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In Search of El Dorado: Current Trends and Strategies in the Development of Novel Anti-Tubercular Drugs

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*And, as his strength
Failed him at length,
He met a pilgrim shadow --
"Shadow," said he,
"Where can it be --
This land of El Dorado?"
"Over the Mountains
Of the Moon,
Down the Valley of the Shadow,
Ride, boldly ride,"
The shade replied --
"If you seek for El Dorado."
Edgar Allan Poe, "El Dorado"*

1. Introduction

1.1 The enemy, the battlefield and the death toll

Mycobacterium tuberculosis, the ethiological agent of human tuberculosis, is still one of the most effective human pathogens, and along with the causative agent of malaria, *Plasmodium falciparum*, and the HIV virus, conform a triad of killers that merciless strike the human race. Current statistics show that in 2007 these three pathogens took the life of almost 5 million people; the majority of the cases (nearly 3 million people) affecting Africa and specially children (1,8 million deaths). Of these three agents, the tubercle bacilli is perhaps the one that spreads with more efficiency since it infects humans by aerial route, through aerosolized drops produced by coughing tuberculosis patients. Measures for intervention can be designed in the case of malaria (fighting against the transmission vector and its environment) and HIV (proper sex conduct, condom usage) but are much harder to elaborate in order to prevent people suffering of tuberculosis from coughing. Having a very

low infective dose (1-5 live bacilli), *M. tuberculosis* is able to infect a person very efficiently, overcoming the disadvantage of not having so far, an identified reservoir in nature.

Once considered an eradicated disease, tuberculosis has been around for centuries; modern genetic techniques allowed to follow and understand the evolution of *M. tuberculosis*, from an hypothetical ancestral strain that evolved from being an environmental strain to the contemporary human pathogen (Brosch, R. *et al.*, 2002; Mostowy, S., 2002; Gutierrez, M.C, *et al.*, 2005; Smith, N. H., 2009). Although more detailed publications in the field of mechanisms of pathogenesis and immunology have been published (Kaufmann, S. H. and J. Hess. 2000; Collins, H. L. and S. H. Kaufmann. 2001, Ulrichs, T. and S. H. Kaufmann, 2002; Kaufmann, S. H. 2006) a brief description of the events taking place after the bacilli are inhaled as droplets from the atmosphere is described next. After travelling to the lungs, the microorganisms are phagocytosed by alveolar macrophages, triggering a local proinflammatory response that in turn causes the recruitment of mononuclear cells from adjacent blood vessels. These cells are the basic components of the granuloma, which consists of bacilli-infected macrophages surrounded by foamy (lipid loaded) macrophages and other mononuclear phagocytes, lymphocytes, collagen and other extracellular matrix components that form the periphery of the structure (Russell, D. G., 2009). This description corresponds to a phase of the infection in which there is no transmission of the disease neither clinical signs. Later on, the granuloma thickens due to a fibrous cover, becoming hypoxic. Several natural or disease provoked causes such as age, malnourishment, or conditions that impair the normal immune function, lead to major changes in the granuloma, that liquifies, losing structure and releasing the caseum and large numbers of viable, infectious bacilli into the airways. By this process, the tubercle bacilli leaves the infected host and begin a new journey to the following inhalation victim.

2. Old meets new: A powerful face-lift of anti-tubercular drugs

The objective of this section is to describe the features of several anti-tubercular drugs that are still or have once been used for clinical treatment of tuberculosis as well as novel compounds inspired by research on those drugs. The reader is directed to several reviews in which those drugs are described in detail (Zhang, Y. and D. Mitchison. 2003; Vilcheze, C. and W. R. Jacobs, Jr. 2007).

The advent of chemotherapy in the late 19th and early 20th centuries led to the use of different chemicals as options to empirically treat infectious diseases; tuberculosis was not the exemption and dyes such as trypan red and methylene blue were used for treatment on the basis that they could bind the tubercle bacilli in tissues. In the early 1930, the introduction of sulfonamides and their antibacterial effect led to the testing of several compounds against *M. tuberculosis*, amongst them thiosemicarbazones and sulfones such as Promin and Diasone. The results were discouraging so the discovery of streptomycin by Waksman in 1944 and its activity against the tubercle bacilli brought hope that at last an efficacious drug to kill *M. tuberculosis* had been found. Shortly after the onset of the treatment, resistance to streptomycin began to develop, but a new drug, p-aminosalicylic acid (PAS), was generated in 1946 on the basis of the known activity of salicylic acid derivatives against *M. tuberculosis*. During those early years of tuberculosis chemotherapy, other drugs were added to the armamentarium, all of them found by broad screening;

among them we may cite Viomycin, Isoniazid, D-Cycloserine and Pyrazinamide in 1951-52, Ethionamide in 1956, Kanamycin in 1957, Ethambutol and Capreomycin in 1962 (Laughon, B. 2007). One of those drugs, the flagship of the anti-tubercular treatment, Isoniazid (isonicotinic acid hydrazide) displayed excellent activity and was well tolerated alone or in combination with Streptomycin and PAS or pyrazinamide. From that point on, only one drug, Rifampicin, was added to improve tuberculosis treatment. This event took place in 1966 and was the last addition of a drug showing the desirable features of high activity, low toxicity and oral route of administration. Both Isoniazid and Rifampicin became the pillars of the anti-tubercular treatment in spite of the little knowledge on their mode and mechanisms of action. Thus, a first-line of defense against *M. tuberculosis* was built, consisting of the so-called first-line drugs: Isoniazid, Rifampicin, Ethambutol, Pyrazinamide and Streptomycin. A second group of drugs included several antibacterial drugs with activity against *M. tuberculosis* such as aminoglycosides, fluoroquinolones and D-Cycloserine, as well as Ethionamide, an Isoniazid analogue with less potency. A third group of less frequently used drugs (such as Isoxyl and Thiacetazone) was later on discarded due to secondary effects and rapid generation of resistance. All the mentioned drugs had to wait over 40 years to have their mechanisms of action partially understood as will be described below in this section.

During the following 30 years there was little interest from the pharmaceutical industry to develop novel anti-tubercular drugs, most likely because there was a general belief that tuberculosis cases were decreasing every year and infecting strains were in the vast majority, susceptible to the available first- and second- line drugs. Along with this perception, an important factor to decide whether or not start an anti-tubercular drug discovery program resided in the poor knowledge of the mycobacterial physiology and cell structure, necessary elements at the moment of deciphering the mechanisms of action of the anti-tubercular drugs and the mechanisms of resistance put forward by *M. tuberculosis* to avoid the activity of those drugs. In turn, that deficit was caused by the lack of genetic tools needed to manipulate mycobacteria, a situation that radically changed in the late '90 due to the combined efforts of research groups in Europe and USA. The tools devised for the analysis of *M. tuberculosis* (Guilhot *et al.*, 1994; Jackson *et al.*, 2001; Bardarov *et al.*, 2002) and the sequencing of its genome started to put the intricacies of this sophisticated pathogen under a spotlight (Cole *et al.*, 1998). Part of those sophistications included a highly specialized genome with a large number of genes involved in synthesis, modification or degradation of fatty acids, underscoring the importance of those components for the metabolism, structure and virulence of the tubercle bacilli (Wayne and Lin, 1982; Munoz-Elias and McKinney, 2005; Russell *et al.* 2009) (10-12). It was also surprising to detect the presence of two fatty acid synthase systems, designated FASI and FASII. FASI is an eukaryotic type synthase, producing as end products, fatty acids of 16-24 carbons in length, while FASII, is a bacterial type synthase that is in charge of the synthesis of very long chain fatty acids known as mycolic acids (Bloch 1975, 1977). The presence of these two systems can be interpreted as a sign of the specialization and co-evolution of *M. tuberculosis*, reflecting the long time interaction with humans. Thus, an increasing knowledge of the structure of the mycobacterial cell wall envelope accentuated the key role played by mycolic acids, involved in cell integrity and responsible in part for the extremely low cell wall permeability displayed by mycobacteria.

