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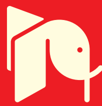
Understanding Tuberculosis

New Approaches to Fighting Against Drug Resistance

Edited by Pere-Joan Cardona



UNDERSTANDING TUBERCULOSIS – NEW APPROACHES TO FIGHTING AGAINST DRUG RESISTANCE

Edited by **Pere-Joan Cardona**

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Meet the editor

After obtaining his MD at the Universitat Autònoma de Barcelona, Pere-Joan Cardona started an internship in Clinical Microbiology in 1994, in the Hospital Germans Trias i Pujol, where he was familiarized with the problem of TB in its clinical and diagnostic challenges. Invited by Ian Orme at CSU, he was trained on the development of TB experimental models. PhD was obtained in 1999. He became Head of the Experimental Tuberculosis Unit at the Institut Germans Trias i Pujol of Badalona and Assistant Professor of Microbiology at UAB. He has been involved for the last 15 years in the study of the pathophysiology of TB infection. Additionally, he has authored 70 peer-reviewed publications in the field and is responsible for the development of different experimental models in mice, guinea pigs, goats and mini-pigs, and in the development of new drug regimens and vaccines against TB.

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Preface

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 Raphaêl, France. This drug was the base for the chemotherapy with Streptomicine, which demonstrated in 1980 to have a 100 per cent efficacy rate after being used together with two or three other drugs during the first two months of treatment in addition to an extra four month treatment combined with Isoniazid. 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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Part 1

Strategies for New Drug Discovering

1

Multi-Drug/Extensively Drug Resistant Tuberculosis (Mdr/Xdr-Tb): Renewed Global Battle Against Tuberculosis?

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1. Introduction

1.1 Background

Tuberculosis is the world's second deadliest infectious disease, with nearly 9.3 million new cases diagnosed in 2007. According to the WHO, an estimated 1.8 million people died from TB in 2007. One-third of the world's population is infected with the TB bacillus and current treatment takes 6–9 months. The current TB vaccine, Bacille Calmette-Guérin (BCG), developed almost 90 years ago, reduces the risk of severe forms of TB in early childhood but is not very effective in preventing pulmonary TB in adolescents and adults — the populations with the highest rates of TB disease. TB is changing and evolving, making new vaccines more crucial for controlling the pandemic. Tuberculosis is now the leading cause of death for people living with HIV/AIDS, particularly in Africa. Multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) are hampering treatment and control efforts. New control measures, diagnostic tools and guidelines for treatment as well as development of new drugs and vaccines have been made a priority and the battle is now raging to restore the grip on the magement and control of MDR/XDR TB. Winning the battle against tuberculosis will depend on the outcomes of the extensive research that is on going to produce new, more effective and fast acting diagnostic tools, drugs and vaccines.

1.2 Drug-resistant TB

Drug-resistant TB is a result of mycobacterial strains that do not respond to drug treatment. Drug resistance has in the recent past become a serious global public health problem especially in the populations of the poor countries of the world. Multidrug-resistant tuberculosis (MDR-TB) refers to organisms that are resistant to at least two of the first-line drugs, isoniazid (INH) and rifampin, (RIF). In recent years, the world has seen a rapidly emerging epidemic of drug-resistant TB or multi drug-resistant (MDR-TB) and/or extensively drug-resistant XDR-TB), which is highly lethal and extremely expensive leave alone being complicated to treat. Extensively drug-resistant tuberculosis (XDR-TB) is a type of multidrug-resistant tuberculosis (MDR-TB) that is resistant to two of the first-line drugs -

isoniazid and rifampicin - as well as to the second-line medications that include a fluoroquinolone such as ciprofloxacin and at least one of the injectable drugs which may be an aminoglycoside such as amikacin or kanamycin, or a polypeptide like capreomycin, or a thioamides such as ethionamide, or cycloserine or p-aminosalicylic acid.

Because the treatment regimen for TB is long and complex, many patients are unable to complete the course of treatment, enabling their disease to develop drug-resistance. Once a drug-resistant strain has developed, it can be transmitted directly to others. XDR TB being resistant to the front-line drugs and two or more of the six classes of second-line drugs, this makes it virtually untreatable and HIV positive people are particularly at a greater risk. Therefore, XDR TB could have a bigger impact on developing nations considering the fact that there is high prevalence of HIV and lack of capacity to quickly and effectively diagnose and identify the disease. To prevent XDR-TB from spreading, there is an urgent need for new diagnostic tools and new and more effective anti TB drugs and vaccines to be developed. An estimated $5bn is required to confront the spread of DR TB.

1.3 Inadequate treatment

The current first-line TB drug regimen of four drugs is nearly 50 years old, takes six to nine months to complete and has significant side effects. Very often, these shortcomings cause patients to default on their treatment which, consequently, results in resistance to TB drugs which then spreads throughout the world. Treatment for MDR-TB or XDR-TB can last up to 30 months, consists of many drugs, (including injectables), many of which have significant side effects, are extremely expensive and resource-intensive to deliver. With the rapid and lethal spread of drug-resistant TB, expediting the development of new, simpler and more effective drug regimens is now a major public health emergency.

1.4 Nature of resistance

In a study conducted by Ioerger et al.,(2009) titled "Genome Analysis of Multi- and Extensively-Drug-Resistant Tuberculosis from KwaZulu-Natal, South Africa", which was designed to investigate the causes and evolution of drug-resistance, it was observed that polymorphisms among the strains was consistent with the drug-susceptibility profiles, in that well-known mutations correlated with resistance to isoniazid, rifampicin, kanamycin, ofloxacin, ethambutol, and pyrazinamide. It was however, realised that the mutations responsible for rifampicin resistance in rpoB and pyrazinamide in pncA are in different nucleotide positions in the multi-drug-resistant and extensively drug-resistant strains, which was taken to be an indication that they acquired these mutations independently, and that the XDR strain could not have evolved directly from the MDR strain though it could have arisen from another similar MDR strain.

The researchers reported that the MDR and XDR strains contain typical mutations in gyrA, rpoB, rrs, katG, and the promoter of inhA that explain resistance to fluroquinalones, rifampicin, kanamycin, and isoniazid. Although susceptibilities to ethambutol and pyrazinamide were not determined clinically, mutations in embB and pncA were observed as well. They further argued that the fact that the MDR and XDR strains have different mutations in rpoB and pncA suggests that they arose separately, and that these mutations were acquired independently after divergence. This observation contradicts the hypothesis

that the XDR strain might have evolved directly from the MDR strain (though it could have arisen from another similar MDR strain). While resistance to streptomycin is usually associated with mutations in rpsL or rrs, the KZN MDR and XDR strains showed a rare 130 bp deletion in gidB. Although recent studies have begun to show that mutations can cause low-level resistance to streptomycin, through abbrogation of ribosomal methylation, this mutation was unique and had never been reported before.

Consistent with what was already known, the researchers found that only the XDR strain KZN-R506 showed a mutation in rrs, the 16S rRNA, at position 1400, which explains the kanamycin resistance as put forward by Suzuki et al., (1998) and that only the XDR strain had the A90V mutation in gyrA responsible for resistance to fluoroquinolones as presented by Aubry et al., (2006). They further reported that the mutation at 1400 in rrs which is the most commonly observed mutation associated with kanamycin resistance, found in 60% of rifampicin-resistant clinical isolates was consitent with findings of Suzuki et al., (1998). The A90V in gyrA, the second-most frequently observed mutation conferring fluoroquinolone resistance, found in 24% of fluoroquinolone-resistant clinical isolates, is also reported to show agreement with the work of van Doorn et al.,(2008) on fluoroquinolone resistance.

With respect to isoniazid (INH) resistance, it is also reported that both strains have the mutation of S315T in katG, the catalase/peroxidase that activates the pro-drug isoniazid as reported by Zang et al.,(1992). The finding that this is the most frequently observed mutation associated with isoniazid resistance was also consistent with the report by Hazbón et al.,(2006) and Pym et al.,(2002). The role of the c-15t inhA promoter mutation, and mutations in katG in ETH/INH co-resistance is also presented as put forward by Morlock et al., (2006)

Resistance to rifampicin (RIF) can be explained by mutations in rpoB (beta-subunit of RNA polymerase). The mutation of Asp 435 in rpoB, was observed to confer rifampicin-resistance as put forward by Ramaswamy and Musser (1998). Ioerger et al.,(2009) further report that this is in the core 507–533 region, in which numerous mutations have been observed to cause resistance to RIF, although they agree that mutations at other sites in this region are more frequent. However, they report that the two Kwazulu Natal strains have different mutations within the same codon, leading to different amino acid substitutions. Strain KZN-V2475 was found to have a G->T substitution in frame 1, producing D435Y, and KZN-R506 with an A->G substitution in frame 2, producing D435G, a case that led the researchers to suggest that the two strains acquired rifampicin resistance independently. They also noted that the XDR strain, KZN-R506, contains two additional mutations in rpoB, L452P and I1106T; the former also being thought to cause RIF-resistance, while the latter does not.

Ioerger et al.,(2009) further contend that streptomycin (STR) resistance is most likely due to a 130 bp deletion in gidB found in both MDR and XDR strains, but not the wild-type. The classic STR-R mutations that have been correlated with streptomycin-resistance in the 530-loop or 915-region of rrs, the 16S ribosomal RNA, or in rpsL, the ribosomal protein S12, were not observed in either strain. However, they also state that mutations in these two genes explain only about 70% cases of STR resistance in clinical isolates (Sreevatsan et al., 1997) implying that there must be other loci that can be responsible. They further add that, despite the mutations in gidB having previously been observed in clinical isolates of M. tuberculosis by Nishimura et al., (2007) that, this 130 bp deletion is distinct from every other gidB

mutation previously reported. They report that the 130 bp gidB deletion observed in the KZN MDR and XDR strains spans amino acids 50–93, which encompasses the SAM-binding site (Romanowski et al., 2002) and causes a frame shift for C-terminal remainder, which is presumed to abbrogate function completely. They report that both strains also show classic mutations in embB, pncA, and the promoter region of ethA, which are associated with resistance to ethambutol (EMB), pyrazinamide (PZA), and ethionamide (ETH), though susceptibility to these drugs was not tested. It is further reported that the M306V mutation in the transmembrane protein embB is one of the most frequently observed mutations in EMB-resistant strains as reported by Sreevatsan et al.,(1997) and that this mutation putatively prevents ethambutol from interfering with biosynthesis of the arabinogalactan layer in the cell wall. In the case of pncA, they further report that the two drug-resistant KZN strains showed different mutations in pncA, a pyrazinamidase, which is thought to be involved in nicotinamide biosynthesis. They futher report that the MDR strain KZN-V2475 has a G132A mutation, and that mutations of this residue have previously been reported to cause resistance to PZA Sreevatsan et al.,(1997). They further report that strain KZN-R506 has a frame-shift mutation in amino acid 152 caused by an insertion of 1 bp, and missense mutations that cause resistance that have been observed downstream of this site and that they believe that the C-terminus of the 186-residue gene product must be important. They add that the two drug-resistant strains also share a mutation at position −8 upstream of the translational start site of ethA, which is a monooxygenase that activates thioamides such as ethionamide, isoxyl, and thioacetazone as pro-drugs as reported by Dover et al.,(2007). The researchers further contend that a mutation in the upstream region could potentially confer resistance by increasing expression although susceptibility of the KZN strains to these drugs was not determined

It is further reported by the study that the MDR and XDR strains contain typical mutations in gyrA, rpoB, rrs, katG, and the promoter of inhA that explain resistance to fluroquinalones, rifampicin, kanamycin, and isoniazid. Mutations in embB and pncA were also observed. It is further argued that the fact that the MDR and XDR strains have different mutations in rpoB and pncA which suggests that they arose separately, and that these mutations were acquired independently after divergence. The researchers further report that, the Kwazulu Natal MDR and XDR strains studied showed a rare 130 bp deletion in gidB although resistance to streptomycin is usually associated with mutations in rpsL or rrs. The researchers coclude by recommending further analysis and comparison of the genome sequences they have reported in order to bring out a better understanding of the nature of the virulence XDR-TB strains.

1.5 Epidemiology of drug-resistant TB

In South Africa, an epidemic of XDR-TB was reported in 2006 as a cluster of 53 patients in a rural hospital in KwaZulu-Natal of whom 52 died - Tugela Ferry case. What was particularly worrying was that the mean survival from sputum specimen collection to death was only 16 days and that the majority of patients had never previously received treatment for tuberculosis. This was the epidemic for which the acronym XDR-TB was first used, although TB strains that fulfil the current definition have been identified since then, though retrospectively. This was the largest group of linked cases ever found; after the initial report in September 2006, cases have now been reported in most provinces in South Africa, the

neghbouring countries and the world at large, with more than 50 countries on all the inhabited continents having reported XDR-TB cases.

MDR/XDR-TB can develop in the course of the treatment of fully sensitive TB and this is always the result of patients missing doses or failing to complete a course of treatment. Although there are reports that these resistant strains appear to be less fit and less transmissible, the high mortality rate especially where there is co-infection with HIV or during use of immunosuppressive drugs, this warrants that the epidemic has to be taken seriously. There is strong evidence that the spread of XDR-TB strains is very much associated with a high prevalence of HIV and poor infection control, and in some countries the upsurge of XDR-TB has been attributed to mismanagement of cases or poor patient compliance with drug treatment.

XDR-TB does not respond to any of the drugs currently available in most developing countries for first- or second-line treatment. Considering the fact the problem is wide spread globally, strict isolation procedures have been suggested to mitigate rapid spread of XDR-TB. The World Health Organization (WHO) recommends improving basic TB care to prevent emergence of resistance, the development of proper laboratories for detection of resistant cases, and when drug-resistant cases are found, it recommended prompt and appropriate treatment to prevent further transmission.

Collaborative care for both HIV and TB is also recommended to help limit the spread of tuberculosis, both sensitive and resistant strains. The spread of drug-resistant cases has also been linked to overcrowding in places such as seen in prison populations, although the major reason for the development of resistance is poorly managed TB care which may be in form of poor patient compliance, inappropriate dosing or prescribing of medication, poorly formulated medications, and/or an inadequate supply of medication.

1.6 Challenges presented by MDR/XDR TB

First, research has revealed that drug-susceptible (regular) TB and MDR/XDR TB are transmitted in the same way. Transmission of XDR TB is in clusters and follows similar transmission patterns as ordinary TB. This makes it difficult to put appropriate barriers to the transmission of the deadly strains. To make matters worse, proper diagnosis involving culture and sensitivity tests is the most commonly used diagnostic method especially in the poor countries. This may take from 6 to 16 weeks, before XDR TB is confirmed during which time it is likely to have spread to other patients and possibly health workers. There have been no new diagnostic tests invented for many years and therefore most laboratories in these areas have limited capacity to respond to XDR-TB. Most laboratories, especially those in developing countries lack the facilities and guidelines for the use of conventional and rapid culture-based or molecular methods for detection of M. tuberculosis and drug resistance and this impedes the widespread use of these tests. The laboratory confirmation of TB in HIV-infected persons is even more difficult and time consuming and highly sensitive and sophisticated and requires technically challenging diagnostic tests that are not universally available in all settings with a high burden of HIV and TB. There is, therefore, poor surveillance especially in the poor developing countries and this presents serious difficulties in identifying and locating the XDR TB cases. A further complication is that TB

affects mostly poor people who live in places where health care is not easily accessible and where the patients have to pay for their own transportation

The next challenge is that there are limited treatment options for XDR-TB especially in the developing countries and this makes the disease virtually untreatable. Considering the fact the majority of patients infected with XDR-TB are co infected with HIV/AIDS and that co-infection has been found to be virtually 100% fatal, this makes the situation more serious. In spite of this serious threat, the world is not responding fast enough and with enough resources as was the case with SARS, avian flu or swine flu. Stop TB estimates that through 2015, it will take about $2.4 billion for further discovery and early-stage development work and another $2.4 billion for clinical trials for new anti TB drugs. Considering the fact that the currently available resources are believed to total about $600 million, this leaves a substantial funding gap. More funding has to be directed towards research and development of new TB drugs and vaccines if the pandemic is to be defeated effectively.

With regard to anti-tuberculosis drugs and vaccines, the world's only vaccine (BCG) is almost 100 years old and only effective in children and for over 40 years there has been no new TB drug put on the market. This may be attributed to the high rates of failures of new drugs at clinical trials but it could also partly be due to complacency that tuberculosis was a defeated disease whose prevalence was on the decline especially in the USA. Another handicap has been that clinical trials required to register a TB drug can take a minimum of 6 years, much longer than trials for other infectious diseases.

A further complication is from drug-drug interactions in patients with TB/HIV co-infection. This is a serious hindering factor in finding treatments for people co-infected with TB and HIV. For example it is reported that rifampicin, which inhibits RNA polymerase, interacts with cytochrome P450 isozyme and causes some HIV drugs to be cleared quickly. To make matters worse clinicians, laboratory technologists, health-care professionals, public health officials, and policy makers do not possess up-to-date knowledge of what constitutes appropriate laboratory capabilities and capacities.

2. The global MDR/XDR response plan 2007-2008

Objectives for the Response were the following: (1) Strengthen basic activities to control TB and HIV/AIDS, as detailed in the Stop TB Strategy; (2) Scale-up the programmatic management of MDR-TB and XDR-TB to reach the targets set forth in the Global Plan; (3)Strengthen laboratory services for adequate and timely diagnosis of MDR- TB and XDR-TB; (4) Expand surveillance of MDR-TB and XDR-TB to better understand the magnitude and trends of drug resistance and the links with HIV; (5) Foster sound infection control measures to avoid MDR-TB and XDR-TB transmission to protect patients, health workers, others working in congregate settings, and the broader community, especially in high HIV prevalence settings; (6) Strengthen advocacy, communication and social mobilization for sustained political commitment and a patient centred approach to treatment; (7) Pursue resource mobilization at global, regional and country levels to ensure that necessary resources are available; and (8) Promote research and development into new diagnostics, drugs, vaccines, and operational research on MDR-TB management to shorten treatment. (Adopted from: WHO Report,2007)

3. Treatment of MDR/XDR -TB

3.1 Monitoring DOTS-plus

The WHO extended the DOTS programme in 1998 to include the treatment of MDR-TB (called "DOTS-Plus"). Implementation of DOTS-Plus requires the capacity to perform drug-susceptibility testing and the availability of second-line agents, in addition to all the requirements for DOTS. DOTS-Plus is therefore much more resource intensive than DOTS, and requires much greater commitment from countries wishing to implement it. Resource limitations mean that the implementation of DOTS-Plus may lead inadvertently to the diversion of resources from existing DOTS programmes and a consequent decrease in the overall standard of care (Dauby et al., 2011;Tam et al.,2009; Li et al., 2006).

Monthly surveillance until cultures convert to negative is recommended for DOTS-Plus, but not for DOTS. If cultures are positive or symptoms do not resolve after three months of treatment, it is necessary to re-evaluate the patient for drug-resistant disease or non-adherence to drug regimen. If cultures do not convert to negative despite three months of therapy, some physicians may consider admitting the patient to hospital so as to closely monitor therapy.

3.2 Management of TB/HIV co-infection

In patients with HIV, treatment for the HIV should be delayed until TB treatment is completed, if possible. The current UK guidance, provided by the British HIV Association, is that for a CD4 count over 200, treatment should be delayed until the six months of TB treatment are complete; for a CD4 count of 100 to 200, treatment should be delayed until the initial two-month intensive phase of therapy is complete ; while for a CD4 count less than 100, the situation is unclear and they recommend clinical trials to examine the issue. There is need for patients in this category to be managed by a specialist in both TB and HIV so that they are not compromised for either disease.

If HIV treatment has to be started while a patient is still on TB treatment, it is recommended that the advice of an HIV specialist should be sought. In general, reports say that there is no significant interactions with the NRTI's. Nevirapine should not be used with rifampicin. Efavirenz may be used, but the dose used depends on the patient's weight (600 mg daily if weight less than 50 kg; 800 mg daily if weight greater than 50 kg). Efavirenz levels should be checked early after starting treatment. The protease inhibitors should be avoided if at all possible because patients on rifamycins and potease inhibitors have an increased risk of treatment failure or relapse. The WHO also warns against using thioacetazone in patients with HIV, because of the 23% risk of potentially fatal exfoliative dermatitis.

3.3 Specific treatment of MDR-TB

The treatment and prognosis of MDR-TB are much more akin to that for cancer than to that for infection. It has a mortality rate of up to 80%, which depends on a number of factors, including: (1) How many drugs the organism is resistant to (the fewer the better); (2) How many drugs the patient is given (patients treated with five or more drugs do better); (3) Whether an injectable drug is given or not (it should be given for the first three months at least); (4) The expertise and experience of the physician responsible; (5) How co-operative

the patient is with treatment (treatment is arduous and long, and requires persistence and determination on the part of the patient) ; and (6) Whether the patient is HIV positive or not (HIV co-infection is associated with an increased mortality).

Treatment courses take a minimum of 18 months and may last for years; it may require surgery, though death rates remain high despite optimal treatment. That said, good outcomes are still possible. Treatment courses that are at least 18 months long and which have a directly observed component can increase cure rates to 69%.

Treatment of MDR-TB must be done on the basis of sensitivity testing since it is impossible to treat such patients without this information. When treating a patient with suspected MDR-TB, the patient should be started on streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide + moxifloxacin + cycloserine (SHREZ+MXF+cycloserine) pending the result of laboratory sensitivity testing. A gene probe for rpoB is available in some countries and this serves as a useful marker for MDR-TB, because isolated RMP resistance is rare, except when patients have a history of being treated with rifampicin alone. If the results of a gene probe (rpoB) are known to be positive, then it is reasonable to omit RMP and to use SHEZ+MXF+cycloserine. The reason for maintaining the patient on INH despite the suspicion of MDR-TB is that INH is so potent in treating TB that it would be irrational to omit it until there is microbiological proof that it is ineffective. There are also probes available for isoniazid-resistance (katG and mabA-inhA), but these are less widely available.

When sensitivities are known and the isolate is confirmed as resistant to both INH and RMP, five drugs should be chosen in the following order (based on known sensitivities): (1) an aminoglycoside such as amikacin, kanamycin or a polypeptide antibiotic such as capreomycin; (2) pyrazinamide; (3) ethambutol; (4) a fluoroquinolones (moxifloxacin is preferred and ciprofloxacin should no longer be used]); (5) rifabutin; (6) cycloserine; (7) a thioamide: prothionamide or ethionamide; (8) PAS; (9) a macrolide such as clarithromycin; (10) linezolid; (11) high-dose INH (if low-level resistance); (12) interferon-γ; (13) thioridazine; and (14) meropenem and clavulanic acid. Drugs near the top of the list are more effective and less toxic while drugs placed near the bottom of the list are less effective or more toxic, or more difficult to obtain.

Resistance to one drug within a class generally means resistance to all drugs within that class, but a notable exception is rifabutin for which rifampicin-resistance does not always mean rifabutin-resistance and the laboratory should be asked to test for it. It is only possible to use one drug within each drug class and if it is difficult to find five drugs to use then the clinician can request that high level INH-resistance be looked for. If the strain has only low level INH-resistance (resistance at 1.0 μg/ml INH, but sensitive at 0.2 μg/ml INH), then high dose INH can be used as part of the regimen.

When counting drugs, PZA and interferon are counted as zero i.e. when adding PZA to a four drug regimen, you must still choose another drug to make five. It is not possible to use more than one injectable (capreomycin or amikacin), because the toxic effect of these drugs is additive: if possible, an aminoglycoside should be given daily for a minimum of three months (and perhaps thrice weekly thereafter). Ciprofloxacin should not be used in the treatment of tuberculosis if other fluoroquinolones are available.

There is no intermittent regimen validated for use in MDR-TB, but clinical experience is that giving injectable drugs for five days a week (because there is no-one available to give the drug at weekends) does not seem to result in inferior results. DOTS Plus strategy has been found to help in improving outcomes in MDR-TB and it is recommended that it should be an integral part of the treatment of MDR-TB.

Response to treatment must be obtained by repeated sputum cultures (monthly if possible). Treatment for MDR-TB must be given for a minimum of 18 months and cannot be stopped until the patient has been culture-negative for a minimum of nine months. It is not unusual for patients with MDR-TB to be on treatment for two years or more.

To be able to contain the spread of resistance, patients with MDR-TB should be isolated in negative-pressure rooms, if possible. Patients with MDR-TB should not be accommodated on the same ward as immunosuppressed patients (HIV-infected patients, or patients on immunosuppressive drugs). Careful monitoring of compliance with treatment is crucial to the management of MDR-TB and hospitalisation should be encouraged for this reason. If possible these patients should be isolated until their sputum is smear negative, or even culture negative, a process that may take many months, or even years. Since keeping these patients in hospital for long periods may not be practicablel, the final decision depends on the clinical judgement of the physician treating that patient. In addition, the attending physician should make full use of therapeutic drug monitoring (particularly of the aminoglycosides) both to monitor compliance and to avoid toxic effects.

Some supplements may be useful as adjuncts in the treatment of tuberculosis, but for the purposes of counting drugs for MDR-TB, they count as zero (if you already have four drugs in the regimen, it may be beneficial to add arginine or vitamin D or both, but you still need another drug to make five). The supplements include arginine (peanuts are reported to be a good source) and Vitamin D.

There are also some drugs which have been used in desperation and for which it is uncertain whether they are effective at all or not, but which are used when it is not possible to find five drugs from the list above. They include imipenem, co-amoxiclav, clofazimine, prochlorperazine and metronidazole.

There is also increasing evidence for the role of surgery (lobectomy or pneumonectomy) in the treatment of MDR-TB, although whether this is should be performed early or late is not yet clearly defined (Mohsen et al, 2007).

3.4 Specific treatment for XDR-TB

Can XDR TB be treated and cured? Yes, in some cases. Some TB control programs have shown that aggressive treatment, using the current drug regimens can make it possible to effect cure for an estimated 30% of affected people. Researchers have shown that a cure is possible with a combination of at least five drugs as is the case with MDR-TB. Tailored treatment in 600 patients in Russia with at least five drugs showed that almost half of XDR-TB patients had treatment cure on completion of the course. The study reported that aggressive management of the disease is feasible and can prevent high mortality rates and further transmission of drug-resistant strains of TB. However, the treatment is extremely labour and resource intensive and has to be done within extremely well structured TB

programmes. It is further reported that successful outcomes depend greatly on the extent of the drug resistance, the severity of the disease, whether the patient's immune system is weakened, and adherence to treatment. There are no newly approved drugs or vaccines specifically for the treatment of XDR-TB although a number of drugs and vaccines are reported to be in various stages of development (Dauby et al., 2011).

3.5 New drugs in the pipeline

There is a desperate need for new and better TB treatments to address today's growing pandemic, which kills nearly 2 million people each year. There have been no new TB drugs for nearly 50 years and, until the past decade, there was no pipeline of TB drug candidates. Now, with increased investments in TB R&D, there are 9 promising TB compounds in the pipeline from six antibiotic classes, making combination testing of new TB drugs possible.

The eperimental drugs PA-824 (manufactured by PathoGenesis Corporation, Seattle, Washington), and R207910 (under development by Johnson & Johnson) are experimental compounds that are not commercially available, but which may be available from the manufacturer as part of a clinical trial or on a compassionate basis because their efficacy and safety are not yet properly understood. There are also reports that a Ukrainian herbal product which has been the subject of several small, open label clinical trials in TB patients and in patients with TB/HIV coinfection has produced promising results. Furthermore, Open Label trials with Dzherelo/Immunoxel have been reported to produce positive results in MDR and XDR-TB patients.

Stirling Products Ltd of Australia has also announced further work on drug-resistant TB and TB/HIV with trials being carried out in Nigeria. V-5 Immunitor (known as "V5"), an oral hepatitis B and hepatitis C treatment vaccine and administered as simple tablets is being developed for patients co-infected with hepatitis C and tuberculosis. It is reported to produce TB sputum clearance within only one month. Further blinded studies at multiple trial centres have reported that V5 is equally effective against multiple drug resistant tuberculosis (MDR-TB).

Currently, about 30 compounds have been identified for potential development of new anti TB drugs. However, new treatment for XDR TB is expected to be available not earlier than 2012. Drugs in the pipeline include, among others, combination regimens containing the fluoroquinolones moxifloxacin and gatfloxacin. Moxifloxacin (from Bayer and TB Alliance) is being looked at as a substitute for isoniazid or ethambutol and should now be undergoing final clinical trials while gatfloxacin (from OFLOTUB) is being developed to replace ethambutol. Other drugs in the pipeline include LL3858 (from Lopin) which should have by now gone through Phase I clinical trials.Work also continues on rifabutin (related to rifampicin) (from Pfizer) which is under study to replace rifampicin and on rifapentine, which was approved in 1998.

PA-824, a nitroimidazole, (from Chiron –part of Norvatis) is under Phase II Clinical trials while OPC-67683, a nitrodihydroimidazo-oxazole derivative (from Japan's Otsuk Pharmaceuticals) is in advanced stages of clinical trials for treatment of MDR TB. TMC-207 (from Johnson & Johnson), an ATP synthase inhibitor that is selective for MTB is under

development at Tibotec for MDR TB and is being considered as substitute for rifampicin and isoniazid to shorten the dosage period for MDR TB.

FAS20013, a sulfonyl tridecamide (from FASgen) is also being developed against MDR TB. It interferes with MTB cell wall synthesis and is expected to be effective against dormant bacteria.

SQ109, a 1, 2-ethylene diamine (from Sequella) is reported to inhibit cell wall synthesis and to have shown synergistic effect with rifampicin and isoniazid. It is also reported to be effective against MDR and latent forms. SQ609, a dipiperidine (from Sequella but got from Sankyo, Japan), which is an inhibitor of translocase, involved in cell wall synthesis, is in pre-clinical studies.

Source: U.S. National Institute of Allergy and Infectious Diseases (NIAID)

3.6 Ongoing research: New paradigm shift

Treating active TB requires a combination of drugs to prevent the development of drug resistance. Traditionally, researchers tested one new drug at a time in a series of lengthy and expensive clinical trials, meaning it would take decades to develop a completely novel drug combination. The individual TB drug candidates were developed and registered separately, by being substituted (or added) one at a time to the existing standard, four-drug-combination TB therapy. Because each substitution (or addition) could take six years or longer, the approval of a new four-drug TB regimen, through successive trials, could take nearly a quarter of a century to develop under this framework. With nearly 2 million people dying of TB each year, the world cannot wait that long for the tools needed to stop this devastating disease.

The push in this direction is because there is a possibility of developing one TB drug regimen capable of treating both drug-sensitive and multidrug- and extensively drug-resistant tuberculosis using combination therapy. Combination drug regimen may especially transform MDR/XDR-TB treatment, resulting in reduction of treatment duration from 2 years to less than six months. This new approach to drug development is expected to expedite the development and production of regimens that can be availed to patients in a much smaller period of time compared to the traditional approach of drug development.

This research approach is being championed by the Critical Path to TB Drug Regimens (CPTR), an initiative established to tackle the regulatory and other challenges associated with TB drug development. CPTR was founded in March 2010 by the Bill & Melinda Gates Foundation, the Critical Path Institute, and the TB Alliance. CPTR focuses on shifting the unit of development from an individual drug to combinations of drugs, which can be tested together and developed as a regimen from early clinical testing. Advances in regulatory science will help clearly evaluate experimental TB drugs both on their own and within the context of a regimen.

This new approach has the potential to shorten the time needed to develop new TB treatment regimens by decades, as well as significantly reduce development costs. However, to be able to test promising combinations together, there must be a change in today's

thinking about TB research— a paradigm shift - and the change must be adopted by everyone: drug sponsors, global regulators, WHO, patients, and other stakeholders throughout the TB landscape. This new approach to drug development enables combinations of previously unregistered TB drugs to be tested together, with the goal of introducing truly innovative regimens in only a fraction of that time. Nearly a dozen pharmaceutical companies, civil society organizations, the European and Developing Countries Clinical Trials Partnership (EDCTP), and others have signed on to the initiative's guiding principles. The US Food and Drug Administration, other regulatory bodies, and the World Health Organization have all shown support for this initiative.

In persuit of this paradigm shift, the 41st Union World Conference, the Global Alliance for TB Drug Development (TB Alliance) in Berlin, Germany on Nov. 8, 2010 announced the launch of the first clinical trial to test a novel tuberculosis regimen designed to expedite new treatments to patients. The novel three-drug combination has shown promising results towards treating both drug-sensitive (DS-TB) and multidrug-resistant TB (MDR-TB), and also being able to alter the course of the TB pandemic by shortening and simplifying treatment worldwide.

The combination now in Phase III clinical trials is called NC001 or New Combination 1. The new combination TB drug candidate being tested contains PA-824 and moxifloxacin in combination with pyrazinamide, an existing antibiotic commonly used in TB treatment today. The developers have reported that preclinical data have revealed that the combination has potential to shorten treatment time for virtually all tuberculosis patients and to harmonize the treatment of drug susceptible tuberculosis (DS-TB) and MDR-TB and possibly XDR-TB treatment with a single three-drug regimen. This is a particularly significant advance for MDR-TB patients, who today must take multiple types of drugs, including injectables, daily for up to two years. It is envisaged that, if successful, the experimental regimen will offer a shorter, simpler, safer, and more affordable treatment option for MDR-TB, an emerging global health threat. The new compounds are being developed by TB Alliance, but with moxifloxacin being developed in partnership with Bayer HealthCare AG.

The trial involves 68 participants at two centers in South Africa, each receiving two weeks of treatment and three months of follow-up to evaluate effectiveness, safety, and tolerability. NC001 is an early bactericidal activity trial and is supported financially by United States Agency for International Development, the Bill & Melinda Gates Foundation, and the United Kingdom's Department for International Development.

NC001, is also testing additional two-drug combinations (TMC207/pyrazinamide and PA-824/pyrazinamide) that may prove to be the building blocks of future regimens. Regimen development may become the new gold standard in TB research and offer lessons for other diseases requiring combination treatment, such as cancer, hepatitis C, and malaria. However, there remains a vital need for funding to bring new TB regimens through late-stage clinical trials.

Table 1 gives a summary of the various compounds and combinations that are in various stages of development.

Discovery			**Pre-clinical Development**	**Clinical Development**		
Target or cell-based screening	Lead identification	Lead optimization		Clinical Phase I	Clinical phase II	Clinical phase III
Natural Products IMCAS	Whole-Cell Hit to Lead Program GSK	Mycobacterial Gyrase Inhibitors GSK	Nitroimidazoles U. of Auckland/ U. Ill Chicago		PA-824 Novartis (NTBRD)	Moxiflox acin (+H, R, Z) Bayer
TB Drug Discovery Portfolio NITD		Pyrazinamide Analogs Yonsei	Preclinical TB Regimen Development JHU/U. Ill Chicago (NTBRD)		Preclinical TB Regimen Development JHU/U. Ill Chicago (NTBRD)	Moxiflox acin (+R, Z, E) Bayer
Topoisomerase I Inhibitors AZ/NYMC	Gyrase B Inhibitors AZ	Diarylqu-inolines Tibotec/U. of Auckland			PA-824/Pyrazinamide (NTBRD)	
	Folate Biosynthesis Inhibitors AZ	Rimino-phenazines IMM/BTTTRI			TMC207/Pyrazina mide (NTBRD)	
	Whole-Cell Hit to Lead Program AZ				PA-824/TMC207 (NTBRD)	
	RNA Polymerase Inhibitors AZ				PA-824/ Moxifloxacin/ Pyrazinamide (NTBRD)	
	Energy Metabolism Inhibitors AZ/U. Penn					

Key: AZ = AstraZeneca, Bayer = Bayer Healthcare AG, BTTTRI = Beijing Tuberculosis and Thoracic, Tumor Research Institute, GSK = GlaxoSmithKline, IMM = Institute of Materia Medica, IMCAS = Institute of Microbiology, Chinese Academy of Sciences, JHU = Johns Hopkins University, Tibotec = Johnson & Johnson / Tibotec, NYMC = New York Medical College, NITD = Novartis Institute for Tropical Diseases, Novartis = Novartis Pharmaceutical, U. of Auckland = University of Auckland, U. Penn = University of Pennsylvania School of Medicine, Yonsei = Yonsei University, (NTBRD) = Novel TB regimen development, Source: Global Alliance for TB Drug Development . June 2011

Table 1. TB Alliance Portfolio for TB drug development

4. TB vaccines and immunizations

Vaccines work by stimulating the immune system to retain a memory of particular molecules from a microbe that will trigger a rapid immune response if the microbe is encountered later. The best candidates for vaccines are those that trigger the strongest response from the immune system. The existing Bacille Calmette Guerin (BCG) vaccine,

which came into the market in 1921, has limited effectiveness in protecting people from TB. BCG is based on a live but attenuated strain of Mycobacterium bovis, which is the species that causes TB in cattle but can also infect humans. It is given at birth throughout the developing world but the problem is that BCG is not very effective - a fact made obvious when you consider that about 2 million people a year still die from TB. It is also reported that the BCG vaccine, which is used to prevent childhood TB, may not be safe for children living with HIV.

The development of a vaccine from a pool of potential candidates through clinical trials to delivery in a healthcare system is a costly and time-consuming process with a very high failure rate along the way. However, this problem not withstanding, a number of groups from around the world are collaborating in major partnerships, on vaccine development where two main vaccine strategies are being pursued: (1) a pre-infection vaccine delivered early in life improved from the current BCG vaccine and (2) a post-exposure vaccine that would invoke immunity to clinical disease after infection. With these stragies in mind, a number of products has reached various phases of clinical trials.

Researchers in the TB-VAC project will select vaccines for TB that work in adults and are suitable for use in resource-poor settings and are safe for HIV-infected individuals. MUVAPRED (Mucosal Vaccines against Poverty Related Diseases) is aimed at stimulating local immunity to neutralise M. tuberculosis where the organism enters the body in the lungs. The focus is on developing vaccines that can be administered orally or as a nasal spray thus avoiding the risks involved in using needles.The momentum to develop a new and more effective vaccine is gathering pace and although there is still much work to be done, there is genuine optimism that a new effective vaccine can be delivered in the next ten years.

4.1 New tuberculosis vaccines in pipeline

TB vaccines under development are designed to work in one or several of the following ways: (1) Prevent infection; (2) Prevent primary disease; (3) Prevent latent infection; (4) Prevent reactivation of latent infection or (5) Shorten the course and improve the response to chemotherapy.

4.1.1 Tuberculosis Vaccine Pipeline - 2010

In the 2010 Tuberculosis Vaccine Pipeline, tuberculosis vaccine candidates are presented in three categories:

a. Candidates Tested in Clinical Trials (Section I): TB vaccine candidates that were in clinical studies in 2010. Certain candidates that have been in clinical studies but are not currently in clinical trials are listed as ‘completed.’
b. Candidates in Preclinical Studies & GMP-2010 (Section II): TB vaccine candidates that as of 2010 were not yet in clinical trials, but have been manufactured under good manufacturing practice (GMP) for clinical use and have undergone some preclinical testing that meets regulatory standards.
c. Next Generation Candidates-2010 (Section III): TB vaccine candidates that are in the research and development stage with some preclinical testing performed to show that they may confer protection.

Type of vaccine	Products	Product description	Sponsor	Indication	Status as of 2010
Recombinant Live	VPM 1002	rBCG Prague strain expressing listeriolysin and carries a urease deletion mutation	Max Planck, Vakzine Projekt Management GmbH,	Prime rBCG, Booster	TBVI Phase Ib
	rBCG30	rBCG Tice strain expressing 30 kDa Mtb antigen 85B; phase I completed in U.S..	UCLA, NIH, NIAID, Aeras	Prime	Phase I [completed]
	AERAS-422	Recombinant BCG expressing mutated PfoA and overexpressing antigens 85A, 85B, and Rv3407	Aeras	Prime	Phase I
Viral Vectored	Oxford MVA85A / AERAS-485	Modified vaccinia Ankara vector expressing Mtb antigen 85A	Oxford Emergent Tubcerulosis Consortium (OETC), Aeras	Boost, Post-infection, Immuno-therapy	Phase IIb
	AERAS-402/ Crucell Ad35	Replication-deficient adenovirus 35 vector expressing Mtb antigens 85A, 85B, TB10.4	Crucell, Aeras	Boost	Phase IIb
	AdAg85A	Replication-deficient adenovirus 5 vector expressing Mtb antigen 85A	McMaster University in Canada.	Prime , Boost	Phase I
Recombinant Protein	M72 + AS01 (GSK M72)	Recombinant protein composed of a fusion of Mtb antigens Rv1196 and Rv0125 & adjuvant AS01	GSK, Aeras, othrers	Boost, Post-Infection	Phase II
	Hybrid-I+ IC31 (SSI Hybrid 1 (H1))	Adjuvanted Recombinant protein (Ag85B plus ESAT 6) fusion molecule with adjuvant (IC31). recombinant protein composed of Mtb antigens 85B and ESAT-6	Statens Serum Institute (SSI), TBVI, EDCTP, Intercell	Prime, Boost, Post-infection	Phase I/II
	Hybrid-I+ CAF01	Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT6	SSI	Prime, Boost, Post-inection.	Phase I –

Type of vaccine	Products	Product description	Sponsor	Indication	Status as of 2010
	HyVac 4/AERAS 404, +IC31 Recombi-nant protein (Ag85B plus TB10.4) fusion molecule with adjuvant (IC31).	Adjuvanted recombinant protein composed of a fusion of Mtb antigens 85B and TB10.4	SSI, Sanofi Pasteur, Aeras, Intercell	Boosting vaccine for prevention of new TB in BCG vaccinated infants.	Phase I
Whole Cell, Inactivated or Disrupted	M. vaccae (Investiga-tional heat-killed preparation derived from rough variant of an environmental isolate).	Inactivated whole cell non TB mycobacterium; phase III in BCG primed HIV+ population completed; reformulation pending	NIH, Immodulon (with Aeras).	Booster to BCG, for HIV infected. Post-infection, Immuno-therapy	Phase III [completed]
	Mw [M. indicus pranii (MIP)]	Whole cell saprophytic non TB mycobacterium	Department of Biotechnology (Ministry of Science & Techno-logy, Govern-ment of India), M/s. Cadila Pharma-ceuticals Ltd.	Immuno-therapy	Phase III
	RUTI	Fragmented Mtb cells Based on detoxified cellular fragments of M. tuberculosis.	Archivel Farma, S.I. Being develop-ed by Germans Trias i Pujol Health Science Research	Targets subjects with latent new TB infection (LTBI).	Phase II

Type of vaccine	Products	Product description	Sponsor	Indication	Status as of 2010
	M. smegmatisa	Whole cell extract; phase I completed in China –	-	Boost, Post-infection, Immuno-therapy	Phase I [completed]

Table 2. Candidates Tested in Clinical Trials (SECTION I)

The vaccine candidates are further subdivided into specific vaccine types: (1) Recombinant Live (2) Viral Vectored (3) Recombinant Protein or (4) Other. A brief description is also provided. The Table lists vaccines intended to be used as a Prime (P) or Booster (B) vaccine, as a Post-infection vaccine (PI) or in immunotherapy (IT). The information contained here was provided and updated by the vaccine developers unless otherwise indicated. In cases where an update regarding a previously listed vaccine candidate was not received in 2010, the 2009 listing was retained.

