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An Approach to the Search for New Drugs Against Tuberculosis

Fernando R. Pavan[1,*], Daisy N. Sato[1] and Clarice Q.F. Leite[1,*]
[1]*School of Pharmacy, São Paulo State University, Araraquara, SP, Brazil*

1. Introduction

The history of *Mycobacterium tuberculosis* (MTB) as the main agent of tuberculosis (TB) goes back a very long time. Fragments of the spinal column of Egyptian mummies from 2400 BCE (Before Common Era) show definite pathological signs of tubercular decay (Tripathi, Tewari *et al.*, 2005). From that time to the present day millions have died of the disease and only a few drugs are active against the bacilli. Current data show an ancient disease more prevalent in the world today than at any other time in human history (Koul, Arnoult *et al.*, 2011). Annually, TB is responsible for the death of two to three million people worldwide and global economic losses around $ 12 billion (Ma, Lienhardt *et al.*, 2010). Approximately a third of the world's population is estimated to be infected with MTB, giving rise to 9.4 million new cases of active TB disease each year (Zwerling, Behr *et al.*, 2011). In addition, the highly resistant Multi-Drug-resistant (MDR) and Extensively-Drug-Resistant (XDR) MTB organisms are virtually untreatable in immunocompetent patients. So when these bacteria enter into contact with highly immunocompromised HIV-infected populations, the mortality rate reaches 100% within a few weeks of infection (Gandhi, Nunn *et al.*, 2010).

No new specific drug against MTB has been developed since 1960 and the emergence of MDR-TB and XDR-TB highlights the ineffectiveness of current treatment (Lalloo e Ambaram, 2010). An effective new drug against TB could: (i) reduce the duration of the treatment, (ii) be active against resistant strains, (iii) not interfere with antiretroviral drugs and (iv) be active against latent bacilli (Ma, Lienhardt *et al.*, 2010). According to Koul et al (2011), the key questions are: how vigorous are current strategies to discover new TB drugs and what measures could be taken to shorten the protracted clinical development of new drugs (Koul, Arnoult *et al.*, 2011).

In this chapter we describe a successfully working pipeline being developed at the "Dr. Hugo David" Laboratory, which is based on the responses that a new drug has to provide, ordered into a sequence of lead-optimization stages that go from initial screening hits to the final *in vitro* and *in vivo* preclinical assays of candidate drugs.

2. Rational pipeline: Phenotypic or genotypic?

The lack of new anti-TB drugs since 1960, when rifampicin (RMP) was added to the therapeutic together with the failure of current treatment against MDR and XDR-TB and

* Corresponding authors

some new public and private investments, have caused an explosion and awakening of many research groups, to develop new strategies to identify new drugs against TB. This research follows two different paths, phenotypic and genotypic, both concerned with the discovery of new drugs and the development of new rapid, inexpensive and reproducible assays.

Owing to the availability of the genome sequence of MTB (Cole, Brosch *et al.*, 1998), the pharmaceutical companies and research institutions have been driven to employ target-based high-throughput assays for the identification of candidate TB drugs. However, the genome- derived target-based approaches have had little success in the antibacterial therapeutic area in general (Payne, Gwynn *et al.*, 2007; Koul, Arnoult *et al.*, 2011). One of the most important targets investigated in the search for new drugs against TB is the group of isocitrate lyases. These enzymes are the key to the glyoxylate-shunt pathway and are essential to the intracellular growth of mycobacteria and their long-term persistence in mice. However, the several high-throughput screening campaigns launched to identify inhibitors of isocitrate lyases were discontinued, owing to their lack of druggability (WGND, 2011). Even when a good bacterial enzyme inhibitor is found, the question of whether it can be converted into a compound that can easily penetrate the highly impermeable mycobacterial cell wall still has to be answered.

With regard to phenotypic assays, at first, new drugs were discovered by randomly testing compounds on whole cells. This strategy identified all the compounds currently employed in first-line therapy, namely, RMP, isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) (Handbook of anti-tuberculosis agents, 2008).

Nowadays, five drugs are undergoing clinical trials: SQ-109 (Sequella Inc.), OPC-67583 (Otsuka Pharmaceutical Company), LL3858 (Lupin Ltd), TMC 207 (Johnson & Johnson) and PA-824 (TB Alliance and Novartis) (Lalloo e Ambaram, 2010; Lienhardt, Vernon *et al.*, 2010) were identified by the whole-cell-screening approach, suggesting that the screening strategy based on whole cells is much more successful than targeting single enzymes (Koul, Arnoult *et al.*, 2011).