The combined effort of several labs shed light into the mechanism of action of the most prominent anti-tubercular drugs in use, namely, Isoniazid (INH), Ethionamide (ETH), Ethambutol (EMB) and Pyrazinamide (PZA). In brief, INH and ETH have a common target, an Enoyl Acyl Carrier Protein (ACP) Reductase dubbed *InhA*, part of the FASII cycle that synthesizes mycolic acids (Banerjee *et al.*, 1994). Interestingly, although both compounds are pro-drugs with chemical similarities their activation step is carried out by two different enzymes; while INH is activated by a catalase-peroxidase encoded by the *katG* gene, a flavin monooxygenase -*ethA*- activates ETH (Zhang *et al.*, 1992; Heym *et al.*, 1995; Baulard *et al.*, 2000; Vannelli *et al.*, 2002). Thus, most of the clinical isolates displaying resistance to each of those drugs are mutants defective in either *KatG* or *EthA* activity (Morlock *et al.*, 2003). These reports generated two experimental approaches based on a rational design, leading to the design of new drugs affecting *InhA*. In the case of INH, a series of compounds showed promising activity against *M. tuberculosis*, inhibiting *InhA* and avoiding the activation step (Sullivan *et al.*, 2006; Freundlich *et al.*, 2009). Those compounds, derived from triclosan, a trichlorinated aryl alkyl ether were subjected to a second round of structure improvement leading to molecules with the desired features of activity and no longer substrate of efflux pump systems (Tonge *et al.*, 2007; am Ende *et al.*, 2008). Similarly, research on the mechanism of action of ETH led to a smart way to improve its potency by increasing its rate of activation. To this end, the partnership between *EthA* and its repressor protein *EthR*, was used (Frenois *et al.*, 2004; Weber *et al.*, 2008). Thus, on the grounds that an inhibition of the activity of *EthR* would leave *EthA* free to act upon ETH, leading to its activation, a set of molecules was synthesized. The results demonstrated that by this approach, chemical “boosters” of ETH activity were obtained, increasing the therapeutic value of this molecule (Frenois *et al.*, 2004; Willand *et al.*, 2009).

Likewise, the elucidation of the mechanisms of action of both EMB and PZA, controversial in both cases, generated a great deal of interest on the possibility of identifying new drugs inhibiting the same targets than the lead compounds. In the case of EMB, an inhibitor of the synthesis of arabinogalactan (a key component of the cell envelope to which mycolic acids are covalently linked) (Takayama and Kilburn, 1989; Khoo *et al.*, 1996; Belanger *et al.*, 1996; Telenti *et al.*, 1997), the search for new anti-tubercular agents led to the identification of SQ109 (Protopopova *et al.*, 2005; Jia *et al.*, 2005). Unexpectedly, this compound, a diamine structurally related to EMB, did not affect arabinogalactan biosynthesis and its true target is still unknown. In spite of that, SQ109 is one of the very few compounds that is currently being tested in clinical trials.

PZA, a pro-drug that is converted to the active Pyrazinoic acid (POA) through the action of an nicotinamidase/ pyrazinamidase (*PncA*), is still a very important component of the anti-tubercular therapeutic scheme. This compound exerts a great activity at low pH, thus targeting the phagosomal bacillar population (Zhang *et al.*, 1999). As described for other pro-drugs, PZA lacks activity if mutations affecting *PncA* are generated. Similarly to what happened in the case of INH, the identification of the mechanism of action of PZA went through a period of uncertainty driven by the conflicting point of view of two laboratories, one supporting the idea of PZA inhibiting FASI (Zimhony *et al.*, 2000), and the second sustaining the hypothesis that PZA acts through “in vivo” generation of Pyrazinoic acid (POA), a weak acid that kills *M. tuberculosis* due to its failure to cope with pH homeostasis efficiently (Zhang *et al.*, 1999; Zimhony *et al.*, 2000). In agreement to that, while most of the *M.*

tuberculosis strains resistant to pyrazinamide are defective in PncA activity, susceptibility to POA is still observed. Although these different points of view did not hold back work on compounds that may mimic the activity of PZA, the fact is that only few derivatives have been proposed until now. Intriguingly some of those compounds, such as 5-chloro PZA, demonstrated a specific inhibition of FASI (Cynamon *et al.*, 1998; Baughn *et al.*, 2010), not shown by the parental PZA or by its derivative POA (Boshoff *et al.*, 2002). Thus, the structure-activity relationship of those compounds seem to be more complex than firstly thought.

Two more anti-tubercular drugs have been studied recently, both Isoxyl (ISO) and Thiacetazone (TAC) are thioureas, sharing the activation mechanism of ETH, thus being in fact pro-drugs that are activated by EthA (Kordulakova *et al.*, 2007; Dover *et al.*, 2007; Nishida and Ortiz de Montellano, 2011). Therefore their use is jeopardized by resistance to ETH since many clinical strains displaying that phenotype showed cross-resistance to the three drugs (Debarber *et al.*, 2000). Both drugs alter the synthesis of fatty and mycolic acids but have different targets and mechanisms of action, so while ISO inhibits unsaturated fatty acid and mycolic acid synthesis, TAC seems to inhibit methyltransferases involved in mycolic acids modifications (Alhari *et al.*, 2007). Several compounds have been made on the basis of the ISO and TAC scaffolds, with some showing good anti-tubercular activity (Bowhurt *et al.*, 2006; Dover *et al.*, 2007). In spite of the new information on these drugs, the fact that the molecular target(s) for each one has not been unequivocally identified yet, is delaying a rational approach to the design of analogues that would overcome both the need for an activator and eliminate the secondary effects of these two compounds.

In summary, after over 50 years of use of drugs included in the clinical treatment of tuberculosis, the research on their mechanisms of actions and the mycobacterial mechanisms of resistance, produced the back-ground information needed to start drug development programs. Notwithstanding this fact, few programs have produced drugs that reached clinical testing, thus keeping a gap between the interest of pharmaceutical companies to invest in drug discovery programs and the social need to have new and better drugs to treat this devastating disease.