Type of vaccine	Products	Product description	Sponsor	Indication
Recombinant Live	Mtb [ΔlysA ΔpanCD ΔsecA2]	Non replicating, Mtb strain auxotrophic for lysine and pantothenate; attenuated for secA2	Albert Einstein College of Medicine	Prime
	MTBVAC [ΔphoP, Δfad D26]	Live vaccine based on attenuation of Mtb by stable inactivation by deletion of phoP and fad D26 genes	University of Zaragoza, Institute Pasteur, BIOFABRI, TBVI	Prime
Recombinant Protein	HBHA	Naturally methylated 21 kDa purified protein from M.bovis BCG	Institute Pasteur of Lille, INSERM, TBVI	Prime, Boost, Post-infection, Immuno-therapy
	Hybrid 56 + IC31	Adjuvanted recombinant protein composed of Mtb antigens 85B, ESAT6 and Rv2660	SSI, Aeras, Intercell	Prime , Boost, Post-Infection.
Other	HG85 A/B	Chimeric DNA vaccines—Ag85A/Ag85B	Shanghai H&G Biotech	Boost, Immuno-threapy
	Spray-dried BCGb	Live attenuated BCG Danish Strain spray-dried for nasal administration	MEND	Prime

Table 3. Candidates in Preclinical Studies & GMP 2010 (SECTION II)

Type of vaccine	Products	Product description	Sponsor	Indication
Recombi-nant Live	HG856, BCG	rBCG overexpressing chimeric ESAT6/Ag85A DNA fusion protein	Shanghai Public Health Clinical Center	Boost, Post-infection, Immunotherapy
	IKEPLUS M. smegmatis with ESX3 deletion/ complementation	Live M. smegmatis with deletion of ESX3 encoding locus and complementation with Mtb locus	Albert Einstein College of Medicine, Aeras	Boost
	paBCG	BCG with reduced activity of anti apoptotic microbial enzymes including SodA, GlnA1, thioredoxin, and thioredoxin reductase	Vanderbilt University	Prime
	Proapoptotic rBCG	Recombinant BCG expressing mutated PfoA and including mutations shown at AECOM to induce macrophage apoptosis	Aeras, Albert Einstein College of Medicine	Prime
	rBCG(mbtB)30	rBCG with limited replication overexpressing the 30 kDa Mtb Antigen 85B	UCLA, NIH, NIAID	Prime
	rBCG T+B rM. smegmatis T+B	rBCG and rM. smegmatis expressing multiple T and B epitopes of Mtb	Finlay Institute, Universiti Sains Malaysia	Prime, Boost, Post-Infection
	rBCG TB Malaria	Expresses multiple epitopes of Mtb fused to malarial epitopes and antigens	Universiti Sains Malaysia	Prime , Boost, Ppost-Infection
	rBCG38	rBCG Tice strain overexpress the 38 kDa protein	Universidad Nacional Autónoma de México	Prime, Boost
	rBCGMex38	rBCG Mexico strain overexpress the 38 kDa protein	Universidad Nacional Autónoma de Mexico	Prime, Boost
	rBCG overexpressing L,D Transpeptidase	Recombinant M. bovis BCG overexpressing an Mtb L,D Transpeptidase	Johns Hopkins University	Prime

Type of vaccine	Products	Product description	Sponsor	Indication
	Replication deficient rBCG	Recombinant BCG expressing PfoA and classical, latency, and resuscitation antigens in live, non replicating background	Aeras	Prime
	rM.microti30 rM.microti38	rM.microti strain overexpress the 30 or 38kDa protein	Universidad Nacional Autónoma de Mexico	Prime
	Streptomyces live vector	Recombinant streptomyces expressing multiple T and B epitopes from M.tb	Finlay Institute, Institute of Pharmacy and Food, Cuba	Prime, Boost, Post-infection,
Recombi-nant Protein	ID93 in GLASE adjuvant	Subunit fusion protein composed of 4 Mtb antigens	Infectious Disease Research Institute	Boost, Post-infection, Immunotherapy
	Latency fusion proteins	Recombinant fusion proteins composed of antigens 85A85B,Rv3407, Rv3407,Rv1733, Rv2626, Rv0867,R1884,Rv2389	Aeras	Boost
		30kDa Mtb Ag85B protein purified from rM. Smegmatis	UCLA, NIH, NIAID	Boost, Post-infection
	R32Kda (recombinant 85A)	Purified recombinant 85A protein from BCG	Bhagawan Mahavir Medical Research Center, LEPRA Society Blue Peter Research Centre	Boost, Postinection ,Immuno-therapy
Viral Vectored	Recombi-nant LCMV	Recombinant lymphocytic choriomeningitis virus expressing Ag85A, Ag85B, or Ag85BESAT6	University of Geneva	Prime, Boost, Post-infection, Immunotherapy
	pND 14 vector	With tpa factor expressing esat6, cfp10, hspx, Ag85A, Ag85B, or Ag85	HEC Pakistan	Prime, Boost,

Type of vaccine	Products	Product description	Sponsor	Indication
Other	Ac2SGL Diacylated Sulfoglycolipid	Mycobacterial lipids with Ac2SGL, a novel glycolipid antigen	Institut de Pharmacologie et Biologie Structurale du CNRS	Prime, Boosr, Post-infection, Immunotherapy
	HG856A	Chimeric DNA vaccines—ESAT6/Ag85A; Ag85A/Ag85B	Shanghai H&G Biotech	Boost, Immun-otherapy
	HG856SeV	Recombinant Sendai virus overexpressing chimeric ESAT 6/Ag85A protein	Shanghai H&G Biotech	Boost
	Hsp DNA vaccine	Codon optimized heat shock protein from M. leprae, a CpG island	Cardiff University, Sequella	Boost
	HVJ Envelope/HSP65 DNA+IL12 DNA	Combination of DNA vaccines expressing mycobacterial heat shock protein 65 & IL12	Osaka University	Boost, Post-infection, Immuno-therapy
	Liporale BCG	Live attenuated BCG Danish Strain in a novel lipid adjuvant and delivery system for an oral vaccine	Immune Solutions Ltd.	Prime, Boost
	Mycobacterial liposomes and proteosomes	Liposomes from M. smegmatis and proteoliposomes from BCG and M. smegmatis	Finlay Institute Universiti Sains Malaysia	Prime, Boost, Post-inection, Immuno-therapy
	NasL3/AM85B conjugate	Nasal vaccine with man capped Arabinomannan oligosaccharide conjugated to Ag85B in Eurocine L3TM adjuvant	Karolinska Institute	Boost
	NasL3/HtkBCG (BCG adjuvant)	Intranasal heat killed whole BCG Copenhagen strain in Eurocine L3TM adjuvant	Karolinska Institute	Prime, Boost, Post-infection, Immunotherapy
	PS conjugate	Subunit Mtb polysaccharide protein conjugate	Albert Einstein College of Medicine	Boost

Type of vaccine	Products	Product description	Sponsor	Indication
	pUMVC6/7 DNA	DNA vaccine plasmid vectors pUMVC6 or pUMVC7 expressing Rv3872, Rv3873, Rv3874, Rv3875 or Rv3619	Kuwait University	Prime
	Recombinant B/HPIV	Recombinant B/HPIV vector encoding fusion of antigens 85A85B,Rv3407, Rv3407, Rv1733, Rv2626, Rv0867, Rv1884, Rv2389	NIH, Aeras	Boost
	TBioVax	Heat shock HspC protein antigen complexes	ImmunoBiology Ltd.	Boost
	TBVax	T cell epitope based DNA prime/peptide boost vaccine	EpiVax , Inc.	Boost, Post-inection

Key: BCG – Bacille Calmette Guérin, IL – Interleukin, GMP – Good Manufacturing Practices, GSK – GlaxoSmithKline Biologicals, M. bovis – Mycobacterium bovis, Mtb – Mycobacterium tuberculosis, NIAID– National Institute of Allergy and Infectious Diseases, NIH – National Institutes of Health, OETC – Oxford Emergent Tuberculosis Consortium, Ltd. SSI – Statum Serum Institute, TBVI – Tuberculosis Vaccine Initiative, UCLA – University of California Los Angeles,
Source: Tuberculosis Vaccine Pipeline 2010

Table 4. Next Generation Candidates – 2010 (SECTION III)

5. Tuberculosis diagnostics

Despite substantial investments and progress made in expansion of the directly observed therapy, short course (DOTS and DOTS Plus) strategy and improved treatment completion rates, inadequate case detection is still a major problem in the efforts to ensure global control of tuberculosis. There is need for health workers to be able to quickly detect resistant forms of tuberculosis and also to be able to distinguish clearly between active and latent forms.Efforts during the past decade to consistently diagnose and treat the most infectious cases have not been very successful. Insufficient access to advanced diagnostic tests has contributed to limited performance in the effort to control tuberculosis in general and MDR/XDR –TB in particular. Up to now, national tuberculosis programmes in disease endemic countries continue to rely largely on antiquated and inaccurate methods such as direct smear microscopy, solid culture, chest radiography, and tuberculin skin testing. There is still no rapid test that can allow early detection of active tuberculosis at health clinics , the biggest challenge bing presented by diagnosis of smear-negative tuberculosis in adults infected with HIV and in children.

To these shortcomings is added the fact that even the existing diagnostics are not used to their full potential because of poor access to health care and failures in health-care delivery systems, including poor coordination between national HIV/AIDS and tuberculosis programmes. There are rampant diagnostic delays, misdiagnoses, and inadequate implementation of existing tests leading to increased morbidity and mortality in patients,

leave alone allowing continued transmission of MDR/XDR strains. The shorcomings of present-day case detection approaches are most pronounced in countries with a high prevalence of HIV infection or MDR/XDR tuberculosis, or both which heralds a bleaque picture for the control of the disease.

5.1 Barriers to development of new tuberculosis diagnostics

The most important barries have been (1)market failure because industry tends to avoid developing and marketing products that will be mainly used for poor patients in resource-limited countries because such products will not generate profits; and (2) health systems in developing countries are generally weak due to poor management, insufficient financial resources, inadequate human resources, and poor laboratory capacity, making them unable to take advantage of tuberculosis diagnostics to achieve best possible performance, and to introduce new advances in diagnostic technologies.

5.2 Tuberculosis diagnostics pipeline

Over the past decade, there has been an unprecedented level of interest and activity focused on the development of new tools for TB diagnosis, with agencies like the Stop TB Partnership's New Diagnostics Working Group (NDWG), the Foundation for Innovative New Diagnostics (FIND), the Global Laboratory Initiative (GLI), the World Health Organization (WHO) and the Special Programme for Research and Training in Tropical Diseases (TDR), several industry partners, non-governmental agencies, and national TB programs being heavily involved. The impetus has been boosted by funding agencies such as the Bill & Melinda Gates Foundation, the Global Fund to Fight AIDS, TB and Malaria (GFATM), and UNITAID that have provided the much needed resources. As a result of this involvement, there is now a strong pipeline of improved or new tools for TB diagnosis.

Fluorescence microscopy is widely used in high income countries since it offers increased sensitivity, and has logistical advantages such as less technician time, but is rarely used in resource-limited countries. Several light-emitting diode (LED) microscopes that can be used in fluorescence microscopy have been developed in the past few years. They are inexpensive, robust, consume little electricity, are highly sensitive, and need less technician time than does Ziehl-Neelsen microscopy. WHO recommended that conventional fluorescence microscopy be replaced by LED microscopy in all settings and that LED microscopy be phased in as an alternative for conventional Ziehl-Neelsen microscopy in both high-volume and low-volume laboratories. Efforts are also underway to minimise diagnostic delays and to improve system efficiency by optimising the number of specimens that are needed and the way in which they are collected (eg, so-called same-day diagnosis, using two sputum smears collected on the same day).

5.3 Limitations of the existing diagnostics pipeline

Although promising work has either been done or is the pipeline we are yet to see a simple, rapid, inexpensive point-of-care test for active tuberculosis that can perform as well or better than conventional smear microscopy, and which can deliver results within minutes without sophisticated equipment or laboratory requirements. Such equipment that can be able to do

point-of-care diagnostic tests are necessary if we are to be able to control diseases such as tuberculosis that need lengthy standardised, decentralised therapy. However, with patient, community, and activist groups providing increased funding and resources to develop such tests, an ideal diagnostic system can be availed in afew years to come.

6. Biomarkers for tuberculosis

6.1 Importance of biomarkers

It is an established fact that biomarkers are important tools for provision of prognostic information about future health status, for individual patients or cohorts in clinical trials. They can be used to indicate normal or pathogenic processes, or pharmacological outcomes of therapeutic interventions. Basing on epidemiological, therapeutic, pathophysiological, or other scientific evidence, biomarkers can form the basis of surrogate endpoints, which can serve as substitutes for clinical endpoints in clinical trials. By using this approach, it possible to use them in drug candidate selection during drug discovery and accelerating dose selection in early clinical research, as well as shortening the time to licensing of new drugs and vaccines. In day-to-day clinical care, biomarkers can allow stratification of individual patients according to outcome risks, thus easing targeted interventions that might not otherwise produce overall benefit. Use of biomarkers can also play a vital role in the advancement of basic knowledge of disease pathogenesis.

With regard to tuberculosis research, the need for biomarkers is paramount in the areas of: (1) patients with active disease, to predict durable or non-relapsing treatment success; (2) patients with latent M. tuberculosis infection, to indicate reactivation risk and predict treatment success; and (3) people other than those with active disease, to indicate protection from tuberculosis by new vaccines. Although a number of studies have been undertaken in this area outstanding breakthroughs are yet to be made.

7. Existing gaps in research on XDR TB

In the majority of areas where XDR -TB has been identified, the actual prevalence of resistance to first- and second-line drugs among TB cases is unknown. The figures being used are maily estimates and, therefore, there is need to determine the exact global prevalence and incidence rates of XDR-TB.

The risk factors for and transmission dynamics of XDR-TB in domestic and international settings are not completely understood and the survival rates among patients with XDR-TB have not been adequately analyzed. Furthermore, host/pathogen determinants of survival, including the effect of co-morbidities, are yet to be completely elucidated.

In the field of diagnostics, the methods for detecting and documenting outbreaks of XDR-TB both domestically and internationally are not currently optimized to allow a rapid response and to these is added lack of effective and safe treatment regimens for XDR-TB which are yet to be established. Rapid, point-of-care identification of drug-sensitive and drug-resistant pulmonary and extrapulmonary TB among HIV-negative and HIV-positive adults and pediatric populations and reliable early identification of latent M. tuberculosis infection are not yet possible.

Another scientific obstacle impeding progress is that up to now we do not have a icomplete understanding of the biology of M tuberculosis and its nteractions with the human host. These knowledge gaps impede the development of biomarkers that can distinguish between the different forms and stages of tuberculosis especially in immunocompromised patients and in children. The present diagnostic tests for latent M. tuberculosis infection do not adequately distinguish resolved from persistent infection, and are unable to efficiently identify individuals who are at highest risk of reactivation.

Furthermore, studies into the predictive value of Interferon-γ–release assays (IGRAs) have only shown modest outcomes, and several studies show similarly low rates of progression in people with positive tuberculin skin test and IGRA results. Other gray areas are in the area of diagnosis of smear-negative tuberculosis in children and HIV-infected individuals, as well as inablity to carry out rapid and accurate identification of resistance to second-line antituberculosis drugs. Although molecular markers have been identified and can be used as rapid and accurate tests for isoniazid and rifampicin resistance, testing for the resistance that characterises extensively drug-resistant tuberculosis is still a problem. Well-validated surrogate markers to rapidly assess clinical efficacy of new chemotherapeutic agents and regimens against XDR-TB are yet to be put in place although a lot of research is on going.

With regard to treatment of MDR/XDR-TB, effective treatments specifically designed for active or latent MDR/XDR-TB infection have not been established. Coupled with this is the fact that the complete pharmacology of existing and new TB drugs, including interactions with antiretroviral medications commonly used among high-risk populations, has not been adequately assessed.

Furthermore, there are still gaps in the areas of characterisation of the efficacy, safety and pharmacology of TB chemotherapeutics in special populations such as children, injection drug users and persons with HIV/AIDS, among other situations.

Further still, the quality of currently available services and treatments for MDR/XDR-TB patients has not been monitored or evaluated sufficiently and safe and more effective treatment regimens and appropriate follow-up procedures for managing contacts of XDR-TB patients are yet to be properly established. Another area of concern has been the fact that, despite M. tuberculosis having been known for a long time, there have been gaps in the knowledge of its characteristics with regard to growth, physiology, biochemistry, genetics, and molecular biology although this situation is now being addressed, as more and more research has been undertaken in these areas.

Finally, there is need to have in place, a comprehensive and up-to-date estimate of the costs of diagnosing, treating, and managing XDR-TB if cost-effective strategies to prevent XDR-TB are to be established.

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2

Chemotherapeutic Strategies and Targets Against Resistant TB

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1. Introduction

Chemotherapeutic cure for about 40,000 years old lethal disease – TB (Callaway, 2008), was started mere ~65 years ago, with the discovery of antibiotic- streptomycin. A few effective drugs against TB have been developed since then and have been classified mainly as first-line (viz. rifampicin, isoniazid, pyrazinamide and ethambutol) and second-line drugs (e.g. ciprofloxacin, levofloxacin, cycloserine, clofazimine etc.). Drugs like rifambutin, clarithromycin and linezolid may be considered as "third line" drugs. The current course of therapy with the first-line TB drugs is more than 40 years old and is slowly becoming outdated due to emergence of multidrug-resistant tuberculosis (MDR-TB, resistant to the two first line drugs) and extensively drug-resistant tuberculosis (XDR-TB, an MDR-TB that is resistant to fluoroquinolones and also to any one of the three injectable second- line drugs: amikacin, capreomycin or kanamycin) (World Health Organisation, [WHO], 2011). Treatment with the second line drugs is limited due to the associated toxicity which halts therapy prior to cure in more than half of the patients suffering from serious side effects. The "third line" drugs have issues of proven efficacy/effectiveness and impractical cost. Longer duration of treatment, usually for six months, with complex regimens leads to poor compliance. Although poor compliance can be managed to great extent by Directly Observed Treatment, Short course (DOTS) launched by World Health Organization (WHO); but that is possible practically in developed countries only where manpower along with financial needs are met adequately. Apart from these problems, during this long treatment period, the patient and one's family suffer from socioeconomic problems, whereby psychological issues such as risk of depression come in picture. Side effect(s) of drugs, due to long treatment, is another major concern.

Researchers have been trying to find out the answer for why the TB treatment is so long and complex. McCune et al found considerable difference in the efficacy of drugs against *Mycobacterium tuberculosis* (*Mtb) in vitro* and *in vivo* (McCune & Tompsett, 1956; McCune et al 1956). However, other researchers (Barclay et al. 1953; Clark, 1985) showed that bioavailability is not a concern. It was proposed that this persistence of *Mtb* might be due to physiologic heterogeneity of bacteria in the tissues (Mitchison, 1979; Handwerger & Tomasz, 1985).

Mitchison found that the lesions have at least four different populations of *Mtb*:

a. Actively growing bacilli: can be killed by isoniazid
b. Bacilli with spurts of metabolism: can be killed by rifampicin
c. Bacilli with low metabolic activity (reside in acidic pH environment): can be killed by pyrazinamide
d. Dormant bacilli: not killed by any existing drug/regimen.

The actively multiplying bacilli are killed in the first 2 days, the remaining are dormant, which are sterilized very slowly by the existing drugs and thus the treatment period is stretched so long (Jindani et al., 2003).

Bacillus Calmette Guerin (BCG), the only approved vaccine for TB in humans, contains attenuated strain of *M. bovis*. It is generally considered safe; however this vaccination may lead to TB infection in immunocompromised individuals. Moreover, BCG only reliably protects against tuberculosis in newborns and fails in adult pulmonary tuberculosis, the most prevalent form (Kaufmann, 2011).

Due to the associated global health and socioeconomic concerns, the increasing rates of MDR-, XDR-TB, and TB-HIV coinfection, the discovery and development of potent new anti-TB agent(s), without cross-resistance with current antimycobacterial drugs, is urgently needed.

This chapter includes brief discussion on existing TB drugs and covers a comprehensive picture of the anti-TB drug discovery status heading to achieve a goal of better drugs/regimen in terms of the desired properties stated above.

2. Existing TB drugs

After the discovery of Streptomycin in 1944, 15-20 antimycobacterial drugs have been approved and used for TB therapy according to the need, availability, cost and safety profile. These existing TB drugs can be classified into first line, second line and third line drugs (also summarized in Tables 1-3).

2.1 First line drugs

2.1.1 Rifampicin, RMP or R

Rifampicin was discovered in 1966. It is a semisynthetic, intensely red coloured bactericidal antibiotic (MIC 0.05-0.5 μg/mL) derived from *Amycolatopsis rifamycinica*. Its penetration to cerebrospinal fluid makes it useful to treat tuberculosis meningitis (Nan et al, 1992). RMP, should be used in combination with other antibiotics as resistance develops quickly during monotherapy. RMP may be excreted in breast milk, therefore breast feeding may be avoided during treatment. However no serious side effects have been observed in breastfed infants during RMP therapy (Peters & Nienhaus, 2008; Drobac et al 2005).

2.1.1.1 Mode of action

RMP inhibits DNA-dependent RNA polymerase in bacterial cells by binding its β-subunit, thus preventing transcription to RNA and subsequent translation to proteins (Aristoff et al, 2010; Tomioka, 2006). RMP-resistant bacteria produce RNA polymerases with subtly different β subunits which resists drug-inhibition (O'Sullivan et al, 2005)

Rifampicin

2.1.1.2 Dosing

Daily regimen 10 mg/kg (up to 600 mg/day) orally or intermittent regimen 10 mg/kg (up to 600 mg/day) orally, are prescribed. (The American Thoracic Society [ATS], 2006).

2.1.1.3 Adverse effects

The main target organs for side effects of RMP are the liver and the gastrointestinal system. Adverse effects include hepatitis with elevation of bile and bilirubin concentrations, anaemia, leucopenia, thrombocytopenia, bleeding, febrile reaction, eosinophilia, leucopenia, thrombocytopenia, purpura, haemolysis and shock, and nephrotoxicity (International Programme on Chemical Safety [INCHEM] a).

2.1.1.4 Pharmacokinetics

The half-life of RMP is generally 2 h (Acocella, 1978). Its absorption is not affected by antacids (Peloquin et al., 1999 a). RMP ester function is hydrolyzed in the bile by esterase catalyzed high pH. The deacetylated form of RMP can not be absorbed by the intestine and thus eliminated from the body.

2.1.1.5 Interactions

Absorption of RMP is considerably hindered when it is combined with another anti-TB drug, 4-aminosalicylic acid (PAS). Therefore, these two anti-TB drugs must be administered separately (8 to 12 hours interval). RMP affects metabolism of several known drugs, viz. warfarin, oral contraceptives, cyclosporine, itraconazole, digoxin, verapamil, nifedipine, simvastatin, midazolam and HIV protease inhibitors. Other drugs for possible interactions include clarithromycin, lorazepam atorvastatin, antiretroviral agents, rosiglitazone/pioglitazone, celecoxib, caspofungin (Baciewicz et al., 2008).

2.1.2 Isoniazid, INH or H

INH (isonicotinylhydrazine) was discovered in 1952. It is bactericidal (MIC 0.01-0.2 μg/mL) to fast replicating mycobacteria (Singh & Mitchison, 1954) but is bacteriostatic to slow-growing mycobacteria. Since the bacteria may exist in a non growing state (latent) for long periods, therapy for latent tuberculosis with INH is continued for a longer duration (6-12 months). However, INH monotherapy is never recommended to treat active tuberculosis due to the development of resistance.

Isoniazid

2.1.2.1 Mode of action

INH itself is a prodrug and is activated by mycobacterial catalase-peroxidase enzyme KatG which catalyzes the formation of isonicotinic acyl-NADH complex from isonicotinic acyl and NADH. This complex then binds to the enoyl-acyl carrier protein reductase known as InhA, consequently blocking the natural substrate enoyl-AcpM and fatty acid synthase. This results in inhibition of mycolic acid synthesis which is essential for the mycobacterial cell wall formation. A direct role for some INH-derived reactive species, such as nitric oxide, in inhibiting mycobacterial metabolic enzymes has also been shown (Timmins & Deretic, 2006; Suarez et al., 2009).

2.1.2.2 Metabolism

INH is metabolized in liver and its metabolites are excreted in the urine with 75 to 95% of the dose excreted in 24 hours (Ellard & Gammon, 1976).

2.1.2.3 Dosing

In adults, the recommended dose is 5 mg/kg/day (max 300 mg daily). For intermittent dosing (twice or thrice/week), 19-15 mg/kg/day (max 900 mg/day) is a standard dose. For patients with slow clearance of INH are put on reduced dosages. The recommended dose for children is 8 to 12 mg/kg/day (McIlleron et al., 2009; [ATS], 2006).

2.1.2.4 Adverse effects

INH causes acute toxicity in the CNS. It induces generalized convulsions, coma and metabolic acidosis. Death may occur from acute respiratory failure or hypotension. Liver, peripheral nervous and haematologic systems are the main target organs of INH chronic toxicity resulting in acute hepatitis, peripheral neuropathy, haemolytic anaemia (INCHEM, b). Vitamin B_6 (10–50 mg/day) supplements are suggested to compensate its (Vitamin B_6) depletion during treatment which may lead to peripheral neuropathy and CNS related side effects (Yamamoto et al., 2011).

2.1.3 Pyrazinamide, PZA or Z

PZA was discovered in 1952. It acts mainly as bacteriostatic agent but can be bactericidal for replicating *Mtb*. Its MIC is 20-100 µg/mL at pH 5.5 or 6.0. This drug is used in the first two months of treatment to shorten the duration of treatment, since regimens not containing PZA must be taken for nine months or more (Hong Kong Chest Service [HKCS]/ British Medical Research Council [BMRC], 1981). PZA crosses meninges and thus is effective for the treatment of tuberculous meningitis (Donald & Seifart, 1988).

Pyrazinamide

2.1.3.1 Dosing

20–25 mg/kg daily or 30–40 mg/kg thrice a week is a recommended dose. ([ATS], 2006).

2.1.3.2 Pharmacokinetics

PZA is well absorbed orally. It is metabolised by liver and the metabolic products are excreted by kidneys (Lacroix et al, 1989). The overall pharmacokinetics may differ in childrens (Arya et al., 2008).

2.1.3.3 Mode of action

PZA is actually a prodrug. In acidic conditions, the enzyme pyrazinamidase (present in *Mtb*), converts it to the active form, pyrazinoic acid which consequently inhibits the enzyme fatty acid synthase (FAS) I, required by the bacterium to synthesise fatty acids (Zhang & Mitchison, 2003; Zimhony et al., 2007). Mutations of the pyrazinamidase gene (*pncA*) are responsible for PZA resistance in *Mtb* (Scorpio & Zhang, 1996)

2.1.3.4 Adverse effects

Some common adverse effects of PZA treatment include hepatotoxicity, joint pains (arthralgia), nausea, vomiting, anorexia, sideroblastic anemia, skin rash, hyperuricemia, dysuria, urticaria, pruritus, interstitial nephritis, malaise, porphyria and fever (rare) (Forget & Menzies, 2006).

2.1.4 Ethambutol, EMB or E

EMB was discovered in 1961 by Lederle Laboratories. It is a bacteriostatic drug. In spite of a relatively modest MIC of 10 μM like PZA, it is a useful drug for tuberculosis chemotherapy, partly because of very low toxicity and relatively few side-effects (Wilkinson et al., 1961; Thomas et al., 1961).

Ethambutol

2.1.4.1 Adverse effects

Adverse effects may include peripheral neuropathy, red-green color blindness, arthralgia, hyperuricaemia, vertical nystagmus and optic neuritis (Lim, 2006).

2.1.4.2 Mode of action

It blocks formation of *Mtb* cell wall by interfering in the synthesis of arabinogalactan (an essential component for the formation of mycolyl-arabinogalactan-peptidoglycan complex of the *Mtb* cell wall) via inhibiting the enzyme arabinosyl transferase (Belanger et al., 1996; Wiles & Jacobs Jr, 1997).

2.1.4.3 Pharmacokinetics

It is well absorbed in the gastrointestinal tract, and well distributed in body tissues and fluids. 50% of the given dose is excreted unchanged in urine (Peloquin et al., 1999 b).

Drug	Mode of Action	Target	Daily Dose (Max. Dose)	Possible adverse reactions
Rifampicin	Inhibits RNA synthesis	RNA polymerase beta subunit	10mg/kg (600 mg/day)	Pruritus, rash, flushing, redness and watering of eyes, breathlessness, nausea, vomiting, abdominal cramps, diarrhea, jaundice, hepatitis, liver failure (rare and in severe cases), chills, fever, headache, arthralgia, and malaise
Isoniazid	Inhibition of cell wall formation	Acyl carrier protein reductase	5 mg/kg/ day (300 mg daily)	Rash, hepatitis, sideroblastic anemia, metabolic acidosis, peripheral neuropathy, mild central nervous system (CNS) effects, intractable seizures (status epilepticus), headache, poor concentration, weight-gain, poor memory, and depression
Pyrazinamide	Disruption of membrane transport and energy depletion	Membrane energy metabolism	20–25 mg/kg daily (30 mg/kg)	Hepatotoxicity, joint pains (arthralgia), nausea, vomiting, anorexia, sideroblastic anemia, skin rash, hyperuricemia, dysuria, urticaria, pruritus, interstitial nephritis, malaise; porphyria
Ethambutol	Inhibition of cell wall formation	Arabinosyl transferase	15 mg/kg daily (25 mg/kg)	Peripheral neuropathy, color blindness, arthralgia, hyperuricaemia, vertical nystagmus and optic neuritis.

Table 1. First Line Drugs

2.2 Second Line Drugs (SLDs)

A drug may be categorized as second (or as third) line if it includes one or more of the following: i. it has side-effects beyond a tolerance threshold (e.g., cycloserine), ii. its

administration is not oral and at the same time (sub)equivalent/better affordable oral medications are available, iii. it is less effective than the first-line drugs (e.g., *p*-aminosalicylic acid); iv. its cost is impractical for routine treatment.

2.2.1 Classification of SLDs

The available second-line TB drugs (SLDs) can be classified as:

1. Aminoglycosides: e.g. amikacin (AMK), kanamycin (KM), gentamicin etc;
2. Polypeptides: e.g., capreomycin, viomycin, enviomycin;
3. Fluoroquinolones: e.g., ciprofloxacin (CIP), levofloxacin, moxifloxacin (MXF);
4. Thioamides: e.g. ethionamide, prothionamide
5. Oxazolidinone: (Cycloserine, the only antibiotic in its class);
6. *p*-Aminosalicylic acid (PAS or P).

Details of some of these SLDs are provided in the table 2.

2.3 Third line drugs

Apart from the reasons listed under second line drugs, a drug may be considered as a third line if it is useful but lacks sufficient efficacy proofs. Rifabutin, macrolides: (e.g., clarithromycin), linezolid, thioacetazone, thioridazine, arginine, vitamin D may be considered as third line antituberculosis drugs.

Drug (Discovery) Route	**Structure**	**Mode of Action**	**Daily Dose (Max. Dose)**	**Adverse effects**
Amikacin (1972) IM or IV		Inhibits protein synthesis by (binds to the bacterial 30S ribosome)	15 - 30 mg/kg (1 g) MIC 4-8 μg/mL (CDC, 1994) [a]	Auditory, vestibular, and renal toxicity, dizziness
Kanamycin (1957) IM or IV		Inhibitions protein synthesis via S12 ribosomal protein & 16 S RNA.	15 - 30 mg/kg (1 g) MIC 1-8 μg/mL	Auditory, vestibular, and renal toxicity
Capreomycin (1963) IM or IV		Inhibits protein synthesis (binds to ribosomal subunit 16S and 23S rRNA (Johansen et al., 2006)	15 - 30 mg/kg (1 g) MIC 1.25–2.5 μg/mL (Heifets, 1988; Heifets & Lindholm-Levy 1989)	Auditory, vestibular, and renal toxicity
Streptomycin (1944) IM		Same as Kanamycin	15-40 mg/kg (1 g) MIC 2-8 μg/mL	Renal, ophthalmic and respiratory toxicity

Drug (Discovery) Route	**Structure**	**Mode of Action**	**Daily Dose (Max. Dose)**	**Adverse effects**
Cycloserine (1952) Oral		Inhibition of peptidoglycan synthesis (D-alanine racemase)	15 - 20 mg/kg (1 g) MIC 5-20 μg/mL	Psychosis, Rashes, Convulsions Depression
Ethionamide (1956) Oral		Inhibition of mycolic acid synthesis	15 - 20 mg/kg (1 g) MIC 0.6-2.5 μg/mL	GI upset Hepatotoxicity Hypersensitivity Metallic taste
PAS (1946) Oral		Inhibition of folic acid and iron metabolism (unknown target)	150 mg/kg (16 g) MIC 1-8 μg/mL	GI upset Hypersensitivity Hepatotoxicity Sodium load
Clofazimine (1954) Oral		Inhibits bacterial proliferation by binding to the guanine bases of bacterial DNA	100 - 300 mg/day MIC 0.12 - 0.24 μg/mL (Lu et al. 2008)	Eosinophilic enteritis, GI irritation, discoloration of the skin (upon sun exposure)
Ciprofloxacin (1960s) Oral		Inhibition of DNA replication and transcription by inhibiting DNA gyrase	750 - 1500 mg/day MIC 0.4 to 6.2 μg/mL (Trimble et al., 1987)	GI upset Dizziness Headache Hypersensitivity Restlessness
Levofloxacin (1992) Oral		Same to Ciprofloxacin	500 mg/day MIC 0.50 to 0.75 μg/mL (Rastogi et al., 1996)	Same as for Ciprofloxacin
Ofloxacin (1980) Oral		Same to Levofloxacin	600 - 800 mg/day MIC 0.12-2 μg/mL (Vacher et al, 1999)	Same as for Ciprofloxacin

MIC (wherever not referenced) is based on Inderlied & Salfinger, 1999.
IM - intramuscular, IV – intravenous
[a]Centre for Disease Control and Prevention

Table 2. Some Second Line Drugs (Source partly from North Dakota Department of Health, 2011).

3. Drug discovery programme

3.1 Early stage drug discovery

Tuberculosis is not only a health threat in Asian or European countries, but a serious problem globally. There is an ever increasing threat of drug-resistant TB appearing as an epidemic in many countries, particularly because no new classes of drugs have been specifically developed for the treatment of tuberculosis since the introduction of RMP in 1967. To tackle this devastating disease, continued high priority research and great efforts are being made to investigate new classes of drugs all over the world. Bill and Melinda Gates foundation has made a major financial philanthropic contribution in this regard worldwide. Governments and private sectors are also opening new avenues with significant funds to fight this disease. Apart from big industries, great roles are being played behind the curtains by basic and semi-applied researchers who start from scratch and work within financial constraints. Following are such examples of different classes of compounds from early stage screening studies.

Since research in this field gained momentum after the year 2000, selected reports published from the year 2000 onwards are included here. In view of the scope and timelines of this chapter, the focus of the literature cited is medicinal chemistry.

3.1.1 Nucleosides

Nucleosides have been of great interest as antiviral agents since decades back. Soon after the emergence of *Mtb* thymidine monophosphate kinase (TMPKmt) as a potentially attractive target for the design of a novel class of antituberculosis agents in year 2001 (Munier-Lehmann et al., 2001), several series of 2′-, 3′-, and 5-modified nucleosides and nucleotides were synthesized and evaluated for their affinities with respect to TMPKmt. Vanheusden et al, in 2002, reported monophosphates of AZT (**1**) and 2′-chloro-2′-deoxythymidine (**2**), as potent inhibitors of TMPKmt with Ki values of 10 and 19 μM, respectively.

1 **2**

These authors in the following year (Vanheusden et al, 2003) further reported a series of 3′-*C*-branched-chain-substituted nucleosides and nucleotides for the same target. The compounds **3, 4**, and **5** were reported to exhibit *K*i values of 10.5, 12, and 15 *μ*M, respectively, for TMPKmt.

3 4 5

In the year 2003, another series was reported by the same authors (Vanheusden et al., 2003) where 5-substituted-2′,3′,5′-trideoxyuridines (**6**-**8**) exhibited Ki values of 5, 7 and 12 μM, respectively, for TMPKmt.

6 7 8

Vanheusden et al. (Vanheusden et al., 2004) also reported a series of bicyclic analogues of thymidine where compound **9** demonstrated Ki of 3.5 μM for TMPKmt with good selectivity index (SI 200) over TMPKh.

9

In all these reports, however, only enzyme inhibition was described and inhibition of mycobacterial replication was not demonstrated.

A nucleoside antibiotic (CPZEN-45) produced by Streptomyces sp., first described in 2003 by the Microbial Chemistry Research Foundation (MCRF) and Meiji Seika Kaisa Ltd. of Japan, is now undergoing preclinical studies as an anti-TB agent. Details of CPZEN-45 are provided in the preclinical section.

COOH
N-CH3
H
N
O
H3C-N
O
H2N
O
O
O
N
HO OH HO OH

CPZEN-45

The complete genome sequence of *Mtb* has been deciphered (Cole et al., 1998). It encodes many of the enzymes required for DNA and RNA synthesis, and pyrimidine and purine biosynthesis. Our group (Johar et al, 2005) therefore hypothesized that modified nucleoside analogs could target several enzymes involved in nucleic acid metabolism. We were first to investigate and demonstrate potent antimycobacterial activity of 5-substituted pyrimidine nucleoside analogs (Johar et al., 2005). The antimycobacterial activity of test nucleosides was examined by mycobacterial growth inhibition using microplate alamar blue assay (MABA) (Franzblau et al., 1998). We observed that the most potent TMPKmt inhibitors reported earlier (Pochet et al., 2003; Vanheusden et al., 2002; Vanheusden et al., 2003) did not show antituberculosis activity in whole cell based assays. Thus the ability of a compound to function as a selective inhibitor of TMPKmt may not correlate well with its antimycobacterial activity. A cell based assay includes the steps of entry into bacterial cells and metabolism which could otherwise limit the efficacy of test molecules (Johar et al., 2005).

Since the initial report in 2005, our group (Kumar, R. and colleagues) has made a significant contribution in the evaluation of pyrimidine nucleosides as anti-tuberculosis agents. During our studies, we initially investigated the effect of a number of known antiviral and anticancer nucleosides modified in the base and/or sugar moiety against *Mtb*, *M. bovis* and *M. avium*. At concentrations upto 100 μg/ml, none of these agents showed potent inhibition of mycobacterial growth. In our subsequent studies, we designed, synthesized and examined a variety of 2-, 4-, 5- and/or 6-substituted/unsubstituted pyrimidine nucleosides containing various deoxyribose, ribose, arabinose, dideoxyribose and acyclic moieties. During our continued search of novel anti-TB agents, we found that 5-alkynyl substituted pyrimidine nucleosides were very potent inhibitors of mycobacteria (Rai et al., 2005). We (Johar et al, 2007), reported that pyrimidine nucleoside analogs 1-*β*-D-2′-arabinofuranosyl-5-dodecynyluracil (**10**), 1-(2′-deoxy-2′-fluoro-*β*-D-ribofuranosyl)-5-dodecynyluracil (**11**), and 1-(2′-deoxy-2′-fluoro-*β*-D-ribofuranosyl)-5-tetradecynyluracil (**12**) exhibited potent antimycobacterial potency in the series against *M. bovis* and *Mtb*. The MIC_{90} exhibited by compounds **10**, **11**,

and **12** (1-5 μg/mL) against *Mtb* H37Ra was close to that of the reference drug RMP (0.5-1 μg/mL). These compounds were also found to retain sensitivity against a RMP-resistant strain of *Mtb* H37Rv (American Type Culture Collection [ATCC] 35838, resistant to RMP at 2 μg/mL) at similar concentrations. No significant toxicity for these compounds was observed in MTT test *in vitro* against Vero cells and human foreskin fibroblast (HFF cells) up to a concentration of 100 μg/mL (CC_{50} >100 μg/mL).

10 **11** **12**

In the same year, we (Rai et al, 2007) further reported syntheses and evaluation of a series of 5-acetylenic derivatives of 2′,3- dideoxyuridine, and 3′-fluoro-2′,3′-dideoxyuridine for their antimycobacterial activity against *M. bovis*, *Mtb*, and *M. avium*. Compound **13** (among 2′,3′-dideoxyuridine series) and compound **14** (among 3′-fluoro-2′,3′-dideoxyuridine series) demonstrated excellent antimycobacterial activity (MIC 1-2 μg/mL) against *Mtb* H_{37}Ra. The compounds **13** and **14**, were also subjected to determine their antimycobacterial activity against a RMP-resistant H37Rv strain (ATCC 35838, resistant to RMP at 2 μg/mL) of *Mtb* using the radiometric-BACTEC assay. The drug-resistant *Mtb* strain was susceptible to the compounds **13** and **14** (MIC_{90} 1-2 μg/mL). No toxicity was observed *in vitro* against Vero cells (MTT test) up to the highest concentrations tested (CC_{50} > 100 μg/mL).

13 **14**

In a subsequent article in the same year by our group (Srivastav et al, 2007), *in vitro* anti-mycobacterial activities of several 5-substituted acyclic pyrimidine nucleosides containing 1-(2-hydroxyethoxy)methyl and 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] acyclic moieties were investigated against *Mtb* H37Ra, *M. bovis*, and *M. avium*. In this study, 1-(2-hydroxyethoxy)methyl-5-(1-azido-2-haloethyl (**15a**), 1-azidovinyl) analog (**15b**), 1-[(2-

hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-decynyluracil (**16a**), and 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-dodecynyluracil (**16b**) exhibited moderate *in vitro* anti-tubercular activity (100% inhibition @ 50 μg/mL) against these mycobacteria. These compounds did not show any toxicity *in vitro* against Vero cells and HepG2 cells up to a concentration of 100 μg/mL.

15a **15b**

16a **16b**

In continued efforts in drug design and discovery for anti-tuberculosis agents, our group (Shakya et al, 2010) investigated various 2′- or 3′-halogeno derivatives of pyrimidine nucleosides containing uracil, 5-fluorouracil, and thymine bases. Among the compounds tested, 3′-bromo-3′-deoxy-arabinofuranosylthymine (**17**) was the most effective antituberculosis agent in the *in vitro* assays against wild-type *Mtb* strain (H37Ra) which displayed MIC_{50} = 1 μg/mL by the MABA assay. Further, it displayed MIC_{50} = 1-2 μg/mL against drug-resistant (H37Rv) (RMP-resistant and INH-resistant) strains of *Mtb* using BACTEC assay (Collins & Franzblau, 1997). The antimycobacterial effect of potent compounds was also determined against intracellular mycobacteria in a human monocytic cell-line (THP-1) infected with *Mtb* H37Ra strain using the colony-forming units (CFU) assay (Bermudez et al., 2001). Interestingly, the compound **17** demonstrated slightly better activity against intramacrophagic mycobacteria (80% reduction at 10 μg/mL concentration) than extracellular mycobacteria (75% reduction at 10 μg/mL concentration). In contrast, pyrimidine nucleosides possessing 5-fluorouracil base were weak inhibitors of *Mtb* H37Ra. The XTT and ^{3}H incorporation assays were performed to evaluate the toxicity of the investigated compounds *in vitro* against a human hepatoma cell line (Huh7). No cytotoxicity was found up to the highest concentration of compounds tested (CC_{50}> 100-200 μg/mL).

17

Our group in the same year (Srivastav et al., 2010) reported investigation of antimycobacterial activities of several 5-alkyl, 5-alkynyl, furanopyrimidines and related 2′-deoxynucleosides against *Mtb*. Compounds with 5-arylalkynyl substituents displayed potent *in vitro* antitubercular activity against *M. bovis* and *Mtb* (MIC 0.5-5 µg/mL). We found that 5-(2-pyridynylehynyl)-2'-deoxycytidine (18) exhibited potent activity against *Mtb* and showed no cytotoxicity Huh-7 cells up to a concentration of >200 µg/mL using XTT and ^{3}H-thymidine uptake assays. Therefore it was selected to test its potency in a mouse model (BALB/c) of *Mtb* (H37Ra) infection. At a dose of 50 mg/kg for 5 weeks, compound **18** showed promising *in vivo* efficacy in this mouse model. Statistically significant reduction in mycobacterial load was observed in lungs, livers and spleens of the treated mice. Our work provides first evidence of antimycobacterial potential of 5-substituted pyrimidine nucleosides in an animal model as a potential new class of antituberculosis agents.

18a

Recently, Kogler et al (Kogler et al., 2011) reported a series of 5-substituted -2'-deoxyuridine monophosphate analogs as potential inhibitors of mycobacterial flavin-dependent thymidylate synthase (ThyX). Compound N-(3-(5-(2'-deoxyuridine-5'-monophosphate)) prop-2-ynyl)-octanamide displayed selective potent inhibition of ThyX with an IC_{50} value of 0.91 µM. This derivative was found to lack activity against the classical mycobacterial thymidylate synthase (ThyA, IC_{50} >50 µM).

18b

Somu et al (Somu et al., 2006) reported a purine nucleoside compound **19** (MIC$_{99}$ = 0.19 μM) inhibiting siderophore biosynthesis of *Mtb* in H37Rv strain under iron-limiting conditions (Domenech et al., 2005, as cited in Somu et al., 2006). The activity of **19**, according to the authors, was due to inhibition of the adenylate-forming enzyme MbtA, which is involved in biosynthesis of the mycobactins. The cytotoxicity of the potent compounds in the series was evaluated against the P388 murine leukemia cell line. None of the inhibitors displayed any toxicity up to the maximum concentration tested (ED$_{50}$ > 100 μg/mL).

19

Gupte et al (Gupte et al., 2008) demonstrated 2-triazole derivatives of 5′-*O*-[*N*-(salicyl)sulfamoyl]adenosine as inhibitors of aryl acid adenylating enzymes (AAAE) involved in siderophore biosynthesis by *Mtb* H37Rv. Enzyme assays were performed at 37 °C with recombinant MbtA expressed in *E. coli*. On the basis of observed potency (MIC 3.13 μM), selectivity, lack of cytotoxicity, and enhanced lipophilicity, compound **20** was reported as the best candidate. No inhibition of cell growth was observed upto 100 μM when this class of compounds were evaluated for inhibition of cell viability against Vero cells using the MTT assay. The compound **20** was also evaluated against MEL, OCL-3, and REH human cancer cell lines. Cell proliferation of OCL-3 and REH lines were not affected at 100 μM, while in the MEL line approximately 25% inhibition was shown at 100 μM.

20a

Adenosine (Ado) kinase is a purine salvage enzyme that phosphorylates adenosine to adenosine-monophosphate. A large number of adenine modified nucleosides were evaluated as substrates and inhibitors of Ado kinase from *Mtb* (Long & Parker, 2006) The best substrates were 2-aza-adenosine, 8-aza-9-deazaadenosine and 2-fluoroadenosine and the most potent inhibitors were N-1-benzyladenosine (Ki = 0.19 μM), 2-fluoroadenosine (Ki = 0.5 μM), 6-cyclopentyloxy purine riboside (Ki = 0.15 μM) and 7-iodo-7-deazaadenosine (Ki = 0.21 μM). Several of these adenosine analogs showed promising antitubercular activity when MIC studies were performed.

20b

In an extension of their work (Long et al, 2008) modifications to the base and ribofuranosyl moiety or modifications to the glycosidic bond positions of adenosine were analyzed against *Mtb* Ado kinase. In this study, the best substrates identified were carbocyclic adenosine, 8-aza-carbocyclic adenosine and 9-[a-L-lyxofuranosyl]-adenine.

3.1.2 Carbohydrates

Sugar derivatives have also been examined as antimycobacterial agents. Although many reports have been published, most of them did not include toxicity data. Some representative examples of this class are summarized here.

Pathak et al (Pathak et al, 2003) synthesized several octyl 5-O-(α-D-arabinofuranosyl)-α-D-arabinofuranoside disaccharide analogs substituted at the 5-position of the non-reducing end of sugar and tested *in vitro* (Suling et al., 1998, as cited in Pathak et al, 2003) against *Mtb* (H37Ra, ATCC 25177), *M. avium* complex (MAC) as well as in a cell free assay system for arabinosyltransferase acceptor/inhibitor activity (Lee et al., 1997, as cited in Pathak et al, 2003). Compound **21** displayed IC_{50} of 1.56 mM in cell free assay and MIC 8 μg/mL against *Mtb*. No toxicity data was reported.

$(C_6H_{11}H_2C)_2N$ HO OH O HO OH OC_8H_{17}

21

Tripathi et al (Tripathi et al., 2005) reported bis-glycosylated diamino alcohols with the most active compound **22a** showing MIC of 3.12 μg/mL against *Mtb* H37Ra as determined by MABA assay. But this compound displayed MIC > 50 μg/mL against *Mtb* H37Rv by Agar microdilution method (Saito et al., 19991, as cited in Tripathi et al., 2005). In this series, they discovered the next active compound **22,** exhibiting activity against *Mtb* H37Ra (MIC 12.5 μg/mL by MABA assay) and against *Mtb* H37Rv (MIC 6.25 μg/mL by Agar microdilution method) that was considered to test further. The compound **22** was also found to be active against MDR strain and showed mild protection in mice. According to the report, this compound seems to possess efficacy against *Mtb* infection in mice at non-toxic concentration (25 mg/Kg). However, at higher doses it caused toxicity.

OH OH O N H NH O OCH_2Ph PhH_2CO

22a, n=10, 22b, n=12

Chiba et al (Chiba et al., 2007) synthesized sugar derivatives of stachyose, and evaluated them for antibacterial activity against *Mtb*, *M. avium*, and *S. aureus* using broth dilution methods (Takii et al., 2002, as cited in Chiba et al., 2007) in MiddleBrook 7H9 broth. The compound **23** (OCT359) was identified as the most active compound in the series with MIC 3.13 μg/mL against *Mtb* H37Rv. OCT359 was also tested against various drug-sensitive and -resistant clinical isolates of *Mtb*. Among them 25 clinical isolates of drug-resistant *Mtb* and 19 drug-sensitive *Mtb* were sensitive to OCT359. The MICs of OCT359 for these clinical isolates ranged from 3.13 to 25 μg/mL. No toxicity data was reported on any host cell lines.

23

Liav et al (Liav et al., 2008) prepared derivatives of thiocarlide (THC), a previously known antitubercular drug, for their evaluation against *Mtb* H37Rv using MABA assay. The most active compound reported was **24** having MIC in the range of 1.56-3.12 μg/mL. No toxicity data for this compound was presented on any host cell line.

THC

24

In a recent report, Horita (Horita et al, 2011) described modification of their previously reported lead compound OCT313 (Glc-N-Ac -DMDTCB) (MIC 25 μg/mL against *Mtb* H37Rv by Broth dilution method). The resultant compound Glc-NAc-pyrrolidine dithiocarbamate (**25**, OCT313HK, Glc-NAc-PDTC) exhibited potent anti-tubercular activity with MIC of 6.25 μg/mL. OCT313HK was also effective against *Mtb* clinical isolates, including MDR and XDR strains at similar concentrations (MIC 6.25-12.5 μg/mL). No toxicity data was reported on mammalian cell lines.

25

3.1.3 Heterocyclic compounds

3.1.3.1 Quinolines and quinoxalines

Quinolines have also been of interest for evaluation as antibacterial agents since fluoroquinolones are already used as antibiotics (e.g. ciprofloxacin, laevofloxacin, ofloxacin). Moxifloxacin and Gatifloxacin from this class are in Phase III clinical trial for tuberculosis treatment (see details in the section describing drugs in Phase III). Many research articles are available in literature on quinoline as anti-TB agents.

