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Antitubercular *In Vitro* Drug Discovery: Tools for Begin the Search

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1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* continues being a big public health problem around the world. The total number of cases of TB worldwide in 2009 was 9.4 million of which 1.8 million died of this disease, reported as the higher in history (Lawn & Zumla, 2011), World Health Organization (WHO) estimates that the one third of global population is infected latently by *M. tuberculosis* (LTBI), however 10% will develop active disease (Zumla et al., 2011). Although several strategies and programs have been implemented and anti TB drugs have been available for 50 years, many TB patients are not diagnosed and treated at time (Ghanashyam, 2011; Sosnik et al., 2010). These mismanaged patients, with non-optimal treatments are the principal source of multidrugresistant TB (MDR-TB), which is resistant to the first line drugs isoniazid and rifampicin, as well as extensive drug resistant TB (XDR-TB), that in addition of isoniazid and rifampicin is resistant to any fluoroquinolone and any aminoglycoside second line anti TB injectable drugs (Koul et al., 2011). Other aspect that aggravates the situation is the coinfection with Human Immunodeficiency Virus (HIV) disease, which increases the TB incidence rates three to five times and affected 1.1 million of TB cases in 2009 (Lawn & Zumla, 2011).

The most important control measures in TB are the prevention and chemotherapy. The current TB therapy has difficulties in controlling effectively the disease, due to inadequate adherence to treatment course caused by the length of time of medication and adverse reactions (Ginsberg & Spigelman, 2007). New antitubercular drugs should comply with following characteristics with the aim of reduce the low adherence that induce therapeutic failure and resistance: be active against MDR and XDR isolates, be active in less time to shorten the therapy, not interact with antiviral drugs, effectivity against latent TB infection, low toxicity and high bioavailability (Mitchinson & Fourie, 2010; Sosnik et al., 2010).

For those reasons the design of an antitubercular drug discovery initiative should have a strong *in vitro* screening program with the ability of optimize the current process and to identify in high degree chemical scaffolds with potent *in vivo* activity for clinical development. The aim of this chapter is offer different tools to perform a rational search for new anti TB drugs improving *in vitro* screening as a powerful source of selection of new compounds.

1.1 Antitubercular drug resistance versus the discovery and development of new antitubercular agents

The drug resistant TB (DR-TB) emergence and spread is a multifactorial problem produced by health mismanagement attention; inadequate therapy courses, antibiotic misuse, insufficient socioeconomic conditions, presence of immunodeficiency disorders and low patient compliance (Haydel, 2010). In addition, coinfection TB-VIH complicates the current treatment regimen because: decrease compliance and increase drug interactions producing toxic side effects (Koul et al., 2011). The need for more effective and less toxic anti TB drugs is really urgent, but the antibiotic drug discovery and development is a long and expensive process with very few compounds making it to the market (Vaddady et al., 2010). The current anti-TB drugs were developed since 1950s until 1980s which represented a missed period in TB drug research that contributed greatly to new challenges for improving treatments for DR-TB and prevent LTBI (Ginsberg, 2010). Actually, the biggest challenge for discover and develop a new era of TB medicines is prevention of drug resistance, which is necessary for treat the patients under ineffective therapeutic regimens (Ginsberg, 2010). Because of this, all efforts between sponsors, TB drug researchers, regulators and funders should be directed to the development of new and optimized portfolio of multidrug treatments.

1.2 Antitubercular *in vitro* drug discovery program design

In vitro experiments seeking to assess the interaction between the drug and the bacteria, which validates the selection of candidate compounds and the determination of the target drug concentrations for further testing (Vaddady et al., 2010). Is a fact that drug candidates fail in the stage of clinical development, in the Tuberculosis Antimicrobial Acquisition and Coordinating Facility Program (TAACF) were evaluated 88601 compounds and finally were selected five potential leads (Lenaerts et al., 2008), which is a high cost drug discovery program. An *in vitro* antitubercular drug screening strategy should consider and integrate several aspects as whole cell screening; single enzyme targets, toxicity testing and the inclusion of *in vitro* pharmacological tests for optimize the selection of promissory new drugs and predicts their clinical behaviour (Koul et al., 2011). In Fig. 1. is shown the design of an *in vitro* drug discovery program with the major phases looking for evaluate and select the largest possible number of novel antitubercular molecules.

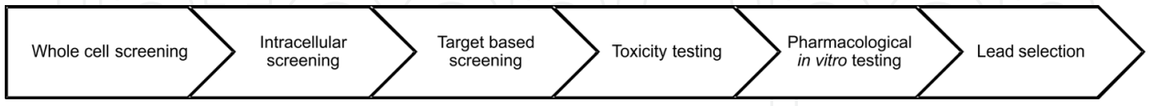

Fig. 1. *In vitro* anti TB drug discovery program components, each phase is an important step in the selection of promising anti TB drugs

2. Screening methods

In the 1950s, Canetti et al. described the first Drug Susceptibility Testing (DST) method for *M. tuberculosis*, which was a agar dilution method, involving the preparation of a concentration series of drugs against *M. tuberculosis* complex in Lowenstein-Jensen medium, inoculation of the bacterial cultures on the slants, and reading of the inhibition of growth by drugs at different concentrations (Canetti et al., 1963). The agar dilution tests permit to

determine the Minimum Inhibitory Concentration (MIC), however, none of its worked out modifications was repeatedly used over a longer period of time. Disadvantage is the high need of amounts of test compounds (20 mg/plate to test 1.000 mg/mL), which restricted its use to easily available test materials (Bueno & Kouznetsov, 2010). Although Canetti test is a reproducible method with high clinical correlation (ability to give a diagnosis consistent with the signs and symptoms), not comply with the rules of an ideal screening method, which must be very simple, robust, preferably homogeneous and amenable to miniaturization and automation (Sethala & Zhang, 2009), correct validation of initial screening assays guarantee the selection of molecules with bactericidal activity, using a template in a multiwell plate for *in vitro* screening as proposed in Fig.2. by Cos et al. (Cos et al., 2006), the vast majority of the following techniques have these characteristics.

