from the *M. bovis*. Unfortunately, that story is *not true*. In fact, genetic studies reveal that it is *M. bovis* which evolved from *M. tuberculosis*, and that via *M. africanum*[1]. The first infection of our ancestors with the ancestral *M. tuberculosis* strain almost likely happened in Africa, but it is unclear what the original source was, or when this first happened[1,2]. A study of 170 samples from mummies in Thebes and Abydos in ancient Egypt have demonstrated infection with *M. tuberculosis* via identification of DNA in 22-28% of suspected TB patients who lived between 3200 and 500 BCE[3]. Genotyping revealed that from 3200-2800 BCE patients were infected with the ancestral *M. tuberculosis*. However 2000-1600 BCE, *M. africanum* as well as the ancestral strain were encountered. From 1500-500 BCE, the ancestral strain gave way to "new" *M. tuberculosis* strains. No *M. bovis* was encountered. Around 460 BCE, Hippocrates described the symptoms of tuberculosis (TB) in Greece and called phthisis the most common and most fatal disease of his time[4]. As regards Americans, humans carried the ancestral *M. tuberculosis* strain from northern China-Siberia into the Americas via the Bering land bridge during the last glacial maximum period 17,000–20,000 years ago[5]. *M. tuberculosis* DNA has been identified in a 1,000-year-old spontaneously mummified Peruvian who had pulmonary TB[6]. Subsequent analysis of 483 skeletons from Chile demonstrated an overall 1% prevalence rate for TB between 2000 BCE and 1500 CE so that TB flourished in the Americas in the pre-Columbian era[7]. In the space age, 2 out of the current 6 billion people on earth have been infected with *M. tuberculosis,* and 9 million develop the disease every year. Rates are *increasing* at 2% per year. TB is the number one infectious diseases killer on earth, and kills 3 million people each year.

Hippocrates warned physicians to stay away from attempts at curing TB patients; they would always fail and ruin physicians reputations. Galen of Perganum (131-201 CE) sent TB patients to Pompeii to inhale the sulphurous volcanic vapors, while others prescribed the vapor of boiling tar, leeches, and the ground sole of a worn out shoe. This was followed by the sanatoria and sunshine movement. With lung collapse therapy, surgical resection of the lung and "plombage," patients' breathing was sacrificed to cure the infection. The first attempts at chemotherapy came after Koch discovered *M. tuberculosis* in 1861. He obtained a crude filtrate of the bacillus, mixed it with glycerin, and injected it into his own arm. He developed a severe blistering reaction. He then tested it on his girlfriend, Hedwig, at lower doses! There was no adverse reaction. He proclaimed he had a cure. Trains in Europe were overbooked as TB patients flocked to Berlin. In a trial of 1769 patients the therapy was found to be ineffective, and in fact *killed* some patients! In 1925 Mollgaard introduced gold based sanocrysin, but this too was toxic. The first real success was p-amino salicylic acid (PAS), developed by Lehman in 1943. However, the most dramatic success came a few months later from streptomycin, developed by Waksman and Schatz in 1943. Unfortunately, drug resistance effectively terminated the effectiveness of streptomycin monotherapy. Further efforts by Domagk led to thiacetazone and isoniazid in 1946 and 1952, pyrazinamide by Kushner et al in the late 1940s, and rifampin in the 1960s[8-10]. These are our current standard drugs.

Mycobacterium tuberculosis is a successful pathogen.

M. tuberculosis are slightly curved non-motile rods that are 2-5 μm long and 0.2-0.5 μm wide. *M. tuberculosis* multiplies rapidly in ambient air, is much more slowly under acidic conditions, and does not replicate under anaerobic conditions. This bacillus has one of the most formidable cell walls in nature, of which >60% is composed of lipids. These lipids are mainly mycolic acids which are composed of 2-branched, 3-hydroxy fatty acids with 76-90 carbons. This protects the bacteria from many immune generated insults such as complement, oxygen radicals, cationic proteins, and lysosomal enzymes.

M. tuberculosis is in fact not one species, but a complex of species that share 99.9% similarity at nucleotide level. The complex comprises of *M. tuberculosis [typus humanus]*, *M. canettii*, *M. africanum*, *M. bovis*, and *M. microti*. *M. tuberculosis, M. canettii* and *M. africanum* exclusively infect humans while *M. bovis* has a wide host spectrum, but *M. microti* is restricted to rodentia. In evolutionary terms, the ancestor *M. tuberculosis* (which is still around) developed into the new *M. tuberculosis* and *M. canettii*[1]. *M. africanum* split from the *M. tuberculosis*, after which *M. bovis* and *M. microti* split from *M. africanum*[1]. It is believed that the *M. tuberculosis complex* (MTC) spread out of Africa when humans left the cradle, and was actually re-introduced into the continent when the current Africans back migrated from Asia[11,12]. However, even the new *M. tuberculosis* has continued to evolve, and genotyping of recent clinical isolates from around the world has identified at least 62 clades/lineages, with the most common being Beijing, Beijing-like, Central Asian, East-African Indian, Haarlem, Manu, T, X, and Latin-American Mediterranean (LAM)[13]. These clades have different virulences and lead to different disease severities. In North America, the most common are T (~25%), X (~20%), Beijing (~15%), and LAM (~10%).

Host Genes Increase Susceptibility to Tuberculosis.