3. Tug-of-war at the pharmaceutical industry: To discover and produce novel anti-tuberculosis agents or not

The development of new anti-tubercular drugs has been slowed down by several obstacles of which we may mention three as the most important ones. In first place, the TB drug market is considered by pharmaceutical companies to be characterized by little profit opportunity or investment return. As a matter of fact, the cost of development of a new drug is estimated at \$115 to \$240 million US dollars (Gardner *et al.*, 2006), thus to reach a reasonable level of profit, market prices of new drugs should be relatively high, contrasting with the current cost of the standard regimen, US \$11 per patient (O'Brien and Nunn, 2001). A very comprehensive analysis of this matter has recently been discussed (Chang Blanc and Nunn, 2000). Importantly, government agencies are fully aware of the need to engage in the battle for the development of new anti-tubercular drugs. This awareness is shown by the several initiatives and programs established since 1994, such as the contracts awarded by the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) to centers of

remarkably high scientific level in the tuberculosis field, such as the Southern Research Institute, (SRI), the Hansen's Disease Centers, and Colorado State University (Orme, 2001), the creation of the Tuberculosis Structural Genomics Consortium (Goulding, 2002), fundamental for the generation of a large set of data on putative mycobacterial targets amenable for drug design (Chim *et al.*, 2011). Action TB, a multinational, interdisciplinary research initiative, was funded by the pharmaceutical company GlaxoSmithKline (GSK); although successful in promoting collaborations and translating research findings into drug screens and vaccine development, Action TB ended years ago. Various other research consortia are testing new drugs in preclinical and clinical trials, large funding agencies, such as the European & Developing Countries Clinical Trials Partnership (EDCTP) and the Bill & Melinda Gates Foundation are supporting these initiatives. The Global Alliance for TB Drug Development (TB Alliance), created in 2000 with support from the Rockefeller Foundation and the Gates Foundation (Pablos-Mendez, 2000; Gardner *et al.*, 2005), has the goal of developing drugs that could shorten the treatment of active tuberculosis, being active on multi-drug resistant strains and on latent stages of the disease.

In conclusion, although several major initiatives were launched some 20 years ago, with a considerable impact on the gathering of knowledge required to achieve the major goal of anti-tubercular drug development, pharmaceutical companies either had relatively low involvement or gave up after a few years.

In second place, besides the profit considerations of drug making, anti-tubercular drug development faces a serious difficulty simply by the nature of the pathogen itself; as mentioned earlier, *M. tuberculosis* is present in sub-populations in the infected individual, each one in different cellular or extracellular locations. Moreover, not only the tubercle bacilli has the ability to enter a dormant state in which its metabolism diminishes to a minimum, but also can form biofilms (Wayne, 1994; Ojha, 2008). In both situations, the metabolic changes help the pathogen to evade the action of the anti-tubercular drugs. Ideally, clinical anti-tubercular regimes should kill both the rapidly growing mycobacteria and the persisting mycobacteria in lesions. The major problem is that the molecular mechanisms behind dormancy (characterized by a very low metabolic activity of the mycobacteria) and tolerance (drug-susceptible *M. tuberculosis* that survive in spite of continuous exposure to anti-tubercular drugs) are not yet fully deciphered (Zhang, 2004). Thus, from the point of view of the information available to rationally design new anti-tubercular drugs, although more essential pathways are identified, they are not understood in full.

The third challenge resides on the fact that there are currently no animal models that can be used with accuracy to test new anti-tubercular drugs and predict treatment duration (Druilhe *et al.*, 2002; Mitchinson and Chang, 2009). At this point, the guinea pig model exceeds the mouse model since it displays pathology characteristics of the disease more closely resembling those of the infected human. In spite of that factor, the need to rely on the correct extrapolation of results from the animal model to the human led to an interest in developing a non-human primate animal model (Flynn *et al.*, 2003; Flynn, 2006). It is not a minor point to state that this choice of an animal model to test the new anti-tubercular drugs implies a large difference in costs that has an obvious impact on the total investment required. All of these problems have already been pointed out by Lenaerts and co-workers (Lenaerts *et al.*, 2005), who mention that from over 85,000 compounds tested for their anti-

tubercular activity at Colorado State University (USA), only about 8% (7,500) showed reasonable activity as measured by Minimum Inhibitory Concentration (MIC), 700 had an acceptable selectivity index (reflecting the concentration required to inhibit *M. tuberculosis* vs. the concentration having toxicity on cultured eukaryotic cells), 11 showed “in vivo” activity and only 5 compounds were considered potential leads and pursued further.

Animal studies required 100–150 mouse each, translating into a cost of US \$ 400,000/study. Last but not least, the “gold standards” to evaluate efficacy of an anti-tubercular regimen in phase II (sputum culture conversion from positive to negative after two months of treatment) and III (relapse rate 2 years after completing clinical testing) are either controversial or lengthy processes that add to the paucity in the anti-tubercular drug development processes (van den Boogaard *et al.*, 2009; Perrin *et al.*, 2010).

Thus, it is clear that anti-tubercular drug development is hampered by the lack of a small animal model that would be cost effective, display the characteristics of a natural infection to humans and produce an immune response upon infection comparable to that of humans.

4. New molecules that may renew hopes of defeating *M. tuberculosis*

It has briefly been described above how the information gathered on the mechanisms of action of drugs already in use helped to propose new molecules such as ENR inhibitors that do not require activation, boosters of ETH activity, and ETH analogues. Although those are promising steps forward in the race to prevail over the tubercle bacilli, there are several other compounds that are under clinical testing, some of which may reach the key stage of human use. As of May 2011, the Global Alliance for tuberculosis drug development shows in its webpage (<http://www.tballiance.org/home/home.php>) that three drugs are in clinical stages I and II: moxifloxacin (a fluoroquinolone), PA-824 (a nitropyran) and TMC207, a diarylquinolone. A second nitropyran, OPC-67683 is being studied in phase I clinical trials. With the exception of fluoroquinolones (since they were generated by programs not directed at the development of specific anti-tubercular drugs but aiming at general anti-bacterial drugs), these drugs will be briefly described below:

Diarylquinolones. Diarylquinolines have been identified by broad screening of chemical libraries as having anti-tubercular activity (Diacon *et al.*, 2009; Matteelli *et al.*, 2010). The most active member of the set (TMC207, also called R 207910) is currently being evaluated in phase II clinical trials. The importance of this compound stems from its target, which is the essential mycobacterial ATP synthase enzyme (Koul *et al.*, 2007; Haagsma *et al.*, 2009). Because of that, it is not surprising that until now, there is no report of cross-resistance with available drugs and that the compound is equally efficient on MDR- *M. tuberculosis* strains. However, the fact that resistant mutants were isolated “in vitro”, having mutations in the *atpE* gene (encoding a subunit of ATP synthase) dampens to some extent the expectation of having a novel powerful drug (Koul *et al.*, 2008). TCM 207 has a long half-life in plasma and so far, no drug-drug interactions with INH or PZA were detected. Unfortunately, plasma levels of TCM207 are reduced to 50% by RIF since it strongly induces a cytochrome P-450 system (CYP3A4) that metabolizes TCM207, although a great deal of activity is still maintained. Thus, in this case, drug-drug interaction may not reach a relevance level that would avoid the use of these novel drugs. Addition of TCM207 to standard drug regimes improved efficacy of the treatment and specifically synergy with PZA was noticed in a

mouse model (Ibrahim *et al.*, 2007; Ibrahim *et al.*, 2009). Promising results were also observed in a guinea pig model with sterilization after six weeks of TCM207 monotherapy. Studies on human patients revealed good activity but due to the interaction with RIF, TCM207 activity is currently addressed in treatments not including this drug.

