**MUTATION-INDUCED RESISTANCE: A MAJOR CHALLENGE OF TUBERCULOSIS TREATMENT SHORT-COURSE**

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**ABSTRACT**

Tuberculosis has remained a global health challenge for centuries and is currently the leading cause of death from a single infectious agent. According to WHO report in 2016, the prevalence rate was about 8.6 million with 1.3 million deaths in 2012. The causative organism, *Mycobacterium tuberculosis* (and sometimes other species of *Mycobacteria*) is well adapted in human host to cause active and latent tuberculosis in two-third of world population. The intrinsic attributes of the pathogen enable it to counteract host’s defensive mechanisms, causing diseases and perpetuating infection within human population. The use of various anti-tubercular chemotherapies has met with stiff antibiotic resistance due mainly to mutations in the coding and regulatory regions of the gene responsible for sites of action of the drugs. This, together with patients’ non compliance to the prolonged treatment regimen has, given rise to multi-drug, extended-drug and recently totally-drug resistant strains of the organism. Although nitroimidazoles have been used in the treatment of anaerobic infections, including latent tuberculosis, low redox potential derivatives have proven to be effective against both anaerobic and aerobic phase of the organism. The introduction of this class of drug did not only raise hope of effective treatment but reduction in treatment duration. The activation of the prodrugs is an intrinsic ability of Mycobacteria through the action of Deazaflavin (F420)-dependent nitroreductase (Ddn). The enzyme co-factor (F­420) is responsible for the organism’s emergence from hypoxia. Both drug activation and respiratory activities of the Ddn/F420 ­system is encoded in the same gene. Mutation-induced resistance to these drugs is expected to be lethal to the organism itself due to these coupled activities. However, experimental mutations on synthetic Ddn orthologs revealed loss of drug-activation ability without affecting the native respiratory activity of the co-factor. This, together with discovery of pretomanid-resistant strains without pre-exposure to the drugs has raised questions regarding the possibility of emergence of resistant strains and its fitness cost to the organism.

Key words: Tuberculosis, Treatment regimen, Antibiotic resistance.

**Introduction**

Tuberculosis has remained one of the most global health challenges for centuries and is currently the leading cause of death globally from a single infectious disease (WHO 2016). According to reports, about 8.6 million cases were recorded in 2012, resulting in about 1.3 million deaths. More than 500,000 cases of this incidence occurred among children. The organism is now adjudged one of the most successful pathogenic bacteria having adapted successfully within its only reservoir, the human subject for about 70,000 years (Corbett et al, 2003). A total of about two billion people worldwide (about one third of world population) is said to have active or latent tuberculosis. This fit is due to the pathogen’s ability to resist host’s immune pressure using reactive radicals during disease prognosis and emergence of resistance to most antibiotics developed against it (Russell, 2007; Nathan, 2012; Nguyen and Pieters 2009).

The development of resistance to most antibiotics by *Mycobacteria* remains the most worrisome aspect of the disease management. Even more worrisome is the emergence of certain strain that totally resist all known antibiotics used against them. This classifies the organism into multi-drug resistant (MDR), extensive drug resistant (XDR) and totally drug resistant (TDR) according to their spectrum of resistance to the drugs.

**Pathogenesis of Tuberculosis**

Tuberculosis is caused primarily by *Mycobacterium tuberculosis (Mtb)* but can also result from the infection of other Mycobacterial species collectively called *M. tuberculosis* complex (MBTC). *M. tuberculosis* is spread from active TB patients to healthy individuals through aerosol during coughing, sneezing or spiting. Infection in a new host occurs when the aerosolized droplets containing the pathogen are drawn into the lungs and engulfed by the alveolar macrophages, preventing phagosome maturation and fusion with lysozyme (Russell, 2001; Jordao *et al,* 2008). This may result in active TB, during oxidative stress, where the organism will be dividing rapidly, or latent TB infection during hypoxic stress, where the organism is viable but slowly dividing with minimal signs or symptoms (Getahun *et al,* 2015). In the case of latent TB, cell number gradually overbears the macrophages and a granulomatous inflammation occurs, recruiting more macrophages, ‘T’ and ‘B’ cells to form multinucleated human tubercular lesions (Tsai *et al*, 2006). The resulting complications such as fibrosis, brochostenosis, brochiectasis etc lead to significant lung impairment and breathing difficulty (Maguire *et al,* 2009; Jordan *et al,* 2010).

