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Review

Mechanisms by which Thioridazine in Combination with Antibiotics Cures Extensively Drug-resistant Infections of Pulmonary Tuberculosis

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Abstract. *Advances recently introduced into the Clinical Mycobacteriology Laboratory of the Institute of Hygiene and Tropical Medicine, such that a multi-drug resistant infection of pulmonary tuberculosis (MDR TB) can be identified within one day of receiving the sputum specimen, have greatly contributed to the reduction of the frequency of these infections. However, approximately 50% of reduced infections exhibit a phenotype that is consistent with that presented by an extensively drug-resistant (XDR) infection. More effective agents were required and hence attention was attributed to the possibility that the old neuroleptic phenothiazine thioridazine (TZ), previously shown to inhibit the growth of all encountered strains of Mycobacterium tuberculosis (Mtb) regardless of their antibiotic resistance profile, could be eventually used for therapy of problematic MDR/XDR TB infections. This mini-review discusses the mechanisms that render TZ an effective adjuvant to antibiotics to which the initial infective agent Mtb was resistant.*

As a consequence of the two most effective anti-tuberculosis agents, isoniazid (INH) and rifampicin (Rif), global pulmonary tuberculosis was believed in the late 1950' to be on its way to become an extinct disease. However, as a consequence of famine created by political strife and war, large numbers of people from countries that still had a significant frequency of tuberculosis (TB) migrated to countries of the west Western Europe, North America, *etc*., and consequently, TB became a problem, at first thought to be restricted to the migrant population. Moreover, as a consequence of emerging HIV infections during the early 1980's and subsequent progression to AIDS, transmission of TB in cities of the West increased to alarming levels. No better example can be found than that experienced in New York City during the early 1990's when the rates of new infections of TB nearly quadrupled and more than 50% of infections were multi-drug resistant (MDR) (1, 2). The coordinated response of New York City and New York State during the early 1990's was quick and a plan was quickly developed and implemented which provided the means by which the diagnosis of TB could be made much quicker than that previously possible. This plan included the "Fast TB Track" programme (3, 4) which on the basis of two positive acid fast stains of a sputum specimen gave the patient's specimen priority over all other specimen whose TB status was not supported by the acid fast stain. Priority included laboratory assays that could render a positive culture within 12 days and susceptibility to INH and Rif within another 7 to 12 days. However, although this Fast Track program was a vast improvement over that of the past which could take as long as two or more months, this period of "turn-around time" of reporting was still far from ideal. Obviously, ideally, what was needed was a "Faster TB track programme.

In 1999, Lisbon, Portugal presented the highest TB frequency of any city of Western Europe (5, 6). Moreover, as much as 28% of all positive TB cultures exhibited an MDR phenotype (6). In 2001, the Institute of Hygiene supported the creation of the most modern TB laboratory in Western Europe which could provide a guaranteed turn-around time of 12 days for the identification of Myrobacterium tuberculosis susceptibility to INH and Rif for

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sputum specimen that satisfied the requirements of the Fast TB programme that had been implemented with the cooperation of the 12 major hospitals of the greater Lisbon area that constituted the TB Task Force of Lisbon (7). However, although the delivery of urgently-needed laboratory information that could assist the pulmonary physician in therapy of the TB infection was significantly reduced from an excess of three months to 12 days, it was still not good enough.

Consequently, the mycobacteriology laboratory developed a plan to replace the old Bactec 460 with the newly-introduced fluorescence-based Bactec 960/MGIT for culture and antibiotic sensitivity assays and added methods that provided the direct identification of *Mycobacterium tuberculosis* (*Mtb*) and identified mutations within the *rpo b* gene that rendered the strain resistant to Rif (8). Because in Lisbon, Portugal, mono-resistance to Rif is extremely rare, whenever resistance to Rif is evident, it is accompanied with resistance to INH. In other words demonstration of a mutation within the *rpo b* gene can serve as a surrogate for the identification of a MDR *Mycobacterium tuberculosis* strain. The new TB laboratory programme afforded the identification of an MDR *Mtb* strain within the same day of receiving the positive acid fast sputum (7, 8). By the end of 2008, the frequency of MDR Tb in Lisbon had been reduced to less than 8% of positive TB specimen (8). However, out of this 8% more than half were deemed to be extensively drug-resistant (XDR) *Mtb* (9). Obviously, the continuation of MDR forms of pulmonary tuberculosis required more than rapid laboratory service and other avenues were required if tuberculosis could be reduced to an acceptable level.