Sriram (Sriram et al, 2006) reported a series of 7-substituted derivatives of gatifloxacin and evaluated them for antimycobacterial activity *in vitro* and *in vivo* against *Mtb* H37Rv and MDR-TB. The compounds were also tested for their ability to inhibit the supercoiling activity of DNA gyrase from *Mtb*. Among this series, compound **26** was found to be equally active (IC_{50} of 3.0 μg/mL) as gatifloxacin in the inhibition of the supercoiling activity of wild-type *Mtb* DNA gyrase. The compound **26** was also found to be the most active *in vitro* with an MIC of 0.0125 μg/mL against *Mtb* and MDR-TB. Activity evaluation *in* animal model showed that this compound decreased the bacterial loads in lung and spleen tissues by 3.62- and 3.76-log10, respectively. After 72 h exposure with the test compounds, viability of Vero cells was assessed using MTT assay to determine their cytotoxicity. The compounds were found to be non-toxic up to a concentration of 62.5 μg/mL. The compound **26** showed selectivity index (IC_{50}/MIC) of >1250.

26

Sriram and coworkers (Dinakaran et al, 2008 a) also synthesized novel ofloxacin (OFX) derivatives and evaluated them for *in vitro* and *in vivo* antimycobacterial activities against *Mtb* H37Rv , MDR-TB, and *M. smegmatis* using agar dilution method. These compounds were also tested for their ability to inhibit the supercoiling activity of DNA gyrase from mycobacteria. Among the synthesized compounds, **27** exhibited most potent activity (MIC_{99} of 0.19 μM and 0.09 μM against *Mtb* and MDR-TB, respectively). The compound **27** decreased bacterial loads (strain ATCC 35801) in lung and spleen tissues by 1.91 and 2.91 -log10, respectively, at 50 mg/kg dose when evaluated in a mouse model. This compound was reported to possess a selectivity index (IC_{50}/MIC) of >1467.

27

Another publication by the same group (Dinakaran et al, 2008 b) described various 2-(sub)-3-fluoro/nitro-5,12-dihydro-5-oxobenzothiazolo[3,2-a]quinoline-6-carboxylic acid derivatives. Among the reported compounds, **28** displayed the most potent activity *in vitro* with MICs of 0.18 and 0.08 μM against *Mtb* and MDR-TB, respectively. In a mouse model of *Mtb* infection, **28** decreased bacterial loads in lung and spleen tissues with 2.78 and 3.12 _ log10, respectively, at the dose of 50 mg/kg. The selectivity indices (IC_{50}/MIC) of the compound **28** were reported to be 1576 against MDR-TB and 700 against *Mtb*. Phototoxicity evaluation was also performed (Mayne et al., 1997, as cited in Dinakaran et al, 2008 b) and no significant phototoxicity was recorded.

28

Senthilkumar et al, 2009, published synthesis of various 1-(substituted)-1,4-dihydro-6-nitro-4-oxo-7-(sub-secondary amino)-quinoline-3-carboxylic acids. Among the compounds investigated, **29** was found to be the most potent compound *in vitro* with MIC values of 0.08 and 0.16 μM against *Mtb* and MDR-TB, respectively. In the *in vivo* studies, **29** significantly decreased bacterial load in lung and spleen tissues, at 50 mg/kg dose. The SI (IC_{50}/MIC) of **29** was stated to be 793 against MDR-TB and 1586 against *Mtb*. No significant phototoxicity was described for **29**.

29

Other groups have also been exploring quinoline derivatives as anti-TB agents. Vicente et al (Vicente et al., 2009) published a series of 3-phenylquinoxaline 1,4-di-N-oxide against *Mtb* H37Rv using MABA assay. The compounds exhibiting fluorescence were tested in the BACTEC 460-radiometric system. The compounds affecting <90% inhibition in the primary screen (MIC >6.25 μg/mL) were not evaluated further. Thirty-four of the seventy tested compounds showed MIC values less than 0.2 μg/mL. The most active compound reported was **30** (MIC <0.2 μg/mL) with an IC_{50} >100 (SI >500).

30

Ancizu et al (Ancizu et al., 2010) described a series of 3-methylquinoxaline-2-carboxamide 1,4-di-N-oxide derivatives. Many of the tested compounds showed MIC values less than 1 μg/mL. In this report, compounds **31** and **32** displayed most significant inhibition of *Mtb* H37Rv (MIC <0.2 μg/mL). Cytotoxicity evaluation indicated that **31** and **32** were non-toxic with IC_{50} value of >100 and SI >500.

31, R=Cl, n=1; 32, R=H, n=2

Carta et al (Carta et al., 2007) reported antimycobacterial evaluation of 3-methyl-9-substituted-6-oxo-6,9-dihydro-3H-[1,2,3]-triazolo [4,5-h]quinolone-carboxylic acids and their esters against wild-type H37Rv and 11 clinically isolated strains of *Mtb*. Several derivatives inhibited mycobacterial replication with MIC_{90} in the range of 0.5–3.2 μg/mL. The most

potent compound **33** (MIC_{90} = 0.5 μg/mL) showed no cytotoxicity (CC_{50} > 50 μg/mL), when tested against human macrophages and Hep-2 cells.

33

Upadhayaya et al (Upadhayaya et al., 2011) identified indeno[2,1-c]quinoline derivatives which were considerably active (MIC 0.39-0.78 μg/mL) but had solubility problems. Ester derivatives of the lead compound indeno[2,1-c]quinolines were synthesized, which showed 2- to 4-fold improved anti-TB activities, with increased solubility and superior selectivity index (SI) over their respective parent compounds. In this study, compound 34 was described to be the most potent agent with MIC of <0.39 μg/mL. In general, no cytotoxicity was observed in Vero cells.

34

Jaso et al (Jaso et al., 2005) evaluated a series of 6(7)-substituted quinoxaline-2-carboxylate 1,4-dioxide derivatives against *Mtb* H37Rv. Fourteen compounds were selected to test for their activity against intramacrophagic mycobacteria. It was found that ethyl and benzyl 3-methylquinoxaline-2-carboxylate 1,4-dioxide derivatives with a chlorine group at position 7 of the benzene moiety (compound **35**, MIC 0.1 μg/mL, SI 470) and the unsubstituted derivative (**36**, MIC 0.1 μg/mL, SI 76) have good antitubercular activity, including activity in macrophages (EC_{90} 0.15 μg/mL and 0.0005 μg/mL, respectively). The compounds **37** and **38** of the series were also active against drug-resistant strains of *Mtb* H37Rv with MIC 0.39-1.56 and 3.13-12.5, respectively.

35, R1= H, R2 =H, R3 = Ph; **36**, R1= Cl, R2 =H, R3 = Ph;

37, R1= R2 = R3 = CH3; **38**, R1= Cl, R2 =Cl, R3 = Ph

Lilienkampf et al (Lilienkampf et al., 2009) revealed several potent quinolines bearing an isoxazole containing side-chain as anti-TB compounds. These compounds were first tested for their activity against the *Mtb* strain H37Rv using MABA assay. The compounds showing good anti-TB activity were further evaluated for their potency against non replicating persistent TB (NRPTB) in a low oxygen recovery assay (LORA). The most active compounds, **39** and **40**, exhibited MICs of 0.77 μM and 0.95 μM, respectively against the replicating bacteria. These compounds, in general, also had good potency against the nonreplicating persistent bacteria without toxicity on Vero cells up to 128 μM. The compounds **39** and **40** also retained anti-TB activity against RMP-, INH-, and streptomycin resistant *Mtb* strains.

39, R=CF_3, X=CH_2; 40, R=H, X= m-Ph

3.1.3.2 Pyrimidine and purines

Khoje et al (Khoje et al, 2010) synthesized various purine analogs and evaluated them *in vitro* against *Mtb* H37Rv using MABA assay. The 8-aza-, 7-deaza- and 8-aza-7-deazapurine analogs displayed good antimycobacterial activities. The 7-deazapurine analogs exhibited MIC values between 0.08 and 0.35 μM; comparable or better than the reference drugs (RMP, MIC 0.09 μM; INH, MIC 0.28 μM and PA-824, MIC 0.44 μM). The most active compound among 7-deaza purines was **41** with MIC 0.11 μM and SI 1063. The 7-deazapurines were slightly more toxic towards mammalian cells, but still had good selectivity indices. In this study, five most active compounds were also evaluated against a panel of drug-resistant *Mtb* strains, where they all were found to retain activity. However, these compounds were significantly less active when tested against non-replicating persistent *Mtb*.

41

Trivedi et al (Trivedi et al., 2010) examined a series of dihydropyrimidines for their *in vitro* activity against *Mtb* H37Rv. All compounds were initially screened for their *in vitro* activity

at 6.25 μg/mL. The compounds exhibiting 90% inhibition in the initial screen were re-examined at and below 6.25 μg/mL using two-fold dilutions to determine the actual MIC. Two compounds, **42** and **43** were found to be the most active agents with MIC of 0.02 μg/mL. These compounds were more potent than the reference drug INH. In Vero cells, they exhibited IC_{50} >10 μg/mL (SI >500).

42, R=F, 43, R=NO_2

3.1.3.3 Pyrrole derivatives

Biava et al (Biava et al., 2006) reported design and synthesis of pyrrole analogues of BM212. The compounds were preliminarily screened for their activity toward *Mtb B814 and M. fortuitum CA10*. Compounds showing MIC values of 16 μg/mL or lower were further tested against *Mtb CIP 103471* and a panel of atypical mycobacteria, such as *M. marinum* CIP *6423*, *M. avium CIP 103317*, and *M. smegmatis* CIP *10359*. Cytotoxicity was examined in Vero cells to determine the maximum nontoxic dose ($MNTD_{50}$) defined as the drug concentration that decreased cell multiplication to less than 50% of the control. The best compound reported in this series was **44** with MIC of 0.4 μg/mL, $MNTD_{50}$ of 64 μg/mL and a high protection index (MNTD/MIC, 160) that was better than BM212, INH, and streptomycin (6, 128, and 128, respectively).

BM 212 **44**

In the year 2009, the same group (Biava et al., 2009) further investigated new diarylpyrroles on the basis of SAR analysis of pyrroles, reported by them previously. The compound **45** emerged as the most potent agent (MIC 0.25 μg/mL) with protective index (maximum non toxic dose in Vero cells/ MIC) > 512.

45

Biava et al (Biava et al., 2010) also identified 4-((1-(4-fluorophenyl)-2-methyl-5-(4-(methylthio)phenyl)-1H-pyrrol-3-yl)methyl)thiomorpholine (**46**) as a potent antimycobacterial agent against *Mtb* 103471 and H37Rv strains (MIC values of 0.125 μg/mL comparable to streptomycin and RMP), with a cytotoxicity (CC_{50}) value of >128 μg/mL and protection index of >1000.

46

3.1.3.4 Furan

5-Nitrofuran-2-yl derivatives (Sriram et al. 2009) were investigated against tubercular (H37Rv) and various non-tubercular mycobacterial species in log-phase and 6-week-starved cultures. The compound **47** exhibited MIC of 0.22 μM. This compound showed 3 times more activity than INH and equal activity as RMP in log-phase culture of *Mtb*

H37Rv. It inhibited starved *Mtb* H37Rv with MIC of 13.9 μM and was 50 times more active than INH and slightly more active than RMP. It displayed an IC_{50} of 139 μM in Vero cells.

47

3.1.3.5 Azoles

Azoles are one of the major classes of compounds which have been probed for anti-TB activity, but unfortunately, many of the publications emerging on azoles did not provide toxicity data, making it difficult to analyze their potential. Following are some of the representatives studies found worthy to summarize here.

Shiradkar et al (Shiradkar et al, 2007) published synthesis and antituberculosis activity of a series of N-{4-[(4-amino-5-sulfanyl-4H-1,2,4-triazol-3-yl)methyl]-1,3-thiazol-2-yl}-2-substituted amide derivatives against *Mtb* H37Rv (ATCC 27294) using MABA and BACTEC 460 assays where compounds **48** and **49** demonstrated MICs of 0.78 and 0.39 μM, respectively. The cytotoxicity analysis by neutral red uptake assay in Vero-C-1008 cell line showed that none of this class of compounds was toxic up to a concentration of 50 μg/mL.

48, R = CH_3; 49, R = Ph

Velaparthi et al (Velaparthi et al., 2008) reported 5-*tert*-butyl-*N*-pyrazol-4-yl-4,5,6,7-tetrahydrobenzo[*d*]isoxazole-3-carboxamide derivatives as novel and potent inhibitors of *Mtb* pantothenate synthetase (PS). Pantothenate is a key precursor of coenzyme A and acyl carrier protein, essential for many intracellular processes including fatty acid metabolism, cell signaling, and synthesis of polyketides and nonribosomal peptides. The PS pathway is not present in humans. Compounds **50** and **51** displayed the best inhibition in terms of IC_{50} of < 100 nM.

50, R = Ph; 51, R = 2-naphthyl

N-Aryl-*C*-nitroazoles were investigated by Walczak et al (Walczak et al., 2004) against H37Rv (ATCC 27294) using MABA assay. Compound **52** exhibited MIC 0.39 µg/mL with SI >160.

52

Lee et al (Lee et al., 2011) synthesized econazole-derived nitroimidazoles and reported their antitubercular activity against H37Rv by MABA assay. The MIC against non-replicating *Mtb* was determined by using the green fluorescent protein (GFP) expressing *Mtb* strain in the Wayne hypoxia model (anaerobic conditions) (Wayne et al. 1996, as cited in Lee at al., 2011). The MICs of the most active azoles **53** and **54** was found to be 0.5 µg/mL under aerobic conditions and 4 and 1 µg/mL, respectively, under anaerobic conditions against H37Rv. The IC_{50s} in Vero cell noted for **53** and **54** were 100 and >100, respectively.

53, R=2,4-dichloro; 54, R= 4-Ph

In the year 2009, a series of 2-methylbenzothiazole derivatives was described by Huang et al (Huang et al, 2009). The most potent compounds found in this series were **55** and **56** with MIC values of 1.4 and 1.9 µM, respectively, against replicating *Mtb* H37Rv. All the active compounds in this series were nontoxic toward Vero cells (IC_{50} > 128 µM).

55

56

3.1.3.6 Azines

Palmer et al (Palmer et al., 2010) reported antitubercular activity of biphenyl analogs of PA-824, which is currently under phase II clinical trial (pl. see Phase II section), using MABA and LORA assays. Among these, several of the compounds showed potent in vitro activity with MIC values of <1 μM. The most active compound **57** had MICs of 0.015 and 1.4 μM in MABA and LORA assays, respectively. All the compounds investigated were relatively nontoxic to mammalian Vero cells, with IC_{50} >125 μM. In a mouse model of acute *Mtb* infection, seven of the compounds showed substantially (>10-fold) improved efficacies over PA-824, while three of them were >200-fold more effective than PA-824.

PA-824

57

3.1.3.7 Pyridine hydrazides (INH analogs)

Several INH derived Schiff bases were investigated by Hearn et al (Hearn et al, 2009). These compounds showed high *in vitro* activity against *Mtb* and mycobacteria-infected macrophages. They provided strong protection in tuberculosis-infected mice with low toxicity. The mean of the MIC values determined against *Mtb* H37Rv strain Erdman for

the forty-four compounds tested was 1 μg/mL. A representative cyclohexanone derivative **58** displayed MIC of 0.03 μg/mL (SI >40,000) and exhibited log CFU reduction/lung of 4.65.

58

Lourenco et al (Lourenco et al., 2008) prepared a series of (E)-N′-(monosubstituted-benzylidene) isonicotinohydrazide derivatives and evaluated their antibacterial activity against *Mtb* H37Rv (ATCC 27294, susceptible both to rifampin and INH) *in vitro* using Alamar Blue assay. Compound **59** exhibited significant activity (MIC 0.31 μg/mL). Cellular viability of murine macrophage cells in the presence and absence of test compounds was determined by Mosmanns's MTT(3-(4,5-dimethylthylthiazol-2yl)-2,5-dimethyl tetrazolium bromide; Merck) microculture tetrazolium assay (Souza et al., 2003, as cited in Lourenco et al., 2008) and 100% cell viability was found for the compound **59** @ 100 μg/mL.

59

3.1.4 Metal complexes

Not very many reports are available on metal complexes since metal complexes are generally found to be toxic. A few representatives are summarized here.

Eiter et al (Eiter et al, 2009) described Gold(I) analogues of a platinum-acridine. Compound **60** exhibited an IC_{50} of 0.652 μM and IC_{90} of 1.141 μM against *Mtb* H37Rv in a high-throughput screen. It also demonstrated inhibition of non-small-cell lung cancer cell line (IC_{50} of 3.940 ±0.38) with a selectivity index of 23.66. The compound **60** was selected to test its efficacy *in vivo* but serum samples collected from mice treated at a maximum tolerated dose (MTD) of 300 mg/kg orally did not inhibit *Mtb*. This indicated limited oral bioavailability of the complex.

60

Melnic et al (Melnic et al., 2010) investigated new hetero(Mn, Co, Ni)trinuclear iron(III) furoates where compound **61** $[Fe_2CoO(\alpha\text{–fur})_6(THF)(H_2O)_2].H_2O$ displayed potent in vitro inhibition (MIC = 0.827 μg/mL) of *Mtb* H37Rv (ATCC 27294) with an SI of >36.2 (cytotoxicity assay was performed using Vero cell lines).

In a series of Ruthenium (II) phosphine/picolinate complexes, Pavan et al (Pavan et al, 2010) reported MIC values of 0.78 and 0.26 μg/mL for compounds **62** and **63**, respectively, against H37Rv ATCC 27294 using REMA (Resazurin Microtiter Assay) method (Palomino et al., as cited in Pavan et al., 2010). No toxicity data, however, was reported in the article.

62, x=1; 63, x=2.

3.1.5 Natural products

Natural product research is a tedious, labour-intensive and difficult process. Only a few publications have emerged describing significant anti-mycobacterial activity in this field. Selected reports are presented here.

Torres-Romero et al (Torres-Romero et al., 2011) evaluated new dihydro-β-agarofuran sesquiterpenes, isolated from the leaves of *Celastrus vulcanicola*, and their derivatives against H37Rv ATCC 27294 and multidrug-resistant (clinical isolate, strain 02TBDM039EP097) using the tetrazolium microplate assay (TEMA) method. (Rojas et al., 2006, as cited in Torres-Romero et al., 2011). All of the 25 compounds reported showed MIC values of >25 μg/mL against the sensitive H37Rv strain whereas 1a-acetoxy-6b,9b-dibenzoyloxy-dihydro-b-agarofuran (**64**) had MIC value of 11.9 μM against MDR TB strain, which was comparable to or better than INH or RMP. No toxicity data was included in this article.

64

Nicholas et al (Nicholas et al., 2003) screened 1500 extracts derived from marine plants, invertebrates and terrestrial fungi for their ability to inhibit a newly described mycobacterial detoxification enzyme mycothiol-S-conjugate amidase (MCA) using a fluorescence-based assay that measures the extent of cleavage of the substrate mycothiol bimane by MCA (Newton et al., 2000, as cited in Nicholas et al., 2003). Only compound **65** showed inhibition of MCA (IC_{50} 0.1 μM).

65

Three new aminolipopeptide, trichoderins were isolated by Pruksakorn et al (Pruksakorn et al., 2010) from a culture of marine sponge-derived fungus of Trichoderma sp. as anti-mycobacterial substances. Trichoderins showed potent activity against *M. smegmatis*, *M. bovis* BCG, and *Mtb* H37Rv under standard aerobic growth conditions as well as dormancy-inducing hypoxic conditions using the established methods, (Sobou et al., 2008; Arai et al., 2009, as cited in Pruksakorn et al., 2010) with MIC values in the range of 0.02–2.0 μg/mL. The best compounds **66** and **67** displayed MICs of 0.12 and 0.13 μg/mL, respectively, for aerobic and hypoxic *Mtb*. No toxicity data was included in this report.

66, R=CH3; 67, R=H

Mahapatra et al (Mahapatra et al., 2007) reported a series of synthetic and plant-derived naphthoquinone derivates of the 7-methyljuglone scaffold and their evaluation against *Mtb* H37Rv (ATCC 27294). Several of these compounds have been shown to operate as subversive substrates with mycothiol disulfide reductase. The synthesized compound **68** exhibited MIC of 0.5 µg/mL as determined by radiometric respiratory technique using the BACTEC system. The SI obtained for **68** was 30.22 (cytotoxicity evaluation was done using Vero cells).

68

3.1.6 Miscellaneous

3.1.6.1 Artemisinin analog

Artemisinin also called qinghaosu, is a natural peroxide containing sesquiterpene based on 1,2,4-trioxane, and is a highly active and relatively nontoxic antimalarial agent (Devdutt, C. et al., 2010, as reported by Miller et al., 2011). Miller et al (Miller et al., 2011) reported Mycobactin-Artemisinin Conjugate **69** that had submicromolar activity against different clinical strains of tuberculosis. In H37Rv, it displayed MIC 0.338 µM, and in one XDR strain (HREPKOTh) it exhibited MIC of 0.078 µg/mL. No toxicity data was mentioned, however.

Artemisinin

69

3.1.6.2 Macrolides

Falzari et al (Falzari et al., 2005) reported macrolides and ketolides (descladinose) with substitutions at positions 9, 11, 12, and 6, which were assessed for activity against *Mtb*. Several compounds with 9-oxime substitutions or aryl substitutions at position 6 or on 11, 12 carbamates or carbazates demonstrated submicromolar MICs. Four compounds possessing low MICs also effected significant reductions in CFU in infected macrophages. The active compounds were assessed for tolerance and the ability to reduce CFU in the lungs of BALB/c mice in an aerosol infection model. A substituted 11,12 carbazate macrolide demonstrated significant dose-dependent inhibition of *Mtb* growth in mice, with a 10- to 20-fold reduction of CFU in lung tissue. The compound **70** (RU66252) was found to be a promising compound having MIC of 0.25 μM with SI 99.52.

70

3.1.6.3 Peptides

Jiang et al (Jiang et al, 2011) reported evaluation of a series of α-helical peptides consisting of all D-amino acid residues and synthetic human L-LL37 (L-enantiomer) and D-LL37 (D-enantiomer), against *Mtb* H37Rv and a clinical MDR strain. Not very good activity was observed. The most active analog had MIC of 11.2 and 15.6 μM, against H37Rv and MDR strains, respectively.

3.2 Molecules in pipeline

(Source: Working Group on New Drugs [WGND] and TB Alliance, and Tuberculosis Trial Consortium [TBTC])

After years of vacuum, TB drug development pipeline has begun to enrich during the past decade. The major credit goes to the Global Alliance for TB Drug Development (TB Alliance) which is largely funded by Bill & Melinda Gates Foundation as a philanthropic effort and Working Group on New Drugs (WGND). It is also to be noted in regard of this pipeline that many of the compounds here are either derivatives of existing drugs or are working on the same target as existing drugs. This is obviously a shorter and a quicker method for new drug development, however, this approach may pose a risk of cross-resistance in these future drugs. This risk may be neglected, however, in view of urgent need of effective drugs

to halt TB associated mortalities. Following are the compounds that are at various stages of preclinical and clinical development (summarized in tables 3-5).

3.2.1 Hit to lead

Sponsor/Developer	**Compounds**	**Target**	**Remarks**
The Lilly TB Drug Discovery	Novel synthetic compounds	Unknown	Not much information is available
FAPESP/Brazil	Ruthenium(II)phosphine/picolinate complexes, synthetic (>100).	Unknown	MIC less than 1 µM against H37Rv and resistant strains. *In vivo* assays are underway
AstraZeneca R & D Bangalore	200,000 Synthetic, novel compounds	Not mentioned	Target against H37Rv strain
GlaxoSmithKline, TB Alliance:	Synthetic compounds	Not mentioned	Whole cell microorganism screen
University of Illinois, TB Alliance	Total 1,21,0000 compounds. 66,000 synthetic and semisynthetic	Whole cell	Approximately 1500 hits have been identified and confirmed
Shaw Environmental and University of Illinois at Chicago	30 Indole-based combinatorial biosynthetic compounds (Several compounds showed activity comparable to first line drugs).	Under investigation	Whole cell microorganism screening against replicating and nonreplicating *Mtb*.
Mycosynthetix, University of Illinois at Chicago	15,000 Natural product extracts as fungal metabolites	Not mentioned	Not much information is available
University of Illinois at Chicago, Myongji University	Actinomycete metabolites purified and derived from 70,000 natural products extract	Not mentioned	Several samples showing MIC of less than 0.5 µg/mL.
Vertex Pharmaceuticals, Incorporated	315,000+ Compounds	*Mtb* Protein Kinase Inhibitors	The screening assay uses a basic protein kinase assay.

Sponsor/Developer	Compounds	Target	Remarks
AstraZeneca R & D Bangalore, India	500,000 Synthetic compounds.	Enzyme(s) involved in DNA synthesis	Not much information is available
AstraZeneca, TB Alliance	Synthetic compounds are under screening via high throughput assay	Folate Biosynthesis Inhibitors	Not much information is available
GlaxoSmithKline, Texas A & M University, TB Alliance	Library of 1.4 million synthetic compounds has been screened via High throughput screening	Malate Synthase Inhibitors	Hits have been identified.
Colorado State University, TB Alliance	Synthetic, known already and evaluated as cholesterol synthesis inhibitors.	Menaquinone Synthase (MenA) Inhibitors	Project aims to "retro-design" and evaluate derivatives of the known compounds
UPenn and TB Alliance	110,000 Synthetic compounds derived from natural products	Inhibitors of *Mtb* energy metabolism, electron transport chain	About 100 hits have been identified

Table 3. Various Compounds at Lead Identification stage

3.2.2 Lead optimization

Sponsor/Developer	Compounds	Target	Remarks
TB Alliance, Institute of Materia Medica, The Beijing Tuberculosis and Thoracic Tumor Research Institute and University of Illinois.	Riminophenazine (clofazimine) derivatives >500 synthetic compounds	Considered to inhibit energy metabolism in *Mtb* (*Mtb*	Riminophenazines (clofazimine) have been employed to treat leprosy. *In vivo* studies are underway
GlaxoSmithKline, TB Alliance	>2 Million synthetic compounds.	InhA Inhibitors	------
Anacor Pharmaceuticals	>1000 Synthetic boron-containing compounds	LeuRS inhibitors, Protein synthesis	------
TB Alliance, GlaxoSmithKline	>1 Million synthetic compounds	Mycobacterial Gyrase Inhibitors	Several lead compounds have been identified and are being evaluated further.
AstraZeneca, TB Alliance	Synthetic	Mycobacterial Gyrase Inhibitors	------

Table 4. Various Compounds at Lead Optimization stage

3.2.3 Pre clinical

3.2.3.1 CPZEN-45

Sponsor/developer: Microbial Chemistry Research Foundation, Tokyo, Japan Lilly TB Drug Discovery Initiative NIAID, IDRI, Lilly, YourEncore.

CPZEN-45

Synonyms: Caprazene, caprazamycin, nucleoside antibiotic

Summary: CPZEN-45 is a nucleoside antibiotic produced by Streptomyces sp. first described in 2003 by investigators at the Microbial Chemistry Research Foundation (MCRF) and Meiji Seika Kaisa, Ltd of Japan. CPZEN-45 possesses MIC of 1.56 µg/mL against *Mtb* H37Rv and 6.25 µg/mL against a MDR strain of *Mtb*. This compound is active against both replicating and non-replicating *Mtb in vitro*, suggesting it could be efficacious against latent organisms *in vivo*. CPZEN-45 has shown efficacy against both drug sensitive and XDR *Mtb* in a mouse model of acute tuberculosis (TB). Recent data by NIAID using the gamma interferon gene-disrupted (GKO) mouse model of acute tuberculosis in which infection was achieved by aerosol exposure to *Mtb* (Erdman) also demonstrated efficacy of CPZEN-45 with 1-1.5 log CFU reduction in lungs of infected mice. Its mode of action is not specified (Hirano et al., 2008; WGND)

3.2.3.2 Quinolone DC-159a

Sponsor or developer: Japan Anti-Tuberculosis Association, JATA Daiichi-Sankyo Pharmaceutical Co.

DC-159a

Summary: DC-159a exhibited the highest activity against drug-susceptible (MIC = 0.03 µg/mL), quinolone-resistant (QR) MDR-TB and non-tuberculous mycobacteria isolates

compared to that of moxifloxacin, gatifloxacin, levofloxacin and RMP. The potent activity of DC-159a is ascribed to the inhibition of DNA gyrase from wild-type and MDR-*Mtb*. In the drug-susceptible-*Mtb* infection model, it exhibited better early bactericidal activity (EBA) and higher log reduction of CFU in lungs, compared to moxifloxacin, levofloxacin, INH and RMP. In the QR MDR-TB infection model, it showed 2~3 times longer "mean survival days" which was superior to moxifloxacin, levofloxacin, INH and RMP. Pharmacokinetic study of DC-159a in a monkey model after an oral dose of 5 mg/kg of body weight, showed that it achieved a higher peak concentration (C_{max}; 2.20 μg/ml) and area under the concentration-time curve from 0 to 24 h (AUC 0–24; 16.9 μg.h/ml) than the MIC against *Mtb*, and showed better pharmacokinetic properties than levofloxacin (C_{max}, 1.68 μg/ml; AUC 0–24, 15.3 μg.h/ml). DC159a lacked interaction with cytochrome P450 3A4 (WGND; Disratthakit, & Doi, 2010; Sekiguchi et al., 2011), suggesting a better safety profile.

3.2.3.3 SQ-609

Sponsor/developer: Sequella

SQ-609

Summary: Sequella screened >100,000 molecules for anti-mycobacterial activity and identified SQ609 as the most potent (MIC = 4 μg/mL) and promising candidate among a new series of potential cell-wall inhibiting dipiperidines that are structurally different than any existing antitubercular drugs/candidates. Precise mode of action of SQ 609 is unknown (WGND; Bogatcheva et al., 2011).

3.2.3.4 SQ-641

Sponsor/developer: Sequella

Target**:** Translocase 1 (TL1) enzyme Inhibitors

Compounds: **>**7000 compounds synthetic compounds derived from natural products

SQ-641

Summary: Translocase 1 (TL1) enzyme, which is absent in eukaryotic cells, is an essential enzyme in bacteria for the biosynthesis of the peptidoglycan layer of the cell wall. The semi-synthetic nucleoside Capuramycin has been studied as inhibitor of TL1 enzyme. The lead candidate SQ-641 (MIC = 0.5 μg/mL) is under preclinical development for the treatment of TB. Its mycobactericidal rate is faster than any existing TB drugs. SQ-641 possesses activity against MDR clinical strains of *Mtb*. It has shown efficacy in a mouse model of chronic TB by reducing CFU in lungs of infected mice by 1.0 to 1.5 log (WGND; Bogatcheva et al., 2011).

3.2.3.5 Benzothiazinone (BTZ-043)

BTZ-043

Summary: BTZ-043 belongs to a new class of antimycobacterial agents. It is highly active against *Mtb* (MIC = 1-10 ng/mL) and other actinobacteria. It also possesses activity against MDR- and XDR-TB strains. It showed *in vitro* bactericidal activity comparable to INH. It is non-mutagenic and has good oral bioavailability. BTZ-043 inhibits cell wall biosynthesis, and targets the DprE1 (Rv3790) subunit of the enzyme decaprenylphosphoryl-beta-D-ribose 2'-epimerase.

3.2.3.6 Q-201

Sponsor/developer: Quro Science, Inc.

It is an imidazopyridine compound. Not much detail is available about this compound.

Phase	Compound	Sponsor/developer	Mode of action
I	AZD5847	Astrazeneca	Protein synthesis inhibitor
II	PNU-100480	Pfizer	Protein synthesis inhibitor
	LL3858	Lupin Pharmaceuticals Inc.	Not yet known
	SQ-109	Sequella, NIH	Not yet known
	PA-824	TB Alliance	Protein synthesis and cell wall lipids inhibitor
	OPC67683	Otsuka Pharmaceutical Co. Ltd.	Protein synthesis and cell wall lipids inhibitor
	TMC 207	Tibotec	Affects proton pump of ATP synthase
	Linezolid	Tuberculosis Trials Consortium (TBTC), Pfizer	Protein synthesis inhibitor Novel unique
II/III	Rifapentine	CDC, Sanofi-aventis	Inhibits DNA dependent RNA polymerase

Phase	**Compound**	**Sponsor/developer**	**Mode of action**
III	Moxifloxacin	University College London	Inhibits bacterial replication
	Gatifloxacin	Institut de Recherche pour le Développement, WHO, European Commission (primary developers)	Inhibits bacterial replication

Table 5. Compounds in phase I-III clinical trials

3.2.4 Phase I

3.2.4.1 AZD-5847

Sponsor/developer: Astrazeneca

Summary: AZD-5847, an oxazolidinone antibiotic (structure is not disclosed), originally developed for staphylococcal infections, is currently in Phase 1 clinical trials. It possesses MIC_{90} of 1 µg/mL against laboratory *Mtb* strains and clinical isolates resistant to INH, RMP, streptomycin, EMB or OFX (Abstract Balasubramanian et al., 2011). Studies to examine safety, tolerability and blood levels of AZD-5847 in healthy volunteers are underway.

3.2.5 Phase II

3.2.5.1 PNU-100480

Sponsor/developer: Pfizer

PNU-100480

Summary: PNU-100480 is a structural analogue of linezolid (see details in Phase II section). It is more active than linezolid against TB (Williams, et al., 2009 as cited in Alffenaar et al., 2011) and possesses similar efficacy to that of INH and RMP (Cynamon et al., 1999, as cited in Alffenaar et al., 2011). Its MIC was found in the range of .0625-0.5 µg/mL in drug-susceptible and drug-resistant clinical strains of *Mtb* (Alffenaar et al., 2011). When added to a first-line regimen in a murine model, PNU-100480 had a synergistic bactericidal effect, while linezolid had an antagonistic effect (Williams, et al., 2009 as cited in Alffenaar et al., 2011). 14 day dose-escalation and 28 day dose study in healthy volunteers have been completed (WGND, 2011).

3.2.5.2 Pyrrole (LL-3858) or Sudoterb.

Sponsor/developer: Lupin Pharmaceutical Inc.

BM 212 **LL3858**

Summary: Deidda et al. (Deidda et al., 1998) first reported the activity of the pyrroles against *Mtb*. The most potent compound identified was BM212 (MICs = 0.7 to 1.5 µg/mL against several strains of *Mtb*). This work by Deidda et al. later on inspired Lupin to synthesize a series of pyrroles and one of their leads LL3858 is currently in clinical development for the treatment of TB (Arora et al., 2004). The MIC90 of LL3858 for *Mtb* is reported to be 0.25 µg/mL (Tuberculosis**.** 2008. Ll-3858, as cited in van den Boogaard et al., 2009). LL3858, in combination with current anti-TB drugs, is reported to sterilize the lungs and spleens in lesser time than the conventional therapy (Sinha et al., 2004). The mechanism of action for this class of compounds has not yet been established.

3.2.5.3 Diamine (SQ-109)

Sponsor/developer: Sequella, NIH

SQ-109

Summary: SQ109, or N-adamantan-2-yl-N'-(3,7-dimethylocta-2,6-dienyl)-ethane-1,2-diamine, is being developed by Sequella. It was the most potent compound (MIC = 0.1–0.63 µg/mL) in the series (Lee et al., 2003). *In vivo* studies showed 1 to 2.0-log reduction in CFU counts in the lung and spleen at 25 mg/kg. Its oral bioavailability is only 4% (Jia et al., 2005). Preclinical toxicology studies have been completed and further phase 2 clinical studies are underway.

3.2.5.4 Nitroimidazoles (PA824 AND OPC67683)

3.2.5.4.1 PA-824

Sponsor/developer: TB Alliance

PA-824

In 1970s Ciba-Geigy in India screened a series of nitroimidazoles as radiosensitizers. Many of them were later found to possess antimicrobial activity, (including anti-*Mtb* activity). However, further development was discontinued after the lead molecule CGI-17341 was found to be mutagenic. In 1995 a pharmaceutical company, PathoGenesis, modified Ciba-Geigy's molecules and screened around 700 compounds against *Mtb* and found PA824 as the most active (Stover et al., 2000) and non mutagenic (Ginsberg & Spigelman, 2006). After PathoGenesis, Chiron Corporation obtained the rights and finally the Global Alliance for TB Drug Development acquired its rights for its clinical development. It has potent *in vitro* activity against *Mtb*, as evidenced by an MIC range of 0.015 to 0.25 mg/ml, and retains this activity against isolates resistant to a variety of commonly used anti-TB drugs. PA-824 kills *Mtb* bacilli by inhibiting the synthesis of protein and cell wall lipids (Stover et al., 2000). In mouse model it was highly active for latent TB in combination with moxifloxacin (Nuermberger et al.; 2005). It is suggested, however, that PA-824 is a prodrug and requires reductive activation of the aromatic nitro group (Manjunatha et al., 2006). PA-824 showed good tissue permeability in rat studies. Its minimum bactericidal dose (to reduce the lung CFU count by 99%) was found to be 100 mg/kg/day in murine studies. PA-824 in combination with INH prevents selection of TB mutants resistant to INH. It is effective against replicating and persistent TB bacilli. It is also effective against MDR strains and *Mtb* grown under oxygen depletion (Tyagi et al., 2005; Lenaerts et al., 2005). It has completed phase 1 studies in healthy volunteers (Spigelman, 2005).

3.2.5.4.2 OPC-67683 (Delamanid)

Sponsor/developer: Otsuka Pharmaceutical Co. Ltd.

OPC-67683

Another nitroimidazole compound, OPC-67683 (MICs 0.006 µg/mL) is being developed by Otsuka Pharmaceutical. It was found to be potent against *Mtb in vitro* and *in vivo* (Matsumoto et al., 2005). In a mouse model, its efficacy was reported to be superior to that of currently used TB drugs. The effective plasma concentration of OPC-67683 was 0.100 µg/mL (achieved with an oral dose of 0.625 mg/kg). It showed no cross-resistance with the current anti-TB drugs. The mechanism of action of OPC-67683 is suggested to be similar to PA-824 (Kawasaki et al., 2005).

3.2.5.5 Diarylquinoline (TMC-207 or R-207910 or Bedaquiline)

Sponsor/developer: Tibotec

TMC-207

TMC-207 is owned by Johnson & Johnson (J&J) and is being developed at its research subsidiary Tibotec. TMC-207 not only showed very potent *in vitro* activity against both MDR and drug-susceptible strains of *Mtb* but also has potent activity against other Mycobacterial species (*M. avium*, *M. marinum*, *M. fortuitum*, and *M. abscessus M. smegmatis*). Its MIC ranges from 0.002 to 0.06 µg/mL for drug susceptible and drug resistant strains (Andries et al., 2005; Huitric et al., 2007). It is active *in vitro* against TB organisms resistant to INH, RMP, streptomycin, EMB, PZA, and moxifloxacin. It has no cross-resistance with current anti-TB medications (Andries 2004). In mice, a single dose had bactericidal potency for about eight days. When used as monotherapy, a single dose of TMC-207 was as potent as the triple combination of RMP, INH, and PZA and was more active than RMP alone. It works on the proton pump of ATP synthase (Andries et al., 2005). The effective half-life was found was ~24 h. Single ascending dose and 14-day multiple ascending dose studies in healthy human males showed no severe adverse effects. Further clinical trials are underway.

3.2.5.6 Linezolid for the Treatment of Multi-Drug Resistant Tuberculosis

Sponsor/developer: Tuberculosis Trials Consortium (TBTC), Pfizer

Linezolid

Linezolid is an approved antibacterial drug without a TB indication. It was discovered in 1990s and approved in 2000 for the treatment of Gram positive bacterial infections. It is active against most Gram-positive bacteria with MIC_{90} 1-2 µg/mL (Alcalá et al., 2003). It works as a protein synthesis inhibitor. Lack of information on its efficacy is one of the major concerns for its use as anti-TB agents (Migliori et al., 2009). Long-term use has been associated with thrombocytopenia, neuropathy and haematopoietic suppression (Gerson et al., 2009).

3.2.5.7 Rifapentine (TBTC study)

Sponsor/developer: CDC, Sanofi-aventis

Rifapentine

Rifapentine is a cyclopentyl derivative of the first-line TB drug RMP. Its MIC was found to be 0.03 µg/mL by 7H12 broth radiometric assay (Heifets et al., 1999). Its mechanism of action is the same as of RMP (Williams et al., 1998). It induces the CY450 system to a lesser extent than RMP (Weiner et al., 2004). It can also be used for latent TB as a part of regimen with either moxifloxacin or INH (Nuermberger et al., 2005). The aim of the clinical trial is to examine antimycobacterial activity and safety of an experimental intensive phase (first 8 weeks of treatment) tuberculosis treatment regimen in which RMP is substituted by rifapentine.

3.2.6 Phase III

3.2.6.1 Fluoroquinolones

In the past few years, attention has been focused on the use of fluoroquinolones for shortening the treatment duration of *Mtb*. Most of the credit for the use of fluoroquinolones goes to a clinical trial by the Tuberculosis Research Centre, Chennai, India (Tuberculosis Research Centre [TRC], 2002). In this trial, newly diagnosed pulmonary TB patients were randomly divided to receive one of four regimens containing a fluoroquinolone – ofloxacin (OFX). The rates of sputum conversion by this treatment at 2 months ranged from 92%-98% (superior to ~80% conversion rate by conventional therapy) (Tuberculosis Trials Consortium [TBTC], 2002).

3.2.6.1.1 Moxifloxacin

Sponsor/developer: University College London

Moxifloxacin

Moxifloxacin ("Avelox" by Bayer) is a broad-spectrum antibiotic (400 mg/day dose) and is active against both gram positive and gram negative bacteria. It exhibits MIC of 0.5 μg/mL against *Mtb* (Shandil et al., 2007). It displayed early bactericidal activity comparable to INH and rifampin in humans (Pletz 2004; Gosling 2003). It affects bacteria by binding to the DNA gyrase and topisomerase IV, which are involved in bacterial replication. It has no cross-resistance to other antituberculosis drug classes; therefore, it might be useful against MDR-TB and XDR-TB. Further, it has been shown to display good activity profile against MDR strains (Tortoli et al., 2004). However, it has CNS side effects and drug interactions with other fluoroquinolones. Moxifloxacin has not been reported to be safe or effective in children younger than 18 or in pregnant or lactating women (Bayer, n.d.). Nuermberger et al. (2004) found that substituting moxifloxacin for INH shortens the duration of therapy for active disease much better than does substituting moxifloxacin for EMB.

3.2.6.1.2 Gatifloxacin

Sponsor/developer: Institut de Recherche pour le Developpement, WHO, European Commission (primary developers)

Gatifloxacin

Gatifloxacin ("Tequin" by Bristol-Myers Squibb) is also a broad-spectrum antibiotic (dosage of 400 mg/day). It works by the same mechanism as moxifloxacin. It is active against occasionally dividing *Mtb*, but not for dormant bacteria (Paramasivan et al., 2005). Gatiflozacin in combination with ethionamide and PZA was most effective to sterilize the lungs and prevent relapse (Cynamon & Sklaney, 2003). Gatifloacin can cause CNS toxicity and has been associated with increases in insulin levels among diabetics. It has not been shown to be safe or effective in children younger than 18 or in pregnant or lactating women. Gatifloxacin has completed a phase 2 study on randomized patients receiving 8 weeks of

therapy with either conventional treatment or the combination of INH, PZA, and RMP with either OFX or moxifloxacin, or gatifloxacin. In this study, serial sputum colony count measurements indicated that the patients in the moxifloxacin and gatifloxacin arms cleared their sputum more quickly than the patients receiving conventional therapy or the regimen containing OFX (Lienhardt et al., 2005).

3.2.7 Experimental compounds

The following experimental compounds are not commercially available. Their efficacy and safety are unknown.

3.2.7.1 A herbal product from Ukraine has been subjected to many open label clinical trials, with promising results in TB and TB/HIV coinfected patients (Zaitzeva et al., 2009; Nikolaeva et al., 2008a, 2008b). Open label trials with adjuvant Dzherelo (Immunoxel) have also been positive in MDR-TB and XDR-TB patients (Prihoda et al., 2007).

3.2.7.2 V-5 Immunitor or "V5", is an oral vaccine available in tablets for hepatitis B and hepatitis C treatment. TB sputum clearance was unexpectedly noted within a month, in hepatitis C-TB co-infected patients. Blinded studies suggest that V5 is also effective against MDR-TB (Olga et al., 2010; Butov et al., 2011).

4. Conclusion

After decades of reluctance in the TB drug discovery, several groups/institutions such as TB Alliance, Working Group on New Drugs (WGND) and New Medicines for Tuberculosis (NM4TB) have rekindled hope for new anti-tuberculosis drug(s) which may offer promise against MDR- and XDR-TB, and HIV-TB co-infection. The new drugs may also have capability of shortening the treatment duration of drug susceptible TB. Apart from the above big organizations, smaller research teams worldwide including our laboratory are actively involved in the search of new classes of potent and safe anti-tuberculosis drug(s).

The current TB drug pipeline (Table 6), no doubt, is the richest we have ever seen, but still it will take a long before any new drug hits market with approval. There are hurdles on the way ahead. Fund constraints, slow pace trial designs, insufficient infrastructure to validate the drug(s), validation and approval mechanism of Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA), and most importantly, the strong political will power, are the crucial issues ahead. The TB Trials Consortium (TBTC) (funded by The Centers for Disease Control and Prevention), National Institutes of Health (NIH) and European and Developing Country Clinical Trials Program (EDCTP) have to play better and expanded roles along with the ongoing efforts to accelerate the drug development. Governments, regulatory agencies, pharmaceutical and biotechnology companies, involved international agencies and communities, and basic and applied researchers worldwide all have to work together to achieve the goal of eradicating TB, like other big burden disease such as HIV.

It is worth to mention here lastly, that not only cure by drugs, but prevention measures and awareness steps by Governments and social bodies are also crucial and play very important role to stop any such infectious devil. Particular area on alert which need drastic improvements are imprisonment, health care systems, sex workers, travel and transportations,

and mass gathering activities such as festivals and events. The most important but neglected part of prevention program, which might be addressed and implemented urgently and effectively, is a separate and intense educational program designed for families having a member with diagnosed active TB. Together with a successful drug hunt and preventive measures, we can soon hope of the world without fear of millions of yearly deaths from tuberculosis.

Discovery Classes, (Sponsor/developer)			**Preclinical**	**Clinical**			**Existing Drugs**
Screening	**Lead Identification**	**Lead Optimization**		**Phase I**	**Phase II**	**Phase III**	
Natural Products (IMCAS)	Whole-Cell Hit to Lead Program (GSK)	Mycobacterial Gyrase Inhibitors (GSK)	Nitroimidazoles (U. of Auckland/ U. Ill Chicago)	AZD5847	PNU-100480	Moxifloxacin (Bayer)	**First Line** Rifampicin Isoniazid Pyrazinamide Ethambutol
Topoisomerase I Inhibitors (AZ/NYMC)	Folate Biosynthesis Inhibitors (AZ)				PA-824 (Nova-rtis)		
	RNA Polymerase Inhibitors (AZ) Energy Metabolism Inhibitors (AZ/U. Penn)	InhA Inhibitors	CPZEN-45				
Nucleosides	Ruthenium(II)pho-sphine/picolinate complexes	Pyrazinamide Analogs (Yonsei)	Preclinical TB Regimen Develo-pment (JHU/U. Ill Chicago)		Lnezolid; low dose	Gatifloxacin	**Second Line** Amikacin Kanamycin Capreomycin Streptomycin Cycloserine Ethionamide, PAS Clofazimine Ciprofloxacin Levofloxacin Ofloxacin
Carbo-hydrates	Whole-Cell Hit to Lead Program (AZ)						
Metal Complexes	Folate Biosynthesis Inhibitors	Diarylquinolines Tibotec/U. of Auckland	DC-159a		TMC 207 (Tibotec)		
Hydrazides and hydrazones	Menaquinone Synthase (MenA) Inhibitors						
Hetero-cyclics Quinolines, Quinoxalines, Pyrimidines, Purines, Pyrroles, Azines	Protein Kinase Inhibitors	Riminophenazines (IMM/BTTTRI)	SQ-641		LL3858	Rifape-ntine	
	Enzyme(s) involved in DNA synthesis				SQ-109 OPC67683		
			SQ-609				**"Third Line"** Rifabutin, Macrolides: (e.g., clarithromycin); Linezolid, R207910, Thioacetazone; Thioridazine; Arginine; vitamin D; .
Chalcones	Malate Synthase Inhibitors		BTZ-043				
Artemisinin derivatives	Actinomycete metabolites				Rifapentine		
	Fungal metabolites						
Macrolids			Q-201 (Quro Science Inc.)				
Peptides							

Table 6. Drug discovery: Screening to Existing Drugs.