In the case of oxidative stress (OS), during active TB, cascade of oxidative processes are triggered, leading to the production of reactive oxygen species (ROS), which serve as antimicrobial defense against *Mtb* and other microbial cells. *M. tuberculosis* often overwhelms ROS through its anti-oxidant process and continues to multiply within the host (Ezraty *et al*, 2017; Van-Acker and Coenye, 2017; Jamaati *et al,* 2017).

**Treatment Background and Mechanism of Action of Antimycobacterial Agents**

Many drugs have been introduced at various points in an attempt to overcome the ever-twisting resistant mechanisms developed by tubercule organisms.

**Rifampicin:** This drug is effective against actively growing and dormant (or slow growing) bacilli. Since its introduction in 1972, it has been regarded as one of the most effective anti-TB and in combination with other drugs constitutes a multi-drug regimen against tuberculosis (Mitchison, 1979). Rifampicin activity is through its binding to β-subunit of RNA polymerase to inhibit messenger RNA enlongation (Blanchard, 1996). Most Rifampicin resistant clinical isolates emerge through mutations in the gene (*rpoB* gene) coding for the β-subunit of the polymerase thereby reducing affinity to the drug (Telenti *et al,* 1993).

**Isoniazid:** The use of Isoniazid as an anti-TB agent dates back to 1952. It is being used together with Rifampicin as a treatment regimen for the disease. It is effective against only actively replicating organisms. Upon its activation by the catalase/peroxidase enzyme *KatG*, isoniazid inhibits the mycolic acid synthesis through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by *inhA* (Zhang *et al*, 1992; Rawat *et al*, 2003). Resistance to the drug has been found to be associated with mutations at the two genes (*KatG* and *inhA*) relevant to its mechanism of action (Ramaswamy *et al*, 2003; Hazbon *et al,* 2006).

**Ethanbutol:** The use of Ethanbutol to treat TB started in 1966 as a first line drug. It acts through its interference with the biosynthesis of arabinogalactan in the cell wall, bringing a bacteriostatic effect on the multiplying bacilli. The genes *embCAB*, code for arabinosyl transferase, which is responsible for the synthesis of arabinogalactan, producing the accumulation of the intermediate D-arabinofuranosyl-P-decaprenol (Takayama *et al*, 1989; Mikusova *et al,* 1995). Resistance occur when there is mutation in the gene especially at *embCAB*306 position. This mutation also predisposes the organism to develop transmissible resistance to other drugs (Sreevatsan, 1997; Ahmad, 2007).

**Pyrazinamide:** This was introduced in 1950 and is part of first line anti-TB regimen. It inhibits semi-dormant bacilli found in TB lesions (Mitchison, 1985). Being an analogue of nicotinamide, it is converted ito its active form, pyrazinoic acid by the enzyme pyrazinamidase/nicotinamidase coded by *pncA* gene. The pyrazinoic acid inhibits membrane transport through disruption of membrane energetic. Pyrazinamide after conversion is excreted outside the cell by weak efflux pump. In an acidic environment, the protonated pyrazinoic acid is reabsorbed and accumulated inside the cell due to an inefficient efflux pump, resulting in cellular damage. It is also known to inhibit fatty acid synthesis in replicating *M. tuberculosis* cells (Scorpio et al, 1996; Zhang *et al*, 2003; Zimhony *et al,* 2007). A recent study had proposed an inhibition of a process of ribosome-sparing in *M. tuberculosis* by pyrazinoic acid (Shi *et al,* 2011)*.* Resistance to Pyrazinamide develops following mutations in *pcnA* gene. Occurrence of resistance without mutation in this gene has been reported, suggesting mutations in other yet to be known gene(s) (Cheng *et al,* 2000; Jureen *et al,* 2008).

**Streptomycin:** Streptomycin was the first antibiotics used against TB but became ineffective as soon as it is used due its monotherapeutic use. It acts against growing bacilli by inhibiting initiation of translation in protein synthesis at the level of 30S ribosomal subunit and 16S rRNA controlled respectively by the genes *rpsL* and *rrs* (Crofton *et al,* 1948; Finken *et al,* 1993). Mutations in these genes account for up to 70% resistance recorded (Gillespie, 2002).