Nitroimidazopyrans. The nitroimidazopyrans derive from bicyclic nitroimidazofurans that were initially developed for cancer chemotherapy (Stover *et al.*, 2000; Denny and Palmer, 2010). Their anti-tubercular activity against growing and dormant *M. tuberculosis* put two of these compounds (PA-824, a nitroimidazo-oxazine, and OPC-67683, a dihydroimidazo-oxazole) into clinical testing (Lenaerts *et al.*, 2005). There is a good deal of information on the mechanism of action of PA-824, which is -like several other anti-tubercular agents- a pro-drug; in this case it is activated by the coupled system glucose-6-phosphate dehydrogenase (FDG1)- coenzyme F420 (Choi *et al.*, 2001, Bashiri *et al.*, 2008). Thus, mutations affecting the mycobacterial genes *fbiA*, *fbiB*, and *fbiC* cause a defect in coenzyme F420 synthesis and subsequently, resistance to PA-824 (Choi *et al.*, 2001; Choi *et al.*, 2002). Also, mutations in Rv3547, a deaza flavin dependent nitroreductase, have been described and associated to resistance to this compound (Manjunatha *et al.*, 2006). Once activated, PA-824 exerts its activity shutting down the synthesis of proteins and cell wall lipids, although it seems that the main effect on non replicating bacilli is mediated by generation of reactive NO radical by reduction (Singh *et al.*, 2008). As expected from its molecular features and activation step, PA-824 shows equal activity of drug susceptible and drug resistant strains with MICs in the sub-micromolar order. Importantly, there is no cross-resistance with the classical anti-tubercular agents. The animal and human clinical studies performed recently assigned good pharmacokinetic features to PA-824: in mice it reached high serum concentrations rapidly, without any detectable undesired interaction with other anti-tubercular drugs (Neumrberger *et al.*, 2006; Gissenberg *et al.*, 2009). Its powerful bactericidal activity puts it in the same level of efficacy than INH or RIF, thus converting PA-824 in a surrogate candidate to replace RIF in those cases where the *M. tuberculosis* clinical isolate is resistant to RIF. Combined with moxifloxacin, PA-824 showed activity on mouse models of latent tuberculosis, which makes this compound a very attractive candidate to treat human latent tuberculosis (Singh *et al.*, 2008). In spite of those valuable features, PA-824 failed in shortening treatment times, and although it did not display any antagonism, it did not show any synergy.

The second nitropyran, OPC-67683 is also a pro-drug that acts by inhibiting the synthesis of two families (methoxy- and keto-) of mycolic acids, essential components of the mycobacterial cell wall (Sasaki *et al.*, 2006; Matsumoto *et al.*, 2006). Interestingly, the mycobacterial mechanism of resistance to PA-824 is also used to confer resistance to OPC-67683, thus mutations in the *M. tuberculosis* Rv3547 gene are also behind the resistance to this new inhibitor. There is no drug-drug interaction with any of the currently used anti-tubercular drugs and recent studies indicate that OPC-67683 has good intracellular killing ability and high sterilizing activity even on drug tolerant (persistent) sub-populations of *M. tuberculosis* (Saliu *et al.*, 2007). These features, along with its lack of interactions with the liver microsomal enzymes (thus not being affected by them) strengthen the chances of this molecule to be added to the therapeutic regime. As a disadvantage, its Early Bactericidal Activity is low although along the treatment time this compound exerts a high sterilizing activity (Saliu *et al.*, 2007). There is still a long way to go to reach that point and clearly, more

evaluation of its activity, adverse effects and drug formulation is required. Nonetheless, OPC- 67683 remains as a new choice to treat MDRTb and XDR-TB infections, targeting at the same time the dormant and persistant sub-populations.

Other drugs under study. This brief description is by no means complete and it does not intend to describe the whole set of compounds that are being studied by different public and private ventures. It only presents those compounds which are already part of clinical trials and thus, are the most promising candidates to join the therapeutic anti-tubercular regimes. Amongst several other molecules which are under vigorous studies now, we may cite proposed inhibitors active on the FASII dehydratase (i.e. NAS 91 and derivatives) (Bhowrut *et al.*, 2008), inhibitors of FtsZ, a critical protein involved in cell division (Huang *et al.*, 2007; Slayden and Belisle, 2009; Kumar *et al.*, 2010), inhibitors of AccD6, an essential acetyl-CoA carboxylase necessary for the synthesis of malonyl-CoA required for fatty acid biosynthesis (Lin *et al.*, 2006; Kurth *et al.*, 2009), and compounds such as 1,3-benzothiazin-4-one (inhibitor of the enzyme decaprenylphosphoryl- β -d-ribose 2'-epimerase involved in cell wall arabinans) (Makarov *et al.*, 2009; Manina *et al.*, 2010), a serious contender to reach the podium of novel anti-tubercular medicine. Finally, clinical studies on other novel compounds displaying anti-mycobacterial activity are being pursued at different pace, there is not enough information available to assess their possible impact; like in the case of SQ109 (Protopopova *et al.*, 2005; Jia *et al.*, 2005), and pyrrol derivatives such as LL-3858 (Ginsberg, 2010).

5. Available drugs that deserve an opportunity

In the current context of drug development, when the quest is so time consuming and so demanding in terms of the funds required, it brings a beacon of light and hope the fact that there are some drugs with proven activity against *M. tuberculosis* that have been in clinical use for long time. Although those compounds are effective against different non bacterial infections and even on non infectious diseases, the strength of the information gathered lately, underscoring the potency of their effect on the tubercle bacilli is impossible to ignore. The compounds I am referring to are a- the efflux pumps inhibitors, verapamil and reserpine; b- the antifungal azoles, and c- the neuroleptics phenothiazines. A brief overview of each of these compounds is given below.

5.1 Efflux pumps in *M. tuberculosis*, their role in tolerance to drugs and a simple way to prevail over them

The amazing complexity of mycobacteria reveals at least two mechanisms to undermine the power of anti-mycobacterial drugs, one is the intrinsic resistance presented by its cell wall envelope, characterized by a very low permeability (Nikaido and Jarlier, 1991; Liu *et al.*, 1996); the second one is the presence of several systems that actively pump out drugs (De Rossi *et al.*, 2002; Viveiros *et al.*, 2003).

Bacterial drug efflux pumps are classified into five groups, two of them, the ATP-binding cassette superfamily and the major facilitator superfamily, contain a large number of members while the other three (the small multidrug resistance family, the resistance-nodulation-cell division family and the multidrug and toxic compounds family) although increasing, are less populated. It is not the subject of this manuscript to dissect the molecular

biology of those families, but it is one of its goals to mention the role of all those families that are represented in the *M. tuberculosis* genome (as well as in other mycobacterial genomes) in the drug discovery process.