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3

A New Hope in TB Treatment: The Development of the Newest Drugs

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1. Introduction

TB chemotherapy is made up of a cocktail of first-line drugs: isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM), given for six months. If the treatment fails as a result of bacterial drug resistance, or intolerance to one or more drugs, second-line drugs are used, such as para-aminosalicyte (PAS), kanamycin, fluoroquinolones, capreomycin, ethionamide and cycloserine, that are generally either less effective or more toxic with serious side effects. Treatment becomes quite difficult by the presence of metabolically silent, persistent or dormant bacteria within host lesions, which are not susceptible to the antimycobacterial drugs that usually kill growing bacteria, but not persistent bacteria (Zhang, 2004). Therefore, in the search for improved drugs to treat drug sensitive, active tuberculosis, the target product profile might include (Zhang, 2006; Ginsberg, 2008): (1) the ability to shorten treatment duration to 2 months or less (typically defined as potency greater than the most active first-line drug, isoniazid, against *M. tuberculosis* growing under aerobic conditions, and/or potency greater than the best current drug, rifampin, under conditions where *M. tuberculosis* is slowly replicating; the latter serves as a model of the "drug-persistent" state and therefore as a marker of a compound's potential to shorten treatment-duration); (2) safety at least as good as that of current first-line TB drugs; (3) a novel mechanism of action for TB treatment; (4) oral bioavailability; (5) pharmacokinetic-pharmacodynamic profile consistent with once-daily or less frequent dosing; (6) minimal or no interactions with hepatic cytochrome P450 enzymes (and therefore minimal potential for drug-drug interactions, especially with antiretroviral therapy); and (7) low cost of drugs. Seven candidate TB drugs representing five different chemical classes are currently known to be undergoing clinical evaluation (Ginsberg, 2010). This review will provide a brief update on the latest developments in current TB drug discovery efforts.

2. PA-824

A series of bicyclic nitroimidazofurans originally investigated as radio sensitizers in cancer chemotherapy, were found to possess activity against replicating *M. tuberculosis in vitro* and

had also significant *in vivo* activity in a murine infection model. The nitroimidazo-oxazine PA-824, exhibited a low MIC (0.015 to 0.025 µg/ml) against *M. tuberculosis* and became a leading compound. PA-824 was first identified and its anti-*M. tuberculosis* activity characterized (Stover et al., 2000) in the mid-1990s by Pathogenesis Corporation, later purchased by Chiron (now Novartis). In 2002, Chiron out-licensed this compound and its analogs to the Global Alliance for TB Drug Development (TB Alliance), granting it a worldwide exclusive license to develop it for TB. Since then the TB Alliance has brought PA-824 through preclinical development, filed an Investigational New Drug Application (IND) in April 2005, conducted Phase Ⅰ clinical evaluations, its safety, tolerability, pharmacokinetic properties, and efficacy in drug-sensitive, sputum smear-positive, adult pulmonary TB patients. PA-824 is currently undergoing Phase Ⅱ clinical trials (Ginsberg, 2010).

In vitro studies showed MICs of PA-824 against fully susceptible and MDR strains ranging from 0.015 to 0.25 µg/ml. PA-824 activity is concentration dependent (Stover et al., 2000; Lenaerts et al, 2005; Tyagi et al., 2005). The bactericidal activity of PA-824 (25 to 50 mg/kg) was comparable to that of isoniazid (25mg/kg) in mice and guinea pigs (Stover et al., 2000; Lenaerts et al, 2005; Tyagi et al., 2005) and to those of rifampin (20mg/kg) and moxifloxacin (100mg/kg) in mice. PA-824 showed greater activity than isoniazid and moxifloxazin *in vitro* and in mice and comparable activity to combination therapy with rifampin and isoniazid (Lenaerts et al., 2005; Tyagi et al., 22005; Hu et al., 2008)). PA-824 (100mg/kg) has been incorporated in the standard regimen in mice to evaluate its potential to shorten treatment duration. Only the regimen in which isoniazid was replaced with PA-824 achieved faster lung culture conversion and a lower CFU count after 2 months of treatment than the standard regimen. However, relapse rates were similar in these regimens (Nuermberger et al., 2006). The sterilizing activity of a regimen containing PA-824 (100mg/kg), moxifloxacin (100 mg/kg), and pyrazinamide (150 mg/kg) was recently found to be better than that of rifampin (10 mg/kg), isoniazid (25 mg/kg), and pyrazinamide (150 mg/kg) in mice, indicating that PA-824 could be incorporated in a rifampin-free regimen to treat MDR-TB Nuernberger et al., 2008). PA-824 (100 mg/kg) was highly active in a mouse model for latent TB when combined with moxifloxacin (100 mg/kg) (Nuermberger et al., 2005). PA-824 was spray-dried into porous particles containing a high drug load and possessing desirable aerosol properties for efficient deposition in the lungs (Sung et al., 2009). Pharmacokinetic parameters were determined in guinea pigs after the pulmonary administration of the PA-824 powder formulation of three doses (20, 40 and 60 mg/kg of body weight) and compared to those after the intravenous (20 mg/kg) and oral (40 mg/kg) delivery of the drug. Animals dosed by the pulmonary route showed drug loads that remained locally in the lungs for 32h after exposure, whereas those given the drug orally cleared the drug more rapidly. Therefore, pulmonary delivery may achieve the same efficacy as oral delivery at the same body dose, with a potential improvement in effectiveness related to pulmonary infection.

Ginsberg et al. (Ginsberg et al., 2009a) evaluated the safety, tolerability, and pharmacokinetics of PA-824 in two escalating-dose clinical studies, one being a single-dose study (50, 250, 500, 750, 1000, 1250, 1500mg) and the other being a multiple-dose study (200, 600, 1000, 1400mg, up to 7 days of daily dosing). In 58 healthy subjects dosed with PA-824 across these studies, PA-824 was well tolerated with no significant or serious adverse events. In both studies, following oral administration, PA-824 reached maximal plasma levels in 4 to

5 hours, independent of dose. Maximal blood levels averaged approximately 3 µg/ml (1500mg dose) in the single-dose study and 3.8 µg/ml (600mg dose) in the multiple dose study. The steady state was achieved after 5 to 6 days of daily dosing, with an accumulation ratio of approximately 2. The elimination half-life averaged 16 to 20 hours. Overall, PA-824 was well tolerated following oral doses once daily for up to 7 days, and pharmacokinetic parameters were consistent with a once-a-day regimen. The results of these studies, combined with the demonstrated activity of PA-824 against drug-sensitive and multidrug-resistant *M. tuberculosis* with no influence of serum concentration by coadministration of RIF, INH, PZA in various combinations (Nuermberger et al., 2006; Ginsberg et al., 2009a), support investigation of this novel compound for the treatment of tuberculosis. Since multiple doses of 1000mg were associated with a moderate, reversible increase in creatinine, Ginsberg et al. made a further assessment (Ginsberg et al., 2009b) of the effects of PA-824 on renal function in healthy subjects. The results suggests that PA-824 causes creatinine level to rise by inhibiting renal tubular creatinine secretion and such an effect is considered clinically benign since it has been described for several marketed drugs.

Recently, Ahmad (Ahmad et al., 2011) found that PA-824 exhibited time-dependent activity in a murine model of tuberculosis. Diacon (Diacon et al., 2010) concluded that PA-824 demonstrated bactericidal activity over the dose range of 200 to 1200mg daily over 14 days by evaluation of its early bactericidal activity and pharmacokinetics in smear-positive tuberculosis patients. Garcia-Contreras et al. (Garcia-Contreras et al., 2010) evaluated the effects of PA-824 therapeutic aerosols on the extent of TB infection in the low-inoculum aerosol infection guinea pig model. Four weeks after infection by the pulmonary route, animals received daily treatment for 4 weeks of either a high or a low dose of PA-824 dry powder aerosol. The lungs and spleens of animals receiving the high dose of inhaled PA-824 particles exhibited a lower degree of inflammation, bacterial burden, and tissue damage than those of untreated or placebo animals. Their studies indicate the potential use of PA-824 dry powder aerosols in the treatment of TB.

It has been found that PA-824 is a prodrug that needs the mycobacterial glucose-6-phosphate dehydrogenase (FDG1) or its cofactor, coenzyme F420, to be transformed into an active form (Stover et. al., 2000; Manjunatha et al., 2006a; Singh et al., 2008). Activated PA-824 inhibits the synthesis of proteins and cell wall lipids. PA-824 activity is limited to *M. tuberculosis* complex (Manjunatha et al., 2006b) and is active in susceptible and resistant *M. tuberculosis* strains. No cross-resistance with standard anti-TB drugs has been observed. Mutations in the Rv3547 gene have been described in PA-824 resistant strains. Complementing these mutants with intact Rv3547 fully restored the ability of the mutants to metabolize PA-824 (Manjunatha et al., 2006a). In a further study by Barry et al. (Singh et al., 2008) revealed that Rv3547 was a deazaflavin-dependent nitroreductase (Ddn) that converted PA-824 into three primary metabolites; the major one is the corresponding des-nitroimidazole (des-nitro). When derivatives of PA-824 were used, the amount of des-nitro metabolite formed was highly correlated with anaerobic killing of *M. tuberculosis*. Dea-nitro metabolite formation generated reactive nitrogen species, including nitric oxide (NO), which are the major effectors of the anaerobic activity of these compounds. Furthermore, NO scavengers protected the bacilli from the lethal effects of the drug. Thus Barry et al. (Singh et al., 2008) concluded that PA-824 might act as intracellular NO donors and could augment a killing mechanism intrinsic to the innate immune system.

In summary, PA-824 has three key characteristics: (1) a unique mechainsm of action, (2) a narrow spectrum of activity and (3) no cross-resistance with current antituberculosis drugs. Thus, PA-824 seems a most promising drug to treat latent TB together with second-line or new anti-tuberculosis drugs and shorten treatment duration.

3. OPC-67683

OPC-67683, a novel nitro-dihydro-imidazoooxazole active against *M. tuberculosis*, is structurally related to PA-824 and discovered and being developed for TB by Otsuka Pharmaceutical Co. OPC-67683 is a mycolic acid biosynthesis inhibitor (Sasaki et al., 2006). It possesses highly potent activity against TB, including MDR-TB, as shown by its exceptionally low minimum inhibitory concentration (MIC) range of 0.006~0.024 μg/ml *in vitro* and highly effective therapeutic activity at low doses *in vivo* (Sasaki et al., 2006; Matsumoto et al., 2006). Additionally, the results of the post-antibiotic effect of OPC-67683 on intracellular *M. tuberculosis* showed the agent to be highly and dose-dependently active also against intracellular *M. tuberculosis* H37Rv after a 4h-pulsed exposure, and this activity at a concentration of 0.1 μg/ml was similar to that of the first-line drug rifampicin at a concentration of 3 μg/ml. The combination of OPC-67683 with rifampin and pyrazinamide exhibited a remarkably quicker eradication (by at least 2 months) of viable TB bacilli in the lung in comparison with the standard regimen consisting of rifampicin, isoniazid, ethambutol and pyrazinamide (Matsumoto et al., 2006). Furthermore, OPC-67683 did not affect, nor was affected by, the activity of liver microsome enzymes, suggesting the possibility for OPC-67683 to be used in combination with drugs, including anti-retrovirals, that induce or are metabolized by cytochrome P450 enzymes (Matsumoto et al., 2006). The early bactericidal activity of 400mg OPC-67683 in patients with pulmonary TB was low during the first 4 days. From day 4 onwards, a significant decrease in CFU was seen (Kaiser Family Foundation, 2009; Boogaard et al., 2009). OPC-67683 in multiple doses up to 400mg was tolerated well by healthy volunteers. No serious adverse events were reported (Kaiser Family Foundation, 2009; Boogaard et al., 2009). In summary, OPC-67683 is a promising new anti-TB drug with bactericidal and sterilizing activity *in vitro* and in mice. This drug is currently in Phase II clinical testing in MDR-TB patients and expected to be a powerful therapeutic.

4. TMC 207

The discovery of diarylquinoline as a promising TB drug that can shorten therapy (Andries et al., 2005) has generated much excitement. Andries et al. identified diarylquinoline compounds that were highly active against mycobacteria in *in vitro* drug screening using fast-growing *Mycobacterium smegmatis*. Modification of the diarylquinolines led to the identification of diarylquinoline TMC207 (R207910, J compound), as the most active agent, with minimum inhibitory concentration (MIC) of 0.003 μg/ml for *M. smegmatis* and 0.030 μg/ml *M. tuberculosis*. TM207 is much less active against other bacterial species, such as *E. coli* and *S. aureus* (MIC>32 μg/ml). *M. tuberculosis* and *M. smegmatis* could develop resistance to diarylquinoline at a frequency of 1×10^{-7} to 1×10^{-8} .Diarylquinoline-resistant *M. smegmatis* and *M. tuberculosis* strains were found to harbor mutations in the subunit C encoded by *atpE* gene (D32V for *M. smegmatis* and A63P for *M. tuberculosis*) in the FO moiety of mycobacterial F1F0 proton ATP synthase, which is a key enzyme for ATP synthesis and

membrane-potential generation. Complementation studies confirmed that the mutations in *atpE* were responsible for resistance to diarylquinoline. The target for diarylquinoline was proposed to be the mycobacterial F1F0 proton ATP synthase, which was a new drug target in mycobacteria. In fact, TMC207 is also active against MDR-TB strains. Based on transposon mutagenesis analysis, F1F0 ATP synthase seems to be an essential enzyme in *M. tuberculosis* although the enzyme is not essential for *E. coli* because mutants of F1F0 were viable but grew at a reduced rate and were attenuated for virulence in mice Zhang, 2003). TMC207 was more active than INH and RIF in the mouse model (Andries et al., 2005) and could shorten TB therapy from four months to two months in mice with an established infection model. Of particular interest is the synergy between diarylquinoline and PZA, which seems to be the most effective drug combination in sterilizing infected spleens and lungs. This finding is consistent with the previous observation that N,N′-dicyclohexylcarbodiimide (DCCD) -- which also inhibits the same C chain of the FO moiety of F1F0 ATPase as diarylquinoline—has synergy with PZA against *M. tuberculosis* (Zhang, 2003). Thus, the observed synergy of diarylquinoline with PZA [24] could be explained the same way as the synergy of DCCD with PZA. TMC207 had excellent early and late bacterial activity, good pharmacokinetic and pharmacodynamic properities with a long half life, absence of significant toxicity in mouse and preliminary human safety testing, raising the hope that diarylquinoline might be used for shortening TB therapy in humans (Andries et al., 2005).

There are several unusual features of TM207 that require further explanation. First, antimycobacterial drugs usually do not show the same degree of activity against fast and slow growing mycobacteria. Drugs like INH, RIF, and PZA are more active against slow growing *M. tuberculosis* but less active against fast growers like *M. smegmatis*, which has higher efflux activity and is better able to maintain its energy status compared with *M. tuberculosis*. However, in this case, diarylquinolines are even more active against fast growing *M. smegmatis* than against *M. tuberculosis*, which is quite unusual. Second, the high early and late bactericidal activity in mice is unusual because other TB drugs show either early or late sterilizing activity but not both. Third, the selective activity of diarylquinolines against the mycobacterial enzyme F1F0 Atpase (present in all mycobacteria, but also in host cell mitochondria) without apparent toxicity is quite remarkable. Finally, mycobacteria would be expected to have alternative means, such as the electron transport chain, to produce energy or ATP without F1F0 ATPase, and thus the inhibition of F1F0 ATPase by diarylquinoline would not be lethal unless TMC207 also interferes with other drug targets in the mycobacteria.

An extended early bactericidal assay (Rustomjee et al., 2008ab) was conducted with human patients who were treated for 7 days with TMC207 given at 25mg, 100mg, and 400mg per day. Patients treated with either 600mg per day of RIF or 300mg per day of INH were used as controls. This study showed that TMC207 given at 400mg per day revealed a significant decrease in CFU counts in the sputum of treated patients in comparison with the pretreatment levels. A drug-drug interaction study was conducted in 16 healthy volunteers who received a single dose of 300mg of TMC207 alone and seven daily doses of 10mg/kg of body weight of RIF. The area under the concentration-time curve (AUC) from time zero to 336 h for TMC207 after its coadministration with RIF was about half that when it was dosed alone, indicating that the metabolism of TMC207 is induced by coadministration with RIF. Lounis et al. (Lounis et al., 2008) assessed the impact of reducing the dose of TMC207 on its

efficiency when TMC207 was combined with a background regimen of INH, RIF and PZA. Addition of 25mg/kg of body weight or 12.5mg/kg TMC207 to the background regimen resulted in faster bacterial clearance and culture negativity. The difference in efficacy between the two doses was not statistically significant. The minimal bactericidal dose of TMC207 when it was tested as a part of the combination was identical to that when it was tested as monotherapy. Because of the drug-drug interaction in human, the activity of TMC207 in human could be less than that expected from studies with mice. Data from the mouse model demonstrate that TMC207 has significant activity, even when its exposure is reduced by 50% and when it is added to a strong background regimen of INH, RFP, and PZA (Lounis et al., 2008). In killing kinetic studies, the bactericidal effect of TMC207 in mice was modest during the first week of treatment, but it increased in the following 3 weeks, while the bactericidal activity of isoniazid was limited to the first week of treatment.

Because of its potent activity against *M. tuberculosis*, its distinct mechanism of action, and its impressive activity at what appear to be human-equipotent dosages in the murine model, TMC207 is a particularly promising new drug candidate. Recently, Zhang (Zhang et al., 2011) used an established experimental model of latent TB infection chemotherapy in which mice are aerosol-immunized with a recombinant BCG vaccine prior to low-dose aerosol infection with *Mycobacterium tuberculosis*, the efficacy of TMC207 alone and in combination with rifapentine was compared to currently recommended control regimens as well as once-weekly rifapentine + isoniazid and daily rifapentine +/- isoniazid. Parameters used were monthly lung CFU counts and relapse rates. Lung CFU counts were stable around 3.75 log10 for up to 7.5 months post-infection in untreated mice. Rifamycin-containing regimens were superior to isoniazid monotherapy. TMC207 exhibited sterilizing activity at least as strong as that of rifampin alone and similar to that of rifampin + isoniazid, but daily rifapentine +/- isoniazid was superior to TMC207. Addition of TMC207 to rifapentine did not improve rifapentine's sterilizing activity in this model. That signifies that TMC207 has substantial sterilizing activity and may enable treatment of DR-LTBI (drug-resistant latent tuberculosis infection) in 3-4 months.

5. Moxifloxacin and gatifloxacin

The fluoroquinolones are a promising class of drugs for the treatment of TB. In particular, they are distributed broadly throughout the body, including within cells, which explains their efficacy against mycobacteria. Moxifloxacin and gatifloxacin are candidates for shortening TB treatment, since they have the lowest MICs and greatest bactericidal activity, as expressed in the rate of fall in CFU count (Paramasivan et al., 2005; Shandil et al., 2007).

Moxifloxacin is a broad-spectrum 8-methoxy fluoroquinolone with activity against both gram-positive an The fluoroquinolones are a promising class of drugs for the treatment of TB. In particular, they are distributed broadly throughout the body, including within cells, which explains their efficacy against intracellular mycobacteria. Moxifloxacin and gatifloxacin d gram-negative bacteria, including anaerobes. It inhibits bacterial DNA gyrase, an enzyme that is essential for the maintenance of DNA supercoils, which are necessary for chromosomal replication (Shindikar & Viswanathan, 2005). The development of mycobacterial resistance to fluoroquinolones has been described in MDR strains and in strains from HIV-infected TB patients with a low CD4 count (Shandil et al., 2007). Fluoroquinolone resistance is due to stepwise mutations in the quinolone resistance-

determining region of the mycobacterial *gyrA* and *gyrB* genes (Ginsberg, 2008). No cross-resistance with the first-line anti-TB drugs has been shown (Hu et al., 2003). Moxifloxacin is metabolized by glucuronidation and sulfation (Phase Ⅱ metabolism) rather than by CYP450-mediated (Phase Ⅰ) metabolism (Nijland et al., 2007). *In vitro* studies with moxifloxacin show MICs of 0.25 to 0.5mg/l (Shandil et al., 2007). *In vitro* studies and studies in mice showed enhanced bactericidal activity of moxifloxacin and isoniazid when coadministered (Yoshimatsu et al., 2002). Moxifloxacin efficacy has also been shown in humans. Early bactericidal activity (EBA) studies in newly diagnosed pulmonary TB patients showed comparable activity of moxifloxacin (400mg) and isoniazid (300mg or 6mg/kg) (Pletz et al., 2004). The regimen with moxifloxacin caused the fastest decrease in CFU during the early phase of a biexponential fall (in a nonlinear model that differentiates between quickly and slowly eliminated bacilli) (Rustomjee et al., 2008b). A multicenter three-armed trial in which the standard regimen is compared to a regimen of 2RHZM/2RHM and a regimen of 2RMZE/2RM has recently started (Rosenthal et al., 2006). Moxifloxacin could be of use in the treatment of latent TB (Hu et al., 2008). The combination of 3 months of once-weekly moxifloxacin and rifapentine was as effective as 6 months of isoniazid monotherapy in a mouse model for latent TB (Ginsberg et al., 2009b). A single dose moxifloxacin of up to 800mg was tolerated well but little is known about the long-term tolerability in TB patients. In February 2008, Bayer distributed a "Dear Doctor" letter warning physicians about rare but severe hepatological and dermatological adverse events associated with moxifloxacin. Therefore, the adverse events of moxifloxacin require extended evaluation (Boogaard et al., 2009).

Gatifloxacin and moxifloxacin show cross-resistance. The MICs of gatifloxacin against *M. tuberculosis* range from 0.2 to 0.5mg/l (Rodriguez et al., 2002). *In vitro* studies and studies in mice showed improved activity of rifampin and isoniazid when gatifloxacin was added and even more when the regimen also included pyrazinamide (Kubendiran et al., 2006). A multicenter trial is enrolling patients at five African sites. It compares the efficacy and tolerability of a 4-month regimen of 2 months of rifampin plus isoniazid plus pyrazinamide plus gatifloxacin followed by 2 months of rifampin plus isoniazid plus gatifloxacin (2RHZG/2RHG) to the standard 2RHZE/4RH regimen. An increased risk of dysglycemia was described in elderly patients using gatifloxacin for a variety of bacterial infections (Chen et al., 2006). Elderly patients with hypoglycemia or hyperglycemia were 4 or 17 times more likely to have used gatifloxacin than controls. Therefore, the risk of mycobacterial resistance development and the recently found association between gatifloxacin and dysglycemic events are concerns. If the phase III trials demonstrate safety and efficacy, a 4-month, fluoroquinolone-based treatment for DS-TB could be registered for use by 2015 (Ginsberg, 2010).

6. SQ109

SQ109 is an investigational new drug candidate that was identified from a library of over 60,000 combinatorial compounds, based on a 1,2-ethylenediamine pharmacophore from ethambutol (Kaiser Family Foundation, 2009). However, only the diamine nucleus remains and studies to date suggest that SQ109 should not necessarily be considered a second-generation EMB analogue. Although its mechanism of action involves cell wall inhibition, the specific target of SQ109 remains unknown. In vitro, it has an MIC range of 0.11-0.64

µg/ml against *M. tuberculosis*, including strains resistant to INH, RIF or EMB [41]. It inhibits growth of *M. tuberculosis* in macrophages to a similar extent as INH and to a greater extent than EMB (Protopopova et al., 2005; Jia et al., 2005). *In vitro*, at sub-MIC concentrations, SQ109 demonstrates synergy with RIF and INH and addictive activity with streptomycin, but neutral effects with EMB and PZA. Some synergy between SQ109 and RIF is also evident against RIF-resistant strains (Chen et al., 2006). SQ109 has demonstrated activity in murine models, where it is at least four times as potent as EMB, as 25mg/kg of SQ109 and 100mg/kg of EMB have similar effects (Protopopova et al., 2005). Substitution of SQ109 for EMB enhances the activity of the standard four-drug 2-month initial regimen of HREZ (Nikonenko et al., 2007). The activity of SQ109 in the mouse is particularly remarkable, given the low serum concentrations. This is presumably because the drug has a rapid tissue distribution that results in sustained concentrations in lungs and spleen that exceed the MIC (Jia et al., 2005). In summary, SQ109 is a potential anti-TB drug that has entered Phase Ⅰ / Ⅱ clinical trials. It has low MICs against both susceptible and resistant *M. tuberculosis*. SQ109 has different and more favorable properties than ethambutol, suggesting that it should be regarded as a truly new diamine, and not just as an ethambutol analogue. SQ109 could be included in regimens containing RIF and INH, since synergism with both drugs has been shown. Clinical trials are ongoing to establish its future role in TB treatment.

The combination of SQ109 with TMC207 improved an already excellent TMC207 MIC for M. tuberculosis H37Rv by 4- to 8-fold and enhanced the drug postantibiotic effect by 4 h (Reddy et al., 2010). Thus, SQ109 can be used as combination of other anti-TB drugs in the near future.

7. LL3858

Pyrrole derivatives have demonstrated activity against *M. tuberculosis in vitro* (Protopopova et al., 2007). Recently, a substituted pyrrole derivative, LL3858 has advanced to Phase Ⅰ testing for TB. Preliminary data suggest that LL3858 has potent *in vitro* activity, with an MIC range of 0.06-0.5 µg/ml against *M. tuberculosis*, including MDR strains (Arora, 2004). Monotherapy in a murine model of TB yielded bactericidal activity at doses well below the toxic threshold. Moreover, addition of LL3858 significantly enhanced the sterilizing activity of the standard HRZ regimen (Arora, 2004). Further information on this compound is eagerly awaited.

8. Linezolid

The oxazolidinones are a new class of synthetic antibiotics with broad activity against gram-positive bacteria and mycobacteria through a unique mechanism of ribosomal protein synthesis inhibition. Other positive attributes include high oral bioavailability and lack of cross-resistance with existing antibiotics. Linezolid is the first oxazolidinone to be used clinically, although it is not approved for use in TB. Its MIC for *M. tuberculosis* is 0.125-1µg/ml (Alcala et al., 2003). It is reported that 100mg/kg once daily appeared to be bacteriostatic or weakly bactericidal, causing approximately 1~1.5 log reduction in bacterial counts over 28 days and that 600mg of linezolid orally twice daily in salvage regimens for MDR-TB has been associated with sputum culture conversion and cure, albeit with frequent dose- or treatment-limiting side effects such as anaemia, thrombocytopenia, and peripheral

or optic neuropathy (Fortum et al., 2005; Von der Lippe et al., 2006). Linezolid acts as an inhibitor of bacterial ribosomal protein synthesis. *In vitro*-selected linezolid-resistant *M. tuberculosis* (MIC 4~32 μg/ml) were reported to harbor 23S rRNA gene mutation (Hillemann et al., 2008), but Richter et al. claimed that they had found the first linezolid-resistant clinical isolates of *M. tuberculosis* (MIC 8 μg/ml) with no such kind of mutation, suggesting different mechanisms of resistance (Richter et al., 2007).

When interaction between linezolid and *M. tuberculosis* was examined using an experimental *in vitro* model, linezolid seems an alternative as far as generation of resistance is concerned in the treatment of multi-resistant tuberculosis (Cremades et al., 2011).

Drug	Class	Sponsor(s)[a]	Mechanism of action	Target	Mechanism of resistance	Development of stage
PA-824	Nitroimidazo-oxazine	GATB	Inhibition of protein and cell wall lipid synthesis, while anaerobic activity by the generating reactive nitrogen species, including nitric oxide	F420-dependent nitroreductase	*Rv0407, Rv3547, Rv3261, Rv3262* gene mutations	Phase II
OPC-67683	Nitroimidazo-oxazine	Otsuka	Inhibition of cell wall lipid synthesis, inhibition of mycolic acid biosynthesis	a nitroreductase	*Rv3547* gene mutations	Phase II
TMC207	Diarylquinoline	Tibotec	Inhibition of ATP synthesis and membrane potential	F1F0 proton ATP synthase	*atpE* gene mutation	Phase II
Moxifloxacin	Fluoroquinolone	Bayer, GATB CDC, NIH, FDA	Inhibition of DNA synthesis	DNA gyrase	*gyrA* gene mutation	Phase III
Gatifloxacin	Fluoroquinolone	OFLOTUB, NIH consortium[b]	Inhibition of DNA synthesis	DNA gyrase	*gyrA* gene mutation	Phase III
SQ109	Diethylamine	Sequella	Inhibition of cell wall synthesis	unknown	unknown	Phase I/II
LL3858	Pyrrole	Lupin	unknown	unknown	unknown	Phase I
Linezolid	Oxazolidinone	NIH, Pfizer	Inhibition of protein synthesis	Ribosomal inhibition	23S rRNA mutation	lead optimization

[a]GATB, Global Alliance for TB Drug Development; CDC, US Centers for Disease Control and Prevention TB Trials Consortium; NIH, US National Institutes of Health; FDA, US Food and Drug Administration.
[b]Lupin Ltd, NIH National Institute of Allergy and Infectious Diseases TB Research Unit, TB Research Center (Chennai), World Health Organization Special Programme for Research and Training in Tropical Diseases.

Table 1. Promising new drug candidates and their drug targets

9. Conclusion and future prospective

A number of potential candidate drugs with novel modes of action have entered clinical trials in recent years, and these are likely to be effective against resistant strains. This review summarizes, first, how to identify *M. tuberculosis* among many acid-fast bacilli and second,

how to evaluate drug resistance against isolated *M. tuberculosis*. Lastly the latest information about these candidate drugs, including PA-824, OPC-67683, TMC207, moxifloxacin, gatifloxacin, SQ109, LL3858 and linezolid, and describes their activity, pharmacokinetics, mechanisms of action, and development of resistance against them. Promising new anti-TB drug candidates and their drug targets are summarized in Table 1.

For the first time in 40 years, several new drugs with promising attributes have entered the clinical development pipeline for the treatment of TB. With good fortune, one or more of these agents will fulfill or exceed its potential demonstrated in animal models and provide a new cornerstone for the treatment of drug-sensitive and drug-resistant TB. Additional candidates are percolating up through discovery and preclinical development programs. In spite that many challenges must be overcome before any of these new drugs contributes meaningfully to control of TB, TB drug research and development today is in a stronger position to successfully meet the urgent public health need for improved TB therapies than it has been for half a century due to renewed interest, scientific and technological advances, and the combined efforts of the public and private sectors. These efforts must be further enhanced to ensure ultimate success in discovering, developing and delivering radically improved therapies for TB patients.

PNU100480 (Pfizer) and AZD5847 (AstraZeneca) are being repurposed for TB (Ginsberg, 2010). Addition of PNU100480 to first-line drugs shortened the time needed to cure murine tuberculosis significantly. They are now in phase I testing of multiple dose safety, tolerability and pharmacokinetics when administered as an oral suspension over 14 days in healthy volunteers. The results are not yet available at the time of writing this chapter.

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4

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, loosing 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 efficaceous 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-aminosalycilic acid (PAS), was generated in 1946 on the basis of the known activity of salycilic 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 *kat*G gene, a flavin monooxygenase -*eth*A- 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 tri-chlorynated 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 *atp*E 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 *fbi*A, *fbi*B, and *fbi*C 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 asigned 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; Gisnberg *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 mycobaterial 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 (persistant) 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) strenghten 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 *kat*G and *eth*A (encoding the activator enzymes) and *inh*A (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 *msh*A, *msh*B and *msh*C (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 *pcn*A 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 *pcn*A have been reported (Raynaud *et al.*, 1999). Furthermore, the identification of *mmp*L7, 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 *mmp*L7, 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 *lfr*A 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 *mmp*L and *mmp*S) 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 *mmp*L 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 *mmp*L7 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 *mmp*L 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 *mmp*L 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 *mmp*L7) 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 (*drr*A-*drr*B-*drr*C, 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 Erythromicin) 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 erythromicin, 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 mamalian 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 enconding 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 14α-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 explicited 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 (*cyp*128A1) was essential for "in vitro growth" (Sassetti *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 *cyp*125A1 as the only *cyp* gene needed for a successful mycobacterial propagation (Sassetti *et al.*, 2001). Although *cyp*121A 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 *cyp*121A (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 (*cyp*121A1, *cyp*123A1, *cyp*125A1, *cyp*127A1 *cyp*128A1 and *cyp*137A1) 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 14α-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 (Sassetti *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 14α-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, *GLPs* 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 *mmp*S5-*mmp*L5 were responsible for the resistance phenotype. This pump was susceptible to CCCP, as this treatmente 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 demostrated 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, its 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 anti-bacterial 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 compasionate 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.

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5

An Approach to the Search for New Drugs Against Tuberculosis

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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

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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* pre-clinical.

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

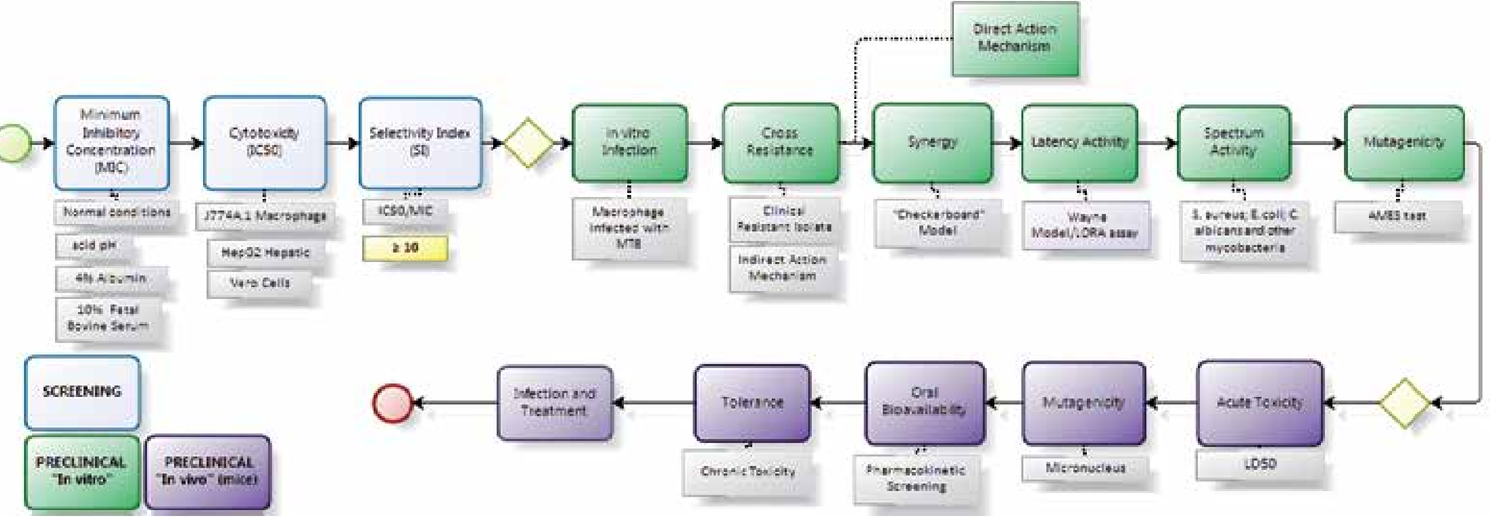

Fig. 1. Pipeline to select new drugs against TB created at the "Hugo David" Laboratory, FCFAR/UNESP, Araraquara, Brazil.

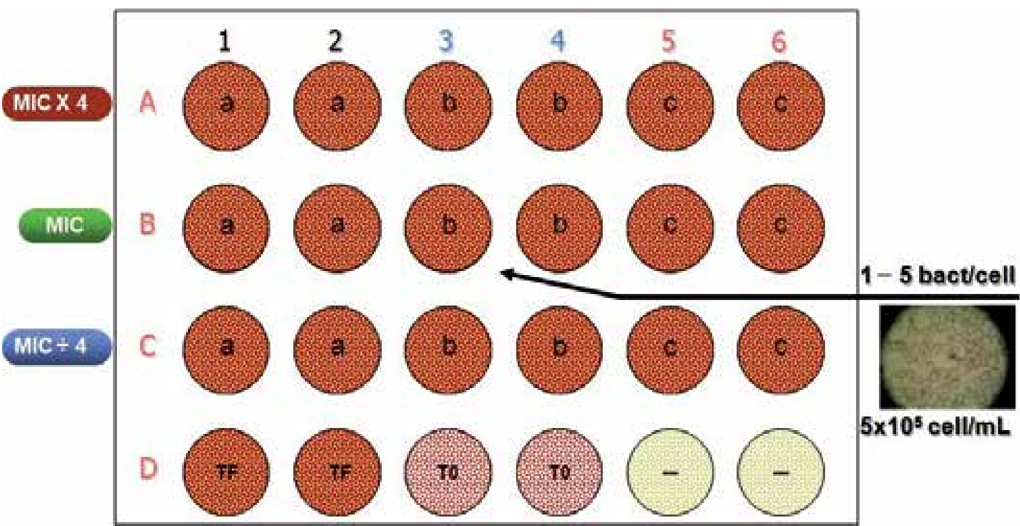

Fig. 2. *In vitro* **infection model:** a, b, c – Different compounds tested in duplicate and at three different concentrations on cells infected with MTB (ATCC 27294) containing the luciferase plasmid (pFCA-luxAB); T0 – Initial Control: After the macrophage infection, the cells were lysed to confirm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

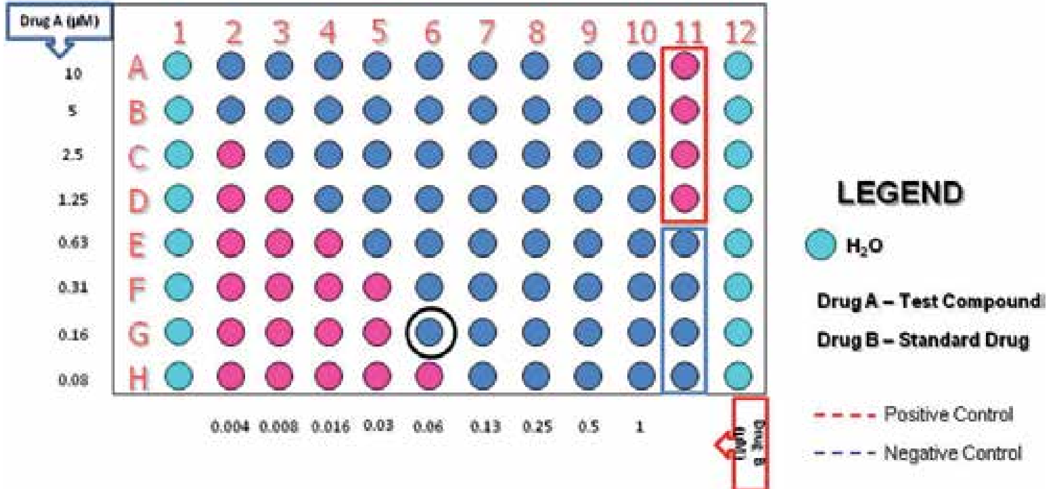

Fig. 3. **2D Checkerboard:** FIC index = (MIC [A] + MIC [B]) combination/(MIC [A] + MIC [B]) alone = ≤ 0.75 synergy; 0.75-4 no interaction or > 4 antagonism. Black circle, example of lower FIC. Resazurin assay: pink=growth, blue=no growth.

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

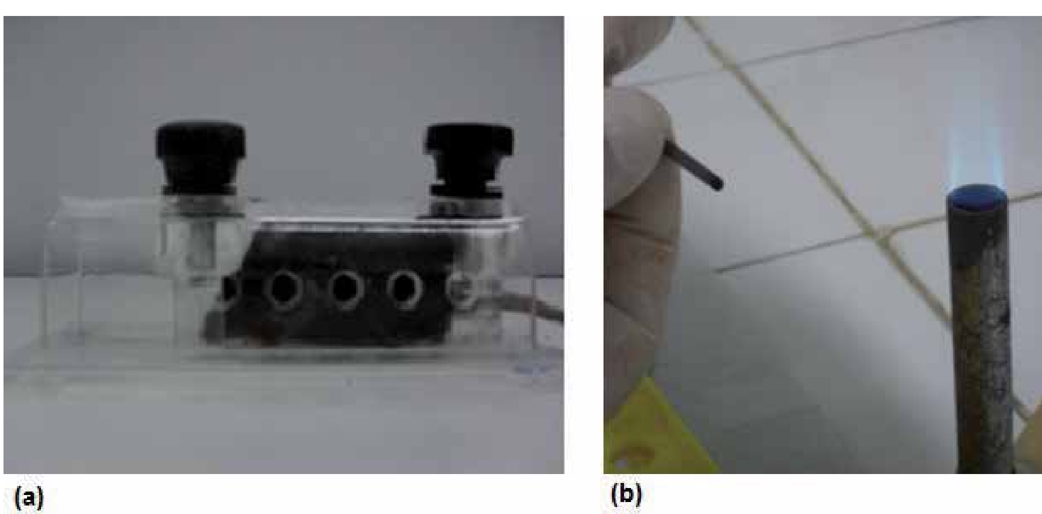

Fig. 4. **Oral bioavailability sample collection**: **(a)** C57BL/6 mouse held in a retainer to cut the tail and collect blood; **(b)** Blood dripping from the tail near the Bunsen burner to enable dilatation of vein and sterilize air.

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.

5. Acknowledgements

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6

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.

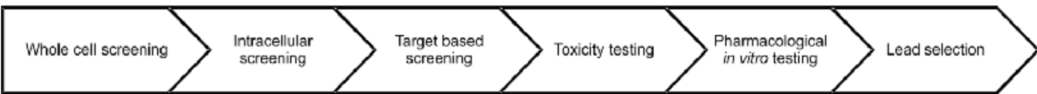

Fig. 1. *In vitro* anti TB drug discovery program components, each phase is an important step in the selection of promising anti TB drugs

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.

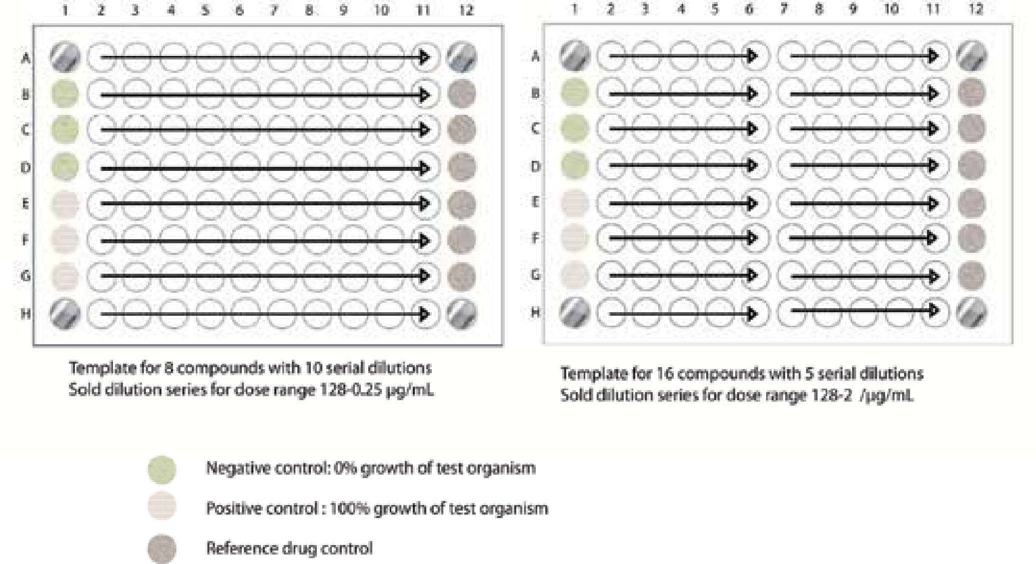

Fig. 2. *In vitro* screening template in a multiwell plate, the ideal concentrations for testing should be selected following the Food and Drug Administration requirements (Enna & Williams, 2007; Enna, 2001; Food and Drug Administration, 2009).

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).

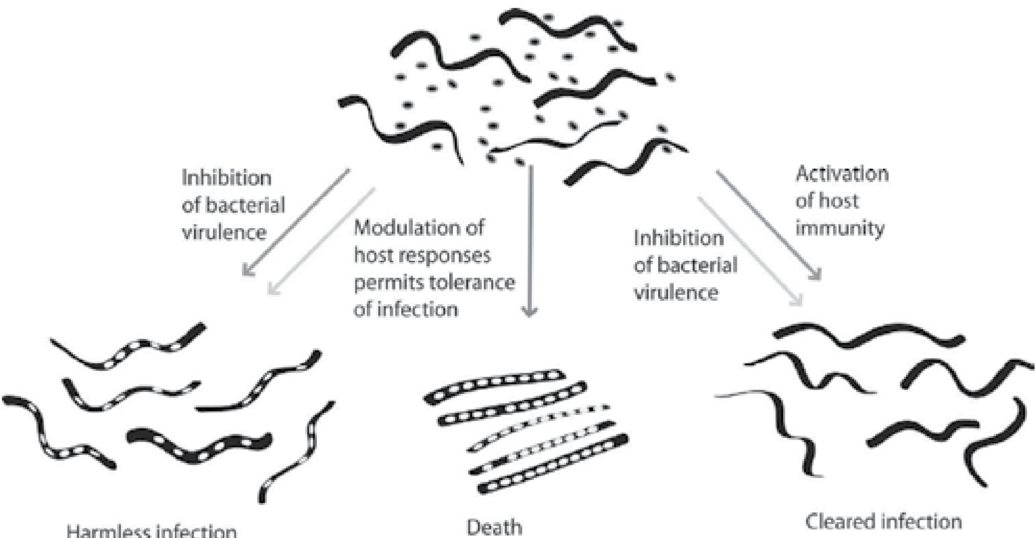

Fig. 3. *In vitro* screening using an infection model with *C. elegans*, the worm shape predicts the protective effect of antimicrobial agent (rigid posture for death, and sinusoidal posture for surviving) (Bhavsar & Brown, 2006).

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.