**Fluoroquinolones:** This is a second line drug used against multidrug resistant TB (MDR-TB) eg Ciprofloxacin, ofloxacin etc. Some new generation quinolones are however being tried and proposed as firstline drugs (Goss *et al,* 1965; Palomino *et al,* 2013). They act by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV. Only topoisomerase II (coded by *gyrA* and *gyrB*) is present in Mtb and so, the only target of Fluoroquinolones (Aubry *et al,* 2004; Fabrega, 2009). Resistance occurs when there is mutation in the quinolone-resistance determining region of *gyrA* or *gyrB*. However, a hypersusceptility to several quinolones has been reported when there is simultaneous occurenece of mutation in *gyrA* (Aubry *et al*, 2006; Sun *et al,* 2008; Maruri *et al*, 2012).

**Ethionamide:** This is structurally similar to isoniazide. When activated by the enzyme monooxygenase (encoded in *ethA* gene), it interferes with synthesis of mycolic acid by inhibiting the activities of the enoyl-ACP reductase enzyme. The transcriptional repressor *ethR* regulates *ethA*. Resistance to ethionamide arises upon mutations in *etaA*/*ethA*, *ethR* and also mutations in *inhA*, which cause resistance to both isoniazid and ethionamide (DeBarber *et al,* 2000; Brossier *et al*, 2011; Carette *et al,* 2012).

**Para-Amino Salictlic Acid (PAS):** This is one the foremost anti-TB drugs but is currently considered as a second line drug, part of regimen for MDR-TB. Being an analogue of para-amino benzoic acid, it is believed to act by competing with dihydropteroate synthase and interfering with foliate synthesis. Resistance occur following mutations in *thyA* gene associated with resistant isolate to PAS and *folC* gene encoding dihydropteroate synthase (Rengarajan *et al,* 2004; Zhao *et al*, 2014).

A lot of other antibiotics have been variously applied in the formulation of anti-TB regimen. These include Cycloserine, Theoacetazone, Clarithromycin, Clofazimine, Linezolid, Kanamycin, Capreomycin, Amikacin, Viomycin. Their major mechanisms of specific resistance also anchor on mutations at various points of synthetic or regulatory genes. However, other intrinsic mechanisms of antibiotic resistance such as efflux pump, permeability barrier, target alteration, target mimicry, drug modification, among other mechanisms are still very relevant in the resistance to anti-TB drugs.

**Nitroimidazole for TB Treatment**

The emergence of multidrug resistant (MDR), extensive drug resistant (XDR) and recently, totally drug resistant strains of *M.* *tuberculosis* has challenged scientists to continue the search for drugs that will contain the stubborn strains. The contribution of gene mutation to the evolution of these resistant strains has lead to continuous search for new drugs to keep up with the new mutants. These mutations follow natural selection processes and have given birth to emergence of stable strains capable of withstanding the environmental conditions and subsequently spreading in human population.

Nitroimidazoles have been in use as anti-infective drugs against other bacterial and protozoal infections. It was later employed against anaerobic phase of *M. tuberculosis*. Some of its derivatives are currently advanced stage of clinical trial as promising anti-tubercular agents. The first drug in this group is Metronidazole (1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole), which is a derivative of azomycin-2 (known as 2-nitroimidazole), an extract from streptomycete 6670 and a potent drug against *Trichomonas vaginalis (*Roe, 1997; Roe, 1999)*.* Nitroimidazoles require bioreduction to yield active components that were found to be mostly effective against anaerobic organisms including hypoxic non-dividing *M. tuberculosis.* Derivatives with lower reduction potentials (5-nitroimidazole) can utilize the redox system of the organism without interfering in those of mammals, thereby specifically killing the target organisms. This is as against the higher reduction potential derivatives (2-nitroimidazole) which are within the reach of aerobic cells including those of mammals(Schmid and Schmid, 1999; Tathagata and Helena, 2011). Following the emergence of MDR and XDR strains of *Mtb*, a lot of structural modifications have taken place and are still on-going to combat the resistant mutants. Some new drugs in this class such as pretomanid, delamanid and various derivatives of 4-nitroimidazole are currently in various stages of clinical trials. They prove to be effective against both actively dividing and latent hypoxic *Mtb* cells. This review seeks to evaluate the potential and shortcomings of these drugs in reducing the spread of TB among human population.