The overall resistance to the drugs that have been part of the classical anti-tubercular therapy, share a common characteristic, the existence of a variable percentage of resistant *M. tuberculosis* strains that do not contain mutations neither in the genes identified as molecular targets nor in the genes encoding the activators in the case of pro-drugs; so, while mutations in *katG* and *ethA* (encoding the activator enzymes) and *inhA* (encoding the target enzyme) cover most of the strains resistant to INH and ETH described in the literature, there is a percentage of those strains that does not show any mutations in the mentioned genes. The body of evidence gathered over the last years, uncovering the role of other genes such as *ndh* (encoding the NADH dehydrogenase) (Miesel *et al.*, 1998; Vilcheze *et al.*, 2005) and *mshA*, *mshB* and *mshC* (involved in the biosynthesis of mycothiol) in the resistance to INH and ETH in *M. tuberculosis* and *M. smegmatis* (Vilcheze *et al.*, 2008, 2011; Xhu *et al.*, 2011) cannot account for a fraction of resistant strains that do not have any mutation in those genes. A comparable situation is found in the case of the resistance to PZA, another pro-drug which has *pcnA* as its activator. Mutations in this gene, encoding the amidase required to produce the active drug, Pyrazinoic acid (POA), accounts for a large fraction of the resistance in *M. tuberculosis* (Jureen *et al.*, 2008). In spite of that, PZA resistant strains without mutation in *pcnA* have been reported (Raynaud *et al.*, 1999). Furthermore, the identification of *mmpL7*, a lipid transporter, as an efflux pump capable of mediating resistance to INH puts forth the role of efflux pumps in the resistance to drugs in mycobacteria (Pasca *et al.*, 2005). Thus, the resistance to several anti-bacterial (including Streptomycin, Aminoglycosides, Fluoroquinolones, Tetracycline, Rifampicin) and anti-tubercular drugs (Ethionamide, Isoniazid, Ethambutol) has been associated to the efflux pumps encoded in the mycobacterial genome. This is not surprising considering the number of genes encoding efflux pumps in mycobacteria, some of them, such as *mmpL7*, having a physiological role unrelated to antibiotic elimination from the cytoplasm. A relevant point is that although some efflux pumps have been characterized, there are few publications reporting a comprehensive testing of anti-bacterial and anti-tubercular drugs on efflux pump gene expression (Jiang *et al.*, 2008; Gupta *et al.*, 2010), so it is possible that any given pump may be involved in resistance to drugs that have not been tested. A second important point is that a systematic deletion of efflux pump genes in *M. tuberculosis* along with examination of the level of resistance to tubercular inhibitors has not yet been accomplished. This would be the only method that would clearly correlate drug resistance to each efflux pump. Finally, in several cases, the identification of *M. tuberculosis* efflux pumps stems from their molecular cloning and expression in a surrogate model (*M. smegmatis*) which, although similar to *M. tuberculosis* has intriguing differences in the number and nature of these pumps (Liu *et al.*, 1996; Li *et al.*, 2004). An example of that is the existence of *LfrA*, that eliminates Fluoroquinolones from the *M. smegmatis* cytoplasm (Sander *et al.*, 2000). The *lfrA* gene is not present in the *M. tuberculosis* chromosome; in spite of that, two different systems, one encoded by *Rv1634* (De Rossi *et al.*, 2006), and the second by the operon formed by *Rv2686c*-*Rv2687c*-*Rv2688c*, take part in the elimination of Fluoroquinolones in this pathogen (Pasca *et al.*, 2004).

According to recent literature, there are 15 genes (named *mmpL* and *mmpS*) present in the *M. tuberculosis* genome, that are classified as members of the RND family (De Rossi *et al.*, 2006). In order to assess the role of those proteins in drug resistance, Domenech *et al.* constructed deletion mutants in each one of the 11 *mmpL* genes present in the *M. tuberculosis* genome

(Domenech *et al.*, 2005) Interestingly, a previous report by Pasca *et al.* pointed out that over-expression of *mmpL7* conferred INH resistance to *M. smegmatis*, a phenotype that decreased upon addition of the efflux pump inhibitors, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ortho-vanadate, reserpine, and verapamil (Choudhuri *et al.*, 1999, Pasca *et al.*, 2005). In spite of this evidence, Domenech *et al.* reported that the deletion of this gene failed to increase the susceptibility to this anti-tubercular drug. Moreover, none of the *mmpL* genes seemed to participate in resistance to drugs, as no compelling decrease in the MICs was observed in the *M. tuberculosis* mutants lacking these genes (Domenech *et al.*, 2005). Current evidence favours the idea that *mmpL* proteins are devoted to the transport of complex lipid molecules (Camacho *et al.*, 2001; Converse *et al.*, 2003; Jain and Cox, 2005), thus it is possible that because of that function, over-expression of some of these genes (such as *mmpL7*) may affect biophysical characteristics of the cell envelope, leading to a secondary phenotype of mild resistance to some anti-tubercular drugs.

Heavily represented in the mycobacterial genome, ABC transporters account for 2,5% of the chromosomal genes (Braibant *et al.*, 2000)(7). Bioinformatic analysis revealed 25 complete ABC transporters for which potential substrates could be postulated in many cases, leading to the prediction of 9 importers and 16 extruders. Comparison to transporter sub-families in other bacteria allowed for the finding of nine of them in *M. tuberculosis*, of those, three were linked to drug transport and one of them was postulated to encode three macrolide transporters members (Rv1473, Rv2477 and Rv1667c-Rv1668c) (Braibant *et al.*, 2000). A second sub-family grouped four transporters similar to multidrug resistance (MDR) proteins of eukaryotes and prokaryotes. Three of them are encoded by two genes arranged in tandem (Rv1273c-Rv1272c and Rv1348-Rv1349) while the remaining two transporters are encoded by single genes (Rv0194 and Rv1819). Lastly, the third sub-family includes six transporters with different gene organization, three containing three genes clustered in the genome (*drvA-drrB-drrC*, Rv1458c-Rv1457c-Rv1456c, Rv2688c-Rv2687c-Rv2686c), two formed by two genes (Rv1218c- Rv1217c and Rv1687c- Rv1686c) and one by a single gene (Rv1747) (Braibant *et al.*, 2000). The identification of an ABC transporter encoded in the Rv 2686c-2687c-2688 operon that confers resistance to fluoroquinolones led to explore its susceptibility to known inhibitors of transporters systems. Three of the compounds that have been mostly used in this analysis are CCCP (a Proton Motif Force uncoupler), reserpine (an inhibitor of ATP dependent efflux systems) and verapamil (an inhibitor of the mammalian P-glycoprotein drug transporter).

Like the ABC transporter family, the MSF family is also a large one; early work by de Rossi *et al.* postulated through bioinformatics, the presence of sixteen candidate genes (Rv3239c, Rv3728, Rv2846c, Rv1877, Rv2333c, Rv2459, Rv1410c, Rv1250, Rv1258c, Rv0783c, Rv1634, Rv0849, Rv0191, Rv0037c, Rv2456c, and Rv2994) (De Rossi *et al.*, 2002), although cloning and expression in the surrogate *M. smegmatis* of ORFs Rv0037c, Rv0783c, Rv0849, Rv1250, Rv1877, Rv2333c, Rv2459, Rv2994, and Rv3239c failed to confer significant levels of resistance to a panel of drugs. Surprisingly, expression of the Rv2686c-Rv2687c-Rv2688c operon, not included in the list mentioned above, conferred resistance to fluoroquinolones in *M. tuberculosis* (Pasca *et al.*, 2004). It is important to mention that a member of this list, Rv1410c (also known as P55 (Silva *et al.*, 2001)), confers resistance to gentamicin, tetracyclin and streptomycin when over-expressed and that its function is abrogated by the addition of the efflux pump inhibitors verapamil and reserpine. In agreement with the proposed role,

work carried out by Ramon-García *et al.* showed that the deletion of Rv1410c caused increased susceptibility to rifampicin, novobiocin, vancomycin and econazole (Ramon-Garcia *et al.*, 2009). Importantly, those authors found out that the *M. tuberculosis* P55 knock-out mutant became more susceptible to oxidative stress and failed to form normal size colonies, stressing the key role of this protein in the proper assembly of the cell envelope. These results were very recently confirmed by Bianco *et al.*, who demonstrated that the LprG-P55 operon is required for proper cell wall assembly (Bianco *et al.*, 2011).

Intriguingly, only one gene (*mmr*) belonging to the SMR (Small Multidrug Resistance Family) family and related to drug efflux (in this case Erythromycin) has been located in the *M. tuberculosis* chromosome (De Rossi *et al.*, 1998).