TB discriminates on grounds of gender. There is a 70% excess of male patients with TB compared to females. When Bellamy *et al* performed a genome wide scan in 81 sib pairs in the Gambia and South Africa, markers on chromosomes 15q and Xq showed linkage to the development of TB, so that an X chromosome susceptibility gene probably contributes to the excess of TB cases among males[14]. Another common motif is the linkage of TB with genes associated with iron metabolism. In the mouse, the natural resistance–associated macrophage protein 1 *(Nramp1)* is a transmembrane protein that transports divalent cations and exports iron out of bacteria harboring phagosome. The effect is to withhold Fe^{2+} from *M. tuberculosis*, which needs iron for intracellular replication. Patients in the Gambia were studied for the relationship between the development of TB and the human homologue of the mouse *nramp-1*, solute carrier family 11, member 1 (SLC11A1)[15]. Subjects who were heterozygous for two specific *SLC11* polymorphisms in intron 4 and the 3' untranslated region of the gene were four times more likely to develop TB compared with those with the most common SLC11A1 genotype[15]. Results have since been confirmed in the Danish, Korean Japanese, Guinea-Conakry, and Canadian populations[16-21]. These SLC11A1alleles are associated with

increased bacillary replication in macrophages. Moyo *et al* have demonstrated that genes associated with iron overload syndromes have an allelic frequency of 0.03-0.05 among the southern Bantu (my own people), who constitute 80% of people in southern African countries[22]. These genes enable people to retain more dietary iron than they need. Since men have no physiological mechanisms to get rid of the excess iron they develop iron overload syndromes typified by excess iron deposition in the reticulo-endothelial system[23]. The authors demonstrated that patients with this iron overload phenotype have a greater risk of developing TB, and are more likely to die than those without iron overload even when standard anti-TB treatment is administered[24]. Lounis *et al* examined the pharmacodynamic effects of iron overload in BALB/c mice with TB they demonstrated that there was shorter survival in mice with iron overloaded versus normal mice[25]. There was a dramatic reduction in the efficacy of standard anti-TB drugs in mice with iron overloaded versus normal mice[25]. A closely related study examined the role of haptoglobin, a protein important in the uptake and clearance of heme associated iron. In humans, haptoglobin has 3polymorphs: Hp 1-1, Hp 2-1, and Hp 2-2. When TB patients were followed for 18 months after start of standard anti-TB therapy, Hp 2-2 (Hp^1 and Hp^2 allele frequency 0.55 and 0.45, respectively) was associated with a 6 fold greater risk of death compared to Hp 1-1[26]. Finally, studies of patients in Sicily, Spain, and South Africa have shown an increased risk of TB in patients with genetic defects in the IL-12-IFN-γ signaling pathway[27].

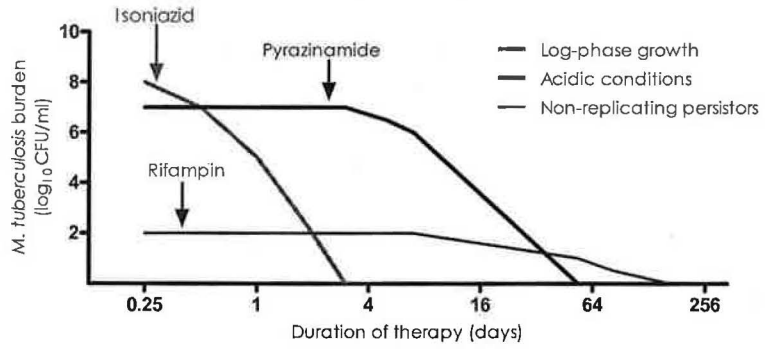
The Immune System and Re-activation Disease.

Infection is established after humans inhale 1-5 bacilli from a close source with cavitary TB. Curiously, even in sputum positive patients infectious aerosols usually come from a small specific group of patients. In one study, only 4% of TB patients contributed 75% of all infection, and in another only 13% of patients infected the entire 100% of secondary cases[28,29]. Size distributions suggest that most of the viable particles in these cough-generated aerosols are immediately respirable. The inhaled bacilli infect alveolar macrophages, which then carry the bacilli to lymph nodes. There the bacilli undergo logarithmic phase (log-phase) growth, and then undergo hematogenous dissemination to the whole body. Starting 3-6 weeks post infection, there is sequential recruitment of NK cells, δγ T lymphocytes, CD4 lymphocytes and CD8 lymphocytes. The Th1 cytokine pattern (IL-12, γ-IFN, TNF-α, IL-18, etc) eventually helps brings the infection under "control" and healing occurs leaving behind Ghon complex-calcified granulomata with dormant *M. tuberculosis.* However, in about 10% of infected people the immune system eventually wanes and they develop re-activation TB, which is the most common clinical form of TB. The most common reasons include old age, diabetes mellitus, chronic renal failure, HIV-infection, malignancy, immunosuppressive therapies and malnutrition. The patients usually present with cavitary and non-cavitary pneumonia, pleural TB, TB meningitis, and TB of the vertebrae.

The basis of modern chemotherapy: Mitchison's three population hypothesis.