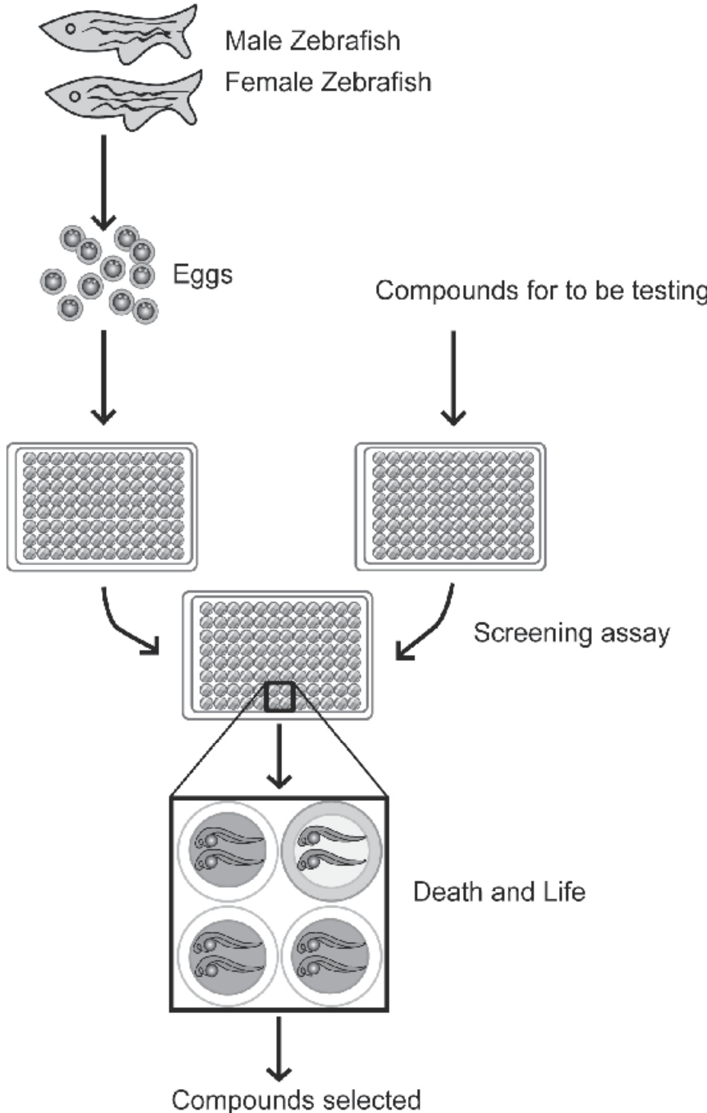

Fig. 4. *In vitro* screening using an infection model with zebrafish embryos, these organisms are optically transparent, permitting visualization of pathogens and lesions in real time (Carvalho et al., 2011).

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).

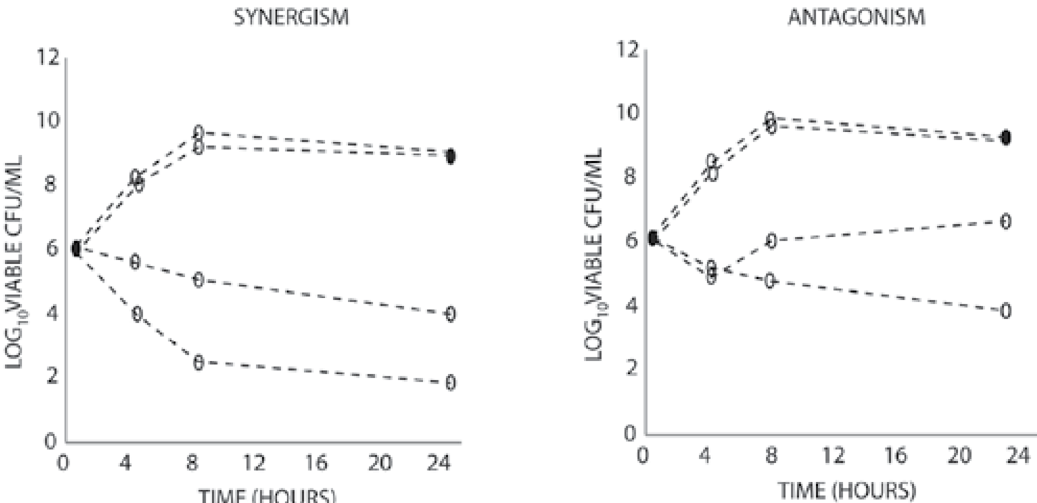

Fig. 5. Time kill curves for study drug interactions. Synergism is defined as a ≥2 log_{10}(CFU/mL)-fold decrease. Antagonism is defined as a ≥2 log_{10}(CFU/mL)-fold increase.

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.

6. Acknowledgment

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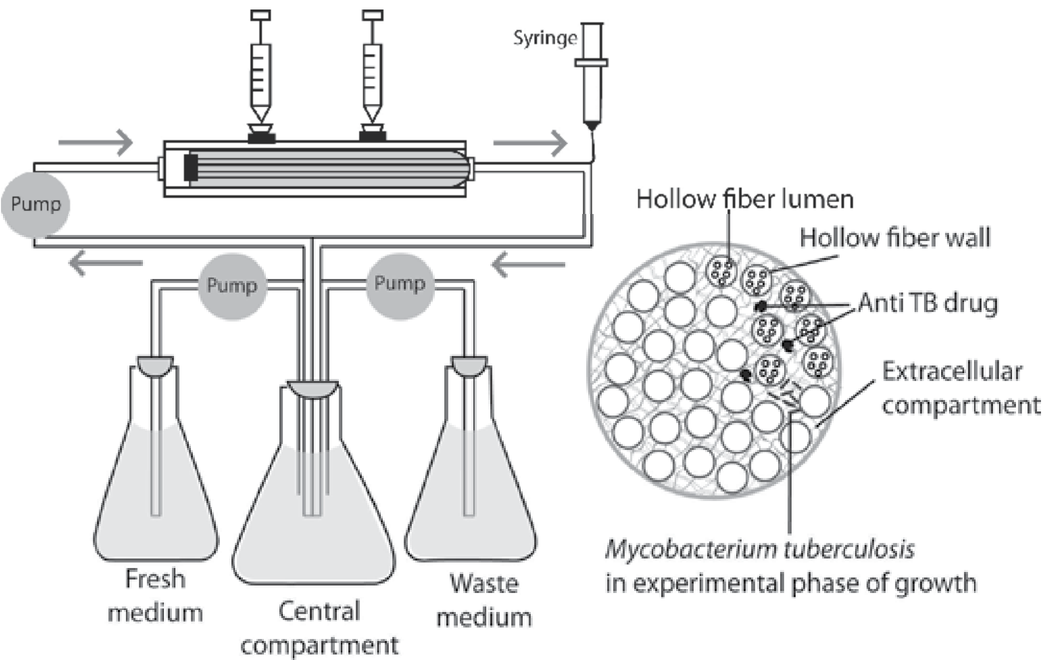

Fig. 6. Hollow-fiber pharmacodynamic model of tuberculosis *Mycobacterium tuberculosis* grow in the extracapillary space but are too big to cross into the central compartment. Anti TB drug is administered to the central compartment. Medium circulates in the central compartment in the direction shown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

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7

New Antitubercular Drugs Designed by Molecular Modification

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1. Introduction

Tuberculosis (TB) is an illness that results from infection with Mycobacterium tuberculosis (MTB). This aerobic bacillus have the cell wall with a high lipid contend which result in a high degree of lipophilicity and resistance to alcohol, acids, alkali and some disinfectants. TB is the leading worldwide cause of mortality resulting from an infectious bacterial agent. The World Health Organization (WHO) estimates that almost one-third of world's population is infected with MTB, with 8.9-9.9 million incident cases by year (WHO, 2010).

MTB is epidemiologically characterized by high rate infectivity, so the one-third of latent infection population which remains a reservoir from mycobacterium is the major obstacle to the total control of the disease. In normal conditions, the bacteria has the ability to live in balance with immune response but in situations such as genetic impaired, intercurrent diseases (i.e. AIDS), malnutrition and medical interventions could occur an imbalance, and the MTB multiplies rapidly developing the disease (Gideon & Flynn, 2011).

The multidrug-resistant tuberculosis (MDR-TB) is another important problem to control TB worldwide. It has been reported that include patients who have never been treated with any TB drug demonstrated resistance. According to WHO, MDR-TB is responsible for approximately 460 thousand new cases per year and for about 740 thousand new patients infected by both MTB and HIV/AIDS. Recent estimates show that 10% of all new TB infections are resistant to at least one anti-TB drug.

The actual drug therapy for tuberculosis has involved administration of multiple drugs because it was clear that monotherapy led to the development of resistance (Barry & Blanchard, 2010). Short course chemotherapy involves taking isoniazid and rifampicin for 6 months with pyrazinamide and ethambutol supplement in the first 2 months (Ma et al., 2010).

For multidrug-resistance (MDR) and extensively drug resistance (XDR) are used the combination of first line drugs and seconde line drugs as aminoglycosides (amikacyn and kanamicyn), polypetides(capreomicyn, viomycin, envyomicin), fluoroquinolones (ofloxacin, levofloxacin, moxifloxacin, gatifloxacin), thioamides (ethionamide, prothionmide),

cycloserine, terizidone, para-aminosalicylic acid. This chemotherapy is less effective, longer , expensive and more toxic than the short course therapy (Ma et al., 2010).

Third line drugs include rifabutin, macrolides (clarithromicin), linezolid, thiacetazone, thioridazine, arginine, vitamin D are still being developed, have less or unproven efficacy and are very expensive (Laloo & Ambaram, 2010).

Since the discovery of rifampicin in 60′ there is no more drugs developed to treat tuberculosis. Considering the increase of resistant the discovery of new antitubercular drugs is urgent. A new anti-TB drug must possess some characteristics such as wide spectrum of action, adequate posology to allow the patient compliance, short duration of treatment and adequate pharmacokinetic properties (half-life, decreased drug-drug interaction among others).

Among the strategies to introduce a new drug in the market, the molecular modification approach has showed to be promising. Several drugs in the market was developed using this strategy. This chapter aiming to discuss some strategies of molecular modification such as prodrug approach, molecular hybridization and biosisoterism in order to design and develop new drugs against M. tuberculosis.

2. Molecular modification

Molecular modification is an chemical alteration in a molecule which could be a lead compound or a drug aiming to enhance its pharmaceutical, pharmacokinetic or pharmacodynamics. This strategy has been used by medicinal chemistry by several years allowing the discovery of many available drugs present in the market. Among molecular modification used we can cite: prodrug approach, molecular hybridization and bioisosterism. Each one of this strategy will be introduced highlighting their application in TB drug discovery.

2.1 Prodrug approach

The first definition of prodrug was introduced by Albert in 1958 which define prodrug as "any compound that undergoes biotransformation prior to exhibit its pharmacological effects" (Albert, 1958). In order to improve this definition, Haper (1959) proposed the term latentiation. Drug latentiation is understood as "the chemical modification of biologically active compound to form a new compound that, upon in vivo enzymatic attack, will liberate the parent compound". In general the prodrugs could be classified into two main classes: bioprecursors and carrier prodrugs.

Bioprecursors is a molecular modification strategy that generates a new compound-substrate for the metabolizing enzymes that after this biotransformation demonstrate biological activity. This approach generally does not use carriers. Several examples of drugs are available in the market used this strategy such as sulindac, acyclovir, losartan among others (Silva et al., 2005).

Carriers' prodrugs are designed using labile linkage between a carrier group and an active compound. This prodrug after chemical or biological biotransformation releases the parental drug responsible for the biological activity (Figure 01). The prodrug, per se, is usually inactive or less active than parental drug.

The carrier selection could explore two strategies: the first one is use an inactive carrier (non-toxic) and the second one use active compounds in order to obtain synergic effect. In the last strategy we could classified as mutual prodrugs or codrugs. In both situations is expected that then active compound (s) should be release with adequate kinetic hydrolysis reaction (Silva et al., 2005).

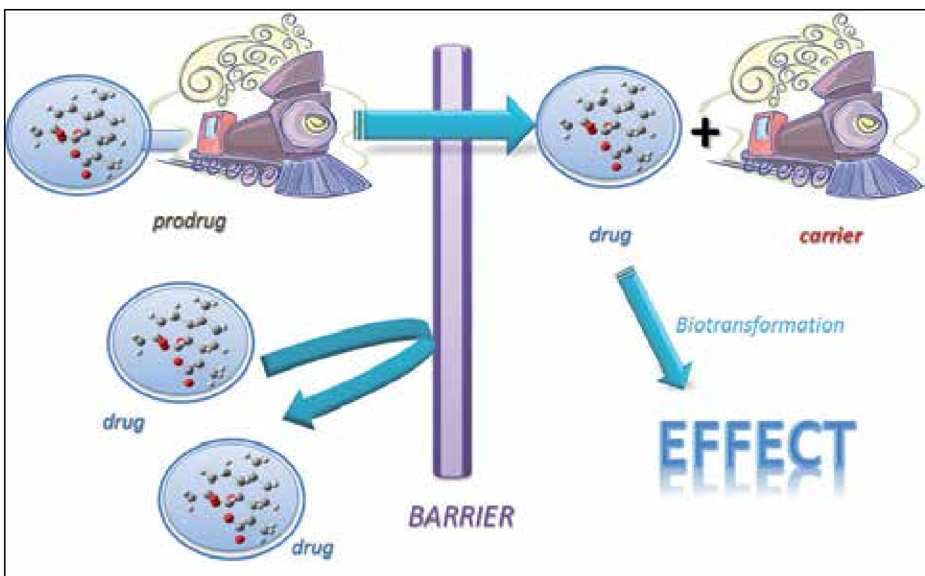

Fig. 1. Prodrug approach. The drug could present some inadequate characteristic related to pharmaceutical, pharmacokinetic or pharmacodynamic phase. As the drug is not able to win barriers due this inadequate characteristic the effect is not observed or is decreased. The use of the prodrug approach can solve this problem. After biotransformation the drug can act demonstrating the optimization of the effect.

The prodrug approach has been used by several researches in order to find new antitubercular compounds (Chung et al., 2007).

Some antitubercular drugs such as pyrazinamide, isoniazide and ethionamide could be considered as bioprecursor prodrugs.

Pyrazinamide is bioconverted by intracellular antimicobacterial pyrazinamidase to pyrazinoic acid (Figure 2). This last one can decrease the pH surroundings the M. tuberculosis preventing growth. Furthermore, pyrazinoic acid can across through mycobacterial membrane allows lowering of cytoplasmatic pH leading to the disruption of membrane transport and energy depletion (Zhang, 2005). Pyrazinamide resistance could be found in tubercle bacillus that present mutation encoding pyrazinamide/nicotinamidase (pncA).

Fig. 2. Pyrazinamide conversion to pyrazinoic acid.

Several pyrazinoic prodrugs with increased lipophilic properties have demonstrated activity against M. tuberculosis. Some substituted pyrazinoic esters demonstrated 100-fold more active than pyrazinamide against M. tuberculosis with high plasma stability (Figure 3) (Cynamon et al., 1995).

Fig. 3. Chemical structures of some substituted pyrazinoic esters.

Other pyrazinoic and quinoxaline esters derivatives were prepared and evaluated against M. tuberculosis. The compounds 4-acetoxy-benzyl esters of pyrazinoic acid and 4'-acetoxybenzyl 2-quinoxalinecarboxylate demonstrated MIC values of 1-6,25 µg/mL (Seitz et al., 2002) (Figure 4).

Fig. 4. Pyrazinoic and quinoxaline esters derivatives.

Another interesting study about pyrazinoic prodrugs was performed by Simões and co-workers (2009). The authors synthesized and compared a series of esters and amides prodrugs based on pyrazinamide structure (Figure 5). All compounds demonstrated higher lipophilicity (log P) than pirazinamide .The esters derivatives demonstrated better in vitro activity against M. tuberculosis (MIC = 10-20 µg/mL) than amide derivatives (MIC = > 800 µg/mL) with suitable stability in presence of plasma.

Fig. 5. Ester and amide derivatives of pyrazinoic acid synthesized by Simôes and co-workers (2009).

The use of prodrug approach to obtain new esters prodrugs such as pyrazinamide analogues seems to be an important strategy to discovery new drugs with improved pharmacokinetic and pharmacodynamic properties.

The isoniazide, a first line drug to the treatment of M. tuberculosis, was discovered in 1950. Isoniazid is a prodrug that is activated through an oxidation reaction catalyzed by the enzyme katG which demonstrate catalase-peroxidase activity. After conversion, the drug is

biotransformed into reactive species capable to acylate an enzyme system found in the mycobacterium. It has been proposed that one of this system is the enzyme inhA which is involved in the biosynthesis of mycolic acids (Mdluli et al., 1996). So, after activation by KatG the isonicotinoyl radical couples with NADH leading to adduct and inhibiting Inha.

One of the most problems with antitubercular therapy is the patient compliance and the long term therapy involving the use of several drugs. In order to decrease problems with therapy adhesion mutual prodrugs (or codrugs) have been reported. Using the prodrug approach it was reported the combination of 4-amino-salicylic acid (PAS) – a second line drug in the treatment, with isoniazide. The PAS treatment presents some inconvenience such as gastrointestinal irritation effects and inadequate bioavailability due to rapid phase II metabolism. In the other hand, isoniazide is rapidly absorbed after oral administration and quickly metabolized to inactive products (acetylhydrazide, diacetylhydrazide, N-acetylisoniazide and hydrazine) (Katleen, 1999). This metabolism reaction (acetylation) is reduce when PAS is previously administered, increasing half-life of isoniazide. So, using the prodrug approach it was proposed the association of both drugs into mutual prodrug in order to reduce gastrointestinal toxicity and extensive metabolism of PAS, reduce intestinal acetylation of isoniazid and increase the duration of drugs actions. All these hypotheses were confirmed after in vitro and in vivo studies (Figure 6) (Prateek et al., 2007).

Fig. 6. Chemical structure of the mutual prodrug proposed by Prateek and co-workers (2007).

In order to decrease the toxicity and prolonged half-life of isoniazide, polymeric prodrugs were synthesized and evaluated. Micellar systems of polyethyleneglycol-poly(aspartic acid) copolymer and N-methylene phosphonic chitosan were used as carrier to obtain isoniazid prodrugs. The micellar prodrugs demonstrated activity against M. tuberculosis (Silva et al., 2001).

The same polymeric prodrugs approach was also explore to design norfloxacin derivatives. It was considered that the use of mannosyl ligands as carriers that can be used to target macrophages increasing the drug levels in this cell (Gordon & Rabinowitz, 1989; Roseeuw et al., 2003).

Ethionamide is a prodrug which similar mechanism of action of isoniazide. After oxidation by catalase-peroxidase the drug is bioconverted to an active acylating agent, ethionamide sulfoxide, which inactive inhA enoyl reductase (Figure 7) (Johnsson et al., 1995).

Fig. 7. Mechanism of action of ethionamide.

The association of TB and HIV infection is dramatic. The immune system is weakened in HIV patients allowing reactivation of latent TB and makes these patients more susceptible to drug-resistant strains. It has been estimated that two-thirds of the patients diagnosed with TB are also HIV seropositive (Shindikar, 2005). The usual treatments of HIV use nucleoside analogs (i.e. zidovudine) associated with other drugs. In this context, mutual prodrugs of antimycobacterial agents (such as isoniazid, norfloxacin and ciprofloxacin) and HIV nucleoside analogs (such as zidovudine, stavudine and lamivudine) were synthesized and evaluated. The zidovudine prodrugs were assayed at 6.25 μg/mL against M. tuberculosis strain H37Rv and demonstrated 99% inhibition (with fluoquinolones derivatives) and 90% of inhibition (with isoniazide) (Figure 8). The compounds also have inhibited HIV-1 replication (Sriram et al., 2005; Sriram et al., 2004).

Fig. 8. Zidovudine prodrugs.

2.2 Molecular modification

The "one-target-one-drug" paradigm conducted the drug design on 20th century. Although very drugs used in therapy have been designed using this approach for a one drug for a single target, many diseases remains inadequately treated today. Considering that this approach fails to treat some diseases, the drug discovery explores the hybridization between molecules in order to modulate multiple targets. The molecular hybridization can be useful to improve the main unsuccessful causes of fail in drug discovery such as lack of efficacy and poor safety (Morphy & Rankovic, 2006).

Nowadays there are different approaches to multiple target therapy used mainly in unresponsive patient's conditions such as: a) use of two or more individual tablets (or other formulation); b) use of fixed dose combination therapy which two or more drugs are

combined in a single tablet (or other kind of formulation); c) use of single molecular entity which combine multiple targets actions. The first strategy has the disadvantage of inadequate compliance for the patients once they will have to use several drugs during the day in order to control some symptoms. Example of this kind of situation could be represented by hypertension or diabetes treatment. When someone compares the second and third strategies one important difference is that the use of a single chemical entity reduce the risk of drug-drug interaction and allows obtaining new compounds that can be patented. However, the challenge to design a compound multiple ligand with adequate ratio to different receptors is high. On the other hand, the combination of drugs in the same formulation favours fast development in order to obtain new commercial product by the pharmaceutical companies and it allows prolonged patent life of some old drugs. Superiority of combination and drug-drug interaction studies must be performed in this kind of strategy (Morphy & Rankovic, 2007).

Schematically, the Figure 9 shows the use of molecular hybridization strategy. The drug A interacts only with the receptor A. The drug B interacts only with the receptor B. It is prohibitive the interaction between drug A and receptor B (and vice versa) but is possible to design compounds that can interact with both receptors contributing synergically for a desire effect.

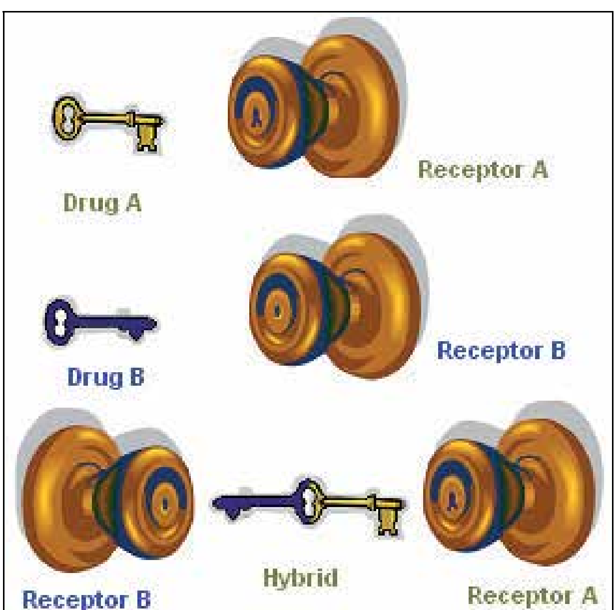

Fig. 9. Molecular hybridization strategy – analogy to “lock-key” model.

The drug design of hybrid compound must consider three different situations: a) the desire subunits are linked by a spacer agent; b) both subunits are linked without spacer agent and they are fused; c) the desired activities are merged in a new structure. The Figure 10 represents these different situations in order to design a new drug.

The combination of multiple actions in the same drug is an interesting strategy in tuberculosis treatment in order to contribute to therapy compliance, improve the activity

and reduce resistance. One fruitful example using this strategy could be represented by Figure 11 which demonstrated the use molecular hybridization of isoniazid and one quinolone derivative to increase the antimycobacterial activity of the novel compounds. This compound was able to maintain high survival rate reducing in vivo the colony-forming unit (CFU) with little few lung lesion and reduce splenomegaly (Shindikar & Viswanathan, 2005).

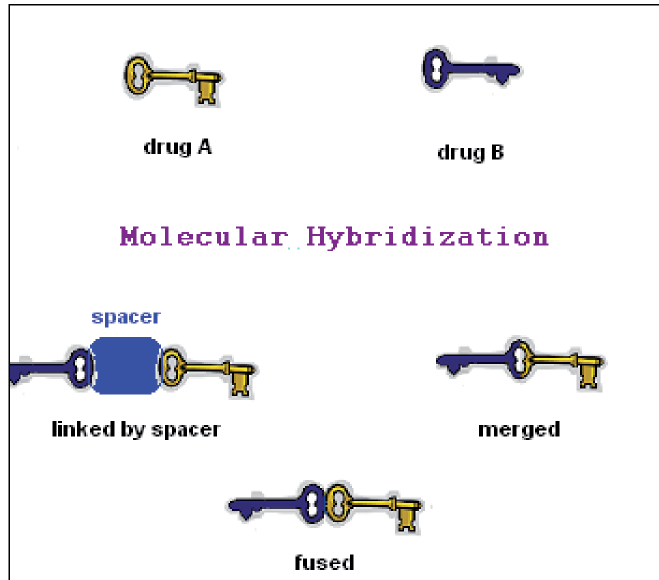

Fig. 10. Different hybrid compounds obtained by molecular hybridization.

Fig. 11. Molecular hybridization between fluorquinole and isoniazid.

Similar molecular hybridization approach was performed using a fluorquinole derivative and pyrazinamide through Mannich bases. The compounds obtained demonstrated in vitro and in vivo antitubercular activity. The compound presented in the Figure 12 demonstrated higher log P than pyrazinamide. The log P is an important property to be evaluated in novel effective compounds due to lipophilic characteristics of the M. tuberculosis wall. In vitro studies showed that the compound was more active than pyrazinamide demonstrating important activity against multi drug resistant M. tuberculosis. Furthermore, the compound

was able to decrease thorough in vivo studies the bacterial load in lung and spleen tissues (Sriram et al., 2006). This result shows that the use of this approach can increase the activity of compounds.

Fig. 12. Molecular hybridization between fluorquinole and pyrazinamide.

Some phthalimide derivatives obtained through molecular hybridization between thalidomide and dapsone designed to be active against *M. leprae* was evaluated against *M. tuberculosis*. The compounds demonstrated in vitro activity with selective index higher than 80. This hybridization strategy shows to be promising in discovery compounds with wide spectrum of action in *Mycobacterium sp* (Santos et al., 2009).

2.3 Bioisosterism

The term isosterism was first definied by Languimuir in 1919 as atoms or organic or inorganic molecules which possess the same number and/or arrangement of electrons examples such as C=O and N=N; CO2 and NO2 (Burger, 1991). Grimm formulated in 1925, the hydride displacement law which explain that the addition of hydrogen to an atom confers properties of the next highest atomic number (i.e. the fluorine anion F- and the hydroxyl anion HO- present some analogies according to Grimm law) (Grimm, 1925). Erlenmeyer proposed the definition of isosteres as elements, molecules or ions which present the same number of electrons at the valence level expanding the definition (Erlenmeyer, 1932).

Currently bioisosteres is understood as groups or molecules which have a chemical and physical similarity producing broadly similar biological effects (Thornber, 1957). Burger classified and subdivided bioisosteres in two broad categories: classic and non-classic (Burger, 1970). The classical bioisosteres is subdivided in: a) monovalent atoms or groups; b) divalent atoms or groups; c) trivalent atoms or groups; tetravalent atoms and e) ring equivalents (Table 1).

The non-classical bioisosteres do not present the steric and electronic definition of classical isosteres, furthermore they do not have the same number of atoms of the substituent or moiety replaced. Among non-classical bioisosteres we could cite: functional groups, non-cyclic or cyclic and retroisosterism.

Monovalent	Divalent	Trivalent	Tetravalent
-OH, -NH2 – CH3, -OR	-CH2-	=CH-	=c=
-F, -Cl, -Br, -I, -SH, -PH2	-O-	=N-	=Si=
-Si3, -SR	-S-	=P-	$=N^{+}=$
	-Se-	=As-	$=As^{+}=$
	-Te-	=Sb-	$=Sb^{+}=$
			$=P^{+}=$

Table 1. Classic biososteres atoms and groups.

The bioisosterism approach is an important molecular modification tool that allows the discovery of several drugs in the market. The drugs discovered using this strategy that are in the markets usually known as “me too” (Lima & Barreiro, 2005).

This strategy has been used to discovery new compounds to treat tuberculosis. One example is the class of fluoquinolones. Fluoquinolones demonstrated, besides Gram-negative and Gram- positive activity, antitubercular activity. This class of drugs is known to inhibit bacterial DNA replication and transcription by binding to DNA-gyrase-DNA complex. The use of fluorquinolones occurs mainly in patients with multidrug-resistance (MDR). The most actives quinolones for the treatment of TB are: ciprofloxacin, sparfloxacin, ofloxacin, moxifloxacin and levofloxacin (Renau et al., 1996). Studies comparing the bactericidal activity of various fluoroquinolones against Mycobacterium tuberculosis in the latent and exponential growth phases.has demonstrated that most promising drugs are moxifloxacin and levofloxacin (Cremades et al., 2011). All these fluorquinolones drugs were obtained using bioisosteric replacement (Figure 13).

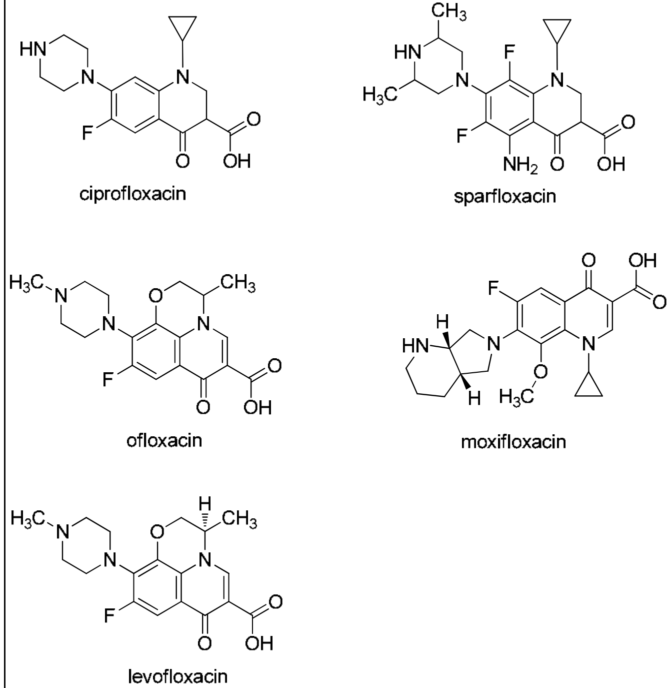

Fig. 13. Fluorquinolones active against Mycobacterium tuberculosis.

Linezolid is oxazolidinone drug belonging of antibacterial agents used in the treatment of nosocomial pneumonia and uncomplicated and complicated skin and soft tissue infections caused by select Gram-positive bacteria (Ford et al., 2001). The drug has been evaluated in the treatment of MDR tuberculosis showing interesting results (Pinon et al., 2010). Based on this interesting result, some oxazolidinone biosisoteres have been developed. PNU-100480 is an analog of linezolid in clinical trial to be used in tuberculosis treatment (Leach et al 2011). Others linezolid derivatives such as radezolid and torelozid are obtained by isosteric replacement and these drugs are under clinical trial (Figure 14) (Leach et al 2011).

Fig. 14. Chemical structure of linezolid bioisosteric derivatives.

Fig. 15. Some active and inactive oxazolidinone isosteres derivatives.

The isosteric replacement not always leads to equal or more active compounds. Sometimes isosteric replacement leads to inactive compounds. Snyder and co-workers do not found relationship between some heterocyclic rings with oxazolidinone present in linezolid. However, the same authors demonstrate an interesting relationship with other active compounds (Figure 15). In general, with bioisosteric approach it is possible to find at least one (or sometimes more) equivalent systems.

Mycobacterium avium – intracellulare complex is one of the most common bacterial opportunistic in patients with AIDS. Clarithromycin and azithromycin are first-line agents for prevention and treatment. They are macrolide antibiotics which bacteriostatic activity due to bind to the 50S ribosomal unit (Wright, 1998). Azithomycin is a bioisoster of clarithromycin (Figure 16).

Fig. 16. Chemical structure of azithromycin and clarithromycin.

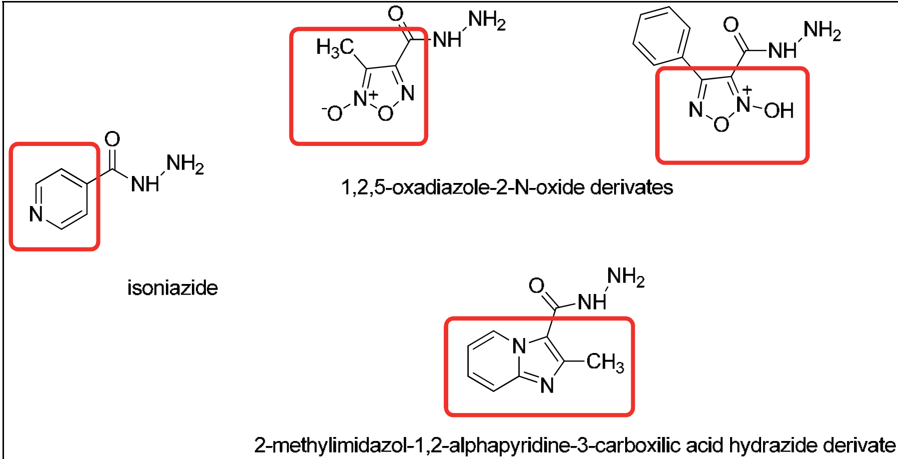

Fig. 17. Ring bioisosteres of pyridine presenting in isoniazid.

Bioisosteres of isoniazid has been designed exploring the bioisosteric replacement of pyridine ring to imidazo[1,2-α]-pyridine. However, the compound 2-methylimidazo[1,2-α]pyridine-3-carboxylic acid hydrazide demonstrate less antitubercular activity than

isoniazide (Kasimogullari & Cesur, 2004). The same results were observed by Santos and co-workers after biosisoteric replacement of pyridine to 1,2,5-oxadiazole-2-oxide (furoxan) (unpublished results) (Figure 17).

3. New drugs candidates for tuberculosis treatment

Tuberculosis drug development efforts have emerged in the last years. Despite the progress, none new drugs were found in this last years. Ryfampicin, discovered 40 years ago, was the last novel antibiotic introduced for the first treatment of tuberculosis. The search for new targets in M. tuberculosis that might be inhibited to eliminate all known strains is a global pursuit.

Several targets in M. tuberculosis have been reported. The inhibition of these targets could act in the growth and latent phase. Some targets in growth phase are GIgE (maltose metabolism), mycolic acid (mycolic acid metabolism), DprE1/DprE2 (cell wall metabolism), MshC (mycothiol ligase), HisG (histidine biosynthesis), AtpE (ATP synthesis), Def (protein processing), methionine aminopeptidase (protein processing). Some targets of dormant phase are isocitrate lyase (energy metabolism), proteosome complex (protein processing), L,D-transpeptidase (peptidoglycan metabolism), DosR (DevR) (regulation of dormancy) and CarD (stringent response) (Lamichhane, 2011). The most advances in new TB targets identification have been directed by the genome sequence of M. tuberculosis, however genome-derived target based approach have had little success until the present moment in the antibacterial class (Payne et al., 2007).

The discovery new antitubercular drugs must possess some desired profile such as: broad spectrum of action acting against MDR-TB and XDR-TB; adequate and shorten treatment duration reducing pill burden in order to reduce numbers of pills taken; adequate pharmacokinetic profile in order to reduce drug-drug interaction (to be administered with HIV drugs), desired target tissue levels and allows long half-life of the drug (Koul et al, 2011).

Fig. 18. Moxifloxacin and gatifloxacin designed using nalidixic acid scaffold.

Currently the global TB development pipeline has nine candidates in different stages of clinical trial. These pipelines are: PNU 100480 (protein synthesis inhibitor), AZD 5847 (protein synthesis inhibitor), SQ 109 (cell wall and multitarget inhibitor), OPC67683 (cell wall and multitarget inhibitor), PA824 (cell wall and multitarget inhibitor), gatifloxacin (DNA gyrase inhibitor), moxifloxacin (DNA gyrase inhibitor), TMC 207 (ATP synthase inhibitor) and sudoterb (mechanism still unknown). Some of them are active in latent and active form against MDR-TB and XD-TB (Koul et al., 2011).

The most of currently pipeline TB drugs were developed using molecular modifications strategies. The fluorquinolones derivatives gatifloxacin and moxifloxacin were derivatized scaffolds from the parent nalidixic acid using the bioisosterism as molecular modification (Figure 18).

The metronidazole scaffold was used to design PA-824 and OPC-67683 using bioisosterism and molecular hybridization (Figure 19).

Fig. 19. Nitroimidazole derivatives designed by molecular modification of metronidazole.

The oxazolidinone derivative PNU-100480 was designed using linezolid as parent scaffold. This last one explored the bioisosterism as molecular modification tool (Figure 20).

Fig. 20. PNU-100480 derivative of oxazolidinones linezolid.

The second line drug ethambutol was used as scaffold to develop the compound SQ109 using bioisosterism and molecular hybridization (Figure 21).

Fig. 21. 1,2-ethylenediamine derivative SQ109 designed from ethambutol.

4. Conclusion

The molecular modification is an important tool to discover new compounds to treat Mycobacterium tuberculosis infection. The use of this strategy has allowed finding more active and safe compounds with wide spectrum, acting include against MDR and XDR tuberculosis. The most currently pipeline drugs for TB in clinical trial were developed using molecular modification demonstrating the importance of this strategy in antitubercular drug discovery new agents.

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8

The Cord Factor: Structure, Biosynthesis and Application in Drug Research – Achilles Heel of *Mycobacterium tuberculosis*?

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1. Introduction

Recently, it was reported that the tuberculosis mortality in 2009 has dropped to 35% since 1990 (WHO, 2010). Nevertheless, the disease caused by the facultative intracellular bacterial pathogen *Mycobacterium tuberculosis* still remains the leading cause of death from a single bacterial species (Coker, 2004; Russell et al., 2010). The emergence of Multi-Drug Resistant (MDR) and Extreme Drug Resistant (XDR) strains of *M. tuberculosis* leads to prolonged treatment which drastically increases the therapy costs.

M. tuberculosis shows a remarkable property of existing in different states of invasion (infection), colonization and persistence (Casadevall & Pirofski, 2000). It also has outstanding mechanisms to escape from elimination and has a high degree of intrinsic resistance to most antibiotics, chemotherapeutic agents and immune eradication (Brennan & Nikaido, 1995; Coker, 2004). The major obstacle for host defence mechanisms and therapeutic intervention is the unusual robust cell wall which is unique among prokaryotes, and is a major determinant of virulence of the bacterium. The cell wall is critical for long-term persistence of *M. tuberculosis* in the hostile environment of the host's cells and for progression of tuberculosis (Barry et al., 1998). Approximately one-half of the cell wall mass is comprised of mycolic acids (Brennan & Nikaido, 1995). In the cell envelope, mycolic acids are esterified to the terminal pentaarabinofuranosyl unit of arabinogalactan, which is a peptidoglycan-linked polysaccharide. The outer envelope consists of trehalose 6,6'-dimycolate (TDM; cord factor) and TMM (trehalose 6,6'-monomycolate, the biosynthetic precursor of TDM), where the mycolic acids of TDM interact with the mycolyl-residues from the layer beneath (Brennan & Nikaido, 1995). The mycolic acid-containing layers have width of ~10 nm and limit the penetration of hydrophilic substances, whereas the inner saccharide layer inhibits the penetration of lipophilic substances. The high abundance of mycolic acids in the outer cell envelope is the main barrier for water soluble antibiotics (Brennan, 2003; Coker, 2004).

The purpose of this review is to highlight the importance of the cord factor as one of the most unique determinant for *Mycobacterium tuberculosis* virulence. This article will especially focus on the steps of the cord factor biosynthesis, i.e., the transfer of mycolic acid from a

TMM to another TMM to form TDM by Ag85 complex enzymes. The Ag85 complex is one of the promising targets for novel antimycobacterial drugs and vaccines. We present our recently developed high throughput screening (HTS) assays suitable for the identification of potential inhibitors against Ag85.

2. Discovery of the cord factor (Trehalose 6,6´-di-mycolate; TDM)

In 1884, Robert Koch described *M. tuberculosis* bacilli grown in culture as rope-like structures (Koch, 1884). More than half a century later, in 1947 Middlebrook found that the ability to form cords under specific conditions is an "essential accompaniment of virulence" (Middlebrook et al., 1947). In 1950 Bloch extracted the substance responsible for cord formation from virulent organisms and identified it as a "toxic substance" (Bloch, 1950).

The removal of the substance with petroleum ether resulted in somehow avirulent organisms but did not affect the growth of the bacilli. This suggested that the substance was located at the surface and since it was obtained only from "cordforming" organisms it was called "cord factor" (Bloch, 1950; Middlebrook et al., 1947). Six years later the cord factor was finally identified as trehalose 6,6´-di-mycolate (TDM) by Noll (Behling et al., 1993a; Noll, 1956). TDM is the most abundant glycolipid produced by virulent *M. tuberculosis* (Hunter et al., 2006a). TDM molecules consist of trehalose (TDM glycan-head). Trehalose is abundant in mycobacteria as a free component (Elbein & Mitchell, 1973; Elbein et al., 2003). In the cord factor trehalose is esterified to two mycolic acid residues and the residues length is variable from species to species (Fig. 1). Mycobacterial mycolic acids contain generally 20–80 carbons (Spargo et al., 1991).

Fig. 1. Structure of trehalose 6,6´-di-mycolate (TDM, cord factor).

The cord factor is comprised of trehalose which esterifed to two mycolic acid residues through their 6- and 6'-hydroxyl groups.

2.1 Effects of the cord factor on the immune system

TDM inhibits the process of phagosome-lysosome fusion and is thus a key compound for the survival of the bacillus inside the host´s phagosomes (Indrigo et al., 2002). TDM induces a broad range of cytokine secretion in the host´s immune system, especially production of IL-1β, IL-6, and TNF in macrophages (Matsunaga & Moody, 2009). In the recent years it has been shown that TDM is a key driver of secondary and cavitary disease type of tuberculosis (Hunter et al., 2006b). Despite the various severe effects on the host immune system the host

receptor could not been identified. In 2009 Ishikawa et al. could demonstrate that macrophage inducible C-type lectin (Mincle) is an essential receptor for TDM (Ishikawa et al., 2009).

2.2 TDM as vaccine adjuvant

In the last years TDM has been used intensively as immunomodulatory and vaccine adjuvant (Behling et al., 1993b; Noll, 1956). TDM can reproduce several pathophysiologic properties of *M. tuberculosis* infection including granuloma formation and induction of proinflammatory cytokines, such as IL-1β, IL-6, and TNF in macrophages (Matsunaga & Moody, 2009). TNF (cachexin) has several toxic effects on host physiology, including sepsis, fever syndromes and cachexia (Silva & Faccioli, 1988).

Doses as little as 1–5 µg are granulomagenic in the lungs of mice (Bekierkunst et al., 1969). Moreover TDM increases the production of antibodies (Behling et al., 1993b; Perez et al., 1994; Perez et al., 2000) and it up-regulates the expression of MHCII on macrophages (Ryll et al., 2001). TDM also induces *in vivo* production of IL-12 (Oswald et al., 1997). Injected as mineral oil solution (Silva & Faccioli, 1988) TDM forms monolayers, where the mycolic acids are exposed. In the monolayer form TDM is highly toxic (lethal dose in mice: LD_{50} ~30 µg) and kills macrophages in minutes (Hunter et al., 2006b). In aqueous suspension TDM forms micelles, where the mycolic acid groups are completely covered and TDM is non-toxic (lethal dose in mice: LD_{50} >50,000 µg). Micellar TDM prevents phagosome/lysosome fusion and thus promotes the survival of mycobacteria in the macrophage. Nevertheless, until now there is no experimental evidence for the existence or formation of TDM micelles or monolayers *in vivo*. Due to its strong immunstimulatory effect, several studies have used TDM as a potential adjuvant in different vaccination models. In 1976 Saito et al. were the first who described the cord factor as good adjuvant in mice and rats but with only low adjuvant effect in guinea pigs (Saito et al., 1976). Lima et al. could show that microspheres, containing TDM with a Hsp65-encoding DNA plasmid, were able to protect vaccinated mice against virulent *M. tuberculosis* (Lima et al., 2003) and against *Leishmania major* infection (Coelho et al., 2006). The major problem using TDM as adjuvant is the relatively high toxicity of the mycolic acids and the accompanying contaminants during the preparation of TDM. A synthetic analog of the cord factor, trehalose-6,6-dibehenate (TDB), was shown to be an effective and safe alternative (Davidsen et al., 2005). TDB is less toxic compared with TDM and easier to produce, making it a potent candidate in the field of vaccine development.

3. Drug targets in the biosynthesis of the cord factor

The cord factor (trehalose 6,6´-di-mycolate) is composed of a sugar and a mycolic acid component. In the following section we present the trehalose and mycolic acid biosynthesis steps and the target enzymes in their biochemical context. Especially enzymes of mycolic acid biosynthesis, such as methyl transferase (PcaA) (Glickman et al., 2000), ß-ketoacyl-acyl carrier protein synthase (KasAB and FabH) (Bhatt et al., 2007), acyl-AMP ligase (Fad32) (Portevin et al., 2005) and polyketide synthase (Psk13) (Portevin et al., 2004), are regarded as promising targets for anti-TB drug development.

3.1 Biosynthesis of trehalose

Mycobacteria possess three pathways for trehalose synthesis (Kaur et al., 2009). Trehalose can by synthesized from glucose-6-phosphate catalyzed by trehalose-6-phosphate synthase (OtsA, *Rv3490*) (Pan et al., 2002) and trehalose-6-phosphate phosphatase (OtsB2, *Rv3372*) (Pan et al., 2002). The second pathway generates trehalose from glycogen involving the maltooligosyltrehalose synthase (TreY, *Rv1653c*) and the maltooligosyltrehalose trehalohydrolase (TreZ, *Rv1562c*). In the third pathway maltose is converted to trehalose by the trehalose synthase (TreS, *Rv0126*). While all the three pathways are functional and essential for the proliferation *of M. smegmatis* (Woodruff et al., 2004), the OtsAB pathway is predominant and strictly essential in *M. tuberculosis* (Fig. 2). In the genome sequence of *M. tuberculosis* exist two o*tsB* homologues, but only OtsB2 (*Rv3372*) has a functional role in the pathway. OtsB2 has been suggested as an attractive target for novel drugs due to absence of trehalose in mammalian cell (Murphy et al., 2005).

3.2 Trehalose transporters

The LpqY-SugA-SugB-SugCATP-binding cassette transporter is highly specific for uptake of the disaccharide trehalose. Since trehalose is not present in mammals, it is unlikely that this system is used for sugar acquisition from the host. Trehalose release is known to occur as a byproduct of the biosynthesis of the mycolic acid cell envelope by *M. tuberculosis* antigen 85 complex. The mycolyltransferases of the antigen 85 complex transfer the lipid moiety of the glycolipid trehalose monomycolate (TMM) to arabinogalactan or another molecule of TMM, yielding trehalose dimycolate. These reactions lead to a constant release of trehalose from the cell. The LpqY-SugA-SugB-SugC ATP-binding cassette has been suggested as transporter system (Fig. 2), recycling the released trehalose. Perturbations in trehalose recycling strongly impaired virulence of *M. tuberculosis*. (Kalscheuer et al., 2010). These sugar transporters are thought to play an important role in bacterial pathogenesis and have been suggested as target for tuberculosis chemotherapy (Kalscheuer et al., 2010).

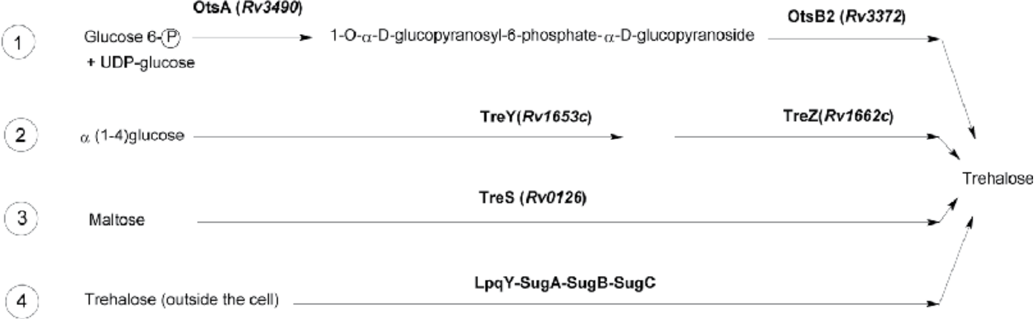

Fig. 2. Trehalose biosynthesis in *Mycobacterium tuberculosis*. 1, OtsAB pathway. 2, TreY-TreZ pathway. 3, TreS-pathway. 4, Trehalose import by an ATP-binding cassette transporter system.