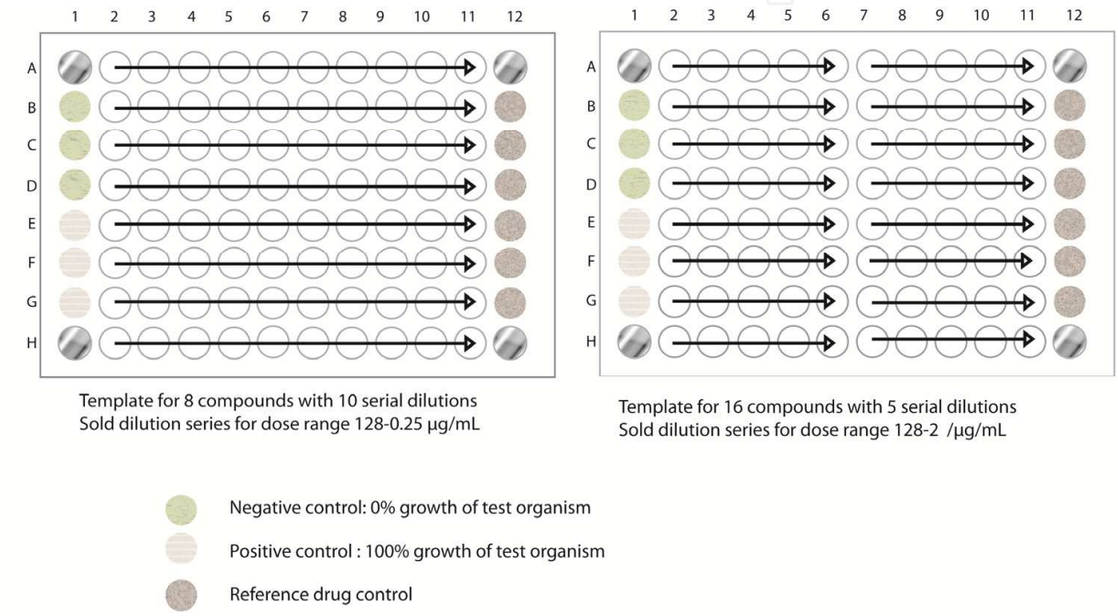

Fig. 2. *In vitro* screening template in a multiwell plate, the ideal concentrations for testing should be selected following the Food and Drug Administration requirements (Enna & Williams, 2007; Enna, 2001; Food and Drug Administration, 2009).

2.1 Colorimetric methods

A number of low-cost colorimetric DST assays using oxidation/reduction indicator dyes have been described, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 2,3,5-triphenyltetrazolium chloride (TTC), and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide (XTT) (Abate et al., 1998; De Logu et al., 2001; De Logu et al., 2003a, 2003b). MTT, XTT and TTC are tetrazolium salts that are reduced to purple formazan crystals in respiratory chain, with which, the growth/inhibition can be read visually; and the reduced form of these dyes can also be quantitated colorimetrically by measuring absorbance at 570 nm. However, these tests have limitations; several compounds can interfere with the formazan production in the assay and give rise to false-negative results and provide an underestimation of activity (Wang et al., 2010). A choice more sensitive is the use of Alamar blue and resazurin assay, which changes

from blue, nonfluorescent and oxidized form to pink and fluorescent upon reduction, can be read visual and fluorimetrically by exciting at 530 nm and detecting emission at 590 nm, and present high correlation with antitubercular gold standards methods (Collins & Franzblau, 1997). But, a more inexpensive colorimetric method, useful for evaluate antimycobacterial molecules in developing countries is using the ability that posses *M. tuberculosis* in to reduce nitrate to nitrite, nitrate reductase-based antibiotic susceptibility (CONRAS) test in liquid medium is perhaps the most cost-effective alternative for an anti TB drug screening program, with excellent results in comparison with other techniques, but is not useful for screening platforms that using nontuberculous mycobacteria nitrate negative (Kumar et al., 2005; Syre et al., 2010).

2.2 Fluorometric testing

The Gold Standard of fluorometric tests is the automated system BACTEC MGIT 960™ (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) which was highly sensitive and specific in the detection of rifampicin-resistant TB, and has been evaluated extensively for DST of anti TB drugs, replacing the BACTEC 460™ system for this task (Ma et al., 2011; Garrigo et al., 2011). BACTEC MGIT 960™ platform contains a modified Middlebrook 7H9 broth with a fluorescence quenching-based oxygen sensor that detects the amount of oxygen consumption by growing microorganisms (Springer et al., 2009). Automated liquid culture system BACTEC MGIT 960™ was designed to measure metabolic activity, and can be a quantitative indicator of bacterial numbers by the use of TB eXiST™ software to perform a quantitative drug susceptibility testing and determinate levels of drug resistance in *M. tuberculosis* (Springer et al., 2009). This measure of growth kinetics in liquid culture will facilitate mycobacterial quantification and will be especially beneficial for evaluating bactericidal activity of new anti-tuberculosis drugs and their combinations.