3. "Hugo David" research laboratory pipeline

The main control and research organizations on neglected diseases in the world (Global Alliance, National Institutes of Health and World Health Organization) suggest several whole-cell biological assays involving *in vitro* and *in vivo* approaches to explore all the features that the new drug must possess, in order to control the disease (Orme, Secrist *et al.*, 2001; Ma, Lienhardt *et al.*, 2010; Koul, Arnoult *et al.*, 2011). On the basis of those suggestions, for more than 10 years, the Mycobacteriology Laboratory of the School of Pharmaceutical Sciences, State University of São Paulo (UNESP), in association with many chemical laboratories around the world, has been seeking new drugs from natural products or synthetic compounds, both organic and inorganic (Do Nascimento, Von Poelhsitz *et al.*, 2008; Higuchi, Pavan *et al.*, 2008; Maia, Pavan *et al.*, 2009; Moro, Mauro *et al.*, 2009; Pavan, Leite *et al.*, 2009; Pavan, Sato *et al.*, 2009; Pavan, 2009; Santos, Yamasaki *et al.*, 2009; Silva, Martins *et al.*, 2009; Carli, Quilles *et al.*, 2010; Honda, Pavan *et al.*, 2010; Maia, Graminha *et al.*, 2010; Pavan, Maia *et al.*, 2010; Pavan, Von Poelhsitz *et al.*, 2010; Tarallo, Urquiola *et al.*, 2010; Miyata, Pavan *et al.*, 2011). While screening over 2,000 compounds, we discovered

promising candidates ("hits") based on MIC values and other biological assays were added to select the lead compounds. After years of investigation, it was possible to assemble a rapid and inexpensive pipeline, to organize the search for new drugs against TB, from screening to the *in vivo* preclinical phase. The pipeline developed in parallel with the promising results for the lead compounds and out of a need to understand better their biological characteristics. By application of this pipeline, we now have some very promising compounds containing ruthenium complexes that exhibit a MIC (Minimum Inhibitory Concentration) comparable to or better than first-line drugs, low cytotoxicity and a selectivity index (SI) much higher than 10 (Do Nascimento, Von Poelhsitz *et al.*, 2008; Pavan, Von Poelhsitz *et al.*, 2010).

The rational phenotypic pipeline mounted in this laboratory is outlined in the flowchart in **Figure 1**. This pipeline is divided into 3 stages: Screening, *in vitro* preclinical and *in vivo* preclinical.

Screening: First of all, the MIC of the putative drug compound against MTB H_{37}Rv ATCC 27294 is estimated by the Resazurin Microtiter Assay (REMA) (Palomino, Martin *et al.*, 2002). Compounds with anti-MTB activity at ≤ 10 μg/mL (or molar equivalent) are selected for the next step, which is to test their cytotoxicity (IC_{50}) on J774A.1 (ATCC TIB67) macrophage cells, HepG2 (ATCC HB 8065) hepatic cells and VERO (ATCC CCL81) normal cells, as described by Pavan et al. (2010) (Pavan, Maia *et al.*, 2010). The last screening step is to evaluate the therapeutic safety of the compound for *in vivo* assays, by calculating the SI, which is the ratio of IC_{50} to MIC (Orme, Secrist *et al.*, 2001). Compounds with SI ≥ 10 are selected for the next stage.

In vitro **Pre-Clinical Stage:** At this stage, the first step is to test if the compounds can act on recombinant MTB Erdman (ATCC 35801) bacteria containing the luciferase plasmid (pFCA-luxAB), inside infected J774A.1 macrophage cells (Snewin, Gares *et al.*, 1999; Pavan, 2009) (**Figure 2**). In the next step of the pipeline, the compounds are tested on clinical isolates with a phenotypic and genotypic profile of resistance to INH and RMP already verified (Miyata, Pavan *et al.*, 2011), by REMA (Palomino, Martin *et al.*, 2002). This assay allows possible cross resistance between the new compounds and the bacteria resistant to INH and RMP to be revealed, throwing indirect light on the mechanism of action or target of the new compounds. After these assays, interactions between the new compounds and the drugs used in current therapy are assessed. Nowadays, TB treatment is based on fixed combined doses of RMP, INH, EMB and PZA in the first two months and RMP and INH in the following four months (Conde, De Melo *et al.*, 2009). Any new drug must be used in combination with other drugs to avoid the appearance of resistant strains. In line with these concepts, the interactions between the new compounds and the current anti-TB drugs are assessed through the 2D Checkerboard experimental design (Moody, 1992; Luna-Herrera, Costa *et al.*, 2007) (**Figure 3**). Next, the compounds are tested for their ability to inhibit dormant bacilli. The Wayne Model (Wayne, 1994) is used to induce the latency stage of the recombinant MTB H_{37}Rv (ATCC 2794) (pFCA-luxAB) and the Low Oxygen Recovery Assay (LORA) to determine the activity of the new compounds against dormant bacilli (Cho, Warit *et al.*, 2007). This assay is important for the discovery of new drugs to use in the control and eradication of TB, owing to the ability of MTB to remain dormant in the individual for months or years in a state of latency (Wayne, 1994; Ma, Lienhardt *et al.*, 2010). Approximately one-third of the world's population is estimated to be infected with latent