**Bioreductive Activation of Nitroimidazoles**

Nitroimidazoles require activation through bioreduction mechanism requiring a low redox potential electron system in order to produce cidal activities. Microorganisms under limiting oxygen condition do not reduce Nitroimidazoles with redox potentials of the electron transport systems in the range of -0.42V and below. This is against those of aerobic cells (including mammalian cells) that reduce those with redox potentials ranging from -0.4V to -0.5V (Edwards, 1993; Mukherjee and Boshoff, 2011). This structure-activity relationship (SAR) necessitates the bioreductive activation to enable low redox potential drugs produce cidal effects on aerobic *Mtb.* The process of activation entails structural modification of the nitroimidazoles. A study of the analogues of 4- and 5-nitroimidazoles reveals the vital role of 2-position oxygen in the determination of aerobic and anaerobic activities of the drugs. While the aerobic activity in the 4-nitroimidazoles is determined by the bicyclic oxazine, the lipophilic tail, and the 2-position oxygen, the bicyclic oxazine and the lipophilic tail never endowed 5-nitroimidazole with aerobic activity except with the addition of 2-position oxygen (philo *et al,* 2009).

The mechanism of activation of the drugs was still unknown until a series of reports emerged. It was originally observed that an F420-dependent glucose-6-phosphate dehydrogenase (FGD1) was responsible for the sensitivity of the aerobic *Mtb* to bicyclic 4-nitroimidazoles, PA-824 and OPC-67683 (Stover *et al,* 2000). This was followed by a report of resistance in isolates that have lost the ability to biosynthesize the enzyme co-factor, deazaflavin (F420) and this lead to the identification of a conserved protein encoded by Rv3547. This protein is essential for susceptibility of the aerobic organisms to the drugs and is now known as Deazaflavin-dependent nitroreductase (Ddn) (Manjunatha *et al*, 2006). F420H2 is the active form of the co-factor, F420. Although the physiological role of Ddn is not known, the F420H­2 – dependent reduction of PA-824 yields three stable products of the imidazole, one of which is des-nitro compound that is further degraded to nitrous oxide (NO). The amount of des-nitro metabolites and corresponding amount of NO correlated with their cidal activity to the aerobic bacterial cells with a poor correlation between aerobicidal and anaerobicidal activities (Singh *et al*, 2008; Philo *et al*, 2009).

**Role of F420H2 in emergence from hypoxic condition**

F420H2 is a redox active enzyme co-factor and is involved in varieties of cellular activities such as methanogenesis, oxygen detoxification, sulphite reduction, antibiotic synthesis and DNA repair in various groups of bacteria with menaquinone reductase activity in *Mycobacteria* (Jacobson and Walsh, 1984; Seedorf *et al*., 2004). In *Mycobacteria,* the role of F420 in emergence from hypoxia has been reported. Menaquinone is the only quinone family present in this organism. It is an electron source which when activated, increases the respiratory activities of the organisms’ cell membrane. The role of Ddn as a quinone reductase was based on its activity with menadione, a synthetic quinone analogue. Here, purified Ddn was shown to catalyze F420H2-dependent reduction of menadione which accepts electrons from so many electron donors and transfer them to terminal oxidases or reductases in Mycobacterial respiration (Gurumurthy *et al,* 2013; Cook *et al*, 2014). A study by Lee *et al,* (2020) revealed a coupling between the reductase activity of Ddn and Mycobacterial respiratory chain. It was observed that the addition of F420, glucose 6-phosphate (G6P), and F420 dependent glucose-6-phosphate dehydrogenase (FGD), which catalyzes the reduction of F420 to F420H2 for use by F420H2-dependent enzymes such as Ddn significantly increased oxygen consumption by mycobacterial membranes when the membranes are activated by NADH. On the other hand, F420-negative strain not only declined growth in-vitro on a culture medium when subjected to oxygen stress but also showed hypersensistivity to other anti-TB agents such as isoniazide, *p*-amino benzoic acid, moxifloxacin and clofazimine (Meera *et al,* 2013).