2.3 Flow cytometry

The modern flow cytometer analyzes and sorts cells or particles at rates up to 50000 per second. A broad range of flow cytometric applications for biotechnology includes applications in diagnostics and vaccine development, genomics, proteomics and protein engineering, drug discovery, reproductive biology, plant and marine biology, toxicology, and single molecule detection (Alvarez-Barrientos et al., 2000). In the 1980s were carried out the first experiments in which flow cytometry was used to study the effects of antimicrobial agents in prokaryotes. In the 1990s, the number of scientific articles addressing at the antimicrobial responses of bacteria (including mycobacteria), fungi, and parasites to antimicrobial agents, were considerably increased (Alvarez-Barrientos et al., 2000). Previous studies have reported that susceptibility testing of *M. tuberculosis* could be accomplished rapidly by using a flow cytometer. Fluorescein diacetate (FDA) staining were used for the flow cytometry susceptibility testing of *M. tuberculosis* (Kirk et al., 1998; Moore et al., 1999). The method is based on the ability of viable *M. tuberculosis* cells to accumulate fluorescein diacetate (FDA) and hydrolyse the compound rapidly to free fluorescein by intrinsic cellular esterases. The fluorescein accumulates in viable cells, while dead cells, or mycobacterial cells inhibited by anti-mycobacterial agents, hydrolyse significantly less FDA (Kirk et al., 1998; Moore et al., 1999). Pina-Vaz et al. stained *M. tuberculosis* in the absence or presence of antimycobacterial drugs with SYTO 16 (a nucleic acid fluorescent stain that only penetrates into cells with severe lesion of the membrane) (Pina-Vaz

et al., 2005). The time needed to obtain susceptibility results of *M. tuberculosis* using classical methodologies is still too long, and flow cytometry is a promising technique in the setting of the clinical laboratory, giving fast results. Multiplication of *M. tuberculosis* is not required, and reproducible results are available within 24h. However, the higher cost of equipment is a limitation of this methodology.

2.4 High Throughput Screening

An important aspect in the discovery and development of new antitubercular agents is search molecular scaffolds that target biochemical pathways and treat DR-TB. One approach in this direction is using the high-throughput screening methods of medicinally relevant libraries against the whole bacterium and develops biochemical, target-specific *M. tuberculosis* drug screening assays. High-throughput screening (HTS) is a method used in drug discovery and relevant to the fields of biology and chemistry. Using robotics, data processing and control software, liquid handling devices, and sensitive detectors, high-throughput screening allows a researcher to quickly conduct millions of chemical, genetic or pharmacological tests (Sethala & Zhang, 2009). In high throughput detection of *M. tuberculosis* a convenient format that permits the rapid determination of bacterial viability, is with the use of genes encoding luciferase enzymes and other fluorescent proteins such as the red fluorescent protein (RFP) and green fluorescent protein (GFP), following their introduction in mycobacteria on plasmids (Collins et al., 1998). The TAACF perform screens of chemical libraries against various biochemical target assays that have been modified, validated, and optimized for a high throughput format. *M. tuberculosis* targets selected by TAACF are as follows (Goldman & Laughon, 2009; Maddry et al., 2009).

- *M. tuberculosis* Dihydrofolate reductase.
- *M, tuberculosis* Enoyl-ACP Reductase.
- *M, tuberculosis* Isocitrate lyase-malate synthase.
- *M. tuberculosis* Pantothenate Synthetase.
- *M. tuberculosis* FtsZ and tubulin.

2.5 Microfluidic testing

Microfluidics research has produced sophisticated nanotechnological techniques for sample processing, fluid handling and signal amplification and detection (Chin et al., 2011). Microfluidics is an attractive platform for rapid single-cell functional analysis. Develop of plugs–droplets of aqueous solution surrounded provide a simple platform for manipulating samples. Microfluidic antimicrobial plug-based assays provide the ability to reduce detection time by confining bacteria into nanoliter-sized plugs (Boedicker et al., 2008). This confinement decreases the detection time by confining the sample into plugs that either have a single bacterium or are empty. This approach increases the effective concentration of the bacterium and allows released molecules to accumulate in the plug. These devices can be performed in the most remote regions of the world and produce a functional low-cost diagnostic device in extremely resource-limited settings. Various strategies for miniaturizing complex laboratory assays using microfluidics and nanoparticles can be useful for to conduct extensive research on bioprospecting in field.

2.6 Biosensing technologies

Current methods for DST of *M. tuberculosis* cannot provide results in real-time and most of these methods are centralized in large stationary laboratories because need complex instrumentation and highly qualified technical staff (Zhou et al., 2011). An interesting alternative is the use of biosensors which are sensors that transduce biorecognition processes via a physico-chemical transducer, with electronic and optical techniques as two major transducers (Song et al., 2006). Biosensors have high sensitive and accuracy. This is because biomolecules often possess high affinity toward their targets and biological recognition is usually very selective. Cell-based biosensors are special devices that employ immobilized living cells as sensing elements, combined with sensors or transducers to detect the intracellular and extracellular microenvironment condition, physiological parameters, and produce responses through the interaction between stimulus and cells (Song et al., 2006). Bulk acoustic wave and quartz crystal biosensors have the ability of determinate the *in vitro* susceptibility of antibiotics through estimation of bacterial growth (Tan et al., 1998). Is important to develop miniaturized biosensors in order to increase portability, an ideal biosensor should be integrated and highly automated with lab-on-a-chip technologies (microfluidics) for develop field studies in anti TB drugs detection.

2.7 Whole infection animal model testing

One hundred year since of magic bullet, the animal model continue being the best way for to find new antimicrobials with clinical potential. The use of live, infected whole animals to screen for antimicrobial compounds advances the established paradigm for identifying antibiotics in several key ways. First, the whole animal approach directly assesses drug efficacy *in vivo*, discarding compounds toxic to the host early in the analysis. Also, unlike conventional *in vitro* screens, this strategy can identify compounds that target the processes by which microbes establish infections, specifically mechanisms that are only manifest when the complex host/pathogen relationship is intact (Moy et al., 2006). But is possible convert this model in a robust and automatable model? With the potential to solve the bottleneck of toxicity/efficacy testing in drug development. Early in the 1960s, Sydney Brenner introduced the soil nematode *Caenorhabditis elegans* as a model organism to study animal development and the nervous system. *C. elegans* is a useful and simple model host that can be infected and killed by a remarkably large number of human pathogens (Bhavsar & Brown, 2006; Sifri et al., 2005). The worms can be stained with SYTOX Orange, which is excluded by living cells but readily enters cells with damaged membranes, specifically staining dead worms (Moy et al., 2006, Moy et al., 2009). With this nematode is possible will develop assays for identifying compounds that promote the survival of organisms persistently infected with the human opportunistic bacterial pathogens (Fig.3.). Other interesting infection model is the indirect study of human TB via the infection of the zebrafish (*Danio rerio*) embryo with *M. marinum* has already led to the clarification of many important processes in the life cycle of the infection, in particular those underlying the mechanisms of granuloma formation (Fig.4.), this model offers practical advantages when compared to *M.tuberculosis*, such as lower biosafety restrictions and faster growth rate (Carvalho et al., 2011).