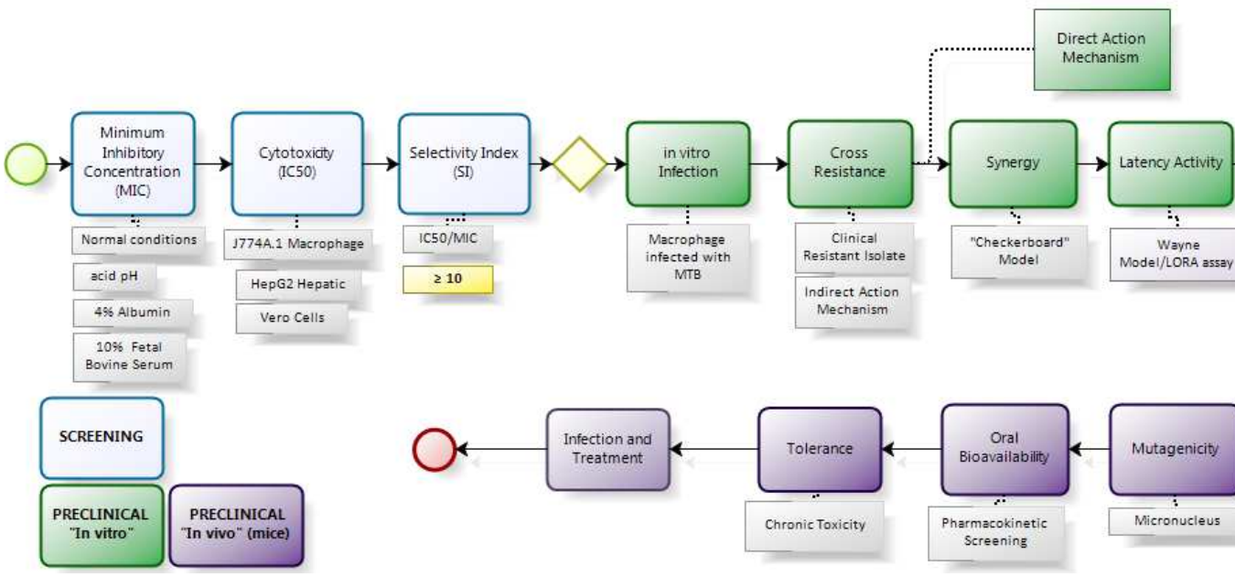

Fig. 1. Pipeline to select new drugs against TB created at the "Hugo David" Laboratory, FCFAR/UN