Finally, to add more complexity to the already intricate scenario, Stephan *et al.* reported that the loss of MspA, a major porin of *M. smegmatis* determined an increase in the resistance to large antibiotic molecules such as rifampicin, vancomycin and erythromycin, results that support the hypothesis that the loss of this porin reduces the permeability of the mycobacterial cell envelope (Stephan *et al.*, 2004).

Thus, although still partial, the knowledge gathered over the last years on the mycobacterial efflux systems points out that even being accessories to the main mechanisms of resistance, efflux pumps play a role in resistance to anti-tubercular drugs, several of those pumps are inhibited by reserpine and/or verapamil, drugs with an extensive history of use in human patients. With that information, novel strategies based on using the mentioned inhibitors associated to anti-tubercular drugs would have been a logic continuation at the level of basic research as well as of clinical trials. Thus, it is surprising that no studies on this topic were carried out until very recently when a paper by Ramakrishnan's group pointed out that the mycobacterial efflux pumps are responsible in part for the drug tolerance in a zebrafish model of infection (Adams *et al.*, 2011). One of such efflux pumps, encoded by the gene Rv1258c, is induced upon macrophage infection, leading to a RIF tolerance phenotype. A mutant strain carrying a transposon insertion in that gene displayed susceptibility to RIF in a macrophage infection model. Verapamil and reserpine are anti-hypertensive drugs that can destroy the activity of mammalian and bacterial efflux pumps, an unexpected side effect (so far without a clear mechanism of action) to their usual clinical use. The treatment of *M. tuberculosis*-infected macrophages with reserpine abolished the tolerance to RIF (Adams *et al.*, 2011). Thus even when not every *M. tuberculosis* efflux pump system is inhibited by reserpine or by verapamil, the use of those compounds and/or any other new inhibitor of efflux pumps may decrease the tolerance to drugs, and shorten the treatment. Although more work is required to study drug-drug interactions and determine the optimal dosage of these anti-hypertensive drugs, it seems to be a promising and fresh starting point.

5.2 Azoles, antifungal drugs with a taste for tuberculosis

The sequencing of the genome of *M. tuberculosis* H37Rv was source of numerous surprises: one of which was the identification of a set of 20 genes encoding Cytochrome P450 (CytP450) dehydrogenases (Cole *et al.*, 1998). This large number is not exclusive of *M. tuberculosis* as there is a comparable number both in related slow growers such as *M. bovis*, as well as in the saprophytic fast-grower *M. smegmatis* that encodes 26 CytP450 genes. A similar collection is present in non mycobacterial Actinomycetes such as *Streptomyces*;

interestingly, *M. leprae*, the pathogenic mycobacterium that excelled in genome decay, has only one *cyp* gene (Cole *et al.*, 2001). Bioinformatic analysis also showed the presence of four genes (CYP121A1, CYP128A1, CYP141A1, CYP135A1) that seem to be unique to the *M. tuberculosis* complex.

The function of these enzymes is complex and versatile. The typical P450 reaction is a mono-oxygenation in which one of the oxygen atoms of molecular oxygen is inserted into an organic substrate while the second oxygen atom undergoes reduction to water. In spite of that, there are other P450-catalyzed reactions, including heteroatom oxidation and epoxidation. The observation that the *M. tuberculosis* was unusually rich in genes encoding enzymes that would be involved in fatty acid modification and degradation, coexisting with the large repertoire of CYP450 dehydrogenases led researchers to hypothesize that at least some of these would be involved in fatty acid metabolism. However, only one, CYP51B1, could be classified considering its important sequence homology to eukaryotic CYP51 enzymes as well as because of its sterol 14 α -demethylase catalytic activity. Curiously, excepting CYP135A1 and CYP135B1, which show 40% identity, the remaining *M. tuberculosis* P450 enzymes display much less similarity (around 30%). In agreement with the idea explicated above, several of the *M. tuberculosis* P450 enzymes have similarities with isoprenoid and fatty acid hydroxylases although functional assays must be performed to confirm the bioinformatics analysis.

A genomic approach based in the analysis of transposon insertion sites (TRASH) suggested that only one gene (*cyp128A1*) was essential for “in vitro growth” (Sasseti *et al.*, 2003), result that was not confirmed by a second independent analysis (Lamichhane *et al.*, 2003). However, evaluation of the *M. tuberculosis* transposon mutants able to replicate in a mouse infection model picked *cyp125A1* as the only *cyp* gene needed for a successful mycobacterial propagation (Sasseti *et al.*, 2001). Although *cyp121A* was placed in a list of essential genes by specific gene deletion these results were opposed by reports indicating that several clinical isolates were mutated in *cyp121A* (Tsolaki *et al.*, 2004). The controversy on the results obtained through those approaches raised again when it was reported that none of the *cyp* genes was essential for growth inside macrophages. Nonetheless, transposon insertions in six *cyp* genes (*cyp121A1*, *cyp123A1*, *cyp125A1*, *cyp127A1*, *cyp128A1* and *cyp137A1*) have variable impact on mycobacterial attenuation. Unfortunately, the substrates for these enzymes have been identified in very few cases; in example, the analysis of CYP121A revealed that it intervenes in the synthesis of a L-tyrosine-L-tyrosine cyclic dipeptide of unknown function. A second case is CYP51B, that shows homology to and activity of 14 α -sterol demethylases, an intriguing observation considering that *M. tuberculosis* does not have a complete sterol biosynthetic pathway in which those enzymes are found. Both CYP125A and CYP128A1 have also been associated to mycobacterial metabolic processes, the first one taking part in the degradation of host cholesterol used by *M. tuberculosis* during infection, and the second one hydroxylating an isoprene unit in the synthesis of a mycobacterial sulfolipid (Rengarajan *et al.*, 2005; Holsclaw *et al.*, 2008). Again, opposing results have been produced, as this sulfolipid is critical for virulence but not for “in vitro” growth in spite of results from TRASH experiments that indicated an essential role for growth under laboratory conditions (Sasseti *et al.*, 2003).

Less characterized, other CYPs such as CYP123A1 seem to be under the control of the PhoP-PhoR operon, a two component system which is strongly involved in virulence to the point

that a mutation in it is the basis of the lack of virulence of *M. tuberculosis* strain H37Ra. The remaining CYPs have not been studied to a point at which significant conclusions may be drawn. Most of the inferences come from analysis of gene location and the nature of nearby genes, evaluation of essentiality by TRASH analysis (although for some genes these results did not match the ones produced by specific gene knock-out), distribution through the mycobacterial genus and other actinomycetes, and biochemical studies.

Regardless of the level of information on mycobacterial CYPs, there is interest on them after the report that azoles, drugs with anti-fungal activity and decades of clinical use, are active on *M. tuberculosis* and other mycobacteria. During analysis of drug able targets in *M. tuberculosis*, six of the top eight genes picked up were the P450 enzymes CYP123A1, CYP124A1, CYP125A1, CYP130A1, CYP140A1 and CYP142A1; moreover, CYP126A1, CYP128A1 and CYP51B1, were placed within the top candidate enzymes, thus nine out of 20 *M. tuberculosis* Cyt P450 enzymes were positioned at the top of a list of drug targets (Aguero *et al.*, 2008). It is reasonable to expect that there will be a great deal of research on this subject.