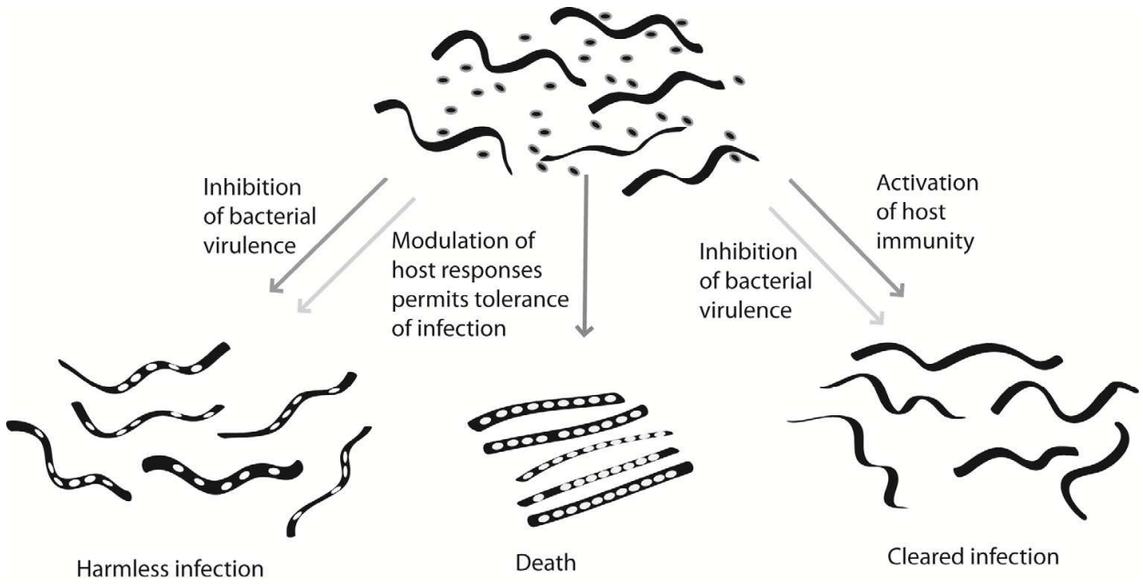

Fig. 3. *In vitro* screening using an infection model with *C. elegans*, the worm shape predicts the protective effect of antimicrobial agent (rigid posture for death, and sinusoidal posture for surviving) (Bhavsar & Brown, 2006).

2.8 Selection criteria for activity

In antimicrobial *in vitro* models, the activity of compounds is generally expressed by numeric values (IC50, IC90, MIC, etc.). For a correct interpretation of these efficacy variables, a profound knowledge of the model and the used protocol are required (Cos et al., 2006). For whole-cell bacteria activity an optimal value being ≤1 μg/mL is required (Enna & Williams, 2007; Enna, 2001; Food and Drug Administration, 2009). The literature reports that "antibacterial" compounds with MICs values greater than 100 μg/mL, which are poorly active and their clinical perspective has little relevance in reality an MIC of less than 10 μg/mL, and ideally less than 2 μg/mL with a selectivity index (SI = IC50Vero cells/MIC) of >10 is considered as being of interest to pharmaceutical industry (Gibbons, 2004, 2008). For validation of activity in FDA should be developed *in vitro* susceptibility test for at least 100 isolates (e.g., range, MIC50, MIC90) of each compound proposed. If the antibacterial drug product is a new molecular entity, its recommend that applicants provide data for at least 500 isolates from broad geographic regions (Food and Drug Administration, 2009).

3. Complementary testing

Compounds with a high activity, so-called "hits", need further evaluation in secondary or specialized *in vitro* bioassays, for increase current data of pharmacological properties and define potential lead-candidate status (Cos et al., 2006). With the end of improve quality of primary screening will focus on complementary *in vitro* testing for anti TB drugs research.