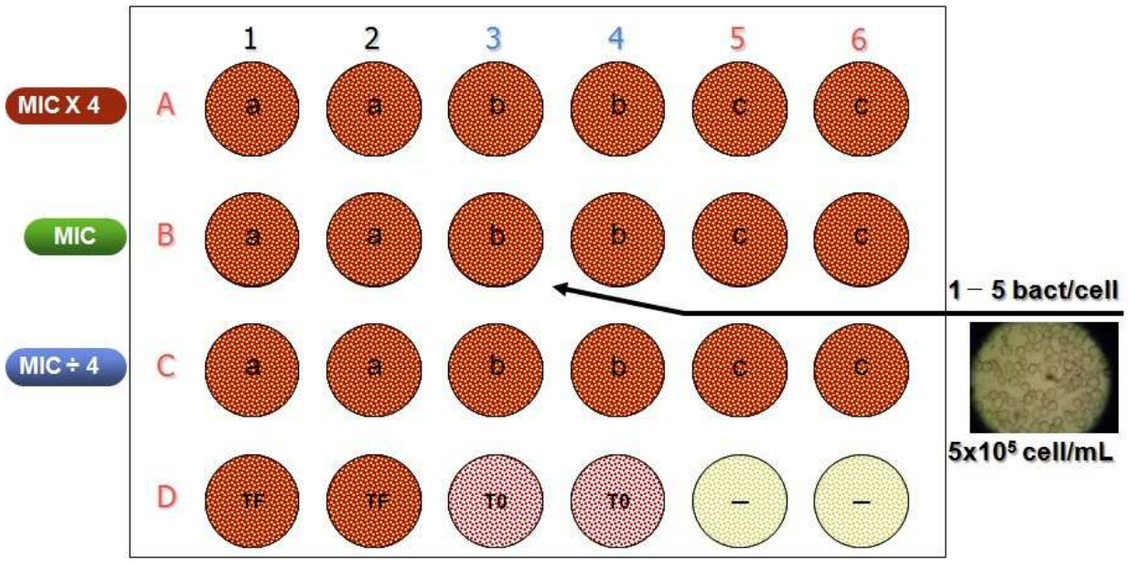

Fig. 2. *In vitro* **infection model:** a, b, c – Different compounds tested in duplicate and at three different concentrations on cells infected with MTB (ATCC 27294) containing the luciferase plasmid (pFCA-luxAB); T0 – Initial Control: After the macrophage infection, the cells were lysed to confirm the infection; TF – Final Control: Cells only infected with MTB; Negative sign: Only uninfected cells.

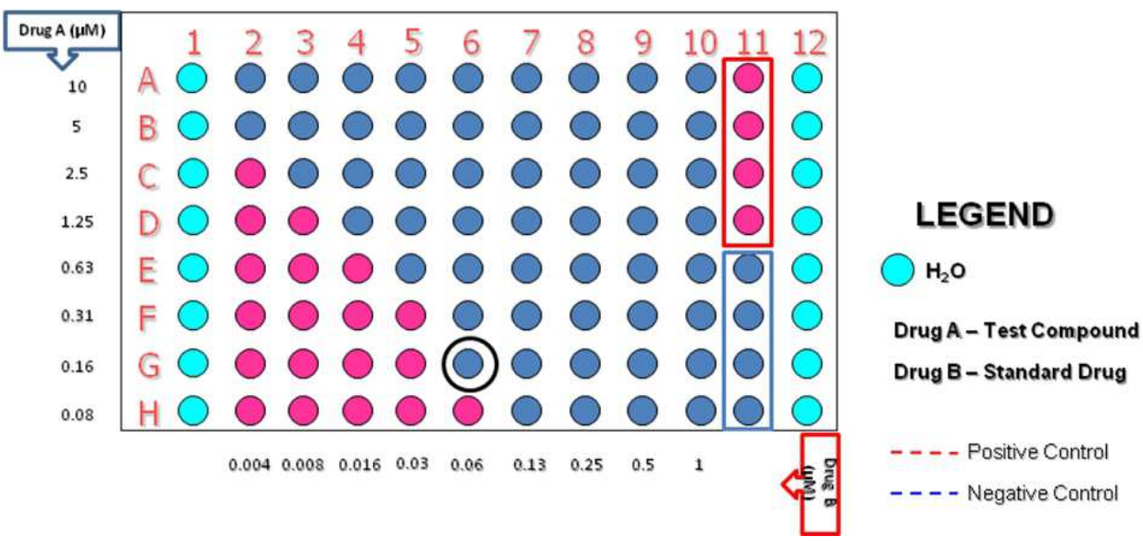

Fig. 3. **2D Checkerboard:** FIC index = (MIC [A] + MIC [B]) combination/(MIC [A] + MIC [B]) alone = ≤ 0.75 synergy; 0.75-4 no interaction or > 4 antagonism. Black circle, example of lower FIC. Resazurin assay: pink=growth, blue=no growth.

bacteria and there are no active drugs against these dormant bacteria. Following this, the spectra of activity of new compounds against various microorganisms: *Staphylococcus aureus* (as a gram-positive organism), *Escherichia coli* (as a gram-negative), *Candida albicans* (as a fungus) and other mycobacterial species (*M. avium* and *M. smegmatis*) are assessed (Moody, 1992). The last step in this stage is to investigate the *in vitro* mutagenic capacity of the compounds via the AMES test (Maron e Ames, 1983). This assay is important as it indicates whether the compound might act as a carcinogen. At the same time the direct mechanism or target of the compounds will be investigated by the genotypic Representational Difference Analysis method (Lisitsyn e Wigler, 1993; Pastorian, Hawel *et al.*, 2000). This assay identifies the genes that are differentially expressed after exposure of MTB to the compounds.