In the 1970s Mitchison hypothesized that in patients with chronic pulmonary TB, *M. tuberculosis* exists as three dynamic metabolic populations[30].This "three population

model hypothesis" forms the basis of current anti-TB therapy[31,32]. In fact the first evidence on the existence of different bacillary populations that had different drug susceptibilities was actually first presented in the 1950s by Canetti, McDermott and others[33-35]. Population A is made of log-phase growth bacilli that are inside aerobic pulmonary cavities, and is the largest population in cavitary TB[30]. Isoniazid (at 300 mg a day) is the primary drug active against this population, and has the highest early bactericidal activity (EBA), which means that it is associated with the fastest and greatest microbial kill during the first two days of therapy. After these two days of isoniazid, it is believed that this large population gets wiped out and EBA ceases. Population B is made of slowly multiplying bacilli that are under acidic conditions, and is the second largest population of bacilli. Mitchison initially considered this population to be within macrophages, but it is now generally considered to be bacilli at the edges of necrotic cavities. Pyrazinamide (at 25 mg/kg) is the most active drug against population B, and eliminates this population in about two months. The end of two months of therapy marks the end of the first phase of anti-TB therapy, at which time >80% of TB patients have already undergone sputum conversion. However, there is still a residual population. Population C is comprised of bacilli that are quiescent but have brief episodes of sporadic multiplication. Population C is generally believed to be in low oxygen tension areas such as inspissated cavities, the center of necrotic cavities and granulomata[36,37]. Rifampin (at 600 mg) is rapidly bactericidal, and is active against this population during the short spontaneous bursts of metabolism. Intermittent isoniazid is concurrently administered with the rifampin against this population for the next four months to prevent resistance development. Just in case of isoniazid resistance either ethambutol or streptomycin is added during early therapy, until absence of drug resistance is demonstrated.



DOTS in trials: DOTS on trial.

Anti-TB therapy is administered via directly observed therapy, short course (DOTS). DOTS is administered in special clinics (e.g., County Health Departments, community health units etc). The idea is to increase adherence to therapy by watching patients take their medications in the correct doses. The primary study to support effectiveness of DOTS came from a study by Weis *et al* in Tarrant County[38]. They collected data on 407 episodes of TB between 1980 and 1986 when no DOTS was administered, and compared them to 581 episodes of TB between 1986 and 1992 when DOTS was implemented in Fort Worth. The rates of primary drug resistance decreased from 13.0% to 6.7 %, acquired resistance declined from 14.0% to 2.1%, and the relapse rate decreased from 20.9% to 5.5%, after the implementation of DOT compared to prior ($P<0.001$ for all

comparisons)[38]. Several other *observational* studies have since confirmed this. However, starting with a randomized controlled study by Walley *et al* in 2001, researchers began to question the utility of supervised administration of anti-TB drugs[39]. In October 2007, Volmink and Garner published a meta-analysis of 11 randomized clinical trials that compared self-administration of anti-TB treatment to family member supervised treatment versus standard DOTS[40]. The studies were performed in low income, middle income and high income countries, and 5609 participants met the inclusion criteria. Cure rates of DOTS versus self administration were similar (RR 1.02, 95% CI 0.86 to 1.21, random-effects model; 1603 participants, 4 trials), with similar results for cure plus completion of therapy. Cure rates for DOTS provided at home were slightly better than those for DOTS provided at clinic (RR 1.10, 95% CI 1.02 to 1.18; 1365 participants, 3 trials). For intravenous drugs users, there was no statistically significant difference between DOTS and self administration (199 participants, 1 trial), even though the DOTS arm had the extra incentive that the IV drug abuser would get $5 at each clinic visit! There was no significant difference detected in clinical outcomes between DOTS at a clinic versus by a family member or community health worker (2 trials), or for DOTS provided by a family member versus a community health worker (1326 participants, 1 trial). Another randomized study compared the costs of DOTS to self-administration in Pakistan, and demonstrated substantially higher costs per case cured with DOTS versus self administration ($310 versus $164)[41]. The long term outcomes once patients "complete" DOTS have not been studied until recently. Cox *et al* followed 213 sputum positive patients in Uzbekistan treated with first line anti-TB drugs for susceptible TB and second line drugs for MDR-TB[42]. The patients were followed for a median of 22 months from diagnosis. At the end of DOTS, 75% of new cases and 46% of previously treated cases were "cured." However, 24% of the patients were dead by the last time of follow up (rate of 15% per year), with 96% of deaths being due to the TB. Among all patients successfully treated and "cured," 36% had recurrence of TB, and many subsequently died. One and half years post-treatment, only 65% of successfully treated TB patients were still alive without recurrence of TB[42]. Thus, in the long term, 4 in 10 patients treated under DOTS have poor outcomes. In my opinion, the fundamental problem is with the pharmacologic basis of modern anti-TB chemotherapy, and will not be solved by such programs as DOTS.

Challenges to Mitchison's three population model hypothesis.

Challenging the hypothesis-population A. According to Mitchison's three population hypothesis, there are three metabolic populations of *M. tuberculosis*, with the largest being organisms in log-phase growth under ambient air. This hypothesis was cemented by Jindani *et al's* pivotal study in Nairobi, in which 27 regimens of (a) monotherapy, (b) dual therapy, and (c) triple therapy of isoniazid, rifampin, pyrazinamide, streptomycin and ethambutol for 2 weeks were administered to TB patients[43]. The study demonstrated that the rapid decrease in sputum bacillary load in patients in the first two days was due to the effect of isoniazid and all other drugs such as rifampin had only small effects during that time. It was deduced that cessation of isoniazid's early bactericidal activity (EBA) during the initial phase of anti-TB therapy was due to depletion of *M. tuberculosis* in log-phase growth, after which there was slower rates of kill which were mediated by