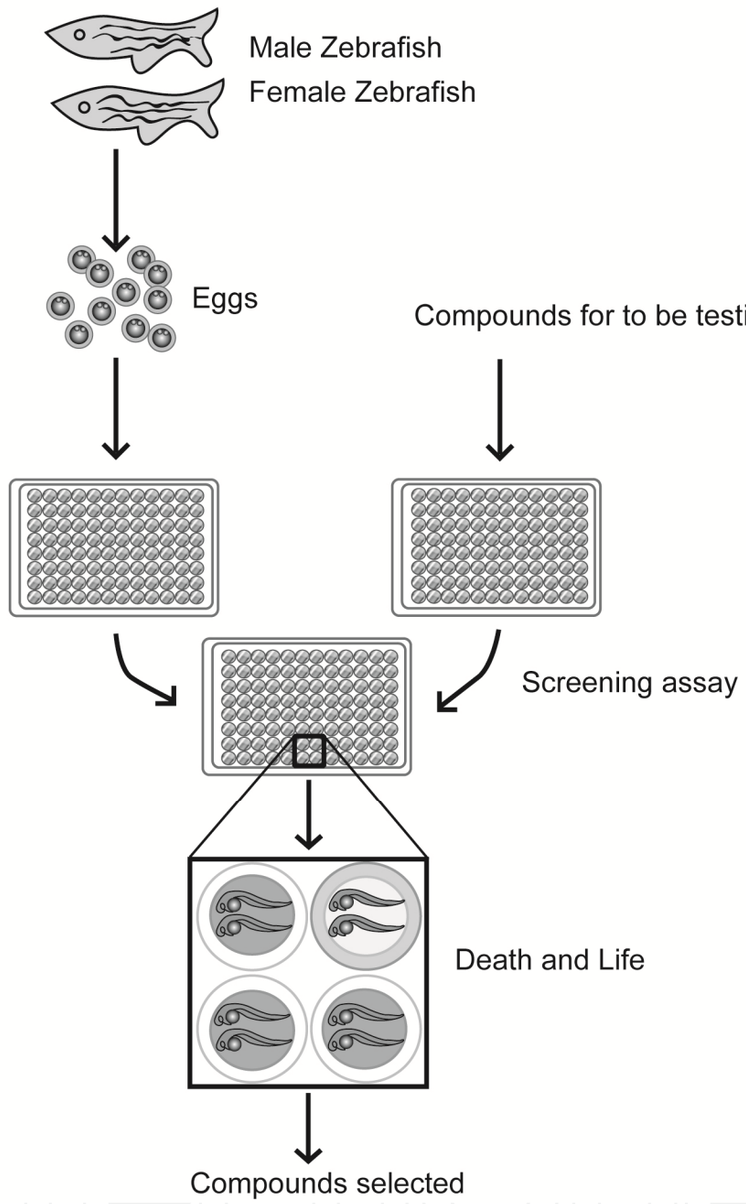

Fig. 4. *In vitro* screening using an infection model with zebrafish embryos, these organisms are optically transparent, permitting visualization of pathogens and lesions in real time (Carvalho et al., 2011).

3.1 Biocidal activity testing

Nosocomial infections by *M. tuberculosis* resulting from contaminated bronchoscopes and medical waste. Antiseptics and disinfectants are frequently used to prevent the spread of mycobacterial infection (Rikimaru et al., 2002). Various species of mycobacteria, including *M. tuberculosis*, show higher resistance against various chemical substances, including antiseptics, than other common bacteria. Non-tuberculous mycobacteria (NTM) are more resistant to common antiseptics than *M. tuberculosis* (Dauendorffer et al., 1999). This has

raised questions about the efficacy of these liquid chemical germicides in killing *M.tuberculosis* and has renewed interest in testing efficacy of new anti TB disinfectants. The currently available methods for testing the tuberculocidal activity of germicides include the AOAC method 965.12, the EPA Tuberculocidal Activity Test Method (TATM), and European Standard EN 14563, this efficacy testing is necessary for to be considered a cleared sterilants and high-level disinfectant by FDA (Hernandez et al., 2008; Rikimaru et al., 2002). The methods are a quantitative assay in function of time (30 seconds-60 minutes) with different concentrations determined in primary screening, performed with cells in suspension and not reflect the chemical sensitivity of cells attached to a surface. Although official guidelines recommend a plate counting, most manufacturers prefer to study the efficacy of their products by using a post treatment growth method using BACTEC MGIT 960™ system instead of a conventional plate counting method (Dauendorffer et al., 1999; Hall et al., 2007).

3.2 Antibiofilm activity testing

A biofilm is a structured formation of bacteria in a polymer matrix consisting of polysaccharide, protein and DNA. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the human immune system (Hoiby et al., 2010). Biofilm-growing bacteria cause chronic infections characterised by persistent inflammation and tissue damage. Chronic infections, including foreign-body infections, are infections that persist despite antibiotic therapy. Biofilms provide an important reservoir of cells that can repopulate colonized sites upon removal of drug treatment. Biofilm has been associated frequently with NTM, which include different species of mycobacteria with common phenotypical characteristics, recent studies has shown that most human NTM infections are biofilm-related (Ortiz-Perez et al., 2011), also been shown by Ojha et al, that *M.tuberculosis* have the ability of form drug-tolerant biofilms, it raises the possibility of *M. tuberculosis* biofilm formation as a potential new target for drugs that facilitate the use of current anti-tuberculosis antibiotics administered in ultra-short regimens (Ojha et al.,2008).

Biofilm formation can be performing on sterile polycarbonate disks or in a polyvinyl chloride (PVC) plastic 96-well microtitre plate containing Middlebrok 7H9 broth. In two weeks is possible obtain biofilms adhered to the wells and can be coloured with violet crystal (Johansen et al., 2009). Other method for evaluate antibiofilm activity is MBEC™ assay system (MBEC™ Biofilm Technologies Ltd. Calgary, AB, Canada). The MBEC™ device consists of a 96-peg lid plate and a ridged trough into which a standardized inoculums is added, this method was used for develop biofilms of *Mycobacterium phlei* (Bardouniotis et al., 2001; Ceri et al., 1999). Although not confirmed that *M. tuberculosis* form biofilms within the lungs, is possible evaluate the activity of various compounds in an *in vitro* screening using Sauton`s broth with specifications of oxygen consumption described by Ojha et al (Ojha et al., 2008).