As noted, the incidence of MDR forms of pulmonary infections in Lisbon were the highest in Western Europe in the year 2000 and although the laboratory did indeed play a major role in the control of TB, by no means could by itself reduce the incidence of MDR infections (MDR/XDR TB) to a level which could be considered "acceptable", namely, a level that would be the lowest possible in the Western world. Foreseeing this limitation, and since after the introduction of streptomycin in the 1960's, no other anti-TB drug was introduced into the market despite growing evidence that resistance of the two most effective anti-TB agents INH and Rif was increasing, there was a great need for an effective anti-TB agent. Given that an old phenothiazine thioridazine (TZ) inhibited the *in vitro* growth of all encountered *Mycobacterium tuberculosis* (*Mtb*) strains regardless of its antibiotic resistance status (resistance to as many as five antibiotics used as "first-line defence" for therapy of pulmonary TB) (10), the studies required for support that indeed this agent could be used for the therapy of MDR/XDR and most-likely, TDR TB infections were begun in 2000. However, it must be understood that the *in vitro* activity of TZ occurs at concentrations in the medium which range from 16 to 32 μg/mL, whereas the maximum plasma concentration that can be clinically achieved in the psychotic patient that is chronically treated with TZ is of the order of 0.5 μg/mL. Nevertheless, there was already reliable information that its sister neuroleptic chlorpromazine (CPZ) was concentrated by macrophages rich in lysosomes (11), and because a pulmonary TB infection is an intracellular infection of the pulmonary macrophage present in the alveolus unit of the lung parenchyma, studies were undertaken to see if intracellular strains of MDR TB could be killed *in situ* by TZ. These studies quickly demonstrated that TZ could enhance the killing of antibiotic-sensitive and MDR strains of *Mycobacterium tuberculosis* by non-killing human macrophages (12, 13) as well as XDR *Mtb* strains (14). Soon thereafter, successful mono-therapy of the *Mtb*-infected mouse was achieved with TZ (15). Because prior studies demonstrated that very small concentrations of TZ could enhance the activity of anti-TB compounds (16), co-administration of TZ with either INH or Rif essentially cured the mouse of an infection by an MDR *Mtb* strain (17). Collectively, these studies provided the basis for the successful therapy of 18 XDR TB patients with TZ in combination with antibiotics to which the initial infective XDR strain was resistant (18, 19).

The first response to an antibiotic or any noxious agent by a susceptible bacterium is to over-express its main efflux pump system which recognises the noxious agent and extrudes it to the environment (20-22). The genes that regulate and code for efflux pump components are progressively more active as the concentration of the noxious agent is serially increased (21-23). However, when the concentration is maintained for a number of serial cultures, the increase in the activity of genes that regulate and code for the efflux pump of the bacterium gradually decrease until the basal level of activity is equivalent to that of its initial wild-type origins, and during this period, mutations of key targets on the plasma membrane, the 30S unit of the ribosome and gyrase begin to accumulate (20, 21). Although both sets of serial cultures result in the appearance of an MDR phenotype (20-23), the addition of a phenothiazine that has activity against efflux pumps, such as is the case for thioridazine, will render bacteria that are exposed to a serial increase of noxious agents concentration susceptible to the antibiotics to which it had become resistant (22, 23), whereas MDR bacteria that result from a prolonged exposure to a constant concentration of the noxious agent will retain its MDR phenotype regardless of the presence of the efflux pump inhibitor (20, 21). These results suggest that long-term ineffective therapy consisting of the same dose of a patient presenting with a bacterial infection will at first initiate an efflux pump response (20, 21) that is eventually followed by an accumulation of mutations, much as predicted by Ian Chopra (24). Moreover, it also explains why a given clinical

isolate obtained from a patient treated consistently with the same dose of antibiotic(s) presents with resistance to a given antibiotic that is 1,000-times greater than its wild-type phenotype (24-26). Consequently, when during ineffective therapy the clinical isolate is obtained, exposure to an EPI may show complete reversal of the efflux pump dependent on the MDR phentotype (20), or a partial reduction of resistance to one or more antibiotics (24), or no effect at all given that the mdr phenotype is solely dependent upon the accumulation of mutations (20, 21).