rifampin and pyrazinamide. This index of microbial kill was developed into the clinical index of EBA, which is the rate of decrease in sputum bacillary burden during the first 2-5 days of therapy. The EBA is the first test utilized to assess the clinical efficacy of new anti-TB drugs. Gumbo *et al* examined the veracity of this cornerstone belief [44]. They utilized an *in vitro* infection model in which *M. tuberculosis* was grown in log-phase in ambient air under constant supply of nutrients and constant removal of waste products. *M. tuberculosis* was exposed to isoniazid concentration-time profiles encountered in patients. Experiments were performed to examine the time-related changes in total bacterial population, isoniazid-susceptible subpopulation, and isoniazid-resistant subpopulation. Cessation of microbial kill occurred between day 3 and 4 of isoniazid therapy, as occurs in patients. There were multiple Logs of log-phase growth organisms remaining at the time bactericidal activity ceased. Mathematical modeling, backed by experimental observations revealed that instead the isoniazid-susceptible subpopulation was replaced by an isoniazid-resistant subpopulation after 80 hours of therapy. The size of the isoniazid-susceptible subpopulation continued to decrease after the total population had ceased to decline, while the resistant subpopulation remained in log-phase growth. Resistance was due to single point mutations in the *katG* gene and to reserpine-inhibitable efflux pumps. This means that there is no need to invoke multiple metabolic populations of *M. tuberculosis* with different isoniazid susceptibilities. The findings lead to three important therapeutic predictions. First, the EBA of each compound will vary depending on how quickly resistance to the compound emerges, and will not just be two days since the size of the bacillary population in log-phase growth will not be a mathematical limitation. Second, EBA will depend on the actual dose used since emergence of resistance is dose dependent. Thus, drugs such as rifampin, deemed to have minimal EBA may actually have high EBAs at higher doses. Third, the suppression or delay of resistance emergence would lead to dramatically rapid sputum conversion.

Mitchison et al's response. Mitchison *et al* counter-argued that numerous EBA clinical studies had failed to demonstrate emergence of isoniazid resistance[45,46]. They argued that instead, "the model lacked realism" because it uses pure log-phase growth organisms, and that the rate of microbial kill in our model was 0.7 $\log_{10}$ cfu/mL/day with 300 mg/day isoniazid, while that in patients was "only" 0.6 $\log_{10}$ cfu/mL/day! They therefore argued that cessation of isoniazid effect should still be attributed to non-replicating persistors and bacilli under acidic conditions. Gumbo *et al*'s rebuttal can be read in full in reference[47].

Siddiqi et al's challenge to Mitchison's hypothesis. Around the same time, Siddiqi *et al* exposed non-replicating *M. tuberculosis* to static concentrations of isoniazid[48]. They used bacillary loads of 10^4 CFU, well below the inverse of the mutation frequency to isoniazid, so that there was no pre-existent isoniazid resistant population. Nevertheless, in these non-replicating bacilli, resistance emerged within 5 days. This not only lends support to Gumbo et al's findings, but effectively decimates the counter argument of Mitchison *et al*, since even the non-replicating persistors themselves develop resistance within the same time frame as log-phase growth cultures.

Rifampin kills log-phase growth bacilli at the same rate as isoniazid. Gumbo *et al* used the same *in vitro* experimental system utilized for isoniazid to study rifampin and

demonstrated that rifampin could achieve the same microbial kill rate as isoniazid, and that the effect of rifampin was peak concentration dependent[49]. Resistance emergence took longer to develop. This predicted that for rifampin, and indeed other rifamycins, high doses would achieve excellent EBA, despite the stipulations of the three population model hypothesis[49]. In fact, since a substantial rifampin-resistant bacillary population takes longer to develop compared to isoniazid therapy, the EBA of rifampin monotherapy was be predicted to be beyond 7 days. This laboratory studies received striking confirmation from Diacon *et al*'s clinical study of patients treated with 1200 mg/day of rifampin versus 600 mg/day for 5 days in Stellenbosch, South Africa[50]. In that study, rifampin's EBA was longer than 2 days (lasted for whole study), and the rate of sputum bacillary decline for the 1200 mg/day was greater than that for 600 mg/day, and was equivalent to that of isoniazid. It is important to remember that non-replicating bacillary population does not show up in sputum cultures. Our inference of this study is that, if the rifampin kills log-phase bacilli at the same rate as isoniazid, but kills for a longer time, then one would get into the logical absurdity of the same log-phase growth bacillary population having two radically different sizes at the same time. In fact, the same conclusions can be reached for moxifloxacin, based on hollow fiber studies and clinical studies by Johnson et al in Uganda[51,52].

...and Pyrazinamide kills non-replicating persistors.