3.3 Intracellular macrophage activity testing

Macrophages activated by T cell cytokines are a critical defense mechanism against intracellular bacterial pathogens (Jayaswal et al., 2010). If tuberculosis therapy is to be

shortened it is imperative that the sterilising activity of current and future anti-tuberculosis drugs is enhanced. Intracellular *Mycobacterium tuberculosis*, phagocytosed by macrophages may be a key subpopulation of bacteria that are less readily eliminated by therapy. *In vitro* models of macrophage infection by *Mycobacterium* spp have been used to assay virulence and the intracellular activity of antimycobacterials (Parish & Brown, 2008). A source of macrophages can vary species, including humans, mice and rabbits. The strain of *M. tuberculosis*, used to infect the macrophages, is another source of variability, e.g., *M. tuberculosis* H37Ra, H37Rv, Erdman, and clinical isolates (Parish & Brown, 2008). Species of mycobacteria other than *M. tuberculosis* have also been tested, e.g., *M. bovis* BCG and *M. avium*. The results of macrophage cytotoxicity are most heavily infected die rapidly and become no adherent (Cai et al., 2006). The activity of selected compounds against intracellular *M. tuberculosis* can be determined using the murine macrophage cell line RAW 264.7 (ATCC TIB-71) infected with *M. tuberculosis* luciferase reporter strain pSMT1 (Protopopova et al., 2006). Measurement of luminescence has shown to provide a rapid alternative to the counting of colonies as a means of evaluate mycobacterial viability (Protopopova et al., 2006). Profiting from mycobacteria expressing GFP, a vast array of recent technologies, based on fluorescence such as confocal microscopy or flow cytometry are now being applied to test new anti-TB drugs intracellular activity (Christophe et al., 2009).

3.4 Anti-dormant tubercle bacilli assays

In people with latent tuberculosis, a group estimated to be one-third of the world's population, *M. tuberculosis* is presumed in dormant state within caseous lesions of the lungs, with hypoxic conditions (Filippini et al., 2010). These non-replicating, dormant bacilli are tolerant to conventional anti-tuberculosis drugs such as isoniazid (Koul et al., 2008). A stage of latency in tubercle bacilli has been found as principle cause for most of the problems associated with the disease (Wayne & Sohaskey, 2001). New drug discovery is dependent on whole cell assays to reliably screen for compounds with anti-dormancy, anti-tubercular activities. There is still no specific drug available in the market, which could effectively kill this latent bacillus (Khan & Sarkar, 2008). The obstacle in the development of novel drugs is caused to the lack of a screening system, which can determine inhibitors of latent bacilli of tuberculosis and the limitations of the currently used colonies forming units (CFU) assay (Khan & Sarkar, 2008). Wayne`s hypoxic model is used for *in vitro* evaluation of new compounds, but posses low throughput capability (Khan & Sarkar, 2008). Using a *M. tuberculosis* pFCA-luxAB strain, which is *M. tuberculosis* H37Rv strain containing a plasmid with an acetamidase promoter driving a bacterial luciferase gene, Cho et al. (2007) implemented a high-throughput, luminescence-based low-oxygen-recovery assay for screening of compounds against nonreplicating *M. tuberculosis* (Cho et al., 2007). An inexpensive alternative is the combination of *in vitro* model of mycobacterial dormancy with colorimetric methods, recently, Khan & Sarkar (2008) have developed a dormant stage specific antitubercular screening protocol in microplate format using Wayne's hypoxic model and nitrate reductase activity in *M. bovis* BCG (Bacillus Calmette-Guérin) culture. In addition, Taneja & Tyagi (2007) used resazurin reduction assay to develop screening for searching anti-dormancy and anti-tubercular compounds (Khan & Sarkar, 2008; Taneja & Tyagi, 2007).

3.5 *In vitro* toxicity testing

Toxicity is a leading cause of attrition at all stages of the drug development process (Kramer et al., 2007; Barile, 2008). A recent analysis has shown that this high attrition is largely caused by lack of efficacy and unexpected safety concerns of new drugs. An important question is therefore how to improve the prediction of drug efficacy and safety. *In vitro* toxicology assays can be divided on the basis of timing and purpose of the application into prospective assays and retrospective assays (Kramer et al., 2007; Barile, 2008). *In vitro* toxicity testing should build upon test models that are relevant for the species to be protected. Proper test development requires well defined test compounds with high quality *in vivo* data (gold standard) and cell systems that mimic *in vitro* the key events that are known to occur *in vivo*. Prospective *in vitro* toxicology assays are those assays that are conducted before *in vivo* toxicology studies, and attempt to predict toxicities that are development-limiting. These include assays for general or cell-type-specific cytotoxicity, genotoxicity, hERG (human ether-a-go-go-related, also known as KCNH2) channel block, drug–drug interactions and metabolite mediated toxicity (Kramer et al., 2007; Barile, 2008). These cytotoxicity assays are often among the earliest toxicity assays to be conducted. *In vitro* cytotoxicity assays can be valuable for interpreting the results of *in vitro* safety and efficacy assays. Instead the Ames assay and micronucleus assay include an assessment of genotoxicity that is critical for the interpretation of the assay results (Kramer et al., 2007; Barile, 2008). Because of its simplicity, cost effectiveness, flexibility, and large validated database, the *Salmonella* assay is an ideal model to consider in the development of equally reliable *in vitro* toxicology assays that can predicts mutagenicity and carcinogenicity of various compounds (Claxto et al., 2010). But, an interesting alternative is to perform toxicity protocols with small animal models (*C. elegans* and *D. rerio*) that are compatible with large-scale screens and permits selection of compounds with a important safety profile (Giacomotto & Segalat, 2010).

4. *In vitro* pharmacologic validation

In vitro pharmacokinetic and pharmacodynamic (PK/PD) model is a pertinent approach for drug discovery. Because in a preclinical validation know the PK/PD parameters of chemical scaffolds allow predict the clinical outcome, adjust the antibiotic doses and prevent adverse reactions (Vaddady et al., 2010). These models can be used for evaluate drug combinations and synergy between them, designing new treatment protocols with early bactericidal activity against *M. tuberculosis*.