Mycobacteria *sp*., as is the case for all living cells, contain efflux pumps whose activity can be over-expressed by differential increases in the activity of genes that regulate and code for the components of the pump (27-31), thereby rendering bacteria with an MDR phenotype. Serial exposure of *Mycobacterium tuberculosis* to increasing concentrations of an anti-tubercular agent such as isoniazid (INH) will promote progressive increases in resistance to the agent which can be totally reversed by the addition of a phenothiazine to the culture (31, 32). As is the case for non-mycobacteria, exposure to increasing concentrations of INH will result in progressive increases in the activity of *mmpL7, p55, efpA, mmr, Rv1258c* and *Rv2459* genes which code for the main efflux pumps of the organism (28). Further exposure to a constant isoniazid concentration resulted in the selection and stabilization of spontaneous mutations and deletions in the *katG* gene along with sustained increased efflux activity (29). Whereas a phenothiazine EPI would reverse induced resistance to INH, it had lesser effect on further induced resistance by a constant concentration of INH, given that at that end-point of the last serial culture both efflux and mutations accounted for the resistance observed (29).

TZ, as is the case for most phenothiazines, inhibits the binding of calcium to calcium-binding proteins (33, 34) and inhibits the transport of potassium through dedicated channels (35, 36). Macrophages require for efflux of calcium and potassium from the phagolysosome for killing of the phagolysosomal trapped bacteria (37, 38). Inhibition of potassium transport by ouabain, a potent inhibitor of potassium transport (39, 40), enhances the killing of intracellular MDR *Mycobacterium tuberculosis* by non-killing human macrophages (41, 42). Consequently, the mechanism by which TZ enhances the killing of intracellular *Mycobacterium tuberculosis* has been proposed to take place as follows: 1. TZ binds to the surface of the plasma membrane and *via* invagination a vacuole forms, that migrates into the cytoplasm of the macrophage. 2. *Mycobacterium tuberculosis* in the medium binds to receptor(s) present on the surface of the plasma membrane promoting engulfment of the bacterium and subsequent invagination results in the phagolysosome which migrates through the cytoplasm. 3. Invagination of the plasma membrane that resulted in the phagolysosome means that potassium pumps present in the plasma membrane which now delineate the phagolysosome, that would normally pump potassium into the cell, now pump the ion from the vacuole to the cytoplasm. 4. The fusion of the phagosome containing the bacterium with a lysosome does not result in killing due to the efflux of potassium from the phagolysosome. Because the vacuolar ATPases require potassium for activity, they remain ineffective thereby the pH of the phagolysosome is not sufficiently lowered for the activation of dormant hydrolases. 5. The fusion of TZ-containing vacuole with the phagolysosome containing the entrapped bacterium makes it possible for TZ to inhibit the efflux pump that would otherwise extrude potassium. Retaining of potassium activates vacuolar ATPase, the pH of the phagolysosome drops and the dormant hydrolases are activated and degrade the bacterium.

The mechanism outlined above has been published in detail (43-45). It should be noted that the described mechanism that results in the killing of intracellular *Mycobacterium tuberculosis* is expected to be assisted by the effect of TZ on the efflux pump of the bacterium thereby rendering the organism susceptible to antibiotics to which it was initially resistant (19, 46-48). We believe that this contribution made it possible for successful therapy of 18 XDR TB patients with combination of TZ and antibiotics to which the infecting strains were initially resistant (18). It should also be noted that the killing effect produced by TZ may also result from the concentrating of TZ by the lysosomes of the macrophage to a level equivalent to that of its *in vitro* minimum bactericidal concentration (11, 33, 36, 49). Because TZ targets the human macrophage that contains the entrapped bacterium, there is no opportunity for a resistance response by the organism. Moreover, because TZ in combination with anti-tubercular agents reverses the initial resistance to these agents, it means that old, cheap and once effective antibiotics whose effectiveness had been compromised by the over-expressed efflux pump of the organism, may again be useful with TZ as adjuvants. The concept of targeting the macrophage for enhanced killing activity presents a totally new therapeutic approach for intracellular infections such as the one produced by *Mtb* (45).

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