3.3 Mycolic acid biosynthesis

Mycolic acids are β-hydroxy fatty acids with a long α-alkyl side chain. They are homologous series of fatty acids differing by a two-carbon unit (Asselineau & Lederer, 1950). The mycolic acids are composed of an α branch at the alpha position in respect to the carboxylic group and a meromycolate branch. The "short" α branch contains species dependent 20-26 saturated carbon atoms. The "long" meromycolate branch has 50-60 carbon atoms and its chemical composition is highly variable, containing cyclopropyl or unsaturated bonds (α-mycolates), methoxy (methoxymycolates) and keto (ketomycolates) groups (Alahari et al., 2007). The confusing denotation "α-mycolates" refers not to their position in the molecule but to their position on thin layer chromatography. The "α-mycolates" (cis, cis-dicyclopropyl fatty acids) are the most abundant mycolic acids in *M. tuberculosis* (~57%), followed by methoxymycolates (32%) and ketomycolates (11%). The methoxy- and ketomycolates can have either the cis or trans configuration on the proximal cyclopropane ring. In summary there are five main classes of mycolic acids in *M. tuberculosis* (Schroeder et al., 2002; Takayama et al., 2005).

In *M. tuberculosis* mycolic acids are essentially provided via conventional fatty acid biosynthesis. Mycobacteria contain both type I and type II FAS fatty acid biosynthesis systems. Fatty acid biosynthesis is initiated by the multifunctional FAS I enzyme (*Rv2524c*), catalyzing the de novo synthesis of long-chain acyl-CoAs (C16:0 and C18:0) from acetyl-CoA and using malonyl-CoA as an extender unit. The domains of the FAS-I multienzyme-complex are organized in the following order: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein (ACP), β-keto reductase, β-ketoacyl synthase (Fernandes & Kolattukudy, 1996). In *M. tuberculosis* the C16:0- and C18:0-S-ACP adducts, are converted either to the CoA derivatives or further elongated by FAS I to produce C26:0 (Kikuchi et al., 1992). In mycobacteria the de novo fatty acid biosynthesis is exclusively carried out by FAS-I, whereas the FAS-II system performs only the elongation of the fatty acids, generated by FAS-I. The FAS I and FAS II systems are connected by a key condensing enzyme, the β-ketoacyl ACP synthase III or FabH, which catalyzes a decarboxylative condensation of malonyl-ACP with the acyl-CoA (C16:0–C20:0) products of the FAS-I system (Fig. 3). The resulting 3-ketoacyl-ACP product is reduced to an acyl-ACP (extended by two carbons) and shuffled into the FAS II cycle.

The ACP cycles the growing acyl chain between four enzymes MabA (β–ketoacyl reductase), β-hydroxyacy dehydrase, InhA (enoyl reductase) and KasA/B (β-ketoacyl synthase). *M. tuberculosis* contains two β-ketoacyl synthases, KasA and KasB, which share 67% identity. KasA seems to be essential for growth, while KasB is not essential but produces longer carbon chains (Bhatt et al., 2005; Slayden & Barry, 2002; Swanson et al., 2009). The deletion of *KasB* in *M. tuberculosis* leads to mycolic acids that are 2-6 carbons shorter in length and a defect in trans-cycloproponation of oxygenated mycolic acids. Phenotypically leads a deletion of *KasB* to a loss of acid-fastness (Bhatt et al., 2007). The most potent inhibitor for mycolic acid biosynthesis is isoniazid (INH). INH is a prodrug which is converted to the isonicotinoyl radical by KatG. INH forms a covalent adduct with NAD. This INH-NAD adduct inhibits FAS-II enoyl-ACP reductase InhA, which in consequence leads to inhibition of mycolic acid biosynthesis, and ultimately to cell death (Mdluli et al., 1998; Takayama et al., 1972; 1975; Wilming & Johnsson, 1999). In *M. tuberculosis*, the C26:0

fatty acids synthesized by FAS I will become the substrate of a dedicated acyl-CoA carboxylase (ACCase) to generate the α-carboxy C26:0 fatty acid used as one of the substrate by the Pks 13 in the biosynthesis of mycolic acids (Gavalda et al., 2009). The last steps of the biosynthesis of mycolic acids are catalyzed by proteins encoded by the *fadD32-pks13-accD4* cluster. Pks13 ultimately condenses the two loaded fatty acyl chains to produce α-alkyl β-ketoacids, the precursors of mycolic acids (Gavalda et al., 2009). FadD32 has been shown essential for growth (Carroll et al., 2011). Double bonds at specific sites on mycolic acid precursors are modified by the action of cyclopropane mycolic acid synthases (CMASs) such as MmaA1-A4, PcaA and CmaA2, which are S-adenosyl-methionine-dependent methyl transferases (Alahari et al., 2007). The antitubercular drug, thiacetazone (TAC) and its chemical analogues acts on CMASs, inhibiting mycolic acid cyclopropanation (Alahari et al., 2007; Alahari et al., 2009).

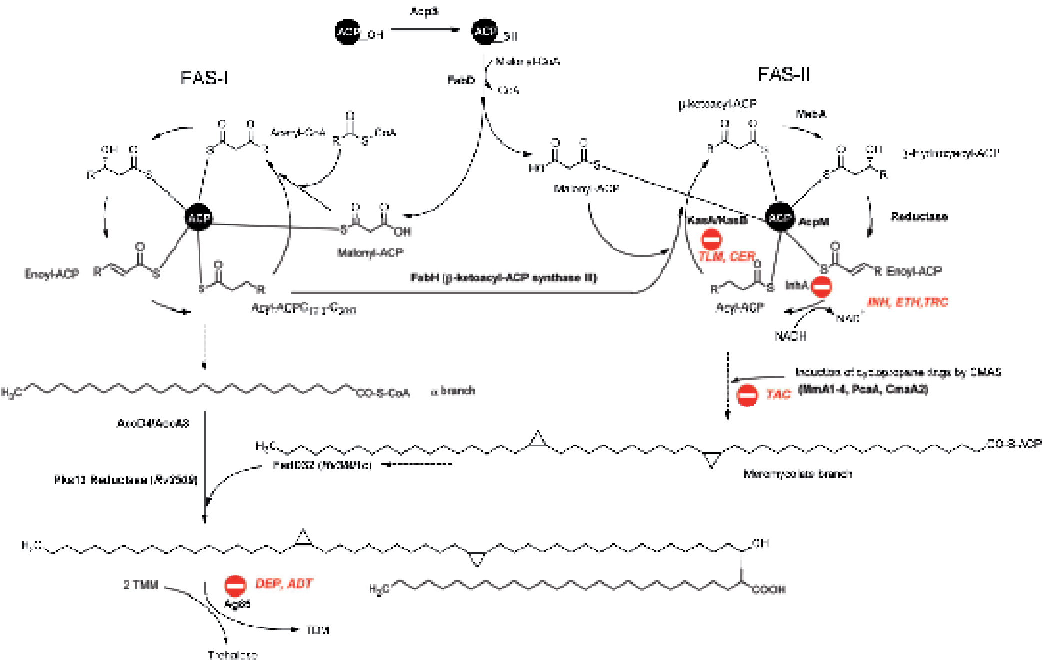

Fig. 3. Biosynthesis of mycolic acids for cord factor synthesis. Enzymes are in bold letters. Selected inhibitors are depicted in red bold letters. TLM, thiolactomycin. CER, cerulenin. ETH, ethionamide. INH, isoniazid. TRC, triclosan. TAC, thiacetazone. DEP, diethyl phosphate. ADT, 6-azido-6-deoxy-α,α'-trehalose (See text for details).

3.4 TMM biosynthesis

TDM is thought to be synthesized exclusively outside the cell and its precursor TMM is transported outside the cell. In addition TMM has to be exported from the cytoplasm, to prevent the degradation of TMM inside the cell by the ubiquitously present Ag85/Fbp (Kilburn et al., 1982; Sathyamoorthy & Takayama, 1987). The mycolyl group is first transferred from mycolyl-S-Pks13 (mycolyl-S-PPB) to D-mannopyranosyl-1-phosphoheptaprenol by a proposed cytoplasmic mycolyltransferase I to yield Myc-PL 6-O-

mycolyl-β-D-mannopyranosyl -1-phosphoheptaprenol (Myc-PL) (Besra et al., 1994). Myc-PL migrates to the inner surface of the cell membrane and docks next to an ABC transporter, with its hydrophobic heptaprenol tail. The mycolyl group is transferred to trehalose 6-phosphate by a proposed membrane-associated mycolyltransferase II to form TMM-phosphate, and the phosphate group is removed by the membrane-associated trehalose 6-phosphate phosphatase, yielding TMM. TMM is transported outside the cell by the ABC transporter (Fig. 4). There should be virtually no accumulation of TMM in the cytoplasm (Takayama et al., 2005).

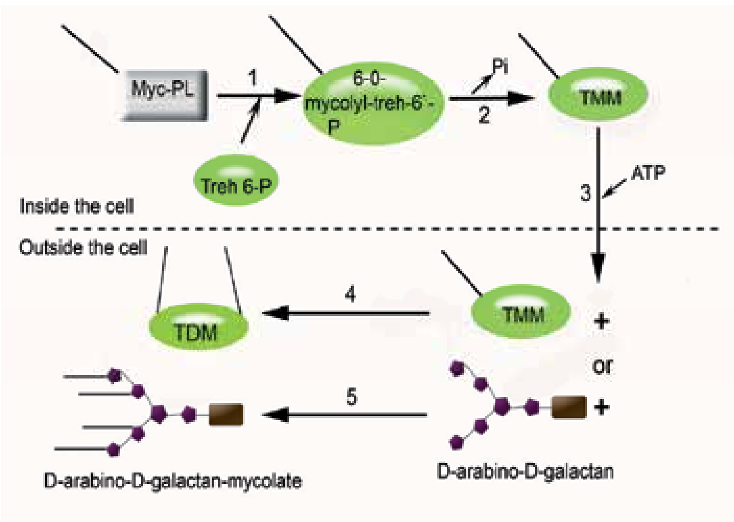

Fig. 4. The proposed process of incorporation of newly synthesized mycolic acids into major cell wall components. The process starts inside the cell. Newly synthesized mycolic acids are transferred to man-P-heptaprenol to produce 6-O-mycolyl-β-D-mannopyranosyl-1-phosphoheptaprenol (Myc-PL) and after that to trehalose 6-phosphate to yield TMM-P by the proposed membrane-associated mycolyltransferase II (reaction 1). TMM is produced by dephosphorylation of TMM-P by the membrane-bound TMM-P phosphatase (reaction 2). The transportation of TMM to the outside is catalyzed by a proposed ABC transporter cassette (TMM transporter) (reaction 3). Outside the cell the Ag85 complex catalyzes the transfer of mycolate to another TMM and arabinogalactan to yield TDM (reaction 4) or arabinogalactan-mycolate (reaction 5) (Takayama et al., 2005).

3.5 TDM biosynthesis by Ag85

The antigen 85 complex is composed of Ag85A (FbpA), Ag85B (FbpB), and Ag85C (FbpC) as the predominant secreted proteins in *M. tuberculosis*. The corresponding genes are *fbpA* (*Rv3804c*), *fbpB* (*Rv1886c*), and *fbpC* (*Rv0129c*) (Belisle et al., 1997; Wiker & Harboe, 1992). The 85 complex proteins share 68–80% sequence identity (Belisle et al., 1997; Ronning et al., 2004). The mycolyltransferases of the antigen 85 complex are located outside the cell membrane and transfer the lipid moiety of the glycolipid trehalose monomycolate (TMM) to another molecule of TMM yielding trehalose dimycolate or to arabinogalactan to form cell wall arabinogalactan-mycolate (Fig. 4) (Sanki et al., 2009a).

There is also evidence, the Ag85 complex proteins bind to fibronectin and the fibronectin-binding property of the Ag85 complex is important for mycobacterium life cycle in the host and macrophages (Klegerman et al., 1994; Ronning et al., 2004). The crystal structures of the three 30–32 kDa proteins (Ag85A, B and C) have been determined (Anderson et al., 2001; Ronning et al., 2004). These proteins contain a carboxylesterase domain bearing the highly-conserved consensus sequence GXXSXXG. The interaction between Ag85 and fibronectin is mediated by the sequence homologous to residues 56–66 (FEEYYQSGLSV) of the recombinant *M. tuberculosis* Ag85C (Ronning et al., 2004). Up to date, the question remains open why *Mycobacterium tuberculosis* has three antigen 85 enzymes sharing the similar sequence and substrate specificity (Daffe, 2000; Ronning et al., 2004).

Ag85 complex members from *M. tuberculosis* belong to the α/β hydrolase superfamily and catalyze the hydrolysis of ester and amide bonds using a catalytic triad comprised of Ser126, Glu230 and His262 in Ag85A/B and Ser124, Glu228 and His260 in Ag85C. All three enzymes contain two carbohydrate binding sites. The active site carbohydrate binding pocket binds TMM to form a temporary mycolate ester with the catalytic serine. The second carbohydrate binding site binds the incoming trehalose monomycolate, which "swings over" to the active site to displace the mycolate from its serine ester (Anderson et al., 2001; Ronning et al., 2004). The second trehalose binding site is separated from the acyl binding pocket by a bulky phenylalanine in Ag85A/B or a smaller leucine in Ag85C. All residues that form the active site carbohydrate binding pocket are 100% conserved in the *M. tuberculosis* antigen 85 proteins, while the surface of the acyl binding pocket, which is supposed to bind the long mycolate chains of TMM, exhibits slight differences. The conserved Leu152 in Ag85A and B is replaced by the bulky Phe150 in Ag85C, which in consequence leads to changes of surface topology in the mycolate binding portion (Fig. 5). The differences may alter substrate specificity and thus Ag85A, B and C might prefer different mycolic acids (Ronning et al., 2000; 2004).

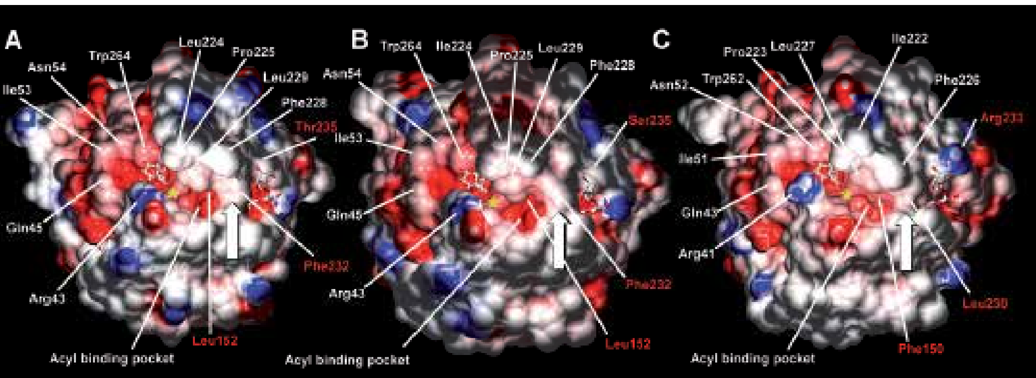

Fig. 5. Surface representation of Ag85A, B and C with two bound trehalose molecules. The trehalose molecules are depicted as ball-and-stick model. The position of the catalytic serine is indicated by a yellow asterisk. The carbohydrate binding pocket of all three proteins is 100% conserved (Arg43, Gln45 Ile53, Asn54, Trp264 in Ag85A and B) and Arg41, Gln43, Ile51, Asn52, Trp264 in Ag85C. Corresponding residues, which differ among the three proteins are shown with red labels. The separation of the second carbohydrate binding pocket from the acyl binding pocket by the Phe232 in Ag85A/B and the corresponding

smaller Leu230 in Ag85C is highlighted by an arrow. Also shown: Arginine 233, which covers the second carbohydrate binding pocket in Ag85C is replaced by smaller polar amino acids Thr235 and Ser235 in Ag85A and B, respectively. The surface is colored by electrostatic potential: The red and blue coloring represent negative and positive electrostatic potential, respectively. For Ag85A, B and C the coordinates from 1SFR, 1F0P and 1DQZ were used, respectively. The position of the trehalose molecules in Ag85A and Ag85C were modeled using the coordinates from 1F0P. The figure was prepared using GRASP (Nicholls et al., 1991).

3.6 Ag85 as a putative drug target for tuberculosis treatment

The ongoing treatment battle of tuberculosis is worsened by the emergence of new strains of *M. tuberculosis* which are resistant to standard antibiotics. In the urgent need of new targets the biogenesis of fatty acids, mycolic acids and glycolipids stay as hotspots. There is hope that the crystal structure of antigen 85A, 85B and 85C shall help in rational drug development for TB (Ronning et al., 2000; 2004).

The treatment by a trehalose analogue, 6-azido-6-deoxy-α,α'-trehalose (ADT) inhibited the activity of all members of Ag85 complex *in vitro* and the growth of *Mycobacterium aurum,* and it also increased the efficacy of various antibiotics, supporting the importance of TDM (Belisle et al., 1997; Mizuguchi et al., 1983). *M. tuberculosis* strain lacking Ag85C has a 40% decrease in the amount of cell wall linked mycolic acid, but with no change in the relative amounts of TMM and TDM (Jackson et al., 1999; Sanki et al., 2009a). Furthermore, an Ag85A knockout strain lost the ability to grow in macrophage-like cell-lines and poor media which highlights the role of Ag85A in virulence and survival of the organism (Armitige et al., 2000). In the last decades several antitubercular drugs have focused on targets in the mycobacterial cell wall (Johnson et al., 2006). Most commonly, ethambutol targets the synthesis of arabinogalactan. Isoniazid and ethionamide inhibit biosynthesis of mycolic acids (Johnson et al., 2006). Obviously, the crystal structure of antigen 85 complex is expected to accelerate the design of new drugs against Ag85 activity and cord factor biosynthesis (Table 1) (Gobec et al., 2004; Sanki et al., 2009b; Wang et al., 2004).

4. Drug development: Novel high-throughput screening assays for mycolytransferase 85A

Since the protein/substrate interactions and co-crystal structure of Ag85 are now known, the search for rapid assays for high-throughput screening (HTS) of large substance libraries has increased considerably. Most of the mycolyltransferase assays previously published are not suitable for HTS, due to their complexity or use of radioactive substances. The first one is a widely used radioassay which monitors enzymatic transfer of mycolic acids from a lipid-soluble TMM molecule to a radioactive water-soluble trehalose. Manipulation of the radioactive products in a two-phase reaction, extraction and thin layer chromatography allows visualization of the products (Kremer et al., 2002; Sathyamoorthy & Takayama, 1987). Another test published uses the substrate analogue p-nitrophenyl-6-O-octanoyl-H-D-glucopyranoside that functions as the acyl donor but it may not represent the natural enzymatic activity (Boucau et al., 2009). Also an excess of D-glucose is added to the reaction to function as an acyl acceptor and to promote turnover of the enzyme. Recently new assay

for Ag85 was developed based on the use of mono and dihexanoyl trehalose substrates, followed by quantitation of the acyl-transfer to the unnatural trehalose by mass spectrometry (Backus et al., 2011).

Synthesis step	Enzyme	Compound / class	References
FAS-I and FAS-II	KasA/KasB	Cerulenin (2R,3S-epoxy-4-oxo-7,10-trans,trans-dodecanoic acid amide	(Schroeder et al., 2002) (Johansson et al., 2008)
FAS-II	KasA/KasB	TLM (Thiolactomycin)	(Douglas et al., 2002; Kremer et al., 2000; Luckner et al., 2010)
FAS-II	KasA/KasB	Platensimycin	(Brown et al., 2009)
	InhA	INH (Isoniazid)	(Slayden et al., 2000)
	InhA	ETH (Ethionamide)	(Kremer et al., 2003)
	InhA	TRC (Triclosan)	(McMurry et al., 1999)
	InhA	alkyl diphenyl ethers (Triclosan derivatives)	(Sullivan et al., 2006)
	InhA	2-(o-Tolyloxy)-5-hexylphenol (PT70)	(Luckner et al., 2010)
Mycolic acid biosynthesis	Unknown	N-octanesulfonylacetamide	(Parrish et al., 2001)
Cyclopro-panation	CMASs (cmaA2, mmaA2 or pcaA)	TAC (Thiacetazone)	(Alahari et al., 2007)
	MmaA4	TAC (Thiacetazone)	(Alahari et al., 2007)
Cord-factor biosynthesis	Ag85C	Methyl β-D-arabinofuranoside	(Sanki et al., 2009b)
	Ag85C	5-S-alkyl-5-thio-arabinofuranoside analogues	(Sanki et al., 2008)
	Ag85 complex	Trehalose analogues	(Wang et al., 2004)
	Ag85 complex	ADT (6-azido-6-deoxy-α,α′-trehalose)	(Belisle et al., 1997; Mizuguchi et al., 1983)
	Ag85 complex	Phosphonate compounds	(Gobec et al., 2004; Gobec et al., 2007)
	Ag85C	DEP (Diethyl phosphate)	(Ronning et al., 2000)

Table 1. Inhibitors of cord factor biosynthesis

The mycobacterial glycolipids and TMM levels in the cell wall might give an indirect indication of the fitness of the cell inside the host cells specially in macrophages. Thus the quantitation of the mycobacterial TMM status after drug treatment may allow the estimation of drug effectiveness. Unfortunately, there is no method for measurement of the amount of glycolipids that is suitable for HTS. We designed an assay (Elamin et al., 2009) based on the use of natural substrate, and this mycolyltransferase assay offers a novel means to determine the TMM status of the mycobacterium cell wall and reflects the natural activity of mycolyltransferase enzyme based on simple steps. The new assay uses the natural substrate TMM, which can be easily purified from mycobacteria (Fujita et al.,

2005) and reflects natural activity, allowing to get the most accurate kinetic parameters. In the reaction Ag85A produces one molecule of trehalose as product per reaction cycle and by adding trehalase the trehalose converted to glucose, which can be easily measured (Fig. 6).

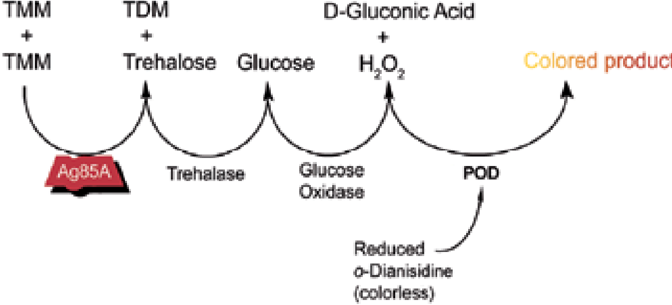

Fig. 6. Scheme for the new mycolyltransferase activity assay. Using trehalose that is produced as one of final products of mycolyltransferase reaction by trehalase to produce glucose, which is oxidized to gluconic acid and hydrogen peroxidase by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to produce a colored product (oxidized o-dianisidine), which will be converted to a stable colored product by sulfuric acid. The colored product is measured at 540 nm (Elamin et al., 2009).

Quantification of glucose is finally achieved by the glucose oxidase assay (Washko & Rice, 1961). The amount of glucose is proportional to the TMM concentration. The assay showed that the antigen 85A can be assayed in the presence of methanol or mixture of chloroform/methanol, which is usually used to extract and purify the glycolipids from mycobacterium cell wall fractions. The results from substrate/solvent experiments showed that the enzyme activity was reduced in the presence of organic solvents than in standard buffer reaction alone. This indicates and proves that this method is useful to quantify the TMM from the total lipid of mycobacterium cells.

One molecule of trehalose produced from TMM processed by Ag85 complex, which by our method is converted to two molecules of glucose. We can calculate the original concentration of TMM and concentration of TDM and trehalose in the reaction by the following equations:

$$\text{Concentration of [TMM]} = \text{concentration of [glucose]} \quad (1)$$

$$\text{Concentration of [TDM]} = \text{concentration of [glucose]}/2 \quad (2)$$

$$\text{Concentration of [trehalose]} = \text{concentration of [glucose]}/2 \quad (3)$$

One has to keep in mind that the extracted total lipids contain free trehalose and glucose and will affect the final calculations. In this case and to calculate the TMM concentration in total lipids one should run different negative controls. The Z′ factor (Zhang et al., 1999) measurement of the current assay in different volumes indicates an excellent signal/noise (S/N) ratio for the assay and its high potential for HTS applications (Table 2).

Reaction volume	200 µl	300 µl	350 µl
Z′ factor	0.67 ±0.021	0.72 ± 0.014	0.73 ±0.012

Table 2. The calculated Z′ factor at different volumes from 96-well plate format assays.

5. Concluding remarks

Large gaps remain in our understanding of mycobacterium pathogenesis and persistence including the critical questions how bacteria survive in host cells and escape from the therapy. Future work on mycobacterial cell wall biosynthesis especially glycolipids and related pathways is expected to reveal *in vivo* drug-resistance mechanism. Perhaps more notably, the described new and low-cost colorimetric method based on use of TMM as natural substrate could brings flexibility and convenience in HT-screening of substance libraries and help in the development of novel drugs against tuberculosis.

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Part 2

New Drugs to Face Resistance

9

Old and New TB Drugs: Mechanisms of Action and Resistance

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1. Introduction

Historically, tuberculosis (TB) has been associated with significant morbidity and mortality, and still remains a major global health problem. It is estimated that 2 billion people are latently infected with *Mycobacterium tuberculosis*, resulting in approximately 3 million deaths worldwide per year. Among the unique features of this organism is its ability to establish persistent infection, requiring prolonged antibiotic treatment in order to achieve clinical cure. The basic goals of anti-tuberculosis therapy include rapid killing of actively multiplying bacilli, prevention of acquired drug resistance, and sterilization of infected host tissues to prevent clinical relapse. Official guidelines recommend a minimum of 6 months of combination antibiotic therapy in order to achieve these goals. Clinical isolates in geographic areas with a high prevalence of drug resistance should be tested routinely for susceptibility to first-line anti-tuberculosis agents if resources permit, in order to optimally guide therapy (Karakousis 2009). The emergence of multidrug-resistant TB (MDR-TB), defined as resistance to the first-line drugs isoniazid and rifampicin, and extensively drug-resistant TB (XDR-TB), defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the injectable second-line drugs (capreomycin, kanamycin, and amikacin), poses formidable challenges to global TB control efforts. The high global incidence of drug-resistant TB, estimated annually to be ~500,000 cases of MDR-TB of which 5% to 7% represent XDR-TB, underscores the need to understand the molecular mechanisms of drug resistance, with the ultimate goals of developing new techniques for rapid detection of drug resistance and identification of new drug targets.

This chapter presents an updated review of the mechanisms of action and resistance of the main old and new anti-tuberculosis agents.

2. Intrinsic and acquired drug resistance

Intrinsic resistance refers to the innate ability of a bacterium to resist the activity of a particular antimicrobial agent through its inherent structural or functional characteristics. Intrinsic drug resistance in *M. tuberculosis* has been attributed to its unique cell wall properties, including the presence of mycolic acids, which are high-molecular-weight α-alkyl,β-hydroxy fatty acids covalently attached to arabinogalactan, and which constitute a

very hydrophobic barrier responsible for resistance to certain antibiotics (Karakousis, Bishai et al. 2004). In addition, *M. tuberculosis* possesses β-lactamase enzymes, which confer intrinsic resistance to β-lactam antibiotics, while efflux mechanisms appear to play an important role in resistance to antibiotics such as tetracycline and the aminoglycosides.

Acquired drug resistance occurs when a microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible. Acquired drug resistance in *M. tuberculosis* is caused mainly by spontaneous mutations in chromosomal genes, and the selective growth of such drug-resistant mutants may be promoted during suboptimal drug therapy (Kochi, Vareldzis et al. 1993). The rate of genetic mutations leading to resistance varies somewhat among anti-tuberculosis drugs, from a frequency of ~10^{-5}-10^{-6} organisms for isoniazid to ~10^{-7}-10^{-8} organisms for rifampin (Karakousis 2009). Since the bacterial burden typically present in pulmonary cavities does not exceed 10^{12} bacilli (Canetti 1965), combination therapy is highly effective for drug-susceptible disease, and the risk for development of acquired drug resistance is minimized.

3. Old TB drugs

3.1 Isoniazid

Isoniazid (isonicotinic acid hydrazide, INH) has been the most commonly used anti-tuberculosis since recognition of its clinical activity in 1952 (Robitzek and Selikoff 1952). Consisting of a pyridine ring and a hydrazide group, INH is a nicotinamide analog, structurally related to the anti-tuberculosis drugs ethionamide and pyrazinamide. Because of its significant bactericidal activity, it has become a critical component of the first-line antituberculous regimens, although in the last two decades resistance to INH has been reported with increasing frequency.

Isoniazid

3.1.1 Mechanism of action

INH appears to penetrate host cells readily (Mackaness and Smith 1952) and diffuses across the *M. tuberculosis* membrane (Suter 1952; Bardou, Raynaud et al. 1998). INH is a pro-drug, requiring oxidative activation by the *M. tuberculosis* catalase-peroxidase enzyme KatG (Zhang, Heym et al. 1992). Although the active metabolites of INH have been reported to inhibit multiple essential cellular pathways, including synthesis of nucleic acids (Gangadharam, Harold et al. 1963), phospholipids (Brennan, Rooney et al. 1970), and NAD metabolism (Zatman, Kaplan et al. 1954; Bekierkunst 1966), the primary pathway

responsible for the killing activity of the drug is mycolic acid synthesis (Winder and Collins 1970; Takayama, Wang et al. 1972; Takayama, Schnoes et al. 1975). Thus, the activated form of the drug binds tightly to the NADH-dependent enoyl acyl carrier protein (ACP) reductase InhA (Banerjee, Dubnau et al. 1994), a component of the fatty acid synthase II system of mycobacteria, which is essential for fatty acid elongation (Quemard, Sacchettini et al. 1995). INH does not directly interact with InhA, as X-ray crystallographic and mass spectrometry data revealed that the activated form of INH covalently attaches to the nicotinamide ring of NAD bound within the active site of InhA, causing NADH to dissociate from InhA (Dessen, Quemard et al. 1995; Rozwarski, Grant et al. 1998). However, the precise mechanism by which INH kills *M. tuberculosis* remains to be elucidated.

3.1.2 Mechanism of resistance

Because INH is the most commonly used antituberculosis drug, resistance to INH occurs more frequently among clinical isolates than resistance to any other agent (Karakousis 2009).

Mutations in INH-resistant clinical isolates are most commonly detected in the *katG* gene, occurring in 50–80% of cases, thus reducing the ability of the catalase-peroxidase to activate the INH pro-drug. The *katG* gene is located in a highly variable and unstable region of the *M. tuberculosis* genome, with missense and nonsense mutations, insertions, deletions, truncation and, more rarely, full gene deletions observed. Depending on the type of mutation, and the degree to which function of the KatG enzyme is preserved, the ensuing minimum inhibitory concentration (MIC) of isoniazid may range from 0.2 to 256 mg/L. Point mutations in *katG* are more commonly observed than other types of mutations, and a single point mutation resulting in substitution of threonine for serine at residue 315 (S315T) accounts for the majority of INH resistance among clinical isolates (Marttila, Soini et al. 1998; Abate, Hoffner et al. 2001). The S315T mutation results in a significant reduction in catalase and peroxidase activity, and is associated with high-level INH resistance (MIC = 5–10 µg/mL) (Rouse, DeVito et al. 1996; Saint-Joanis, Souchon et al. 1999).

INH resistance may also arise from mutations in *inhA*, resulting in reduced affinity of the enzyme for NADH without affecting its enoyl reductase activity (Basso, Zheng et al. 1998), or in the promoter region of the *mabAinhA* operon (Musser, Kapur et al. 1996), resulting in overexpression of the wild-type enzyme. Generally, mutations in *inhA* or in the promoter region of its operon usually confer low-level resistance (MIC = 0.2–1 mg/L) (Wade and Zhang 2004). In addition to conferring resistance to INH, mutations in *inhA* also cause resistance to the structurally related second-line drug ethionamide.

Mutations in the *ndh* gene, which encodes a NADH dehydrogenase, confer resistance to INH and ethionamide in *M. smegmatis* (Miesel, Weisbrod et al. 1998), and have been detected in INH-resistant *M. tuberculosis* clinical isolates, which lack mutations in the *katG* or *inhA* genes (Lee, Teo et al. 2001). Defective NADH dehydrogenase could lead to an increased ratio of NADH/NAD, thereby interfering with KatG-mediated peroxidation of INH, or by displacing the INH/NAD adduct from the InhA active site (Miesel, Weisbrod et al. 1998). Furthermore, mutations in *kasA* and *ahpC* genes have been associated with INH resistance. Nevertheless, as many as a quarter of all clinical INH-resistant isolates do not

have mutations in any of the above genes, suggesting alternative mechanisms of INH resistance (Karakousis 2009).

3.2 Rifampin and other rifamycins

The rifamycins were first isolated in 1957 from *Amycolatopsis* (formerly *Streptomyces*) *mediterranei* as part of an Italian antibiotic screening program (Sensi 1983). Their incorporation into the standard anti-tuberculosis regimen allowed reduction of the duration of treatment from 18 to 9 months. Although the early bactericidal activity of the rifamycins is inferior to that of INH, the former are the most potent sterilizing agents available in TB chemotherapy, continuing to kill persistent tubercle bacilli throughout the duration of therapy (Mitchison 1985; Grosset, Lounis et al. 1998). Rifampin is a broad-spectrum antibiotic and the most widely used rifamycin to treat TB.

Rifampicin

3.2.1 Mechanism of action

Rifamycins contain an aromatic nucleus linked on both sides by an aliphatic bridge. The rifamycins easily diffuse across the *M. tuberculosis* cell membrane due to their lipophilic profile (Wade and Zhang 2004). Their bactericidal activity has been attributed to their ability to inhibit transcription by binding with high affinity to bacterial DNA-dependent RNA polymerase. Although the molecular target of rifampin has been well characterized, the precise mechanism by which this interaction leads to mycobacterial killing remains unclear.

3.2.2 Mechanism of resistance

Although INH monoresistance is relatively common in *M. tuberculosis*, resistance to rifampin alone is rare, and more than 90% of rifampin-resistant isolates are also resistant to INH. Therefore, rifampin resistance has been used as a surrogate marker for MDR-TB. Resistance to rifampin in *M. tuberculosis* arises at a frequency of 10^{-7} to 10^{-8} organisms, most commonly as single point mutations in the *rpoB* gene, which encodes the β-subunit of RNA polymerase (Telenti, Imboden et al. 1993). In over 90% of rifampin-resistant clinical isolates, point mutations cluster in an 81-base pair "hot-spot" region between codons 507 and 533 of the *rpoB* gene, with mutations in codons 531 [Ser] and 526 [His] predominating (Ramaswamy and Musser 1998). However, a small percentage of rifampin-resistant isolates

(<5%) do not contain any mutations in the *rpoB* gene, suggesting alternative resistance mechanisms, potentially including altered rifampin permeability or mutations in other RNA polymerase subunits (Musser 1995).

Higher doses of the rifamycins, especially rifapentine, have the potential to further shorten the duration of TB treatment. Therefore, there is renewed interest in establishing the maximally tolerated dose of these drugs, and a number of clinical trials are planned or underway to examine the safety, pharmacokinetics and efficacy of higher than standard doses of rifampicin or rifapentine in first-line TB treatment (Ginsberg 2010).

3.3 Pyrazinamide

Since the discovery of pyrazinamide (PZA) in 1952 (Yeager, Munroe et al. 1952), and its routine use to treat TB, the duration of treatment required to achieve acceptable relapse rates has been reduced from 9–12 months to the current 6 months (Steele and Des Prez 1988). Although its bactericidal activity is inferior to that of INH and rifampin (Jindani, Aber et al. 1980), the potent sterilizing activity and treatment-shortening potential of PZA has been attributed to the drug's unique ability to target semi-dormant populations of bacilli residing within an acidic environment (Mitchison 1985).

O
N
NH_2
N

Pyrazinamide

3.3.1 Mechanism of action

PZA is an amide derivative of pyrazine-2-carboxylic acid and nicotinamide analog. Despite recognition of its anti-tuberculosis activity six decades ago, the mechanism of action of PZA remains poorly understood. PZA has been hypothesized to act against bacilli residing in acidified compartments of the lung that are present during the early inflammatory stages of infection (Mitchison 1985), since the drug's sterilizing activity appears to be limited to the first 2 months of therapy (1986; 1986; 1991). PZA enters tubercle bacilli passively and via an ATP-dependent transport system (Raynaud, Laneelle et al. 1999). Intracellular accumulation of the drug occurs because of an inefficient efflux system unique to *M. tuberculosis*. PZA, like INH, is a pro-drug, requiring activation to its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PZase) (Konno, Feldmann et al. 1967; Scorpio and Zhang 1996). Uptake and intrabacillary accumulation of POA is enhanced when the extracellular pH is acidic (Zhang, Scorpio et al. 1999). The anti-tuberculosis activity of PZA has been attributed to disruption of the proton motive force required for essential membrane transport functions by POA at acidic pH (Zhang, Wade et al. 2003), although investigation into potential specific cellular targets is ongoing.

3.3.2 Mechanism of resistance

PZA resistance has been attributed primarily to mutations in the *pncA* gene encoding PZase (Scorpio and Zhang 1996). Most mutations, including point mutations, deletions, and insertions, have been reported in a 561-bp region of the open reading frame or in an 82-bp region of its putative promoter (Scorpio, Lindholm-Levy et al. 1997; Jureen, Werngren et al. 2008). The relatively high degree of diversity in *pncA* mutations among PZA-resistant clinical isolates has complicated the development of molecular assays for the rapid and economical detection of PZA resistance. A small percentage of isolates with high-level PZA resistance contain no mutations in *pncA* or its promoter, suggesting alternative mechanisms of resistance such as deficient uptake (Raynaud, Laneelle et al. 1999), enhanced efflux, or altered *pncA* regulation.

The high specificity of PZA for *M. tuberculosis*, with little or no activity against *M. bovis* and other mycobacteria, is attributable to *pncA* mutations, which render PZase inactive in the latter mycobacterial species.

3.4 Ethambutol

Ethambutol (EMB; dextro-2,2.-(ethylenediimino)-di-1-butanol) was initially reported to have anti-tuberculosis activity in 1961 and, together with INH, rifampin, and PZA, constitutes the modern-day short-course for the treatment of drug-susceptible TB. Like INH, EMB primarily kills actively multiplying bacilli and has very poor sterilizing activity.

Ethambutol

3.4.1 Mechanism of action

The primary pathway affected by EMB appears to be that of arabinogalactan biosynthesis through inhibition of cell wall arabinan polymerization (Mikusova, Slayden et al. 1995). EMB also has been reported to inhibit several other cellular pathways, including RNA metabolism (Forbes, Kuck et al. 1962; Forbes, Kuck et al. 1965), transfer of mycolic acids into the cell wall (Takayama, Armstrong et al. 1979), phospholipid synthesis (Cheema and Khuller 1985; Cheema and Khuller 1985), and spermidine biosynthesis (Paulin, Brander et al. 1985).

3.4.2 Mechanism of resistance

Resistance to EMB in *M. tuberculosis* is usually associated with point mutations in the *embCAB* operon (Belanger, Besra et al. 1996). Genetic and biochemical studies have shown that the EmbA and EmbB proteins are involved in the formation of the proper terminal hexaarabinofuranoside motif during arabinogalactan synthesis (Escuyer, Lety et al. 2001),

while EmbC is involved in lipoarabinomannan synthesis (Zhang, Torrelles et al. 2003). As the majority of EMB-resistant clinical isolates contain mutations in the *embB* gene (Sreevatsan, Stockbauer et al. 1997; Telenti, Philipp et al. 1997; Ramaswamy, Amin et al. 2000), EmbB is considered to be the main target of EMB, although X-ray crystallographic data supporting this interaction are lacking. More recently, the most commonly observed mutations in *embB* codon 306 have been reported to be associated with variable degrees of EMB resistance, indicating that such mutations may be necessary but not sufficient for high-level EMB resistance. Other potential mutations involved in EMB resistance include a Gln379Arg substitution in *M. tuberculosis embR*, as well as mutations in the *rmlD*, *rmlA2*, and Rv0340 genes. As many as one quarter of all EMB-resistant *M. tuberculosis* isolates do not harbor mutations in any of the genes described above, suggesting alternative mechanisms of EMB resistance (Karakousis 2009).

3.5 Aminoglycosides

The discovery of streptomycin in the early 1940s represented the first breakthrough in TB chemotherapy. Although relapse rates are comparable when streptomycin is substituted for EMB as the fourth drug in addition to INH, rifampin, and PZA, the poor oral absorption of streptomycin, which necessitates parenteral administration, as well as the toxicity profile of the aminoglycosides, have favored the use of EMB in first-line anti-tuberculosis therapy. Other aminoglycosides with significant antimycobacterial activity include kanamycin and amikacin. Aminoglycosides are used currently as second-line drugs primarily in the treatment of MDR-TB.

Streptomycin

3.5.1 Mechanism of action

As in other bacteria, the mode of action of the aminoglycosides against mycobacterial species is through their binding to the 30S ribosomal subunit, which affects polypeptide synthesis, ultimately resulting in inhibition of translation.

3.5.2 Mechanism of resistance

Resistance to streptomycin and the other aminoglycosides in *M. tuberculosis* usually develops by mutation of the ribosome target binding sites. Interestingly, although cross-resistance is observed between amikacin and kanamycin (Allen, Mitchison et al. 1983), these drugs are not cross-resistant with streptomycin (Tsukamura and Mizuno 1975), suggesting

distinct mechanisms of resistance. Mutations in the *rpsL* gene, which encodes the ribosomal protein S12, account for approximately half of all streptomycin-resistant clinical isolates (Nair, Rouse et al. 1993; Cooksey, Morlock et al. 1996), with the K43R mutation predominating. In about 20% of streptomycin-resistant *M. tuberculosis* clinical isolates, such resistance is associated with mutations in the *rrs* gene, which are usually clustered in the regions surrounding nucleotides 530 or 912 (Douglass and Steyn 1993; Cooksey, Morlock et al. 1996). The particular vulnerability of *rrs* to mutation, leading to streptomycin resistance in *M. tuberculosis* and other slow-growing mycobacteria, can be explained by the fact that these mycobacterial species, unlike other bacteria, contain only a single copy of the *rrs* gene. Generally, mutations in the *rpsL* and *rrs* genes confer high-level (MIC > 1,000 mg/L) or intermediate-level (MIC = 64–512 mg/L) resistance to streptomycin (Sreevatsan, Pan et al. 1996). On the other hand, mechanisms of low-level resistance to streptomycin (MIC = 4–32 mg/L) remain largely undefined but may be attributable to changes in cell envelope permeability or diminished drug uptake (Honore and Cole 1994; Cooksey, Morlock et al. 1996). More recently, it has been shown that mutations in *gidB*, which encodes a conserved S-adenosylmethionine-dependent 16S rRNA methyltransferase, can confer low-level resistance to streptomycin (Wong, Lee et al. 2011).

3.6 Fluoroquinolones

The fluoroquinolones (moxifloxacin, gatifloxacin, sparfloxacin, levofloxacin, ofloxacin, and ciprofloxacin), are bactericidal antibiotics with excellent activity against *M. tuberculosis* and are currently used as second-line drugs in TB treatment. New-generation fluoroquinolones [moxofloxacin, gatifloxacin] are under clinical evaluation as first-line antibiotics with the goal of shortening the duration of TB treatment.

Ciprofloxacin *Moxifloxacin*

3.6.1 Mechanism of action

Fluoroquinolones exert their powerful antibacterial activity by trapping gyrase and topoisomerase IV on DNA as ternary complexes, thereby blocking the movement of replication forks and transcription complexes (Drlica and Malik 2003). Unlike most other bacterial species, *M. tuberculosis* lacks topoisomerase IV, but contains the genes *gyrA* and *gyrB* encoding the A and B subunits, respectively, of DNA gyrase (Cole, Brosch et al. 1998).

3.6.2 Mechanism of resistance

Fluoroquinolone resistance in *M. tuberculosis* is most commonly associated with mutations in the conserved quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB* involved in the interaction between the drug and DNA gyrase (Ginsburg, Grosset et al. 2003). The degree of fluoroquinolone resistance is dictated by the specific amino acid

substitution in the QRDR, as well as the number of resistance mutations present. Therefore, while individual mutations in *gyrA* may confer low-level resistance (MIC > 2 mg/L) (Kocagoz, Hackbarth et al. 1996), high-level resistance to fluoroquinolones usually requires multiple mutations in *gyrA*, or concurrent mutations in *gyrA* and *gyrB* (Kocagoz, Hackbarth et al. 1996; Xu, Kreiswirth et al. 1996). The most frequently observed mutations associated with fluoroquinolone resistance in *M. tuberculosis* are at positions Ala-90 and Asp-94 in the *gyrA* gene. Codon 95 contains a naturally occurring polymorphism (Ser or Thr) that is not related to fluoroquinolone resistance, as it occurs in both fluoroquinolone-susceptible and fluoroquinolone-resistant strains. Interestingly, mutations at position 80 of *gyrA* have been reported to cause hypersusceptibility to fluoroquinolones, especially when present with other resistance mutations (Aubry, Veziris et al. 2006). Since mutations in the QRDR region of *gyrA* are identified in only 42–85% of fluoroquinolone-resistant clinical isolates, alternative mechanisms of resistance are presumed to exist, including the potential participation of efflux pumps. Although full cross-resistance is commonly observed across fluoroquinolones, von Groll et al. (Von Groll, Martin et al. 2009) recently reported a strain with an Asn-533-Thr mutation in *gyrB* that was resistant to moxifloxacin and gatifloxacin but susceptible to ofloxacin.

3.7. Macrolides

3.7.1 Mechanism of action

The macrolides are broad-spectrum antibiotics, which exert their antibacterial effect by binding to the bacterial 50S ribosomal subunit and inhibiting RNA-dependent protein synthesis (Piscitelli, Danziger et al. 1992). However, these drugs have limited activity against wild-type *M. tuberculosis* (Bermudez and Yamazaki 2004). The possible role of macrolides in TB treatment is an area of active investigation, as recent studies have reported synergy of macrolides in combination with other antibiotics.

Clarithromycin

3.7.2 Mechanism of resistance

Intrinsic resistance to the macrolides in *M. tuberculosis* has been attributed to low cell wall permeability and expression of the *erm(37)* gene encoding a 23S rRNA methyltransferase, which is present in all members of the *M. tuberculosis* complex but absent in nontuberculous mycobacteria (Buriankova, Doucet-Populaire et al. 2004). Subinhibitory concentrations of clarithromycin have been shown to cause induction of erm*(37)* expression and a 4- to 8-fold increase in MIC (Andini and Nash 2006).