It has been demonstrated by Zhnag et al that pyrazinamide is converted by *M. tuberculosis* nicotinamidase to pyrazinoic acid (HPOA) [53,54] . HPOA diffuses across the bacillary cell membrane to achieve an equilibrium. HPOA is a weak acid (pKa=2.9) ionizes according to the Handerson-Hasselbach relationship:

$$pH=pKa + \log(POA^-/HPOA).$$

Therefore, when the extracellular pH decreases more POA^- is formed, decreasing extracellular HPOA. This leads to the uncharged HPOA getting trapped inside *M. tuberculosis*. The HPOA contributes intracellular protons and disrupts the proton motive force across the bacterial cell membrane. Wade and Zhang recently demonstrated that non-replicating bacilli that are under anaerobic conditions are efficiently killed by pyrazinamide 2 $\log_{10}$ CFU) in a comparable way to rifampin (3.5 $\log_{10}$ CFU/ml) versus no effect for isoniazid (0 $\log_{10}$ CFU/ml) over 5 days of exposure[55]. The bio-electric basis is straightforward. *M. tuberculosis* is an obligate aerobe so that under anaerobic conditions very little energy is produced by the electron transport chain. The disruptive effect of HPOA on the proton motive force would thus be enhanced under anaerobic conditions. We know from our own preliminary data that pyrazinamide kills log-phase growth *M. tuberculosis* provided an efflux pump blocker is administered, while rifampin, a zwitterionic molecule, kills bacilli effectively at low as well as at neutral pH. Contrary to special selective susceptibility profiles in Mitchison's hypothesis, both rifampin and pyrazinamide have no real selective activity against particular metabolic populations.

The Global Emergence of Resistance Despite DOTS.

Perhaps the biggest failure of modern anti-TB therapy as well as DOTS is the global explosion of multidrug-resistance TB (MDR-TB). MDR-TB is defined as concurrent resistance to isoniazid and rifampin. For example, recently in Mumbai (India), 80% of patients presenting for therapy were infected with an *M. tuberculosis* strain resistant to at least one drug, while 51% had MDR-TB[56]. In a recent global survey by the WHO, the prevalence of MDR-TB was up to 14% of new cases in the Russian Federation, and in countries such as Oman and Kazakhstan the prevalence of MDR-TB in previously treated patients was 60% [57]. MDR-TB exists in all countries on earth. Treatment consists of at least three drugs that the particular isolate is still susceptible to. Common second line drugs include fluoroquinolones [moxifloxacin, levofloxacin, gatifloxacin], PAS, cycloserine, and aminoglycosides [streptomycin, amikacin, kanamycin, capreomycin]. Recently, Chan and colleagues studied 205 patients treated for MDR-TB in Denver[58]. Patients received a median of 6 drugs for 15-18 months (including parenteral aminoglycosides for 3-6 months), which led to a response in 56-75% of patients. One hundred and thirty of the patients underwent surgical resection of the infected lung. The determinants of successful outcomes were fluoroquinolone therapy (OR=3.1, p=0.01) and surgical resection (OR=4.6, p=0.08), while the odds ratio of success with both fluoroquinolone and surgical therapy was 12.7 (p=0.02). In general failure of therapy in patients with MDR-TB occurs in up to 50% of patients[59,60]. MDR-TB increases treatment costs by a factor beyond ten-fold, and TB control programs may spend up to 30% of their budgets on the MDR-TB if only 3% of total TB cases are MDR-TB[61]. The DOTS-plus strategy employed for treatment of MDR-TB means that patients are exposed to toxic and less effective second line drugs for up to 24 months, even though the benefit of this strategy is controversial[62]. To make matters worse pyrazinamide's unique role is now threatened by emergence of resistance in such diverse regions of the world as Canada, South Africa, and Lithuania[63-65].

The standard pharmacological approach to resistance suppression has been based on the thought that the baseline mutation rates to certain critical concentrations of drugs are known, and utilizing these, the minimum likelihood of resistance emerging can be predicted by playing the games of chance. The probability *(P)* of mutants emerging to any one anti-TB drug is described by the equation:

$$P=1-(1-r)^n$$

where *r* is the mutation rate and *n* is the total population of bacilli present in a lesion[66,67]. The mutation rates to first line anti-TB drugs are between 10^{-7} and10^{-10}, so that resistance to any one of the current first-line anti-TB drugs is expected to pre-exist in many patients with cavitary TB who have ~10^9 CFU of bacilli in a 3-cm pulmonary cavity. The current strategy, which grew out of the clinical trials that demonstrated the superiority of streptomycin and PAS combination to streptomycin monotherapy[68], is to use combination chemotherapy. The explanation is that the likelihood that *M. tuberculosis* would develop chromosomal mutations to ≥2 different drugs is the product of two mutation rates (~10^{-17}), which makes probability of emergence to ≥2 drugs vanishingly

small. However, despite the almost universal application of multi-drug therapy and DOTS, resistance prevalence *has* exploded. While it is fashionable to blame the patients ("non-compliance"), in our opinion, there are several biological reasons for the failure. These reasons can be understood by considering the role of (a) mutator alleles and adaptive evolution, (b) induction of efflux pumps and adaptive evolution, and (c) pharmacokinetic-pharmacodynamics (PK-PD) as well as pharmacogenomics.

Mutator alleles and adaptive evolution to the human immune system.