From the foregoing, it can be deduced that the enzyme co-factor, F420 play central roles in the organisms’ emergence from hypoxia as well as its susceptibility to the drugs. In other words, the enzyme co-factor is responsible for the aerobicidal activity of the cyclic nitroimidazoles. Recall that the cidal activitiy of nitroimidazoles is well established in anaerobic organisms (lacking Ddn). Since the presence of Ddn/F420 is the only factor responsible for aerobicidal activity of the drugs in *Mtb*, any alteration in the gene coding for Ddn/F420 system could render the drugs ineffective. It was shown that the menadinone reduction activity of the Ddn is conserved across many species of *Mycobacteria* which however does not couple with nitroimidazole activation activity of the enzyme. While Ddn orthologs purified from species including *M*. *tuberculosis*, *M*. *marinum*, *M*. *smegmatis*, *M*. *vanbaalenii*, *M*. *avium* and *M*. *ulcerans* catalyzed menadione reduction, only *M. tuberculosis* and *M. marium* could activate the drug, pretomanid (Lee *et al,* 2020). This shows that irrespective of the absence of drug activation activity, the Ddn menadione reduction activity is still conserved; suggesting further that an alteration in certain sequences of the Ddn orthologs can confer drug resistance without affecting the native menadione reduction property. According to the report, this in-vitro drugs activation activity corresponded with in-vivo drug susceptibility by the organisms (*M. tuberculosis* and *M. marinum*) when tested with pretomanid.

**Possible spread of variants resistant to 2-position oxygen-bicyclic nitroimidazole**

The role of Ddn/F420 system cannot be over-emphasized. As mentioned earlier, the species lacking F420 could not survive hypoxic stress. This traditional role was conserved across many species of *Mycobacteria.* Ddn orthologs purified from species however lost drug activation abilityunderscoring the possibility of natural alteration in the gene segment controlling drug activation without affecting the traditional menadione reduction activities. Lee *et al,* (2020) reports that the susceptibilities to mutations are based on the molecular structures of the amino acid sequences of the active sites of the enzyme terminals where substitution of one amino acid with another produces significant change in the promiscuous activity of Ddn but not in the traditional menadione reductase activity. This also account for the differences in the degree of activation of different drugs. Although, the above observation was made in an artificial mutation, Lee et al (2020) reported pretomanid resistant strains which have not been exposed to the drugs prior to the test, further pointing to the potential natural emergence of the resistance. The more susceptibility of the promiscuous activity of Ddn to mutation notwithstanding, the more the fitness cost of such mutants is of great interest especially given the possibility of complete knock-out of Ddn activity such as loss of F420 biosynthesis through mutations to F420 biosynthetic genes, loss of F420 reductase activity through knockout of FGD, as well as introduction of stop codons or large genetic insertions/deletions in Ddn.

**Conclusion:**

Tuberculosis has remained a global health challenge due to both intrinsic and extrinsic mechanisms of resistance to chemotherapy by the causative organism. Continuous and unpredictable mutations at various sites of the organisms’ genome have continued to render various chemotherapeutic agents ineffective. It is justified to think that an end to prolonged treatment duration as well as solution to multi-drug resistance is in sight with the discovery of low redox potential bicyclic nitroimidazoles. The ability of these drugs to exert cidal activity against both aerobic and anaerobic phases of *Mycobacteria* seemed to confer an advantage over other anti-TB drugs. Even more exciting is intrinsic attribute of the organism to activate the drug, a property that is coupled with the ability of the pathogen to survive hypoxia. This means that the availability or otherwise of oxygen to the cells does not limit the lethal potential of the drugs. However, the observed mutations at the genes coding for these important enzymatic activities have become an obstacle to the anticipated usefulness of the drugs. With the discovery of the strain resistant to pretomanid prior to exposure and the observation of marked discrepancies in the effects of mutation on the promiscuous and native activities of Ddn/F420 system, the possibility of spread of resistant mutants is not out sight. It is now left to be determined the fitness trade-off of the resistant mutants/variants given the

possibility of mutations at every segment of the gene.

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