3.8 Ethionamide

Ethionamide, a synthetic compound structurally related to INH, is a pro-drug, requiring activation by the monooxygenase EthA (Baulard, Betts et al. 2000; DeBarber, Mdluli et al. 2000; Vannelli, Dykman et al. 2002). Similar to INH, ethionamide inhibits mycolic acid synthesis by binding the ACP reductase InhA. Approximately three-quarters of *M. tuberculosis* isolates with high-level ethionamide resistance (MIC > 50 mg/L) have mutations in *ethA* or *inhA* (Morlock, Metchock et al. 2003). Recently, other potential mechanisms of resistance have been identified, as *M. tuberculosis mshA* deletion mutants were found to be defective in mycothiol biosynthesis and resistant to ethionamide, likely due to defective activation of the drug (Vilcheze, Av-Gay et al. 2008).

S NH2 N CH3

Ethionamide

3.9 Capreomycin

Capreomycin is a macrocyclic polypeptide antibiotic isolated from *Streptomyces capreolus* (Karakousis 2009). Capreomycin, like streptomycin and kanamycin, inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA (Wade and Zhang 2004). Recent studies using site-directed mutagenesis have identified the binding site of capreomycin on 16S rRNA helix 44 (Akbergenov, Shcherbakov et al. 2011). In *M. tuberculosis*, resistance to capreomycin and kanamycin has been associated with mutations in the *rrs* gene encoding 16S rRNA (Taniguchi, Chang et al. 1997; Alangaden, Kreiswirth et al. 1998). Mutations in the gene *tlyA* encoding a 2'-O-methyltransferase of 16S rRNA and 23S rRNA have been implicated in resistance to capreomycin and viomycin (Johansen, Maus et al. 2006), and such resistance is generally associated with the addition of methyl groups to rRNA rather than their loss (Sander, Meier et al. 1996). However, recent studies have shown that capreomycin-resistant strains lack mutations in *tlyA* (Jugheli, Bzekalava et al. 2009).

Capreomycin

3.10 Cycloserine

Cycloserine is a d-alanine analogue, which interrupts peptidoglycan synthesis by inhibiting the enzymes d-alanine racemase (AlrA) and d-alanine:d-alanine ligase (Ddl) (Caceres, Harris et al. 1997). Overexpression of *M. tuberculosis* AlrA and Ddl on a multicopy vector results in resistance to D-cycloserine in *M. smegmatis* and *M. bovis* BCG (Caceres, Harris et al. 1997; Feng and Barletta 2003), although whether similar mechanisms are responsible for cycloserine resistance in *M. tuberculosis* remain to be determined.

Cycloserine

3.11 Paraaminosalicylic acid

Paraaminosalicylic acid (PAS) is thought to inhibit folic acid biosynthesis and uptake of iron (Wade and Zhang 2004). Mutations in the *thyA* gene encoding the enzyme thimidylate synthesis of the folate biosynthesis pathway have been identified in PAS-resistant *M. tuberculosis* clinical isolates, suggesting that PAS may act as a folate antagonist (Rengarajan, Sassetti et al. 2004). However, only slightly more than a third of the evaluated PAS-resistant strains had mutations in *thyA*, suggesting the existence of additional mechanisms of PAS resistance. Thr202Ala has been reported as the most common mutation associated with PAS resistance, although this mutation has also been identified in several PAS-susceptible isolates (Leung, Yip et al. 2010).

Paraaminosalicylic acid

4. New TB drugs

Several new drugs have emerged recently as potential candidates for the treatment of TB. In most cases, their mechanism of action is distinct from that of the classical anti-TB drugs, although strains resistant to several of the novel drugs already have been described even prior to their routine clinical use.

4.1 Nitroimidazoles

Reduced oxygen tension may be an important microenvironmental condition encountered by persistent bacilli within necrotic lung granulomas in the human host (Haapanen, Kass et al. 1959). Interestingly, although *in vitro* exposure to microaerophilic conditions renders *M. tuberculosis* less susceptible to killing by INH and rifampin, the bacilli become susceptible to metronidazole (Wayne and Sramek 1994; Wayne and Hayes 1996) a nitroimidazole drug used to treat anaerobic infections. Metronidazole, which becomes reductively activated by the pyruvate:ferredoxin oxidereductase system under anoxic conditions (Edwards 1993) lacks antituberculous activity in mouse models (Brooks, Furney et al. 1999; Klinkenberg, Sutherland et al. 2008) and in guinea pigs (Hoff, Caraway et al. 2008), but displays activity in *M. tuberculosis*-infected rabbits (Via, Lin et al. 2008). Clinical studies evaluating the activity of metronidazole against MDR-TB are ongoing.

4.1.1 PA-824

PA-824, a small molecule nitroimidazopyran related to metronidazole, exhibits bactericidal activity against actively multiplying and stationary-phase cultures of *M. tuberculosis*, as well as in murine and guinea pig models of TB infection (Stover, Warrener et al. 2000; Lenaerts, Gruppo et al. 2005). In addition, PA-824 is highly active against multidrug-resistant clinical isolates of *M. tuberculosis* (MIC < 1 μg/mL), suggesting no cross-resistance with current anti-tuberculosis drugs (Lenaerts, Gruppo et al. 2005). Like metronidazole, PA-824 is a pro-drug requiring reductive activation of an aromatic nitro group, which involves an F420-dependent glucose-6-phosphate dehydrogenase encoded by Rv0407 (*fgd1*) (Stover, Warrener et al. 2000) and deazaflavin-dependent nitroreductase (Ddn) encoded by Rv3547 (Singh, Manjunatha et al. 2008), in order to exert its antitubercular effect. The activity of PA-824 is at least partially mediated through inhibition of the oxidation of hydroxymycolates to ketomycolates, a terminal step in mycolic acid synthesis (Stover, Warrener et al. 2000). Recently, formation of the des-nitroimidazole metabolite of PA-824 was shown to generate reactive nitrogen species, including nitric oxide, which appears to contribute to the killing activity of PA-824 and may explain the activity of the drug against non-replicating bacilli (Singh, Manjunatha et al. 2008). Similar to INH, resistance to PA-824 is most commonly mediated by mutations that lead to loss of pro-drug activation, including those in the genes Rv0407 and Rv3547 encoding the activating enzymes.

PA-824

4.1.2 OPC-67683

OPC-67683 is a nitro-dihydro-imidazooxazole derivative with potent activity against drug-susceptible *M. tuberculosis* and MDR-TB. The drug exerts its killing activity by inhibiting the synthesis of methoxy- and keto-mycolic acids. The substitution of OPC-67683 for INH and

EMB in the standard regimen alongside rifampin and PZA led to more rapid sterilization of *M. tuberculosis*-infected mouse lungs (Matsumoto, Hashizume et al. 2006). Like the other nitroimidazoles, OPC-67683 is a pro-drug requiring reductive activation by *M. tuberculosis*. As in the case of PA-824, mutations in the Rv3547 gene have been identified in strains resistant to OPC-67683, indicating defective drug activation (Matsumoto, Hashizume et al. 2006).

OPC-67683

4.2 TMC207

TMC207 (also named R207910 or "J compound") is a first-in-class anti-TB diarylquinoline with bactericidal and sterilizing activities against drug-susceptible and drug-resistant *M. tuberculosis in vitro* and in animal models, including in a murine model of latent TB infection (Zhang, Li et al. 2011). Mouse model studies suggest a synergistic relationship between TMC207 and PZA (Matteelli, Carvalho et al. 2010). TMC207 inhibits ATP synthase, a critical enzyme in the synthesis of ATP for *M. tuberculosis* (Andries, Verhasselt et al. 2005). The addition of TMC207 to standard therapy for MDR-TB significantly reduced the time to conversion to a negative sputum culture and increased the proportion of patients with conversion of sputum culture as compared with placebo (Diacon, Pym et al. 2009). Resistance to TMC207 is mediated by mutations in the *atpE* gene encoding the transmembrane and oligomeric C subunit of ATP synthase, typically at positions 63 or 66 (Petrella, Cambau et al. 2006). However, more recent studies have shown that a majority of *in vitro*-generated mutants resistant to TMC207 lacked mutations in *atpE*, indicating alternative mechanisms of drug resistance (Huitric, Verhasselt et al. 2010).

TMC207

4.3 SQ109

SQ109 was identified by screening a large synthesized combinatorial library based on the 1,2-ethylenediamine structure of EMB, and was found to have limited toxicity and potent

activity against intracellular bacilli as well as in a murine model of chronic TB infection (Protopopova, Hanrahan et al. 2005). Early clinical data reveal the drug's potential to enhance the treatment of TB during the first 2 months of intensive therapy and also to treat MDR-TB (Lalloo and Ambaram 2010). Whether upregulation of *ahpC* expression, observed in strains resistant to INH, EMB, and SQ109, plays a role in resistance to SQ109 or merely reflects a compensatory metabolic mechanism remains to be determined (Jia, Coward et al. 2005).

Me Me H N Me N H

SQ109

4.4 Phenothiazines

The antipsychotic phenothiazine drug thioridazine has been reported to be active against drug-susceptible and drug-resistant *M. tuberculosis*, both in macrophages (Ordway, Viveiros et al. 2003) as well as in murine models (van Soolingen, Hernandez-Pando et al. 2010). Although serum concentrations above the MIC for *M. tuberculosis* (8-16 mg/L range) cannot be safely attained in humans, thioridazine still has potential as an antimycobacterial drug because of intracellular accumulation, such that concentrations inside macrophages are at least 10-fold higher than in serum. Despite the favorable toxicity profile of thioridazine relative to chlorpromazine and other phenothiazines, cardiac arrhythmia associated with prolongation of the QTc interval remains a risk. Thioridazine has been used successfully to cure patients with XDR-TB in Argentina and as salvage therapy in similar patients in India (Amaral, Boeree et al. 2010). The mechanism of action of thioridazine is likely multifactorial, as the drug appears to act on enzymes involved in fatty acid metabolism and membrane proteins, particularly efflux pumps (Dutta, Mazumdar et al. 2011), in addition to inhibiting type II NADH:menaquinone oxidoreductase as a phenothiazine (Weinstein, Yano et al. 2005). Mechanisms of *M. tuberculosis* resistance to the phenothiazines remain to be elucidated.

N N S S

Thioridazine

4.5 Oxazolidinones

Oxazolidinones are a new chemical class of synthetic antibiotics related to cycloserine with broad-spectrum activity against gram-positive pathogens through inhibition of protein synthesis.

4.5.1 Linezolid

Linezolid is the first compound belonging to the oxazolidinone class approved for clinical use. Due to its ability to penetrate macrophages, linezolid is active against intracellular bacilli, exerting its activity by binding to the ribosomal 50S subunit and thus inhibiting an early step in protein synthesis (Zhang 2005). Linezolid is most commonly used to treat drug-resistant TB, but its use has been limited by toxicity concerns, particularly hematological disturbances such as leukopenia and thrombocytopenia, as well as peripheral neuropathy, which may be irreversible. While resistance to linezolid in *M. tuberculosis* clinical isolates is rarely reported, *in vitro*-selected mutants with high-level resistance to linezolid (MIC = 16–32 mg/L) have been found to contain mutations at G2061T and G2576T in the 23S rRNA gene (Hillemann, Rusch-Gerdes et al. 2008). On the other hand, mutants with lower level linezolid resistance (MIC = 4–8 mg/L) lack mutations in the 23S rRNA gene, implicating other possible mechanisms of resistance.

Linezolid

4.5.2 PNU-100480

PNU-100480, another oxazolidinone, has been shown to have more potent activity against *M. tuberculosis* than linezolid, as the MIC of PNU100480 is half that of linezolid, and is as bactericidal as isoniazid in an acute model of TB infection in mice (Cynamon, Klemens et al. 1999). Recent studies in the mouse model have shown that the addition of PNU-100480 to the standard first-line regimen of rifampin, INH, and PZA can shorten the duration of treatment necessary to prevent relapse (Williams, Brickner et al. 2009), suggesting that this drug may have sterilizing activity against drug-susceptible and drug-resistant *M. tuberculosis*. Recent Phase I studies have shown that PNU-100480 is safe and well tolerated at all tested doses, and exhibits synergy with PZA in an ex vivo whole-blood culture assay (Wallis, Jakubiec et al. 2011). Resistance mechanisms are expected to be similar to those of linezolid.

4.5.3 AZD5847

AZD5847 was originally designed for treatment of gram-positive infections, but was later repositioned for TB treatment with the goal of improving the toxicity profile associated with linezolid, including inhibition of mitochondrial protein synthesis, thrombocytopenia, and myelosuppression (Koul, Arnoult et al. 2011). Like linezolid, AZD5847 has bactericidal activity against *M. tuberculosis* in macrophages, as well as in murine models of acute and chronic TB infection. Recent Phase I trials revealed that oral administration of the drug up to 800 mg twice daily for 14 days was satisfactorily tolerated in healthy volunteers. Although

bioavailability decreases with increasing dose, this effect may be largely compensated if taken within 2 hours of meals, and the exposures achieved in man correspond to efficacious exposures in the mouse model of TB infection (B. Subramanian, Gordon Research Conference on Tuberculosis Drug Development, July 2011). Phase 2 studies to be conducted in South Africa are in the planning stage.

4.6 Benzothiazinones

The 1,3-benzothiazin-4-ones (BTZs) represent a new class of drugs, which have activity against *M. tuberculosis in vitro*, *ex vivo*, and in murine TB models (Makarov, Manina et al. 2009). BTZs are activated in *M. tuberculosis* by reduction of an essential nitro group to a nitroso derivative, which then specifically reacts with a cysteine residue in the active site of the enzyme decaprenylphosphoryl-β-D-ribose 2'-epimerase (DprE1) (Trefzer, Rengifo-Gonzalez et al. 2010). Inhibition of this enzymatic activity abolishes the formation of decaprenylphosphoryl arabinose, a key precursor that is required for the synthesis of the cell-wall arabinans, thus causing bacterial lysis and death (Makarov, Manina et al. 2009). Although spontaneous BTZ-resistant laboratory mutants were found to have a Ser or Gly substitution at codon Cys387 of *dprE1*, resistance to BTZs has not been reported in clinical *M. tuberculosis* isolates (Pasca, Degiacomi et al. 2010). Recently, a novel resistance mechanism to BTZ was described in *M. smegmatis* involving the overexpression of the nitroreductase NfnB, which leads to the inactivation of the drug by reduction of a critical nitro-group to an amino-group (Manina, Bellinzoni et al. 2010). However, *M. tuberculosis* seems to lack nitroreductases able to inactivate BTZs.

5. Antibiotic tolerance

Antibiotic tolerance refers to the ability of nonreplicating bacteria to resist killing by cell wall-active antibiotics, which target actively multiplying organisms (Tomasz, Albino et al. 1970). This phenomenon is distinct from drug resistance (intrinsic or acquired), since it is not attributable to genetic mutations, and the organisms regain susceptibility to these antibiotics once the stress conditions have been removed and bacterial growth resumes. The prolonged duration of antibiotic treatment required to eradicate TB is believed to reflect the altered physiological state of "persistent" bacilli, which have developed tolerance to standard anti-tuberculosis drugs, particularly to isoniazid, which inhibits mycolic acid synthesis (Karakousis, Williams et al. 2008). One of the major challenges facing current TB drug development programs is to identify compounds with sterilizing activity against antibiotic tolerant "persisters", with the ultimate goal of shortening the duration of TB treatment.

6. Conclusion

The principal etiology of drug-resistant TB remains inadequate and/or incomplete treatment, including poor medical adherence to the standard treatment regimen and the addition of a single active drug to a failing drug regimen (Sharma and Mohan 2006). Given the increasing global prevalence of drug-resistant TB, it is of paramount importance to understand the mode of action of each drug as well as the molecular basis of drug resistance. Novel anti-TB drugs, which are safe, able to shorten the course of treatment,

effective against drug-resistant strains and latent TB infection, are urgently needed, especially in the era of MDR- and XDR-TB.

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10

Pyrazinecarboxylic Acid Derivatives with Antimycobacterial Activity

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1. Introduction

Tuberculosis (TB) is still one of the major causes of bacterial infections and mortality in the world. In 1944, streptomycin was introduced as a first antibiotic remedy to cure TB effectively. After that, in the 1940s and 50s, with the introduction of pyrazinamide (PZA), rifampicin (RIF), isoniazid (INH), ethambutol and streptomycin as well as cocktail-drug treatment of tuberculosis, a cure for tuberculosis was considered reasonable. This period was a period of optimism, as it was believed that TB could be cured and eliminated. Hence, the United Nations targeted elimination of TB by 2025. These expectations were dashed as a worldwide pandemic of tuberculosis began in 1987 and the World Health Organization declared that tuberculosis posed a global emergency in 1993. The estimates of the global burden of the disease caused by *Mycobacterium tuberculosis* in 2009 are as follows: 9.4 million incident cases, 14 million prevalent cases, 1.3 million deaths among HIV-negative people and 0.38 million deaths among HIV-positive people. It is estimated that among TB patients notified in 2009, 250 000 had multidrug resistant TB (MDR-TB). The situation is worsening because of the increasing incidence of single-drug resistant, multidrug resistant and extensively drug-resistant (XDR-TB) TB (WHO report, 2010; The Stop TB Partnership, 2010; Velayati et al., 2009). This chapter deals with pyrazinecarboxamide (amide of pyrazinecarboxylic acid, PZA, see Fig. 1, structure **I**) and its derivatives. PZA was introduced to clinical practice in 1952, and it has been among the first-line antituberculosis (anti-TB) drugs (Kushner et al., 1952; Solotorovsky, 1952; Steele & Des Prez, 1988).

Fig. 1. Hydrolysis of pyrazinamide (PZA, **I**) to pyrazinoic acid (POA, **II**).

PZA is an important component in the intensive phase of short-course treatment of TB owing to its sterilizing effect, ability to act in acidic environments and excellent synergy with rifampicin (Zhang & Mitchison, 2003). PZA exhibits stringent structure-activity

relationships (SAR) requiring the pyrazine ring and the carboxamide moiety (for more details see Section 2.3) for antimycobacterial activity. Pyrazine is a 6π-electron-deficient heteroaromatic compound, in which the inductive effects of the nitrogen atoms induce a partially positive charge on the carbon atoms. Pyrazine, its derivatives as well as other 1,4-diazines, *i.e.* compounds with partial pyrazine structure (e.g. quinoxaline, phenazine, pteridine, flavin and their derivatives), demonstrate unique physico-chemical properties that are caused by a low lying unoccupied π-molecular orbital and by the ability to act as a bridging ligand. Pyrazine and derivatives are known to provide a superexchange pathway for magnetic exchange (Awwadi et al., 2005). Due to these two properties 1,4-diazines, and especially their parent compound pyrazine, possess a characteristic reactivity. A fully comprehensive study of the pyrazines including reactivity and synthesis is beyond the scope of this work but can be found in the literature (Bird, 1992; Brown, 2002; Joule & Mills, 2010). Pyrazines are a class of compounds that occur almost ubiquitously in nature. Their effectiveness at very low concentrations and the ever increasing application of synthetic pyrazines in the flavour and fragrance industry and in the food and pharmaceutical industries are responsible for the high interest in these compounds (Doležal, 2006a). Tetramethylpyrazine (ligustrazine) was discovered as a natural product and is reported to scavenge the superoxide anion and decrease nitric oxide production in human polymorphonuclear leukocytes (Zhang, 2003). In plants or insects, pyrazines play the roles of attractants, pheromones and signal substances. Similar substances were found in food, and therefore their sensoric properties were investigated (Maga, 1992; Wagner et al., 1999). Mutagenic/carcinogenic pyrazines were found in processed foods, smoked-dried bonito meat, roasted coffee beans and charred eggs (Kikugawa, 2004). Synthetic pyrazines are used as "identical" additives in the food manufacturing and tobacco industries (Maga, 1992). Pyrazines are also synthesized by a number of fungi, such as the antibiotic aspergillic acid (White & Hill, 1943; Micetich & MacDonald, 1965) and the fungicidal pigment pulcherriminic acid (MacWilliam, 1959; MacDonald, 1965; Uffen Canole-Parola, 1972). The pyrazine derivative coelenterazine was found in many marine bioluminescent organisms (Thompson & Rees, 1994). Other marine pyrazine alkaloids botryllazines A and B isolated from the red ascidian *Botryllus leachi* possess interesting cytotoxic properties (Duran et al., 1999). The wide occurrence of pyrazines in nature has been already discussed (Woolfson & Rothschild, 1990; Müller & Rappert, 2010; Rajini et al., 2011). Pyrazines are also very important anthropogenic compounds, especially dihydropyrazines are essential for all forms of life due to their DNA strand-breakage activity and/or due to their influence on apoptosis (Yamaguchi, 2007). It is not surprising that many simple pyrazines have potent pharmaceutical activities. Examples are antiviral drugs flutimide (Singh & Tamassini, 2001), favipiravir (Furuta et al., 2009), the potential antineoplastic pyrazinediazohydroxide (Edelman et al., 1998) or acipimox, a niacin analogue used as a hypolipidemic agent (Ambrogi et al., 1980). The introduction of the pyrazine ring in bulky compounds brings specific chemical and physico-chemical properties for the whole molecule, like some weak basicity and aromatic character, examples are the antibacterial sulfonamide sulfadiazine (Roblin et al., 1940) or the second-generation sulfonylurea derivative with hypoglycaemic activity glipizide (Ambrogi, 1972). Such type of modification can be also found in the proteasome inhibitor for the treatment of multiple myeloma bortezomib (Adams, 1996), in the fentanyl derivative with selectivity for the μ opioid receptor mirfentanil (France et al.,

1991), in the drug for smoking cessation varenicline (Coe, 2005) or in the protease inhibitor for treatment of hepatitis C telaprevir (Furuta et al., 2009). Another examples are the diuretic amiloride and its derivative (Cragoe et al., 1964) newly also used for the treatment of cystic fibrosis, elpetrigine, a new chemical entity that combines two mechanisms known to work well in antiepileptic drugs (it is both a sodium channel and calcium channel blocker) (Foreman et al., 2008), and eszopiclone, a short acting nonbenzodiazepine sedative hypnotic (Cotrel et al., 1975). Diallylsulfide (a component of *Allium sativum*) has chemoprotective activity and pyrazine has binding affinity to cytochrome P-450 2E1 (inhibits CYP 2E1 activity), therefore, pyrazine was attached to the allylsulfide radical of diallylsulfide to form 2-allylsulfanylpyrazine to increase the binding affinity of diallylsulfide to CYP 2E1 (Kim et al., 1997). A similar pyrazine derivative, oltipraz, is an antischistosomal agent and a chemoprotective agent increasing the level of glutathione-*S*-transferases (Clapper, 1998). From this point of view the pyrazine ring can be an important part of peptide-mimicking molecules to modulate the interactions between proteins and a drug. The importance of the pyrazine (1,4-diazine) ring for biological activity can be evaluated primarily from the point of view of the molecular size/space and its physico-chemical properties. In relatively small compounds, the pyrazine ring is necessary for biological action due to its resemblance (bioisosterism) to the naturally occurring compounds (in human), for example, nicotinamide or anticancer and antiviral drugs containing the 1,4-diazine ring as position analogues of pyrimidine (1,3-diazine) nucleic bases (Doležal, 2006a).

Three main types of mycobacterial population are found in a patient with active TB, (*i*) actively growing bacilli, (*ii*) slowly growing bacilli inside macrophages, and (*iii*) the persisting semidormant bacilli localised extracellularly in solid caseous lesions. Since the pH is acidic inside the macrophages, PZA is the most active drug acting on this population followed by RIF, both drugs have crucial role in achieving sterilization (Mitchison, 1985; Zhang & Mitchison, 2003). While TB is curable, MDR-TB and XDR-TB may be fatal and the cure rates are frustratingly low. For this purpose, it is necessary to accelerate investigations to identify new types of anti-TB drugs acting on novel drug targets (Vinšová & Krátký, 2010).

2. Pyrazinamide

PZA (see Fig. 1, structure **I**) is indicated for the initial treatment of active tuberculosis in adults and children when combined with other anti-TB agents. PZA is an important sterilising tuberculosis drug that helps to shorten the duration of current chemotherapy regimens for tuberculosis. It is unique among antituberculosis drugs in having no genomic site of action and having greater bactericidal activity as bacillary metabolism slows down; it is remarkably effective in human disease. PZA is an important component in the intensive phase of short-course treatment of TB owing to its sterilising activity, ability to work in acidic environments (in macrophages), and excellent synergy with RIF (Mitchison, 1985; Zhang & Mitchison, 2003). Pyrazinamide appears to kill at least 95% of the semi-dormant bacterial population persisting in a low-pH environment since its activity is present only in the acidic environment found in active inflammation (Mitchison, 1985; Heifets et al., 1992). The development of a new drug with a similar mode of activity might be very fruitful, especially if there were no need for an acid environment (Aldrich et al., 2010). Objectives for

TB drug development are: (*i*) to shorten the total duration of effective treatment and/or significantly reduce the total number of doses needed to be taken under directly observed treatment, short-course (DOTS) supervision, (*ii*) to improve the treatment of MDR-TB, which cannot be treated with INH and RMP, and (*iii*) to provide a more effective treatment of latent TB infection (LTBI). Genomics, the systematic identification of all of the genes in a cell through deoxyribonucleic acid (DNA) sequencing and bioinformatic analysis, also offers great potential in terms of drug target discovery and development of new antibacterial agents, and the recently sequenced genome of *M. tuberculosis* should provide a number of new targets for novel drugs (Cole et al., 1998; Vinšová & Krátký, 2011).

2.1 Metabolism and toxicity of PZA

Pyrazine is a weak diacid base ($pK_1 = 0.57$; $pK_2 = -5.51$), weaker than pyridine (pyrazine $pK_a = 1.1$, pyridine $pK_a = 5.2$) due to the induction effect of the second nitrogen. The absorption of weak amine bases such as pyrazine derivatives is optimal at intestinal pH (5–7). In humans and laboratory rodents, orally administered substituted pyrazines are rapidly absorbed from the gastrointestinal tract and excreted. Methyl-substituted pyrazines are oxidized to yield the corresponding pyrazine-2-carboxylic acids. Pyrazine-2-carboxylic acid derivatives and 5-hydroxypyrazine-2-carboxylic acid derivatives are major urinary metabolites formed by side-chain oxidation and ring hydroxylation of alkyl-substituted pyrazine derivatives. PZA is hydrolyzed to pyrazine-2-carboxylic acid in humans and laboratory animals, while in other animals it can be metabolised also to 5-hydroxypyrazine-2-carboxylic acid (Sak-Bosnar & Kovar, 2005).

Early clinical studies described an unacceptable incidence of hepatotoxicity, but PZA exerts minimal toxicity when used for brief periods (preferably in six-month regimens) (Steele & Des Prez, 1988). The most frequent side effect is nongouty polyarthralgia (Zierski & Bek, 1980), and the phototoxicity of PZA has been described recently (Vargas et al., 2003).

2.2 Mechanism of action of PZA

The mode of action of PZA is unusual and has confused scientists for decades since the discovery of its antimycobacterial activity in 1952. The main reason is that PZA is completely different from common antibacterial chemotherapeutics that are primary active against growing bacteria, since PZA has no apparent activity against growing tubercle bacilli at neutral pH. PZA plays a unique role in shortening the therapy from 9–12 months to 6 months, because it kills a population of dormant/semi-dormant tubercle bacilli that are not killed by other antituberculous drugs (Mitchison, 1985). The basic knowledge about the mechanism of action of PZA was very poor for a long time. PZA as a prodrug form of pyrazinecarboxylic acid (pyrazinoic acid, POA, see Fig. 1, structure **II**) is devoid of significant antibacterial activity. PZA is activated in the mycobacterial cell by pyrazinamidase (PncA) and/or nicotinamidase (Konno et al., 1967), while its action is focused on depletion of membrane energy. Mycobacterial amidase, pyrazinamidase or nicotinamidase (nicotine deamidase, NAMase, EC 3.5.1.19), catalyzes the conversion of PZA to POA and nicotinamide to nicotinic acid (niacin) (Tarnok et al., 1979). POA is only active under acidic conditions (McDermott & Tompsett, 1954; Yamamoto et al., 1995). The cell envelope of *M. tuberculosis*, a Gram-positive bacterium with a G+ C-rich genome, contains

an additional layer beyond the peptidoglycan that is exceptionally rich in unusual free lipids, glycolipids and polysaccharides (Kolattukudy et al., 1997). The biosynthetic pathways generate cell-wall components such as mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan, and several of these may contribute to mycobacterial longevity, trigger inflammatory host reactions and act in pathogenesis. The complete genome sequence of the strain *M. tuberculosis* H37Rv was determined (Cole et al., 1998). Although 40% of protein-coding genes were functionally annotated based on sequence similarity analysis and other analysis methods, 60% of them were reported as unknown functions (Manabe et al., 2000). This situation was improved in 2002 by Camus et al., who re-annotated *M. tuberculosis*'s genome (Camus, 2002). Mycolic acid is the major constituent of the unique mycobacterial cell wall, which comprises arabinogalactan-mycolate covalently linked with peptidoglycan and trehalose dimycolate and protects the tubercle bacillus from general antibiotics and from the host's immune system.

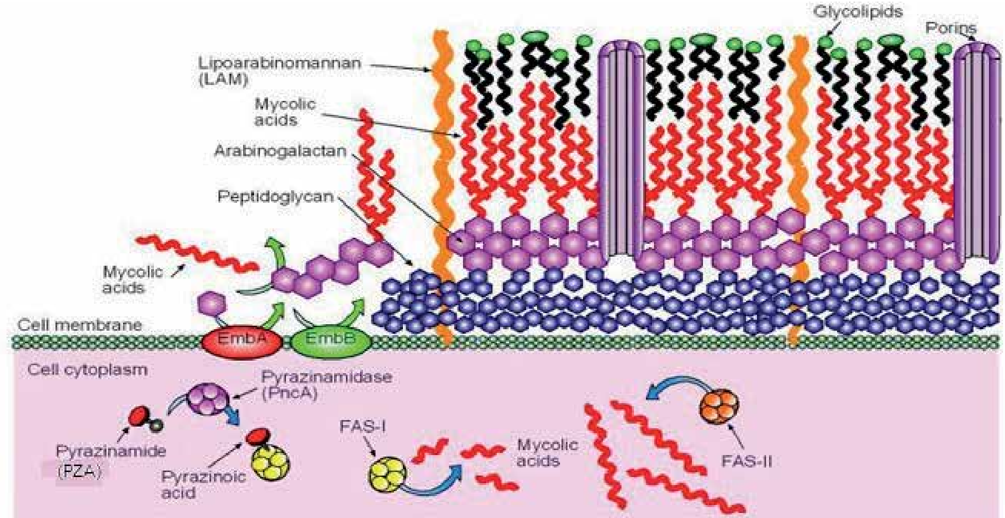

Fig. 2. Mode of action of PZA. The first-line antituberculosis drug PZA interferes with cell wall biosynthesis in *M. tuberculosis*. PZA is a prodrug and is converted to an active form (POA) by a nicotinamidase-peroxidase enzyme known as pyrazinamidase (PncA). POA inhibits the action of FAS I and FAS II. FAS I is involved in the synthesis of short-chain mycolic acids, and FAS II is involved in the synthesis of longer chains. Mycolic acids are essential structural components of the mycobacterial cell wall and are attached to the arabinogalactan layer. (Taken from Immunopeadia.org/First-Line Therapeutic Drugs in <http://www.immunopaedia.org.za/fileadmin/gallery/TB%20Drugs%20-%20Line/pyrazinamide.jpg> with the permission of the Research Administrator of the website.)

The synthesis of mycolic acids has been shown to be critical for the survival of *M. tuberculosis*; therefore, the mycolic acid pathway (MAP) has been of great interest as indicated by a large number of biochemical and genetic studies in the literature. The differences in the composition of free lipids at the cell envelope surface may result in strain-specific *M. tuberculosis-Homo sapiens* interplay modalities that contribute to determination of outcomes of the initial pathogen-host encounters as well as the infection

and disease course (Crick et al., 2008). Large fatty acids constitute a significant proportion of the mass of the cell wall core (see Fig. 2), forming a hydrophobic permeability barrier (Liu et al., 1996). The endogenous resistance of *M. tuberculosis* to many drugs (Brennan & Nikaido, 1995; Liu et al., 1999) is generally attributed to the presence of the permeability barrier generated by the mycolic acids. At least two discrete types of the enzyme system, fatty acid synthase I (FAS I, EC 2.3.1.85) and fatty acid synthetase II (FAS II), are involved in fatty acid biosynthesis in mycobacteria. The fatty acids are elongated by repeated cycles using these enzymes; generally the FAS I system is responsible for shorter chains (up to C_{16}–C_{26}) and the FAS II system, for longer chains found in meromycolates (to C_{56}) (Kolattukudy et al., 1997; Mdluli et al., 1998).

As PZA is a prodrug, hydrolytic activation by pyrazinamidase (PncA) that converts it to POA is required (Scorpio & Zhang, 1996). It was assumed that POA has both specific and nonspecific effects due to an intracellular accumulation of the liberated acid. This accumulation lowers the intracellular pH to a suboptimal level that is likely to inactivate a vital target enzyme (Zhang & Mitchison, 2003).

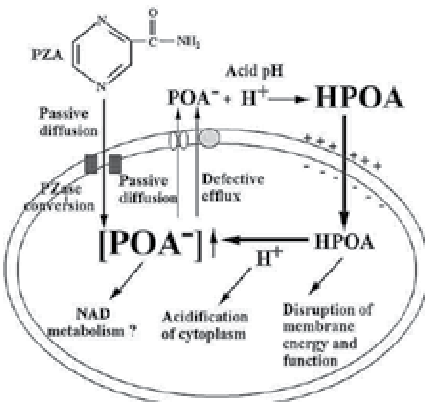

Fig. 3. Mode of action of PZA. PZA diffuses into *M. tuberculosis* in a passive transport, is converted into POA by PncA, and because of an inefficient efflux system, accumulates in huge amounts in the bacterial cytoplasm. (Ref. Wade & Zhang, 2004; reprinted with the editor's permission).

As pK_a of POA is 2.9, close to neutral intracellular pH, POA will be in the anion form and will be transported out of the cell through passive diffusion and deficient efflux to the cell surface (see Fig. 3). Under acidic pH, POA anion will form protonated or uncharged HPOA, which will diffuse back into the cell. The acid-facilitated POA influx is stronger than the weak POA efflux and this causes accumulation of POA in *M. tuberculosis* (Scorpio & Zhang, 1996). As HPOA enters the bacilli at acid pH, it brings in protons and over time could cause acidification of the cytoplasm and disruption of the proton motive force and depletion of energy. The disruption of proton motive force by POA then inhibits the membrane transport function. PZA acts as an ionophore, causing cellular acidification (Hurdle et al., 2011). Patient isolates that are resistant to PZA typically show mutation within the gene encoding PncA (Scorpio & Zhang, 1996). Some weak acids (benzoic, sorbic and propyl hydroxybenzoic acid), UV and various energy inhibitors were found to enhance the activity of PZA *in vitro* against *M. tuberculosis* (Wade & Zhang, 2006).

Raman et al. applied a systems-based approach, Flux Balance Analysis (FBA), to investigate the MAP of *M. tuberculosis* H37Rv for identification of antituberculosis drug targets in 2005 (Raman et al., 2005). The MAP model consists of four sub-pathways: (*i*) production of malonyl CoA, (*ii*) FAS I pathway, (*iii*) FAS II pathway and (*iv*) condensation of FAS I and FAS II products into methoxy- and keto-mycolic acids.

2.3 Modifications of PZA structure

The PZA and POA as potential analeptic drugs were synthesized in Germany (Dalmer & Eugen, 1936) and later in USA as intermediate compounds on the pathway of aminopyrazine synthesis (Hall & Spoerri, 1940), but the antimycobacterial activity of PZA was reported later, in 1952. The use of nicotinamide-related compounds for the therapy of tuberculosis followed the serendipitous observation that nicotinamide was effective for the treatment of murine tuberculosis (Chorine et al., 1945; McKenzie et al., 1949). The initial information that nicotinamide possessed modest antitubercular activity stimulated the evaluation of other analogues. This observation led to the subsequent discovery of not only PZA but also INH (Vinšová et al., 2008) and ethionamide. Of many nicotinamide analogues that were subsequently synthesized and evaluated for antituberculosis activity, only PZA was active *in vivo* (Deassau et al., 1952; Yeager et al., 1952; Malone et al., 1952; Solotorovsky et al., 1952). In the same year (Kushner et al., 1952) the synthesis of 49 PZA derivatives and 14 related compounds was reported, and their antimycobacterial activities in the *in vivo* murine system were briefly referred to, but no information on their *in vitro* antimycobacterial activities was provided. It was soon shown that an enzyme called nicotinamidase inside the mycobacterium hydrolyzed nicotinamide and pyrazinamide to the corresponding carboxylic acids, and these carboxylic acids were the actual active compounds (Konno et al., 1967). However, nicotinic and pyrazinoic acids did not demonstrate activity due to their poor penetration into mycobacterial cells. PZA is still unusual because of its narrow spectrum of activity. Several aminomethylene analogues/prodrugs of PZA were prepared and evaluated including morphazinamide (see Fig. 4, structure **III**), which is a morpholine derivative, Mannich base of PZA (Felder & Tiepolo, 1962); the lack of any demonstrable improvement over PZA *in vivo* led to its abandonment. Surprisingly, it was found that amides of 5-substituted pyrazinoic acid displayed good activity against *M. avium*, PZA-resistant *M. tuberculosis*, and phagocytized *M. tuberculosis*, and these compounds also showed activity over a broad range of pH values (Heifets et al., 1986). *M. avium* became a serious cause of disseminated infection among patients with AIDS. Novel aminomethylene analogues of PZA (see Fig. 4, general structure **IV**) were tested (Chung et al., 2008). The activity of *N*-(pyrrolidin-1-ylmethyl)pyrazine-2-carboxamide was higher than that of PZA when administered with rifalazil or RIF in a mouse model of infection. The activity of these analogues against PZA-resistant strains suggests that development of the second generation PZA analogues may be especially fruitful.

Fig. 4. Structures of aminomethylene analogues of PZA.

PZA exhibits remarkably stringent structure-activity relationships (SAR) demonstrating an absolute requirement for the pyrazine nucleus and the carboxamide moiety in position 2 for the activity. The modifications of the pyrazine ring with pyridazine and/or pyrimidine were not successful (Rodgers et al., 1952). It can be concluded that any isosteric replacement of carbon by nitrogen or shift of nitrogen to another position results in the loss of antimycobacterial activity. Also *ortho*-condensation of the aromatic/heteroaromatic ring with pyrazine nucleus did not lead to active compounds. Similarly, the substitution of the carboxamide group in thioamide, *N*-methyl, *N*-acetyl, hydrazide (analogy with INH), nitrile, tetrazole and free carboxylic acid provided compounds that were completely inactive *in vivo* (Kushner et al., 1952). One of the effective methods that can lead to new drug discovery is the bioisosteric replacement of a functional group. Numerous functional groups have been reported as bioisosteric replacements for the carboxylic acid functionality (Thornber, 1979). The prodrug approach in series of PZA derivatives is already very hopeful. Substituted pyrazinecarboxylic acid esters have been previously reported to have *in vitro* activity against *M. avium* and *M. kansasii* as well as *M. tuberculosis*. Modification of both the pyrazine nucleus and the ester functionality was successful in expanding the antimycobacterial activity associated with PZA to include *M. avium* and *M. kansasii*, organisms usually not susceptible to pyrazinamide. In an attempt to understand the relationship between the activities of the esters and the needed biostability, quantitative structure-activity relationships (QSAR) were found (Fernandes et al., 2010). While POA cannot pass through mycobacterial cell walls due to its low lipophilicity, the esterification of POA is a suitable approach to increase the likelihood of its penetration into the resistant mycobacteria (Cynamon et al., 1992; Cynamon et al., 1995). Thus series of POA esters were prepared and evaluated. PZA-resistant isolates became susceptible *in vitro* to pyrazinoic acid, and *n*-propyl pyrazinoate was the most promising candidate (see Fig. 5, structure **V**). Esters of POA appeared to circumvent the requirement for activation by mycobacterial amidase. The MICs of *n*-propyl pyrazinoate for *M. tuberculosis* isolates were lower than those of pyrazinoic acid. This may lead to a candidate compound with enhanced activity against both PZA-susceptible and PZA-resistant *M. tuberculosis* isolates suitable for clinical development (Cynamon et al., 1992; Speirs et al., 1995). However, efficacy studies in mice failed to show any antitubercular activity likely due to poor stability of the esters in plasma (Zhang & Mitchison, 2003). Another series of more lipophilic ester prodrugs (*i.e.* tetradecyl ester) were found to be active in concentrations 10-fold lower than those needed for PZA to kill sensitive *M. tuberculosis* and also have suitable stability in the presence of plasma (Simoes et al., 2009). These relationships are consistent with the observation that *tert*-butyl 5-chloropyrazine-2-carboxylate and 2-methyldecyl 5-chloropyrazine-2-carboxylate are 100-fold more active than PZA against *M. tuberculosis* and exhibit serum stability 900-1000 times greater than the lead compounds in the series. Some 5-hydroxypyrazine-2-carboxylic acid derivatives (see Fig. 5, general structure **VI**) are up to 1000-fold more active against *M. tuberculosis* and other *Mycobacterium* strains than existing antituberculous agents (Bergmann et al., 1996); synthesis of compounds **VI** is important, because it is a building block for the synthesis of new antituberculous agents. 5-Hydroxypyrazine-2-carboxylic acid can be produced microbiologically by whole-cell biotransformation of 2-cyanopyrazine (Wieser et al., 1997). Various substituted

analogues of PZA with bioisosteric replacements of the carboxylic acid functionality were prepared. Thus 5-aroylpyrazine-2-carboxylic acid (see Fig. 5, general structure **VII**, Doležal et al., 2003) and arylsulfanylpyrazinecarboxylic acid (see Fig. 5, general structure **VIII**, Jampílek et al., 2007) derivatives were investigated as potential antitubercular agents.

Fig. 5. Structures of pyrazine-2-carboxylic acid derivatives.

In order to find more active PZA derivatives, various PZA analogues were synthesized and assayed against *M. tuberculosis* (Yamamoto et al, 1995). In these experiments, four compounds showed high levels of antimycobacterial activities, not only bacteriostatic but also bactericidal, against *M. tuberculosis* as well as *M. avium* complex (MAC). These compounds, namely, pyrazinoic acid pivaloyloxymethyl ester, pyrazinoic acid *n*-octyl ester, pyrazinethiocarboxamide and *N*-hydroxymethylpyrazinethiocarboxamide, may warrant further examinations. 5-Chloropyrazine-2-carboxamide (see Fig. 6, structure **IX**) showed excellent *in vitro* activity against PZA-resistant strains of *M. tuberculosis* (Cynamon et al., 1998). Therefore FAS I and/or FAS II were proposed as a target of this compound, *i.e.* this compound possesses a different mechanism of action (Boshoff et al., 2002). Due to this fact 3-chloropyrazine-2,5-dicarbonitrile (see Fig. 6, structure **X**, Palek et al., 2008) and 6-chloro-5-cyanopyrazine-2-carboxamide (see Fig. 6, structure **XI**, Dlabal et al., 1990; Zitko et al., 2011) and their derivatives were synthesized, and their noteworthy antimycobacterial activities were reported recently.

3. Anti-*Mycobacterium tuberculosis* bioassays

Several *in vitro* bioassays have been developed to evaluate antitubercular activity of chemical compounds. In most of these methods, *Mycobacterium* is cultured in various types of broth- and agar-based media. But, the main problems are long growth time (several weeks) and its pathogenicity, hence containment facilities are required. The common conditions for the anti-TB susceptibility evaluation are influenced by acid-base properties of medium (McDermott & Tompsett, 1954). For nearly two decades the radiometric BACTEC 460TB System provided the most rapid method for antimicrobial susceptibility testing (Siddiqi, 1992).

The Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) was established in 1994 by the National Institute of Allergy and Infectious Diseases (a part of the National Institute of Health of the US Government) in order to encourage the discovery of

new antitubercular drugs. The program managed by Southern Research Institute (Birmingham, Alabama, USA) assisted commercial, academic and government laboratories worldwide in identifying new chemical classes of compounds for evaluation using *in vitro* and *in vivo* models. These screening activities have resulted in several promising agents that have reached advanced stages of testing. TAACF was providing a great service to mankind by way of screening thousands of compounds synthesized in research laboratories across the globe for activity against TB and related diseases. The TAACF program has ended in March 2010 (TAACF, 2010). This *in vitro* screening had several levels.

IX **X** **XI**

Fig. 6. Structures of chloropyrazine derivatives.

3.1 *In vitro* bioassays (% of inhibition)

The primary screening was conducted at 6.25 μg/mL (or molar equivalent of the highest molecular-weight compound in a series of congeners) against *M. tuberculosis* H37Rv (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA) (Collins & Franzblau, 1997). Compounds **1–91** and **104–115** were tested in the BACTEC 460-radiometric system. In general, compounds effecting >90% inhibition in the primary screening (MIC <6.25 μg/mL) were further evaluated. The results are presented in Tables 1, 2 and 4.

3.2 Primary screening (dose response): Determination of 90% inhibitory concentration (IC_{90} values)

The initial screening was conducted against *M. tuberculosis* H37Rv (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA) (Collins & Franzblau, 1997). Compounds **92-103** were tested in ten 2-fold dilutions; typically from 100 μg/mL to 0.19 μg/mL. The IC_{90} is defined as the concentration effecting a reduction in fluorescence of 90% relative to controls. This value is determined from the dose-response curve using a curve-fitting programme. Any IC_{90} value of ≤10 μg/mL is considered "active" for antitubercular activity. For the results see Table 3.

3.3 Secondary screening: Determination of mammalian cell cytotoxicity (CC_{50})

The VERO cell cytotoxicity assay was done in parallel with the TB Dose Response assay. After 72 hours exposure, viability was assessed using Promega's Cell Titer Glo Luminescent Cell Viability Assay, a homogeneous method of determining the number of viable cells in culture based on quantification of the ATP present. Cytotoxicity was determined from the dose-response curve as the CC_{50} using a curve fitting program. Ultimately, the CC_{50} was divided by the IC_{90} to calculate an SI (Selectivity Index) value. SI values of ≥10 were considered for further testing (TAACF, 2010). The results are presented in Table 2.

4. Physico-chemical properties of prepared compounds

It is generally admitted in drug research that the passage of molecules across cellular barriers increases with lipophilicity and that the most lipophilic compounds have the highest intestinal absorption. Most of the drugs behave in solution as weak acids, weak bases or sometimes as both weak acids and weak bases.

4.1 Capacity factor *k* determination (log *k* and distributive parameters π)

A Waters Alliance 2695 XE HPLC separation module and a Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA) were used. A Symmetry® C18 5 µm, 4.6×250 mm (Waters Corp., Milford, MA, USA) chromatographic column was used. The HPLC separation process was monitored by Millennium32® Chromatography Manager Software, Waters 2004 (Waters Corp., Milford, MA, USA). A mixture of MeOH p.a. (70.0%) and H_2O-HPLC – Mili-Q Grade (30.0%) was used as a mobile phase. The total flow of the column was 1.0 mL/min, injection volume 30 µL, column temperature 30 °C and sample temperature 10 °C. The detection wavelength of 223 nm was chosen. The KI methanolic solution was used for the dead time (t_D) determination. Retention times (t_R) were measured in minutes, capacity factors (*k*) were calculated. The calculated log *k* values of all compounds are shown in Tables 1, 3 and 4.

The distributive parameters π characterizing lipophilicity of individual moieties substituted on the skeleton were calculated as the differences of determined capacity factor logarithms of individual unsubstituted and substituted compounds. The calculated π values of all substituents in the amide part of the molecule are shown in Tables 1, 3 and 4.