4.1 Time kill curve

The bactericidal activity of an antimicrobial agent can be expressed as the rate of killing by a concentration in function of time (Schwalbe et al., 2007). Time-kill curves are used to study the efficacy of an antimicrobial agent to a particular bacterial isolate. This rate is determined by counting the number of CFU in various time intervals. They are be used to study the antibacterial effect of single and combination drug compounds and dosing regimens before *in vivo* efficacy studies (Bhuda et al., 2009). Bactericidal activity can be determined from a time-kill curve if a greater than 3 $\log_{10}$-fold decrease in the number of survivors is noted (Shandil et al., 2007). This is equivalent to 99.9% killing of the inoculums. Time-kill

curves can also be used to study drug interactions. Synergy is defined as a ≥2 $\log_{10}$(CFU/mL)-fold decrease by the combination compared with the most active single agent. Antagonism is defined as a ≥2 $\log_{10}$(CFU/mL)-fold increase by the combination compared with the most active single agent (Fig.5.) (Schwalbe et al., 2007).

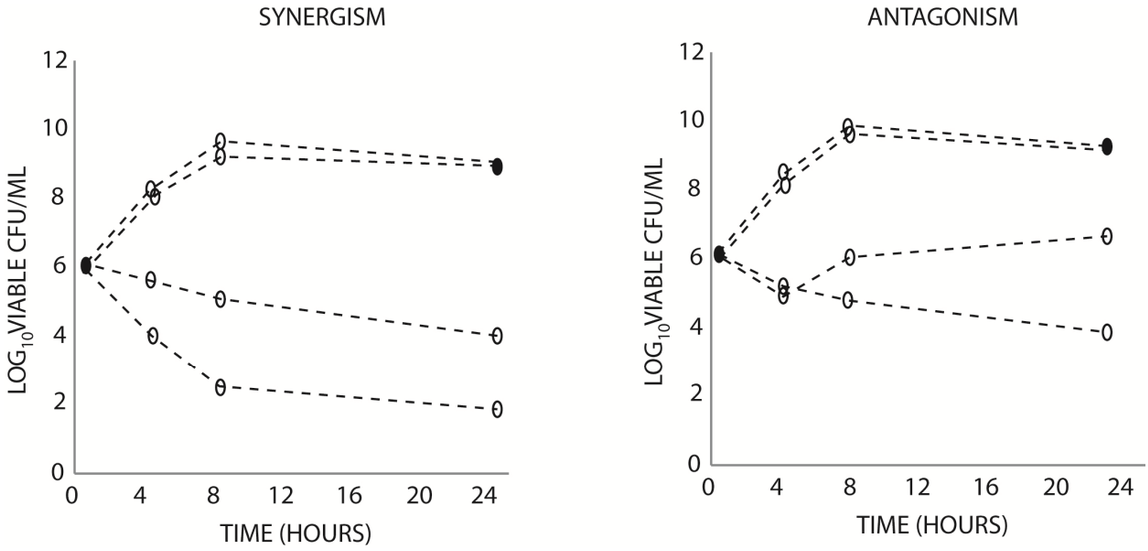

Fig. 5. Time kill curves for study drug interactions. Synergism is defined as a ≥2 $\log_{10}$(CFU/mL)-fold decrease. Antagonism is defined as a ≥2 $\log_{10}$(CFU/mL)-fold increase.

4.2 Mutant prevention concentration

Antimicrobial dosing is currently attracting attention as a way to minimize the emergence of resistance (Zhao & Drlica, 2008). Failure, relapses and the selection of resistant mutants during treatment are complex phenomena associated with the characteristics of the microorganism together with the characteristics and dosage of the drug used (Rodriguez et al., 2004). Mutant prevention concentration (MPC) has been proposed as a new measure of antibiotic potency (Sindelar et al., 2000). This method is characterized by determinate the capacity for prevent/restrict the selection of drug resistant mutants during antibiotic treatment by which should be evaluated (Dong et al., 2000). MPC is estimated as the drug concentration that blocks growth when 10^{10} cells are applied to agar or tested in liquid medium (MIC determination uses 10^4–10^5 cells) (Zhao & Drlica, 2008). MPC studies should be compared by pharmacokinetics *in vivo* parameters, where concentrations in serum of new anti TB drug must be above MPC for the longest period possible (Sindelar et al., 2000).

4.3 Post antibiotic effect

The post antibiotic effect (PAE) is defined as persistent suppression of bacterial growth after a brief exposure (1 or 2 h) of bacteria to an antibiotic (Chan et al., 2004). Factors that affect the duration of the post antibiotic effect include duration of antibiotic exposure, bacterial

species, culture medium and class of antibiotic. Post-antibiotic effect is now a well established pharmacodynamic parameter exerting an antibacterial effect longer than expected from the active concentration at the infection site (Horgen et al., 1999). The standard method to quantitate PAE is to calculate the difference in time required for drug-exposed and control cultures to increase one $\log_{10}$ above the number present immediately after withdrawal of the antibiotic (Vaddady et al., 2010).The PAE using bacterial counts as a parameter is calculated by PAE = T–C, where T is the time required for bacterial counts of drug-exposed cultures to increase one $\log_{10}$ above the counts observed immediately after washing/dilution and C is the corresponding time required for counts of untreated cultures (Vaddady et al., 2010). Theoretically, the ability of an antibiotic to induce a PAE is an attractive property of an antibiotic since antibiotic concentrations could fall below the MIC for the bacterium yet retain their effectiveness in their ability to suppress the growth (Fuursted, 1997). The PAE is an intrinsic characteristic of each antimicrobial agent, and has been shown to exist both *in vitro* and *ex vivo*, and clinical trials have demonstrated a potential role of PAE in dosing regimens (Fuursted, 1997).

4.4 Checkerboard analysis

Obtaining meaningful information about the interaction of antimicrobials in combination, singly or in synergy require *in vitro* testing in the clinical laboratory. This use of antimicrobial combinations to achieve *in vitro* activity and clinical efficacy against organisms resistant to inhibition and/or killing by acceptable concentrations of single agents continues to be of great clinical relevance (Lorian, 2005; Schwalbe et al., 2007). Checkerboard titration is one of the most frequently used techniques to assess drug interactions (Lorian, 2005; Schwalbe et al., 2007). The results are calculated mathematically and expressed in terms of a fractional inhibitory concentration (FIC) index equal to the sum of the FICs for each drug. The FIC for a drug is defined as the MIC of the drug in combination divided by the MIC of the drug used alone. If the FIC index is ≤0.5, the antimicrobial combination is interpreted as being synergistic; between 1 and 4 as indifferent; and >4 as antagonistic (Lorian, 2005; Schwalbe et al., 2007).