M. tuberculosis, like all other organisms, has a narrow baseline rate of chromosomal mutations. This makes sense since too high a mutation rate would lead to the collapse of the genetic information, while too low a rate would not allow fast enough adaptation and survival in response to a changing environment. Mutation rates are kept in check by specific enzyme systems that repair damage to the microbial DNA. There are more than 50 *M. tuberculosis* genes associated with DNA repair, and mutations in any one of these may lead to hyper-mutable strains[69]. For example, the human immune cells release various reactive chemical species targeted at intracellular bacilli. This can result in oxidized guanine (8-oxo-G), which eventually leads to G→T transversions. Mutator (Mut) T (1 to 4) is a bacillary enzyme family that hydrolyzes both 8-oxo-dGTP and 8-oxo-rGTP, and prevents transversions, and thus has anti-mutator effect. It has been demonstrated in the laboratory, for example, that deficiencies of the MutT1 component in *M. tuberculosis* results in a 16-fold increase in spontaneous mutation frequency that leads to increased rifampin resistance, Similarly there was a ~50 times increase in mutations leading to rifampin resistance in MutT4-deficient *M. smegmatis*[70]. The gene *ogt* encodes a methyltransferase that repairs GC to AT transitions and protects against alkylation damage, while *ung* encodes for uracil N-glycosylase that repairs CG to TA transitions and is involved in base excision repair[71,72]. *M. tuberculosis* mutants in which two DNA repair genes *ada/alkA* and *ogt* involved in alkylation damage repair are inactivated have been made in the laboratory[71]. Inactivating the two genes increased mutation frequencies to rifampin 100 fold in the laboratory [71]. In general, mutations in the *mut, ogt*, and similar such genes will lead to higher mutation rates to standard anti-TB drugs, all at the same time! In other words, chances of resistance emergence to two drugs if clinical isolates had these mutations would be real!

The *M. tuberculosis* genotype that comprises 86% of all *M. tuberculosis* isolates from China, and ~40% from Mongolia, South Korea and Thailand is the Beijing strain[73]. The Beijing strain has been demonstrated on every continent, and comprises 20% of strains from Moscow, and 15% of strains from South Africa for example[73]. In Texas, the Beijing strain constitutes 25% of all cases in Houston, Texas[74]. The Beijing strain has been associated with MDR-TB outbreaks, in New York City, in the USA, and throughout the world[75]. Recently Rad *et al* examined *mut* gene variations in 139 MTC strains (*M tuberculosis, M. africanum*, and *M. bovis*) representative of those from 35 different countries[76]. Fifty five strains had the Beijing genotype, and 78% had mutations in *mut* genes and *ogt* genes. Twelve of the 55 strains had the MDR-TB phenotype, and 9 of them had the missense mutations in *mut* genes. Thus, the association of resistance emergence to the Beijing strain can be explained in terms of second-order selection of hypermutable

(mutator) alleles based on alterations in DNA repair genes. On the other hand, the most common *M. tuberculosis* genotype in the world, with the exception of the Far East, is the "T' subtype. When Nouvel *et al* examined for mutations in 55 MDR and 194 non-MDR clinical isolates of *M. tuberculosis* from the Central African Republic, which were mostly of the "T" genotype, *ada/alkA-ogt* mutations were observed in 14 MDR strains and 138 non-MDR strains [77]. In another study of clinical isolates from Argentina and Colombia, which were mostly of the Haarlem genotype, *ogt* and *ung* mutations were demonstrated to be common. It is likely that more mutator phenotypes will be encountered in clinical isolates of different genotypes. Thus, hyper-mutable strains of *M. tuberculosis* are common across the globe, and any therapy regimens that rely on low mutation rates to combination therapy is doomed sooner or later to lead to outbreaks of drug resistance. These mutations are in fact not difficult to explain, they have been demonstrated to offer *M. tuberculosis* adaptive advantages inside the macrophages.

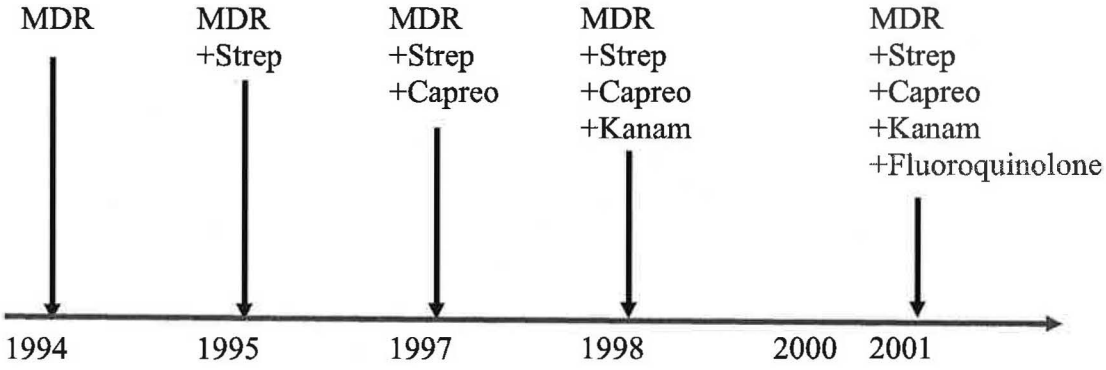
The role of efflux pumps in adapting to chemical environments.

A full 5% of the *M. tuberculosis* genome encodes for efflux pumps and other transporters. We have already alluded to the fact that the reason for the cessation of isoniazid EBA in experiments was resistance emergence due to efflux pumps as well as chromosomal mutations[44,78]. Indeed, a three-gene operon, *iniABC,* induced in *M. tuberculosis* by isoniazid, ethionamide, and ethambutol was recently described by Alland *et al* [79,80]. This operon encodes for part of a reserpine-inhibitable MDR-pump associated with both isoniazid and ethambutol resistance, essentially reducing the effectiveness of two of the four first line agents in one step. In the case of rifampin, Gumbo *et al* recently examined the relationship between rifampin concentration and its entry into the *M. tuberculosis* cytoplasm[49]. Higher extracellular rifampin concentrations led to higher intracellular concentrations, consistent with a saturable rifampin-efflux pump. Piddock *et al* have demonstrated that rifampin accumulation in the bacilli increases in the presence of reserpine, consistent with a low-level efflux pump[81]. The efflux pump protein is encoded by Rv1258c, is the same pump for streptomycin, ethambutol, and orfloxacin[82]. Thus, the same efflux pumps for rifampin are also associated with resistance to other first line and second line anti-TB agents. Other fascinating findings have come from the work of Zhang *et al* who studied *M. tuberculosis* under acidic conditions and demonstrated that *M. tuberculosis* extrudes HPOA from the cytoplasm via an efflux pump[53]. This pump can be inhibited by several efflux pump blockers. Thus, we know that for all first line drugs *M. tuberculosis* develops pumps to reduce the effect of anti-TB drugs. This gives the bacilli an adaptive advantage to grow in the face of these chemical insults and time to adapt via mechanisms such as chromosomal mutations.