In vivo **Pre-Clinical Stage:** In the first *in vivo* test, the therapeutic safety margin is determined in accordance with the OECD (Organization for Economic Co-Operation Development) guideline, by finding the classical acute toxicity (LD_{50}) to mice of a single oral dose (gavage) of the compounds (OECD, 2001). Next, the *in vivo* mutagenic potential of the compounds should be assessed by the micronucleus assay (Hayashi, Morita *et al.*, 1990). If the result is satisfactory, the next step is to understand the pharmacokinetic properties of the compounds. To this end, the oral bioavailability of the compounds is determined (Gruppo, Johnson *et al.*, 2006) (**Figure 4**). This assay allows the quantity of the compound that can be

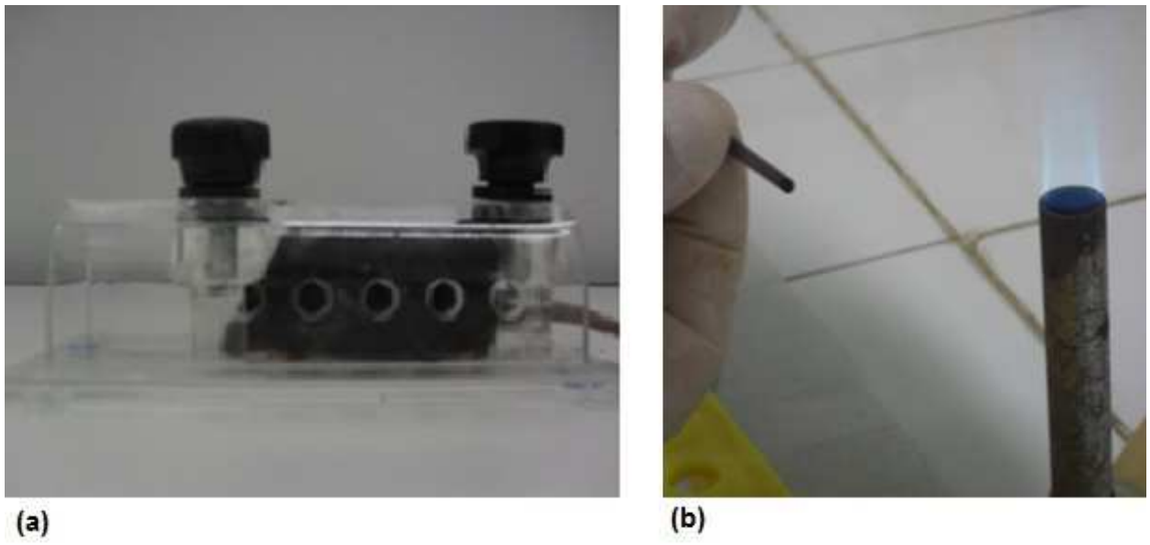

Fig. 4. **Oral bioavailability sample collection**: **(a)** C57BL/6 mouse held in a retainer to cut the tail and collect blood; **(b)** Blood dripping from the tail near the Bunsen burner to enable dilatation of vein and sterilize air.

absorbed into the body of the animal to be measured, as well as its permanence (time of degradation) during animal testing. Compounds that give favorable results are then tested for their safety (chronic toxicity) in animal models, noting the levels of tolerance when they are administered daily for a period at sublethal doses. Finally, the animals are infected intranasally with MTB Erdmann (ATCC 35801) (Pethe, Sequeira *et al.*, 2010) and, after confirmation of lung infection, they are subjected to oral treatment with the new compounds for two weeks and then tested for reduction of the bacterial load in the lungs by cultivation of the viable bacilli in the lungs (Falzari, Zhu *et al.*, 2005). This experiment would reveal the compounds that effectively reach the lungs and remain these long enough to act on MTB internalized in the alveolar macrophages.

4. Conclusion

Many groups are working towards the same goal, to find a new drug against TB, not only to serve the interests of the pharmaceutical industry, but also prioritizing the patient, remembering that the less privileged population is most affected by the disease. The question is how to discover new drugs. Here we have described a successfully implemented phenotypic pipeline evolved at the "Hugo David" Research Laboratory.

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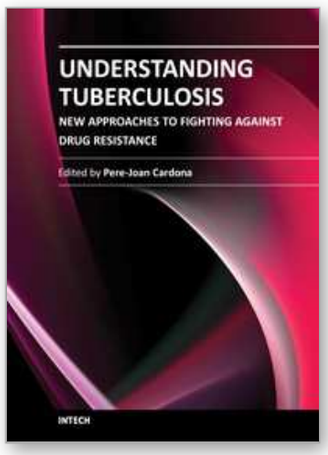

Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance

Edited by Dr. Pere-Joan Cardona

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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 Streptomicine. The euphoria generated by the success of this regimen lead 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.

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