4.5 Hollow fiber system

In vitro PK/PD models can be used to study the antibacterial effect of single and combination drug compounds and dosing regimens before *in vivo* efficacy studies (Gumbo et al., 2007). The advantage of these models is that the appropriate human/animal pharmacokinetic profiles can easily be simulated and the effect of these changing drug concentrations on bacterial growth and emergence of drug induced tolerance and resistance can be assessed. Thus, *in vitro* models offer a safer and more ethical way of assessing the PK/PD relationships of antibiotics compared to animal or human studies. More recently, Gumbo et al. (2007) have published several reports using hollow fiber bioreactors (diffusion models) as *in vitro* models for testing antibacterial activity against *M. tuberculosis* (Gumbo et al., 2007; Gumbo et al., 2009; Pasipanodya & Gumbo, 2011). There are severe limitations associated with the use of hollow fiber bioreactors for *in vitro* culturing of bacteria. As these bioreactors are complex and difficult to sterilize between experiments, new hollow fiber cartridges are recommended for every study which makes a broad-based application of these experiments cost-prohibitive. However, preclinical antimicrobial PK/PD data have great clinical relevance to the treatment of TB, thus,

as new drugs are created, it would be advantageous to have them undergo rigorous PK/PD studies (Pasipanodya & Gumbo, 2011).

5. Conclusion

An imperative urgency for a new antitubercular drug is to reduce the duration of therapy. An ideal drug would have bactericidal activity on replicating and dormancy mycobacteria in an extracellular and intracellular space. Also a new anti TB drug also should be able to have synergistic effects with current therapy. Finally the dosing regimen of new drug should be developed in accordance with pharmacokinetic/pharmacodynamic parameters. *In vitro* screening should go beyond selection of actives molecules and get to be able to predict with high degree of efficiency the activity in animal models and clinical outcome.

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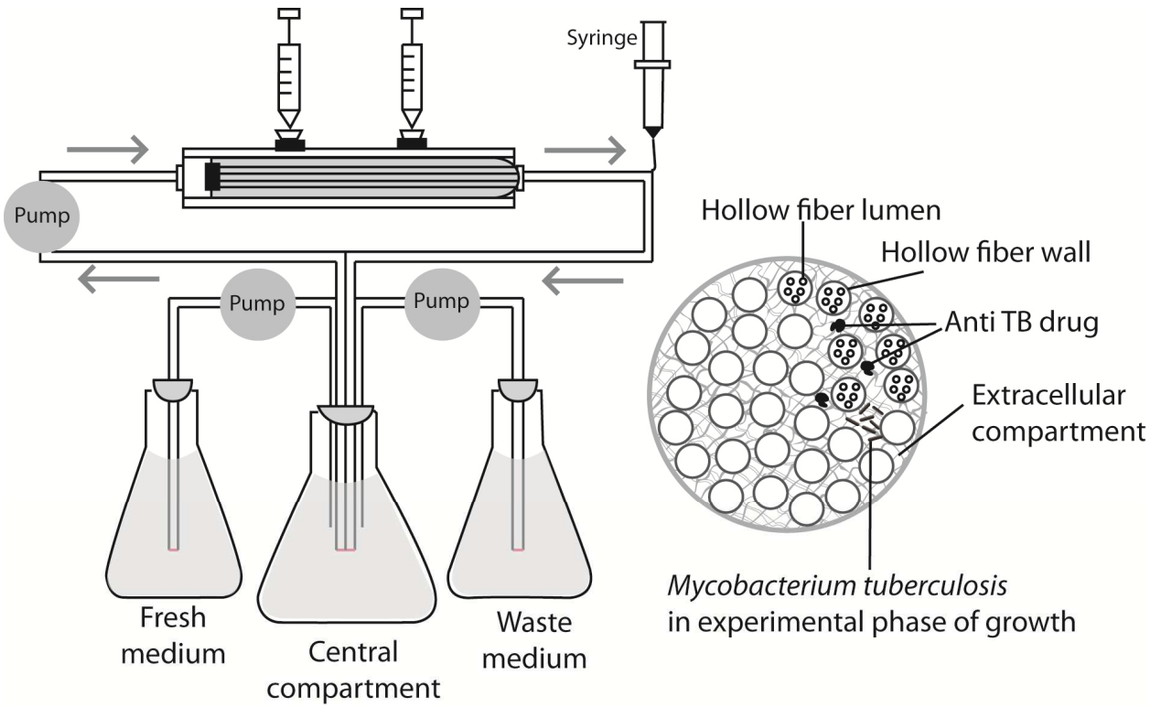

Fig. 6. Hollow-fiber pharmacodynamic model of tuberculosis *Mycobacterium tuberculosis* grow in the extracapillary space but are too big to cross into the central compartment. Anti TB drug is administered to the central compartment. Medium circulates in the central compartment in the direction shown by the arrows (Gumbo et al., 2007)

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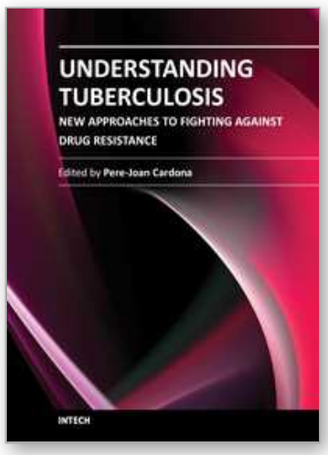

Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance

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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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