The flight of (adaptive) evolution's arrow: The emergence of XDR-TB.

> The hour of fate
> Has come! Let's lift our eyes
> To view new skies!
> The valley of the Hills!
>
> H. Dhlomo's "The valley of a thousand hills (1941).

MDR-TB eventually evolved under antibiotic pressure to become resistant to the second line agents that are used to treat it. The wake up call to this was in Tugela Ferry, in KwaZulu, right there in the famous "Valley of a Thousand Hills." In this valley were Zulu warriors won many a battle, a new interspecies battle was declared in 2005. Physicians noticed that patients were dying rapidly from an unusual type of TB. Fifty three HIV-infected patients were demonstrated to have developed MDR-TB as well as resistance to all second line drugs, including quinolones. This was later termed extensively drug resistant TB (XDR-TB) which was defined as MDR-TB PLUS resistance to fluoroquinolones and at least one of three injectable drugs (i.e., amikacin, kanamycin, or capreomycin). Ninety eight percent of the patients died an average of two weeks after the first positive sputum. Spoligotyping revealed that 85% of the isolates were the LAM KwaZulu Natal sub-strain (LAM/KZN), while 15% were the Beijing strain. Pillay and Sturm have examined isolates from KwaZulu for the decade prior, and their research revealed a rather fascinating evolution picture, depicted below[83].



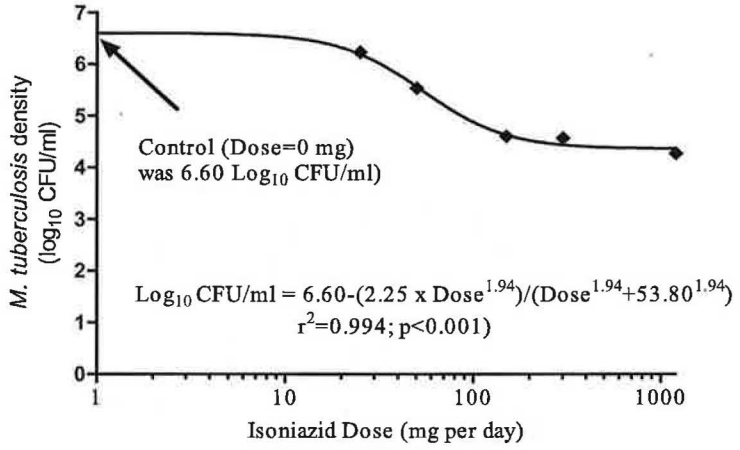
Essentially XDR-TB emerged in this particular locality in two different subspecies at the same time. It turned out that this was not just happening in KwaZulu in isolation, but concurrently in the rest of the world so that the events at Tugela Ferry merely alerted us to the global problem in a dramatic manner. The CDC and the WHO recently surveyed national laboratories on 6 continents and found that one average there 20% of all TB cases worldwide are MDR-TB while 2% are XDR-TB. As a general rule, XDR-TB constitutes 10% of MDR-TB cases. While XDR-TB was ~4% of MDR-TB cases in the USA, it was ~20% in Latvia and 15% in South Korea. On average XDR-TB constitute 1% of all MDR-TB in Africa and the Middle East. Another study has shown that perhaps the proportion of MDR-TB in the world is about 4%, with 60% of all cases coming from China, India, and the Russian Federation. XDR-TB has been documented in Texas.

Most XDR-TB is actually encountered in non-HIV infected patients. Recently, Kim et al examined the impact of XDR-TB in non-HIV infected patients[84]. They reviewed charts of 211 patients with MDR-TB in South Korea, and found that 20% of these were XDR-TB[84]. The XDR-TB patients presented with more extensive pulmonary cavitation compared to those with MDR-TB. The median duration of therapy was 43 months (close to 4 years!) for XDR-TB compared to 25 months for MDR-TB. Fifty six percent of

patients with XDR-TB received surgical therapy. As expected, the risk of therapy failure was 5 times compared to those with MDR-TB[84]. The excess mortality in XDR-TB compared to MDR-TB has been documented by the CDC.

Quo Vadimus: PK-PD and Pharmacogenomics as a Rational Approach to Therapy.