As was mentioned above, the discovery of CYP51B1, a *M. tuberculosis* enzyme homologous to the fungal sterol 14 α -demethylase led to the tempting hypothesis that antifungal azole compounds might also target CYP51B1 and other P450 enzymes with lethal effects for the pathogen. In a short time, the anti-mycobacterial activity on *M. tuberculosis* was demonstrated on “in vitro”, “ex-vivo” and “in vivo” assays (Ahmad *et al.*, 2005; Ahmad *et al.*, 2006 a,b,c) (3-6). Moreover, azoles were active on *M. smegmatis*, with loss of glycopeptidolipid (GPLs) biosynthesis (Burguiere *et al.*, 2005). Unfortunately there are several inconsistent issues in those results: in first place, GPLs are not essential components of *M. smegmatis* as knock-out of the pathway render viable cells with an altered envelope (Deshayes *et al.*, 2010). In second place, GPLs are not present in *M. tuberculosis* suggesting different target(s); in last place Fluconazole, an azole with the highest binding to CYP121A1 is inactive on *M. tuberculosis* although it is very active on fungi. More conflicting data suggesting target(s) other than CYPs for azole drugs stem from microarray analysis comparing a wild-type strain to a bifonazole-resistant strain; the study showed no induction of any CYP gene upon treatment with bifonazole (Milano *et al.*, 2009). Instead, three genes (Rv0678, Rv0677c and Rv0676c), showed higher levels of expression in the Bifonazole mutant compared to the wild-type strain. Not surprisingly, Rv0677c and Rv0676c encode the membrane proteins MmpS5 and MmpL5, predicted to be RND family of transporters, and therefore, very likely involved in mediating resistance to azoles by efflux of the drug (Milano *et al.*, 2009). In agreement with those results, Milano *et al.* confirmed the involvement of efflux pumps in the resistance to azoles by selecting and sequencing azole-resistant mutants of *M. tuberculosis* and *M. bovis* var BCG; their results proved that over-expression of *mmpS5-mmpL5* were responsible for the resistance phenotype. This pump was susceptible to CCCP, as this treatment reduced the resistance to azole drugs back to wild-type levels. Moreover, sequencing of *cyp* encoding genes failed to show any mutation in a *M. bovis* var BCG mutant resistant to the azole drug bifonazole. In summary, there is no proof of a direct link between azole drugs and the inhibition of CYPs as a mechanism of action. Nonetheless, it is possible that additive inhibition of those non-essential CYP targets may bring the accumulation of growth inhibitory intermediates and /or depletion of cellular metabolites of importance.

Although azoles have the great advantage of a large body of information in humans, they also have the disadvantage of their low oral bioavailability, generating a proposal of their use in encapsulated form. They also have a noticeable impact on human metabolism through the inhibition of the liver P450 enzymes, thus drug-drug interactions and changes in pharmacokinetics and metabolization of drugs are expected.

In summary, having a relatively high number of azole compounds with therapeutic use in humans, the fact that there is no known target(s) and thus mechanism(s) of action for those drugs, dims the initially bright possibility of adding them to the anti-tubercular drug portfolio. The simplicity and relative low cost of the currently existing whole genome sequencing techniques should be used to address those points by analyzing azole-resistant *M. tuberculosis* mutants, most likely selecting them in the presence of efflux pump inhibitors.

5.3 The thioridazine story (or how perseverance is also an essential tool for anti-tubercular drug discovery)

In the quest for novel drugs with improved killing activity against *M. tuberculosis*, a number of non-antibiotic molecules have been tested; among them, some compounds that displayed a surprisingly high killing activity belonged to the family of anti-psychotic drugs. Original work carried out by Ehrlich at the end of the 19th. century led to the discovery of the anti-bacterial and neuroleptic activities of methylene blue, a phenothiazine; later on, its activity on the central nervous system was privileged, leading to the synthesis of chlorpromazine as reviewed by Kristiansen, 1989. Paradoxically, anti-bacterial activity of this compound was again proven over the following years but left aside due to the large number of antibiotic options that were marketed at that time as well as due to the strong toxic side effects displayed in large treatments. Although the introduction of the less toxic compounds thioridazine and promazine decreased the toxicity problem, there was no interest in applying these kind of molecules to the treatment of bacterial infections. The emergence of multi-drug resistant *M. tuberculosis* strains triggered an urgent search for new compounds that could kill those strains efficiently. Thus, research on phenothiazines was embraced by a few investigators that were convinced of the possible uses of that family of drugs (Amaral and Kristiansen, 2000; Kristiansen and Amaral, 1997; Viveiros and Amaral, 2001; Amaral *et al.*, 2004). Even when the concentration of phenothiazines needed to kill *M. tuberculosis* “in vitro” were several times higher than the one reached in plasma of patients (20 µg/ml vs 0.4 µg/ml), the observation that these compounds were concentrated by macrophages, suggested that a balance could be obtained between the intracellular concentration reached and the concentration required to destroy *M. tuberculosis* phagocytosed by the macrophages (Crowle *et al.*, 1992; Ordway *et al.*, 2003). It was indeed so, as proven by Crowle’s group, and that helped to potentiate the research of a few groups that enthusiastically showed thioridazine as the phenothiazine with the highest killing effect and the lowest toxicity (Amaral *et al.*, 1996, 2008; Viveiros *et al.*, 2005; Crowle *et al.*, 1992). Moreover, it was clearly demonstrated that this compound was active on MDR-TB and XDR-TB residing inside the macrophages, needing to be present at such a low concentration (0.1µg/ml) that it was devoid of toxicity. The obvious disadvantage of the requirement for a much higher concentration of thioridazine to kill extracellular *M. tuberculosis* when lung damage is produced may be easily compensated by the use of other drugs much more active on those

extracellular bacilli. Even when the infecting strain is a MDR-TB or a XDR-TB strain, thioridazine may be teamed up with some of the novel drugs described in previous sections, drugs that either overcome the resistance mechanisms or target totally new targets and pathways. It is highly promising that Martins *et al.* has published an article describing the action of several derivatives of thioridazine showing extreme ability to kill intracellular *M. tuberculosis* at low dose and needing only one day to do so, contrasting with the three days required for the same level of action by thioridazine (Martins *et al.*, 2007). Almost simultaneously, Bate *et al.* described novel synthetic derivatives of promethazine and promazine; those compounds were not only effective on actively growing *M. tuberculosis* but also on latent *M. tuberculosis* (Bate *et al.*, 2007).

In terms of the identification of the molecular mechanisms of action and mycobacterial components targeted by phenothiazines, it has been shown time ago, that these compounds can inhibit efflux pumps at concentrations lower than those required to inhibit mycobacterial growth (Amaral *et al.*, 2007, 2008, 2010); one of such inhibited efflux mechanisms may lead to the build up of calcium and potassium ions in the phagosome, reverting the mycobacterial driven block to the action of hydrolyases and other calcium-dependent macrophage mechanisms which in turn may destroy the bacilli (Martins *et al.*, 2008, 2009)). Rubin's group demonstrated the inhibition of the type II NADH-menaquinone oxidoreductase (NDH-2), an essential enzyme of the *M. tuberculosis* respiratory chain, by thioridazine and derivatives; its inhibition leads to a blockade in the electron chain transport, thus it is most likely the most important target for these compounds (Weinstein *et al.*, 2005). Ndh-2 is the only NADH dehydrogenase enzyme expressed in this pathogen, importantly it is absent in humans that rely on the type 1 Ndh enzyme. Biochemical, transcriptional and genetic analysis supports the vital role played by Ndh-2 (Yano *et al.*, 2006). At the light of the published information regarding the multiple targets for thioridazine, the synthesis of less toxic derivatives and the fact that these compounds are concentrated in the macrophages, it is reasonable to consider these molecules as promising compounds that would become not only drugs by themselves but helpers to other drugs due to the inhibition of the mycobacterial efflux pumps.