PK-PD science was pioneered by Harry Eagle and William Craig. The first important PK-PD lesson concerns the relationship between antimicrobial exposure and microbial kill, which is best described by the inhibitory sigmoid E_{max} model. An example, from our work with isoniazid and *M. tuberculosis,* is shown in opposite figure[44]. Initially, there is little kill of the bacteria as the drug exposure is increased. The bacterial density at this stage differs little, if at all, from that in non-treated controls. However, an inflection point is reached after which small changes in exposure have a big effect on kill, characterized by the steep portion of the curve. Eventually, there is a second inflection point after which large changes in exposure have little effect on microbial kill. At these high exposures, microbial kill approaches maximum (E_{max}). A central PK-PD concept, which allows translation of pre-clinical data to the clinic, is that bacteria exposed to the same drug exposure in the pre-clinical model will (in general) have the same dose-response relationship in humans, provided differences in protein binding and the immune system are taken into account. Therefore, we can use the inhibitory sigmoid E_{max} relationships derived in these pre-clinical models as well as exposures demonstrated to have optimal suppression of drug-resistance, to design human dosage regimens that optimize microbial kill and resistance suppression.

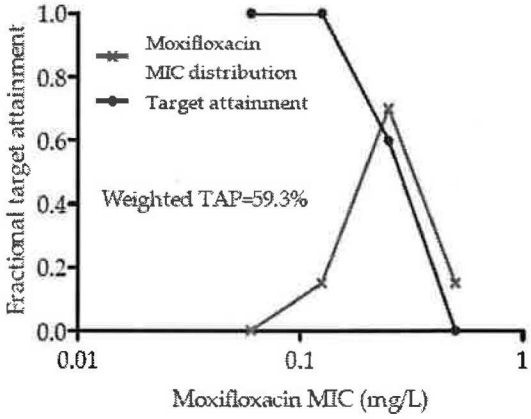


A second central concept is of dose scheduling. For some antibiotics, microbial effect is best achieved if higher peak concentrations (C_{max}) are achieved, so that the index that best explains effect is the C_{max}/MIC ratio. An example from Gumbo *et al* is that of rifampin resistance suppression and post antibiotic effects in *M. tuberculosis*, both of which are C_{max}/MIC linked[49]. This is best achieved by combining doses over several dosing intervals and administering them less frequently. The concentration dependent nature has since been confirmed in patients[50]. On the opposite end are antibacterial agents for which dividing the dose for each dosing interval and administering it more frequently leads to better microbial effect, due to maximization of time that drug concentration is above the MIC (T_{MIC}). The β-lactam antibiotics are an example of T_{MIC} linked drugs. For a third group of antibacterials, as long as a similar cumulative dose is administered, dosing schedule has little effect on microbial effect. Since it is the area under the concentration time curve (AUC) that stays constant during the dosing interval with different dosing

schedules, such antibiotics are AUC/MIC linked. An example is that of others is isoniazid microbial kill and resistance emergence[78,85,86].

Humans have been exposed to many chemicals as they have evolved, particularly chemicals from plants, and have developed enzyme systems for their detoxification[87]. Variability in the genes that control these enzymes has given humans a survival advantage. When we introduce foreign chemicals such as anti-TB compounds to the human body, the same variability is manifest, so that when a fixed dose of a drug is given to group of patients, each patients achieves different concentrations from the other. If the same drug dose is given to the same patients the following day, the patients may achieve different concentrations the following day. When drug concentrations are measured, many people often report these as measures of central tendency and dispersion (e.g. median/mean and SD) for each time point. The technical name for such an approach is the naïve pooled data approach. This is a highly problematic approach. To begin with, the antibiotic concentrations are often not normally, or even log-normally, distributed. The summary measures tell us about the variance observed under the explicit assumption that there is little or no between-subject variability. In estimation of any pharmacokinetic values, it must be recognized that there are several sources of variability and error, including inter-individual variability, intra-individual variability, and "process noise" such as errors in measurement of time to administer the drug to the patient, errors in time to sampling the patient, errors in measuring the quantity of the sample, assay error, and noise (error) in the system introduced by computation methods. This variability and error must be recognized and quantified using non-parametric methods, if dosing predictions are to be accurate. The existence of true-between-subject variability means that a fixed dose of drug will result in a large distribution of drug exposure when that fixed dose is administered to a large population. This means that some patients will respond better than others based on the amount of exposure and some patients will have a higher likelihood of having a concentration-driven toxicity.

Monte Carlo simulations are a mathematical tool that allows use of population pharmacokinetic information to determine the probability that a particular PK-PD exposure value will be achieved by a *specific dose* of the antimicrobial agent administered to patients. Gumbo *et al* have introduced a modification to take into account



Dose (mg/day)	Probability Target attainment
400	59%
600	87%
800	93%

as the distribution of SNPs associated with clinically meaningful pharmacokinetic variability[78]. These pharmacokinetic data, including the covariance matrix, as well as the distribution of *M. tuberculosis* MICs encountered in patients in a particular locale are then examined in the simulations to determine how likely several thousands patients (e.g., 10,000) chosen randomly will achieve the particular PK-PD exposure associated with optimal microbial kill or suppression of resistance in the pre-clinical models such as hollow fibers and animal models. This allows us to chose and optimal dose, and an optimal duration of therapy, prior to conduct of clinical trails[51,78,88]. Using these methods, we have designed new doses and dosing regimens for isoniazid, rifampin and moxifloxacin, published already in the literature. In the last few months we have performed studies with pyrazinamide (as yet unpublished, except in abstract form)[89]. A completely new treatment regimen based on PK-PD and pharmacogenomics, with the ability to completely suppress resistance emergence and reduce duration of anti-TB therapy, has now been designed. This and other pharmacological additions such efflux pump blockers allow us the possibility of very short durations of anti-TB therapy, hopefully weeks rather than months. At that stage, we can then realistically expect patients to be more compliant and resistance emergence to be reduced.

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