As has been stated by Amaral *et al.*, early work from Kristiansen (Kristiansen and Amaral, 1997) postulated that neuroleptics such as thioridazine and chlorpromazine, displayed antibacterial activity by affecting an unknown cell membrane process. Kristiansen went even further coining in 1990 the term "non-antibiotic" to define "medicinal compounds that are used for the treatment of non-infectious pathologies and which also have anti-microbial activity". With tremendous perseverance, Amaral and co-workers have been supporting neuroleptics as drugs that may be used for compassionate reasons in cases of human tuberculosis that are of bad prognosis and difficult treatment due to drug resistance. These researchers insisted for more than ten years that the neuroleptics described above had enough activity *per se* and as "helpers" to be included in the clinical treatment of tuberculosis (Amaral *et al.*, 2004, 2007a, 2007b, 2008, 2010, Martins *et al.*, 2007a,b, 2008; 2009; van Ingen *et al.*, 2009, Viveiros and Amaral, 2001; Viveiros *et al.*, 2003, 2005, 2010). Notwithstanding the body of evidence gathered by them, there has been little receptiveness by the pharmaceutical industry and public health organisms, leading Amaral to put out his frustration through a paper bearing a very challenging title: "Thioridazine cures extensively drug-resistant tuberculosis (XDR-TB) and the need for global trials is now!" (Amaral *et al.*,

2010). Furthermore, their latest work in association with the groups of Dick van Soolingen and Rogelio Hernandez- Pando demonstrated the effectiveness of thioridazine in a mouse model of multi-drug resistant *M. tuberculosis* infection (van Soolingen *et al.*, 2010). Since as has been put forward by Amaral and co-workers, thioridazine and similar molecules already in the market may be described as antipsychotic drugs that are still protected by patents as “new use” (Amaral and Molnar, 2010; Dutta *et al.*, 2011), there are good chances that the actors involved at both public health organizations and private partners will take this matter to a step where these neuroleptics are tested throughout the world.

6. The choice: Broad screening of chemical libraries or rational design based on molecular targets

Along the sections of this chapter I have briefly described the mechanisms of action of several drugs that inhibit growth or kill *M. tuberculosis*. Two opposite approaches for the goal of obtaining new anti-tubercular medicines are based a- on the biological screening of large size chemical libraries (Maddry *et al.*, 2009; Anathan *et al.*, 2009) and b- on structure based design by means of molecular modelling of chemical compounds on the structure of the enzyme under study (Arcus *et al.*, 2006). In between these two options, a third one is to produce derivatives of compounds with known activity and mechanisms of action (such as ISO) but without having structural information of the target enzyme. From the specific anti-tubercular compounds currently used in clinical practice, INH, ETH and PZA have been used as scaffolds for rational drug design. On the basis of the understanding of the nature of the lethal event, different approaches were taken towards that end; i.e. through an increased conversion of the pro-drug to the active drug (ETH boosters), through the inhibition of the identified target by a different molecule not requiring activation (aryl alkyl ethers inhibitors of InhA) or modification of the lead compound (PZA and 5-Cl PZA). The identification of essential mycobacterial enzymes and their intensive characterization at the biochemical and structural level led to propose compounds with activity in the case of cell division (FtsZ) and fatty acid biosynthesis (AccD6). Notwithstanding that, all the compounds that are under phase I and II clinical testing (PA-824, TMC207, LL3858, SQ109 and OPC-67683) have been identified by broad screening (Spiegelman, 2007), although in the case of one of them, OPC-67683, the search was oriented to compounds with a defined mode of action, the inhibition of synthesis of mycolic acid.

So, although logic should have tip the balance towards the utilization of structure-based methods, there is a bias towards screening of libraries of chemical compounds by high throughput methods looking for whole-cell or “in vitro” enzyme inhibition.

7. Summary

There are several reviews in the literature that describe the mechanisms of action of anti-tubercular drugs currently in clinical use, and also a large number of publications summarizing the quest for new drugs and the nature of the novel compounds that may be added to the anti-tubercular treatment in short time. The objective of this review is different from that, and although I have offered a brief account of the recent developments in the field, commenting on the two main approaches (broad screening *vs* structure-based design) for anti-tubercular drug development, I have chosen to focus on few compounds to

demonstrate that there are drugs already produced at pharmaceutical industry levels (namely azole drugs, verapamil, reserpine, and thioridazine) with a large history of use in humans –therefore providing rich information on adverse effects, pharmacokinetics, pharmacology, etc- which are effective on *M. tuberculosis*, not only on pan-susceptible strains but also on MDR-TB as well as on XDR-TB strains; moreover, the effect of some of those drugs reaches as far as the latent population of this pathogen. The fact that the mentioned compounds have already been approved for human use by regulatory agencies shortens the time for the evaluation of their new uses as many aspects have already been addressed. Thus we have a unique opportunity to seize, concentrating effort at academic laboratories to increase our understanding on mechanisms of action of these compounds as well as learning about the ensuing mechanisms of resistance in mycobacteria, testing drug –drug interactions and generating the comprehensive body of knowledge needed to incorporate these drugs to the anti-tubercular portfolio. On a personal basis, I strongly believe that coordinated effort on those compounds by research groups may produce at last the addition of the mentioned drugs to clinical treatment, helping to stop the spreading of human tuberculosis. It is possible that by taking a bold decision on those issues, we will reach our El Dorado: new drugs to defeat tuberculosis.

8. Acknowledgements

HRM is a career member of Consejo de Investigaciones, UNR, Argentina. This work was funded by grants from ANPCyT and UNR.

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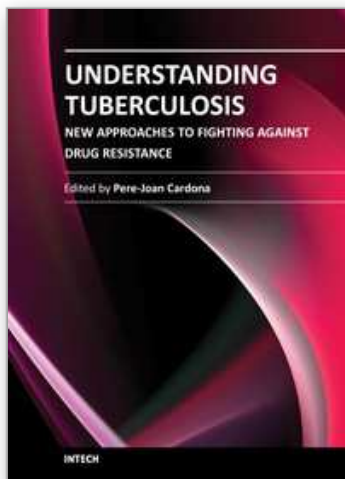
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Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-948-6

Hard cover, 376 pages

Publisher InTech

Published online 15, February, 2012

Published in print edition February, 2012

In 1957, a *Streptomyces* strain, the ME/83 (*S. mediterranei*), was isolated in the Lepetit Research Laboratories from a soil sample collected at a pine arboretum near Saint Raphael, France. This drug was the base for the chemotherapy with Streptomycin. The euphoria generated by the success of this regimen led to the idea that TB eradication would be possible by the year 2000. Thus, any further drug development against TB was stopped. Unfortunately, the lack of an accurate administration of these drugs originated the irruption of the drug resistance in *Mycobacterium tuberculosis*. Once the global emergency was declared in 1993, seeking out new drugs became urgent. In this book, diverse authors focus on the development and the activity of the new drug families.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Héctor R. Morbidoni (2012). In Search of El Dorado: Current Trends and Strategies in the Development of Novel Anti-Tubercular Drugs, *Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance*, Dr. Pere-Joan Cardona (Ed.), ISBN: 978-953-307-948-6, InTech, Available from: <http://www.intechopen.com/books/understanding-tuberculosis-new-approaches-to-fighting-against-drug-resistance/in-search-of-eldorado-current-trends-and-strategies-in-the-development-of-novel-anti-tubercular-drug>

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