4.2 Physico-chemical properties calculations (log *P*, log *S*, pK_a)

For calculation of the basic parameters the ACD/Labs software was used. The values of hydro/lipophilic properties expressed as log *P* were calculated by ACD/LogP DB; solubility data expressed as log *S* were calculated by ACD/LogS DB (aqueous log *S* at pH 7.4), and acid-base characteristics expressed as dissociation constants pK_a were calculated by ACD/pK_a DB. Results are shown in Tables 1, 3 and 4.

5. Antimycobacterial evaluation of substituted pyrazinecarboxamides

The amide function considered in this chapter is based on the bivalent moiety -CONH-, which can form centrosymmetric dimer pairs: (*i*) with another molecule of carboxamide (see Fig. 7, a) or (*ii*) with the carboxamide group of some peptide (see Fig. 7, b). Various compounds possessing -CONH- moiety as a bridging ligand between the heteroaromatic or basic part and the aromatic part of the molecule were found to inhibit the *M. tuberculosis*. The presented study deals with the synthesis of the series of amides prepared *via* anilinolysis of substituted pyrazinecarboxylic acid chlorides with alkylated, hydroxylated and/or halogenated anilines (see Section 5.1) or with substituted aminothiazoles (see Section 5.2). The aim of this work is to find the structure-activity relationships in the mentioned series, *i.e.* to continue studying the substituent variability influence on the biological activity, and to determine the importance of increased hydrophobic properties for biological evaluation of newly prepared substituted pyrazinecarboxamides.

Fig. 7. Possible formation of centrosymmetric dimer pairs of carboxamide group.

5.1 Substituted *N*-phenylpyrazine-2-carboxamides

An effective method that can lead to design of new drugs with higher or modified biological activity is analogy application. Pyrazine skeleton can be substituted by aliphatic, cyclic or hetero/aromatic substituents. Also hydrogens of amidic nitrogen can be substituted. It is important to note that an amidic bond (-CONH-) can mimic a peptide bond. This moiety can form hydrogen bonds to active sites of various enzymes, and therefore only one amide hydrogen should be substituted to maintain this bonding capability (namely by an aromatic (phenyl) or heteroaromatic ring in this series). This approach (radical analogy) provides multiple opportunities for further modifications; therefore a series of 115 carboxamides derived from pyrazine-2-carboxylic acid and various ring-substituted anilines and/or 2-aminothiazoles was designed and prepared. These compounds were primarily designed as potential antimycobacterial compounds, but they also showed noteworthy antifungal activity. The assumption concerning binding to different enzymes through the amide moiety was confirmed, as the mentioned compounds considerably inhibited electron transport in photosystem PS 2 during photosynthesis in spinach chloroplasts, so they can be potentially used as herbicides (Doležal & Kráľová, 2011).

The final compounds **1-103** were prepared by the anilinolysis of substituted pyrazinoylchlorides (Doležal et al., 1999, 2000, 2002, 2006b, 2007, 2008a, 2008b, 2009, 2010; their synthesis and structure are presented in Fig. 8 and in Tables 1 and 3). Their chemical structure, electronic parameters expressed as Hammett's σ parameter (Norrington et al., 1975), hydrophobic parameters (experimentally determined log *k*, experimentally determined distributive parameters π_{Ph} for individual aniline substituents, log *P* calculated by ACD/LogP DB), solubility (log *S* calculated by ACD/LogS DB), dissociation constants (pK_a calculated by ACD/pK_a DB), antituberculotic inhibiting activity and structure-activity relationships (SAR) were studied.

Fig. 8. Synthesis and structure of substituted *N*-phenylpyrazine-2-carboxamides **1-103**.

The following was synthesized in preference: (*i*) compounds with lipophilic and/or electron-withdrawing substituents on the benzene moiety (R^3), (*ii*) compounds with hydrophilic and/or electron-donating groups on the benzene part of the molecule (R^3), and

finally (*iii*) compounds with a lipophilic alkyl chain (R^2), *i.e. tert*-butyl ($-C(CH_3)_3$) and/or halogen (chlorine) substitution (R^1) on the pyrazine nucleus. The data are presented in Tables 1 and 3. The substituents on the phenyl ring were selected according to the principles set by Topliss (Topliss & Costello, 1972).

No.	R^1	R^2	R^3	Inhib. [%]	σ_{Ph}	π_{Ph}	log *k*	log *P*	log *S*	pK_a	Ref.
PZA	**see Fig. 1, structure I**			**	–	–	–	**-0.71±0.28**	**-0.76**	**13.91±0.50** **0.87±0.10**	**
1	H	H	3-CF_3	**99**	0.43	0.19	0.5585	3.05±0.54	-3.23	11.36±0.70 0.14±0.10 -4.48±0.10	b
2	Cl	$(CH_3)_3C$	4-OH	**97**	-0.37	-0.21	0.9236	3.15±0.95	-3.72	11.34±0.30 9.50±0.70 1.30±0.50 -4.79±0.10	f
3	Cl	$(CH_3)_3C$	2-OH	**96**	-0.09	-0.08	1.1148	4.04±0.69	-4.04	11.46±0.35 8.52±0.35 1.57±0.50 -4.88±0.10	f
4	Cl	$(CH_3)_3C$	3-CF_3	**95**	0.43	0.19	1.5271	5.03±0.68	-5.10	9.44±0.70 -0.25±0.10 -4.87±0.10	b
5	Cl	$(CH_3)_3C$	3-F	**88**	0.34	0.03	1.2974	3.20±0.71	-4.35	9.20±0.70 -0.30±0.10 -4.92±0.10	d
6	H	H	4-CH_3	**86**	-0.17	0.07	0.4595	2.70±0.50	-2.68	11.91±0.70 0.80±0.50 -4.27±0.10	b
7	Cl	H	3-CF_3	**77**	0.43	0.19	0.8362	3.44±0.67	-3.88	9.37±0.70 -0.84±0.50	b
8	Cl	$(CH_3)_3C$	3-OH	**75**	0.12	-0.19	0.9473	3.24±0.65	-3.76	10.54±0.10 9.09±0.10 -0.13±0.10 -4.67±0.10	f
9	Cl	H	4-$CH(CH_3)_2$	**73**	-0.15	0.37	0.9920	3.64±0.64	-3.90	9.88±0.70 0.61±0.50	d
10	Cl	$(CH_3)_3C$	4-OH-3,5-Br	**72**	0.41	0.50	1.8895	5.25±1.03	-5.34	8.84±0.70 6.56±0.36 -0.38±0.50	a
11	Cl	$(CH_3)_3C$	3,5-CF_3	**72**	0.86	0.51	1.8982	5.92±0.74	-5.95	8.90±0.70 -0.40±0.10	e
12	Cl	H	4-CH_3	**71**	-0.17	0.07	0.7627	3.10±0.64	-3.34	9.92±0.70 0.64±0.50	b
13	H	$(CH_3)_3C$	3-F	**71**	0.34	0.03	0.9588	2.91±0.59	-3.75	9.87±0.70 1.07±0.10 -3.55±0.10	d
14	H	$(CH_3)_3C$	4-$CH(CH_3)_2$	**71**	-0.15	0.37	1.3281	4.94±0.51	-4.51	10.62±0.70 1.31±0.10 -3.29±0.10	d

No.	**R¹**	**R²**	**R³**	**Inhib. [%]**	**σ_{Ph}**	**π_{Ph}**	**log *k***	**log *P***	**log *S***	**pK_a**	**Ref.**
15	Cl	H	4-OH-3,5-Br	**69**	0.41	0.50	1.1350	3.66±1.02	5.34	8.76±0.70 6.55±0.36 -0.39±0.50	a
16	Cl	H	4-Cl	**65**	0.23	0.16	0.8185	2.23±0.73	-3.37	9.04±0.70 -0.53±0.50	g
17	Cl	H	3,4-Cl	**61**	0.60	0.38	0.9950	3.60±0.81	-4.38	8.37±0.70 -1.33±0.50	a
18	H	$(CH_3)_3C$	4-CH_3	**61**	-0.17	0.07	1.0222	4.40±0.52	-3.98	10.66±0.70 1.32±0.10 -3.29±0.10	b
19	H	$(CH_3)_3C$	4-OH-3,5-Br	**54**	0.41	0.50	1.4758	4.96±0.95	-4.74	9.50±0.70 6.64±0.36 0.88±0.10 -3.75±0.10	a
20	H	$(CH_3)_3C$	3-OCH_3	**53**	0.12	-0.02	0.9146	3.71±0.52	-3.71	10.19±0.70 1.28±0.10 -3.34±0.10	a
21	H	$(CH_3)_3C$	4-$COCH_3$	**53**	0.81	-0.06	0.8573	3.34±0.53	-3.92	10.12±0.70 0.98±0.10 -3.45±0.10	h
22	H	$(CH_3)_3C$	4-OH	**50**	-0.37	-0.21	0.6779	2.86±0.87	-3.12	11.39±0.30 9.64±0.26 1.40±0.10 -3.42±0.10	f
23	Cl	H	4-F	44	0.06	0.05	0.7284	2.14±0.69	-3.29	8.89±0.70 0.19±0.50	b
24	H	$(CH_3)_3C$	3-OH	44	0.12	-0.19	0.7130	2.95±0.51	-3.15	10.79±0.70 9.16±0.10 1.24±0.10 -3.30±0.10	f
25	H	$(CH_3)_3C$	2,4-F	44	0.53	0.14	1.0992	2.99±0.68	-4.24	8.55±0.70 0.90±0.10 -3.73±0.10	d
26	H	$(CH_3)_3C$	3-Br	39	0.39	0.21	1.1516	4.45±0.57	-4.28	9.98±0.70 1.19±0.10 -3.43±0.10	e
27	H	H	2-CF_3	31	–	0.30	0.6511	2.60±0.54	-3.07	11.14±0.70 0.04±0.10 -4.59±0.10	b
28	Cl	$(CH_3)_3C$	2-OH-5-Br	30	0.30	0.17	1.5181	5.27±0.74	-4.92	10.80±0.48 7.86±0.48 0.73±0.50 -4.94±0.10	a
29	Cl	H	2-Br	28	0.71	0.40	1.0014	2.67±0.69	-3.47	8.98±0.70 -0.83±0.50	a
30	Cl	H	3-F	28	0.34	0.03	0.7135	1.61±0.70	-3.1	9.13±0.70 -0.65±0.50	d
31	Cl	H	3-CH_3	28	-0.07	0.07	0.7557	3.13±0.64	-3.35	9.92±0.70 0.24±0.50	e

No.	R^1	R^2	R^3	Inhib. [%]	σ_{Ph}	π_{Ph}	log *k*	log *P*	log *S*	pK_a	Ref.
32	H	$(CH_3)_3C$	2-OH	28	-0.09	-0.08	0.8350	3.76±0.57	-3.44	11.51±0.35 8.67±0.35 1.63±0.50 -3.51±0.10	f
33	Cl	H	2-CF_3	26	–	0.30	0.9102	3.00±0.67	-3.72	9.15±0.70 -1.33±0.10	b
34	Cl	$(CH_3)_3C$	2-Br	25	0.71	0.40	1.7673	4.26±0.70	-4.71	9.06±0.70 -0.39±0.10	a
35	H	$(CH_3)_3C$	4-F	25	0.06	0.05	0.9948	3.44±0.57	-3.94	9.63±0.70 1.20±0.10 -3.42±0.10	b
36	Cl	$(CH_3)_3C$	3-CH_3	24	-0.07	0.07	1.3288	4.71±0.65	-4.59	9.99±0.70 0.25±0.50 -4.57±0.10	e
37	Cl	$(CH_3)_3C$	4-Cl	24	0.23	0.16	1.5015	3.82±0.78	-4.62	9.11±0.70 -0.26±0.10 -4.88±0.10	g
38	H	$(CH_3)_3C$	3-CF_3	23	0.43	0.19	1.1338	4.74±0.55	-4.50	10.11±0.70 1.12±0.10 -3.50±0.10	b
39	Cl	$(CH_3)_3C$	3-OCH_3	23	0.12	-0.02	1.2148	4.00±0.65	-4.31	9.53±0.70 -0.09±0.10 -4.70±0.10	a
40	H	H	2-OH-5-Cl	22	0.28	0.12	0.4541	3.71±0.75	-3.23	11.45±0.70 8.12±0.48 0.93±0.50 -4.67±0.10	f
41	H	$(CH_3)_3C$	2-Br	22	0.71	0.40	1.4057	3.97±0.57	-4.11	9.72±0.70 0.98±0.10 -3.64±0.10	a
42	Cl	$(CH_3)_3C$	4-$COCH_3$	22	0.81	-0.06	1.1676	3.63±0.66	-4.52	9.46±0.70 -0.39±0.10 -4.82±0.10	h
43	Cl	$(CH_3)_3C$	2,4-F	21	0.53	0.14	1.4793	3.28±0.78	-4.84	7.88±0.70 -0.05±0.50	d
44	Cl	$(CH_3)_3C$	3-Br	20	0.39	0.21	1.5502	4.73±0.70	-4.88	9.32±0.70 -0.18±0.10 -4.80±0.10	e
45	Cl	H	3-Br	19	0.39	0.21	0.8452	3.15±0.69	-3.64	9.24±0.70 -0.75±0.50	e
46	H	$(CH_3)_3C$	2-OCH_3	18	0.00	0.04	0.9928	4.17±0.56	-3.87	10.02±0.70 1.28±0.10 -3.34±0.10	e
47	Cl	$(CH_3)_3C$	2-OH-5-Cl	17	0.28	0.12	1.4529	5.69±0.87	-5.14	10.93±0.48 7.99±0.48 0.78±0.50	f
48	Cl	H	2-Cl-5-OH	15	0.79	0.11	0.7801	1.78±0.69	-3.06	10.09±0.18 8.60±0.70 -1.09±0.50	b

No.	R^1	R^2	R^3	Inhib. [%]	$σ_{Ph}$	$π_{Ph}$	log *k*	log *P*	log *S*	pK_a	Ref.
49	H	$(CH_3)_3C$	3,4-Cl	15	0.60	0.38	1.3395	4.90±0.78	-5.01	9.11±0.70 0.83±0.10 -3.80±0.10	a
50	Cl	$(CH_3)_3C$	4-F	15	0.06	0.05	1.3238	3.72±0.70	-4.54	8.97±0.70 0.19±0.50 -4.79±0.10	b
51	H	H	3-Cl	14	0.37	0.14	0.4914	3.24±0.65	-3.22	11.24±0.70 0.09±0.10 -4.53±0.10	g
52	Cl	H	3-Cl	14	0.37	0.14	0.7864	3.63±0.77	-3.88	9.24±0.70 -0.71±0.50	g
53	Cl	H	4-OH	14	-0.37	-0.21	0.5587	1.56±0.95	-2.46	11.33±0.30 9.43±0.70 1.30±0.50	f
54	Cl	H	3-OH	13	0.12	-0.19	0.5758	1.65±0.64	-2.49	10.54±0.10 9.08±0.10 -0.17±050	f
55	Cl	$(CH_3)_3C$	2,6-CH_3	13	0.20	0.20	1.5286	4.23±0.66	-4.56	10.00±0.70 1.45±0.50 -4.74±0.10	e
56	Cl	H	3,5-CF_3	12	0.86	0.51	1.1384	4.33±0.71	-4.77	8.82±0.70 -1.39±0.10	e
57	Cl	H	4-$COCH_3$	11	0.81	-0.06	0.6548	2.04±0.65	-3.29	9.38±0.70 -1.37±0.10	h
58	Cl	H	3,5-OCH_3	11	0.24	0.08	0.7701	2.70±0.66	-3.32	8.99±0.70 -0.43±0.50	a
59	H	$(CH_3)_3C$	3-CH_3	11	-0.07	0.07	1.0191	4.43±0.52	-3.99	10.65±0.70 1.41±0.10 -3.20±0.10	e
60	H	$(CH_3)_3C$	3,5-CF_3	10	0.86	0.51	1.4861	5.63±0.62	-5.35	9.56±0.70 0.96±0.10 -3.66±0.10	e
61	H	H	3,4-Cl	8	0.60	0.38	0.7162	3.20±0.78	-3.72	10.36±0.70 -0.15±0.10 -4.78±0.10	g
62	H	$(CH_3)_3C$	2-CF_3	7	–	0.30	1.2522	4.30±0.55	-4.34	9.89±0.70 1.02±0.10 -3.61±0.10	b
63	H	$(CH_3)_3C$	2-OH-5-Cl	6	0.28	0.12	1.0803	5.40±0.75	-4.53	10.97±0.48 8.14±0.48 0.94±0.10 -3.69±0.10	f
64	Cl	$(CH_3)_3C$	2-CF_3	6	–	0.30	1.6560	4.58±0.68	-4.94	9.22±0.70 -0.35±0.10 4.97±0.10	b
65	H	$(CH_3)_3C$	3,5-OCH_3	5	0.24	0.08	1.0286	4.00±0.54	-3.93	9.73±0.70 1.16±0.10 -3.46±0.10	a

No.	R^1	R^2	R^3	Inhib. [%]	σ_{Ph}	π_{Ph}	log *k*	log *P*	log *S*	pK_a	Ref.
66	H	H	4-Cl	4	0.23	0.16	0.4987	1.84±0.60	-2.72	11.03±0.70 0.13±0.10 -4.49±0.10	g
67	Cl	$(CH_3)_3C$	4-$CH(CH_3)_2$	4	-0.15	0.37	1.7297	5.22±0.65	-5.10	9.96±0.70 0.62±0.50 -4.66±0.10	d
68	Cl	H	3-OCH_3	2	0.12	-0.02	0.6671	2.41±0.64	-3.08	9.45±0.70 -0.17±0.50	a
69	H	$(CH_3)_3C$	2,6-CH_3	2	0.20	0.08	1.1387	3.94±0.52	-3.96	10.67±0.70 1.51±0.50 -3.37±0.10	e
70	Cl	H	2,4-F	1	0.53	0.14	0.8164	1.69±0.73	-3.59	7.81±0.70 -0.05±0.50	d
71	H	H	4-F	0	0.06	0.05	0.4416	*	*	*	b
72	H	H	2,6-Cl	0	1.34	0.32	0.6656	*	*	*	g
73	H	H	2-Cl-5-OH	0	0.79	0.11	0.4527	*	*	*	b
74	Cl	H	2-OH	0	-0.09	-0.08	0.6447	*	*	*	f
75	Cl	H	2-CH_3	0	0.10	0.13	0.7774	*	*	*	e
76	Cl	H	2,6-CH_3	0	0.20	0.20	0.8451	*	*	*	e
77	Cl	H	2,6-Cl	0	1.34	0.32	0.9696	*	*	*	g
78	Cl	H	2-OH-5-Br	0	0.30	0.17	0.8305	*	*	*	a
79	H	$(CH_3)_3C$	2-CH_3	0	0.10	0.13	1.0984	*	*	*	e
80	H	$(CH_3)_3C$	3-Cl	0	0.37	0.14	1.0996	*	*	*	g
81	H	$(CH_3)_3C$	4-Cl	0	0.23	0.16	1.1043	*	*	*	g
82	H	$(CH_3)_3C$	2-OH-5-Br	0	0.30	0.17	1.1070	*	*	*	a
83	H	$(CH_3)_3C$	2-Cl-5-OH	0	0.79	0.11	1.0654	*	*	*	b
84	H	$(CH_3)_3C$	2,6-Cl	0	1.34	0.32	1.2802	*	*	*	g
85	Cl	$(CH_3)_3C$	2-CH_3	0	0.10	0.13	1.4772	*	*	*	e
86	Cl	$(CH_3)_3C$	3-Cl	0	0.37	0.14	1.4896	*	*	*	g
87	Cl	$(CH_3)_3C$	4-CH_3	0	-0.17	0.07	1.3305	*	*	*	b
88	Cl	$(CH_3)_3C$	2-Cl-5-OH	0	0.79	0.11	1.4335	*	*	*	b
89	Cl	$(CH_3)_3C$	2,6-Cl	0	1.34	0.32	1.6631	*	*	*	g
90	Cl	$(CH_3)_3C$	3,4-Cl	0	0.60	0.38	1.7563	*	*	*	a
91	Cl	$(CH_3)_3C$	3,5-OCH_3	0	0.24	0.08	1.3564	*	*	*	a

Table 1. Structures, antimycobacterial evaluation (% of inhibition), logarithms of capacity factors (log *k*), calculated lipophilicity (log *P*), solubility (log *S*) and acid-base properties (pK_a) of compounds **1-91** in comparison with the standard (PZA). Electronic parameters are expressed as Hammett's σ parameter (Ref. Norrington et al., 1975). Compounds are ordered according to their decreasing inhibiting activity (Ref Doležal et al., 2008[(a)], 1999[(b)], 2000[(c)], 2006b[(d)], 2002[(e)], 2010[(f)]; Kutilová, 2007[(g)]). *MIC = 16-50 µg/mL at pH 5.5 (Ref. McDermott & Tompsett, 1954) or MIC = 100 µg/mL at pH 6.0 by the BACTEC method (Ref. Siddiqi, 1992). **ACD values were not calculated for activity 0%.

It can be drawn from Table 1 that growth inhibition of *M. tuberculosis* strain H37Rv at 6.25 µg/mL ranged 0-99%: 0% activity was shown by 21 compounds, 1-49% activity was possessed by 48 compounds and 50-99% activity was shown by 22 compounds, which will be primarily discussed below. According to pyrazine substitution, all compounds in Table 1

can be divided into 4 groups: (*i*) ***series I*** – unsubstituted pyrazine core; (*ii*) ***series II*** – pyrazine substituted by chlorine in position 6 (R^1 = Cl); (*iii*) ***series III*** – pyrazine substituted by *tert*-butyl in position 5 (R^2 = *tert*-Bu); (*iv*) ***series IV*** – pyrazine substituted by chlorine in position 6 and by *tert*-butyl in position 5 (R^1 = Cl and R^2 = *tert*-Bu). When the compounds with the activity above 50% are divided into the individual series, ***series I*** contains 2 compounds with average activity 93%, ***series II*** contains 6 compounds with average activity 69%, ***series III*** contains 7 compounds with average activity 59%, and ***series IV*** contains 7 compounds with average activity 85%. According to this analysis it can be stated that the most favourable is $C_{(5)}$ and $C_{(6)}$ disubstitution of pyrazine R^1 = Cl and R^2 = *tert*-Bu, *i.e.* ***series IV***, followed by unsubstituted pyrazine (***series I***) and $C_{(6)}$ monosubstitution R^1 = Cl (***series II***). The least favourable is $C_{(5)}$ monosubstitution of pyrazine by *tert*-Bu, *i.e.* ***series III***. Particular substituents on benzenes can be ordered according to their efficiency. Benzene substitutions 2,6-Cl, 2-X-5-OH, 2-OH-5-X, 4-X, 3-alkyl, 3-X and 2-CH_3 (X = halogen) appear to be the least advantageous. The most advantageous is substitution 3-CF_3 followed by 4-CH_3, 4-$CH(CH_3)_2$, 3-F and 3,5-CF_3. In all cases this is substitution in *meta* and/or *para* positions, mostly by lipophilic substituents (experimentally determined distributive parameters π_{Ph} = 0.03–0.51 with optimum π_{Ph} = 0.07–0.19). Moieties *meta*-CF_3 and 3-F are electron-withdrawing substituents with σ = 0.43 or σ = 0.34, whereas substituents 4-CH_3 or 4-$CH(CH_3)_2$ with σ = -0.17 or σ = -0.15 are significantly electron-donating.

Substituents with hydrophilic groups on the benzene part of the molecule did not show any inhibiting activity. However, it is worth mentioning that only in ***series IV*** substitutions 4-OH and 2-OH are the most favourable, thus with markedly hydrophilic π_{Ph} = -0.21 or π_{Ph} = -0.08 as well as electron-donating substituents with σ = -0.37 or σ = -0.09. Generally, it can be stated that the optimum lipophilicity value range is log *k* = 0.6–1.5 or log *P* = 1.6–6.8, but as regards antitubercular activity (see Table 1), it can be concluded that lipophilicity is only a secondary parameter, facilitating penetration through highly lipophilic mycobacterial wall. According to Table 1 and log *S* (solubility values) it can be stated that with lower aqueous solubility (lower value of log *S*), antimycobacterial inhibition decreases, except ***series I***. The dissociation constant pK_a is also a very important parameter. According to Table 1 it can be generally stated that higher pK_a (basicity close to PZA) supports higher antitubercular activity.

The selected anilides **1-4** that expressed the highest antitubercular activity are illustrated in Table 2. As mentioned in Section 3, the most effective compounds were tested for their cytotoxicity, and subsequently the Selectivity Index, *i.e.* the ratio of cell cytotoxicity (CC_{50}) to activity (MIC) was obtained. It can be drawn from Table 2 that compound **1** from ***series I*** showed the highest inhibition activity and also minimal toxicity. According to MIC it can be stated that compound **4** seems to be more effective than compound **1**, but unfortunately its determined SI value was not as favourable as in the case of compound **1**. Based on these observations it can be concluded that *N*-[3-(trifluoromethyl)phenyl]pyrazine-2-carboxamide (**1**) can be considered to be a promising agent.

Compounds **92-103** mentioned in Table 3 showed a broad spectrum of antitubercular activity, but these activities are not expressed in concrete numbers, unlike inhibition activities in Table 1, therefore no thorough structure-activity relationships could be

established. Nevertheless, with regard to this fact, it is possible to propose at least general SAR based on the above-discussed observations.

In terms of PZA substitution ***series IV*** is the most advantageous, and the most favourable substitution on the benzene ring is 3-I-4-CH_3. All effective compounds listed in Table 2 possess high lipophilicity; the most active compound **92** has log *k* about 1.9 or log *P* about 6, which enables good penetration to mycobacterial cell. Calculated solubility is relatively low, but under the testing conditions it was probably sufficient, so there was no precipitation during testing.

No.	R^1	R^2	R^3	Inhib. [%]	MIC (μg/mL)	CC_{50} (μg/mL)	SI	Ref.
1	H	H	3-CF_3	**99**	6.25	>62.5	**>10**	a
2	Cl	$(CH_3)_3C$	4-OH	**97**	<12.5	-	-	b
3	Cl	$(CH_3)_3C$	2-OH	**96**	<12.5	-	-	b
4	Cl	$(CH_3)_3C$	3-CF_3	**95**	3.13	>10	>3.2	a

Table 2. Structures, primary antimycobacterial evaluation (% of inhibition), actual minimum inhibitory concentrations (MIC), cytotoxicity (CC_{50}), and selectivity indices (SI) of compounds **1-4** (Ref. Doležal et al., 2008[(a)], 1999[(b)]).

No.	R^1	R^2	R^3	IC_{90} [μg/mL]	σ_{Ph}	π_{Ph}	log *k*	log *P*	log *S*	pK_a
PZA	**see Fig. 1, structure I**			**>20 ***	**–**	**–**	**–**	**-0.71±0.28**	**-0.76**	**13.91±0.50** **0.87±0.10**
92	Cl	$(CH_3)_3C$	3-I-4-CH_3	**0.819**	0.18	0.56	1.9783	6.10±0.71	-5.42	9.43±0.70 -0.11±0.50 -4.92±0.10
93	Cl	$(CH_3)_3C$	4-CF_3	**20.703**	0.74	0.23	1.5611	4.39±0.68	-4.87	9.27±0.70 -0.41±0.10
94	H	H	3-I-4-CH_3	**69.099**	0.18	0.56	0.8118	4.12±0.58	-3.56	11.35±0.70 0.10±0.10 -4.53±0.10
95	H	H	4-CF_3	>100	0.74	0.23	0.6220	2.41±0.54	-3.00	11.19±0.70 -0.02±0.10 -4.63±0.10
96	H	H	2-Br-3-CH_3	>100	0.64	0.63	0.8784	3.22±0.57	-3.32	10.98±0.70 0.17±0.10 -4.45±0.10
97	Cl	H	4-CF_3	>100	0.74	0.23	0.8536	2.81±0.67	-3.65	9.20±0.70 -1.39±0.10
98	Cl	H	2-Br-3-CH_3	>100	0.64	0.63	1.2118	3.62±0.70	-3.98	8.98±0.70 -0.65±0.50
99	Cl	H	3-I-4-CH_3	>100	0.18	0.56	1.1529	4.52±0.70	-4.21	9.35±0.70 -0.11±0.50
100	H	$(CH_3)_3C$	4-CF_3	>100	0.74	0.23	1.1606	4.11±0.55	-4.27	9.93±0.70 0.96±0.10

No.	R[1]	R[2]	R[3]	IC_{90} [μg/mL]	σ_{Ph}	π_{Ph}	log *k*	log *P*	log *S*	pK_a
										-3.65±0.10
101	H	$(CH_3)_3C$	3-I-4-CH_3	>100	0.18	0.56	1.5766	5.82±0.59	-4.82	10.09±0.70 1.08±0.10 -3.55±0.10
102	H	$(CH_3)_3C$	2-Br-3-CH_3	>100	0.64	0.63	1.6327	4.92±0.59	-4.60	9.72±0.70 1.15±0.10 -3.47±0.10
103	Cl	$(CH_3)_3C$	2-Br-3-CH_3	>100	0.64	0.63	2.0821	5.20±0.71	-5.19	9.06±0.70 -0.22±0.10 -4.84±0.10

Table 3. Structures, antimycobacterial evaluation (% of inhibition), logarithms of capacity factors (log *k*), calculated lipophilicity (log *P*), solubility (log *S*) and acid-base properties (pK_a) of compounds **92-103** in comparison with the standard (PZA). Electronic parameters are expressed as Hammett's σ parameter (Ref. Norrington et al., 1975). Compounds are ordered according to their decreasing inhibiting activity (Ref. Doležal et al., 2009).
*MIC = 16-50 μg/mL at pH 5.5 (Ref. McDermott & Tompsett, 1954) or MIC = 100 μg/mL at pH 6.0 by the BACTEC method (Ref. Siddiqi, 1992).

According to pK_a these compounds are little less basic than PZA, which results in lower ionizability and higher penetration to the cell. Both types of active anilides have unsubstituted $C'_{(2)}$ position of benzene, which is also very important for easy anilide hydrolysis and release of active POA. It can be assumed that antimycobacterial activity is dependent on balanced lipo/hydrophilic properties and ionizability tending to the PZA value, but also it is strongly influenced by the facility of anilide hydrolysis to acid, which is facilitated by a free *ortho* position of the benzene ring and electron-withdrawing effect of substituents.

5.2 Substituted *N*-(thiazol-2-yl)pyrazine-2-carboxamides

A unique series of ring-substituted *N*-(thiazol-2-yl)pyrazine-2-carboxamides **104-115** is shown in Fig. 9. This series was prepared by the same method as used for substituted *N*-phenylpyrazine-2-carboxamides **1-103**. Their physico-chemical properties and their inhibition of *M. tuberculosis* H37Rv at 6.25 μg/mL are listed in Table 4. It can be concluded that contrary to benzene analogues, *tert*-Bu (***series III***) or Cl+*tert*-Bu (***series IV***) is more advantageous than PZA substituent. The substitution by methyl in position 5 of the 2-aminothiazole ring is unambiguously the most advantageous. The activity of the most active compound **104** is comparable with the activity of compound **18**; but in general the series 5-CH_3 (**104**, **105**, **112**, **113**) possesses lower activity than 4-CH_3 anilides (**6**, **12**, **18**, **87**). 5-Methylthiazolyl pyrazinecarboxamides expressed higher effect than 3-CH_3 (**31**, **36**, **59**) and

Fig. 9. Synthesis of substituted *N*-(thiazol-2-yl)pyrazine-2-carboxamides **104-115**.

also than 2-CH_3 (**75**, **79**, **85**) anilides. In comparison with anilide analogues, 2-aminothiazoles possess little higher lipophilicity, lower solubility but considerably lower basicity, which in combination with heteroatoms of a thiazole moiety presumably influences the facility of hydrolysis ability and consequently, the resulting activity. Experimentally determined distributive parameters π_{Th} for methylthiazolyl substituents are presented in Table 4.

No.	R^1	R^2	R^3	Inhib. [%]	π_{Th}	log *k*	log *P*	log *S*	pK_a
PZA	**see Fig. 1, structure I**			**	**–**	**–**	**-0.71±0.28**	**-0.76**	**13.91±0.50** **0.87±0.10**
104	H	$(CH_3)_3C$	5-CH_3	**65**	0.14	1.0670	4.50±0.28	-4.66	4.35±0.70 1.98±0.10 -0.67±0.10
105	Cl	$(CH_3)_3C$	5-CH_3	**61**	0.14	1.4425	5.23±0.36	-5.29	3.69±0.70 1.77±0.10 -2.04±0.10
106	Cl	$(CH_3)_3C$	4-CH_3	**52**	0.12	1.4174	5.22±0.36	-5.29	3.69±0.70 1.80±0.10 -2.04±0.10
107	H	$(CH_3)_3C$	H	47	0	0.8987	4.20±0.27	-4.44	4.35±0.70 1.50±0.10 -0.68±0.10
108	Cl	$(CH_3)_3C$	H	42	0	1.2282	4.93±0.36	-5.08	3.69±0.70 1.28±0.10 -2.05±0.10
109	H	$(CH_3)_3C$	4-CH_3	35	0.12	1.0446	4.49±0.28	-4.65	4.35±0.70 2.01±0.10 -0.67±0.10
110	Cl	H	H	32	0	0.6064	3.34±0.27	-4.09	3.61±0.70 1.26±0.10 -3.03±0.10
111	Cl	H	4-CH_3	21	0.12	0.7068	3.63±0.28	-4.29	3.62±0.70 1.78±0.10 -3.02±0.10
112	Cl	H	5-CH_3	15	0.14	0.7213	3.64±0.28	-4.29	3.62±0.70 1.74±0.10 -3.02±0.10
113	H	H	5-CH_3	10	0.14	0.6193	2.80±0.27	-3.58	5.61±0.70 1.96±0.10 -1.65±0.10
114	H	H	H	0	0.00	0.5565	*	*	*
115	H	H	4-CH_3	0	0.12	0.6112	*	*	*

Table 4. Structures, antimycobacterial evaluation (% of inhibition), logarithms of capacity factors (log *k*), calculated lipophilicity (log *P*), solubility (log *S*) and acid-base properties (pKa) of substituted *N*-(thiazol-2-yl)pyrazine-2-carboxamides **104-115** in comparison with the standard (PZA). Electronic parameters are expressed as Hammett's σ parameter (Ref. Norrington et al., 1975). Compounds are ordered according to their decreasing inhibiting activity (Ref. Doležal et al., 2006b). *MIC = 16-50 μg/mL at pH 5.5 (Ref. McDermott & Tompsett, 1954) or MIC = 100 μg/mL at pH 6.0 by the BACTEC method (Ref. Siddiqi, 1992). **ACD values were not calculated for activity 0%.

Based on the above-mentioned results and discussion, it can be concluded that for high antitubercular activity $C_{(5)}$ and $C_{(6)}$ disubstitution of the pyrazine ring by lipophilic moieties is advantageous. Substitution of amidic hydrogen by ring-substituted anilines is more advantageous than by heteroaromatics, for example substituted pyrazine-2-carboxylic acid thiazol-2-ylamides showed only medium or moderate activity, and ring-substituted pyrazine-2-carboxylic acid pyridin-2-ylamides (non-mentioned in this chapter) did not express any antimycobacterial activity (Osička, 2003). High antitubercular efficiency requires substitution in *para* or preferentially in *meta* position of benzene, not in *ortho* position. Two combinations of substituents seem to be favoured: (*i*) lipophilic together with electron-withdrawing effect (3-CF_3, 3-F) or (*ii*) expressively hydrophilic together with electron-donating effect (phenolic moiety), $C_{(5)}$ and $C_{(6)}$ disubstituted lipophilic pyrazine being strongly needed for the phenolic type of substituents. The same as PZA, the discussed amides are prodrugs that possess modified absorbency/permeability due to substitution, and after penetration through mycobacterial wall the amides are probably hydrolyzed by intracellular amidase to POA. The sufficient chemical stability of the mentioned compounds in slightly acidic environment used in testing is important for high antitubercular activity as well as subsequent facile hydrolysis by mycobacterial amidase. It seems that electron-withdrawing substituents in *meta* and/or *para* positions provide adequate stability to the amidic bond and simultaneously do not protect this bond against the attack of amidase. No primary physico-chemical parameter that would obviously influence antitubercular inhibitory activity of the discussed substituted pyrazine-2-carboxamides was found. Apart from the balanced combination of secondary parameters (e.g. lipophilicity, solubility, substituent electronic parameters), a high enzymatic hydrolysis rate seems to be fundamental for rapid acidification of mycobacterial cytoplasm (by POA) or inhibition of some other cellular vital enzymatic systems by means of generated reactive intermediates (e.g. aminophenols, fluorinated anilines).

6. Future research

In the recent past, drug discovery efforts shifted towards the drug design based on docking studies. These docking computational techniques allow investigating the possible binding modes of a substrate to a given receptor, enzyme or another binding site and consequently determining and identifying the precise or different mechanism of action of both PZA and its derivatives, e.g. 5-chloropyrazine-2-carboxamide (**IX**) and similar compounds. Therefore the priority is to isolate the enzymes (from *M. tuberculosis* and subsequently from cells of other *Mycobacterial* strains) responsible for metabolization/activation of PZA and other enzymes that can be influenced by POA generation, e.g. FAS I, FAS II, etc. After isolation and determination of 3D structure by X-Ray structural analysis, it will be needed to crystallize enzymes with PZA and other PZA derivatives, determine 3D structures of these complexes and develop 3D-pharmacophore for systematic virtual screening based on this process. Then it will be possible to evaluate all PZA-like derivatives based on their virtual binding simulation and to carry out their evaluation using a large set of distance-based topological indices. In addition, various molecular descriptors can be used.

As most pyrazine derivatives seem to be prodrugs and their activity is strongly dependent on the rate of hydrolysis to POA, further *in vitro* experiments with isolated enzymes should

be focused on determination of the hydrolysis rate. Also compounds of interest will be subject to *in vivo* studies for determination of their efficacy against murine *M. tuberculosis*.

7. Conclusion

PZA is a cornerstone drug of current TB therapy and emerged as an important building block for regimens with promise to shorten TB treatment. PZA is a prodrug which must be activated by the *M. tuberculosis* enzyme pyrazinamidase within the bacterium in order to exert its antitubercular activity. The project was focused on discovery of PZA analogues with PZA-like efficiency characteristics along with improved potency and increased safety. The main task was search for new antimycobacterial pyrazines – structure analogues of PZA. In summary, 115 compounds were synthesized and screened for their antimycobacterial activity in the project. Chemical synthesis was followed by structure confirmation, experimental lipophilicity determination (log *k*) and theoretical lipophilicity calculation (log *P*). Biological evaluation comprised of antimycobacterial activity screening as the main task. This biological part of the project was successfully implemented in co-operation between TAACF, Southern Research Institute, Birmingham, USA, and the Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Czech Republic, in years 1996—2009. The most active compounds are presented (see Tables 1-4) and discussed. Several very potent compounds (**1-4**, **92**, **93**) were discovered. The compounds relieved into level 2 testing underwent MIC and CC_{50} determination followed by Selectivity Index calculation (SI, ratio of measured CC_{50} to MIC). To be relieved to level 3 (*in vivo* screening) the compound had to exhibit SI>10. Only one of the presented structures, *N*-[3-(trifluoromethyl)phenyl]pyrazine-2-carboxamide (**1**) was relieved to level 3. The *in vivo* screening of compound **1** was not finished yet. The results of this project could be a very good starting point for the advanced drug design and development of new antituberculous agents based on pyrazine.

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The Potential Therapeutic Usage of Dithiocarbamate Sugar Derivatives for Multi-Drug Resistant Tuberculosis

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1. Introduction

After the discovery of streptomycin (SM) and isonicotinic acid hydrazide (INH), the number of tuberculosis (TB) patient dramatically decreased. People believed that TB was already a past-disease. However, World Health Organization (WHO) reported that there were an estimated 9.4 million incident cases of TB globally in 2009, which is equivalent to 137 cases per 100 000 population (World Health Organization, 2010). There are huge difference in perception of TB and the actual situation of TB. The reason for the misunderstanding of current status of TB could come from low rate of TB crisis. After infection with TB, only 5 % people develop the disease within 1~2 year, and 5 % of the remaining develop within their life time. Fortunately, mot people live their life without crisis of TB (Koul et al., 2011).

The typical treatment of TB is now proposed by WHO. The protocol is named as direct observation treatment short course (DOTS), but the period of treatment is not “short”. The period is at least 6 months, which is not “short” compared to the therapeutic period using antibiotics against common infectious diseases. Although, the regimen of TB treatment is the most powerful chemotherapy in the world.

In 2006, we were surprised to hear of the outbreak in South Africa (Cohen, 2006). The case reports recall the worst event, “Spanish Flu”, in 18th century. The name of TB hailed around the world due to the emergence of extremely multi-drug resistance TB (XDR-TB) which caused the abnormally rapid death of human immunodeficiency virus (HIV)-positive patients suffering from XDR-TB (Koenig, 2008). The life time of TB patients without chemotherapy is usually more than 2 years, but in the case of the HIV-positive XDR-TB patient, their lifetimes were within 1 month, like “Spanish Flu”. We feared the out break of “New Type TB” in the World. Subsequently, TB cases classified under XDR-TB had already

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spread around the world. Many investigations suggest that the incredible virulence of XDR-TB could depend on the status of health condition of patient, that is, those having acquired immunodeficiency syndrome (AIDS) or not. This event, "South African Shock", gave us a warning that continuous development of new TB drugs is needed.

The development of strong medicines against TB has progressed at a snail's pace since 1970's (Ma et al., 2010). The derivatives or analogs of currently used TB drugs faced the problem of cross resistance to formerly developed drugs. It is obvious, because these kinds of drug share the same or similar targets, hence, the similar mechanism of escaping from the attack of antibiotics is observed. Therefore, development of "New Face Drug against TB" is strongly desired.

2. Discovery of sugar derivatives as anti-tubercular compounds

2.1 Background

The genus *Mycobacterium* belongs to actinobacteria and consists of mycobacteriaceae including pathogenic pieces *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Goodfellow & Mage, 1998). The meanings of Latin prefix "myco" is "fungus" and also "wax". The cell wall contains huge amount of waxy compounds, of which weight is around 60 % of dried bacilli (Rao & Meena, 2011). The cell wall also consists of peptidoglycan which binds to arabinogalactan chains. *Mycobacterium* species have unique immunogenic sugar lipids compounds in their cell walls, such as trehalose-6,6'-dimycolate (TDM), alias name is cord factor, etc. (Berg et al., 2007; Ryll et al., 2001; Kaur et al, 2009) Trehalose is a natural alpha-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units, which is seen in cell wall of fungi, plants and bacteria(Nehls, 2008). It has high water retention capabilities implicating in anhydrobiosis (Kaushik & Bhat, 2003). Arabinose is synthesized from phosphoribosyl pyrophosphate (pRpp) derived from glucose through hexose monophosphate shunt and used as a substrate of arabinobgalactan (AG) structure in *Mycobacterium* (Crick et al., 2004). Decaprenyl phosphate is transferred to pRpp by a transferase, and forms 5-phospho-decaprenylphospho-ribose (5-pDpR). 5-pDpR is dephosphorylated, and become DpR. DpR is changed to decaprenyl arabinose (DpA) by an epimerase, and then DpA is transferred to AG by an arabinosyltransferase. Recently, the arabinose synthesis pathway is receiving plenty of attention as new drug target of developing new TB drugs (Wolucka, 2008; Manina, 2010). Ethambutol (EB), a first line TB drug, shows the anti-tubercular activity by inhibiting *Emb* enzymes in mycobacteria. Recently, Besra *et al.* indicated that EB bind to the C-terminal region of *EmbC* (Alderwick et al., 2011). The single knockout of *EmbC* and *EmbB* was lethal in *M. tuberculosis*, but not in *M. smegmatis* and *Corynebacterium glutamicum* (Amin et al., 2008; Goude et al., 2008). Thus, these enzymes and the structure catalyzed by *Emb* enzymes are crucial to *M. tuberculosis*.

As seen above, carbohydrate moieties are crucial for the bacilli. So, we expect any damage on the sugar containing structures could destroy the bacilli, we have searched the anti-tubercular activity with random screening method from the sugar based chemical libraries. The library consisted of various substrates and donors of which the sugar chains were modified. After screening more than 200 compounds, 2 compounds showed the positive results.

2.2 OCT359, allyl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1->6)-O-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1->6)-O-2,3,4-tri-*O*-acetylb-D-glucopyranoside

One of the sugar compounds is OCT359 which is obtained from a plant root, *Stachys sieboldi* Miq (Chiba et al., 2007). The plant root possess huge amount of tetrasaccharide, stachyose, consisting of two α-D-galactose units, one α-D-glucose unit, and one β-D-fructose unit sequentially linked as gal(α1→6)gal(α1→6)glc(α1↔2β)fru. The name stachyose is originated from the name of the species, *Stachys sieboldi* Miq. OCT359, allyl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1->6)-O-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1->6)-O-2,3,4-tri-*O*-acetylb-D-glucopyranoside, is obtained when stachyose is hydrolyzed and acetylated. The minimum inhibitory concentration (MIC) of OCT359 to *M. tuberculosis* is 3.13 μg/ml. It is comparable to the MICs of aminoglycoside antibiotics, such as streptomycin and kanamycin, and amycacin. OCT359 is effective not only against *M. tuberculosis*, but also *Mycobacterium avium, Staphylococcus aure*us including MRSA. However, OCT359 is not effective to *Escherichia coli.* So, the antibacterial spectrum of OCT359 seems to be limited to gram-positive bacilli. The structure is very unique and not observed in the cell wall of the bacilli. It would be difficult to use the compound as a substrate to synthesize cell wall components. Hydrophobic property of the compound is critical to show the activity. Other mechanism by which the compound, inhibit bacterial metabolisms, is by inhibiting enzymes in the cell and waxy cell walls, because all hydroxyl groups of OCT359 are acetylated. The mechanism of antibacterial activity of OCT359 has not been elucidated sufficiently. Our preliminary data suggested that OCT359 includes metal molecules and work as inclusion compound. OCT359 is effective to drug resistant bacilli, MDR-TB and MRSA, therefore, it could be potential novel compound for TB drugs.

2.3 OCT313, 2-acetamide-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate

Another sugar derivative, OCT313 (Horita et al., 2009); 2-acet-amido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (DMDC), is the derivative of *N*-acetyl-D-glucosamine (GlcNAc), which is a monosaccharide derivative of glucose. GlcNAc is significantly available in several biological systems (Moussian, 2008). Peptidoglycan in a bacterial cell wall consists of GlcNAc and *N*-acetylmuramic acid (MurNAc), cross-linked with oligopeptides at the lactic acid residue of MurNAc (van Heijenoort, 2001). OCT313, GlcNAc-DMDC, shows the antibacterial activity to slow growing *Mycobacterium* species, *M. tuberculosis* and *M. bovis*, however, weak activity to other *Mycobacterium* species, *Mycobacterium avium* and *Mycobacterium smegmatis*. Furthermore, OCT313 does not show the antibacterial activity to *S. aureus* and *E. coli*. This character is favorable to use for TB therapy.

As the bacteriolytic effect of OCT313 on the bacilli is observed, the first mode of action of OCT313 is a cell wall of *M. tuberculosis*. OCT313 also have bactericidal activity. The dithiocarbamate group at C-1 position of the glucopyranoside ring of OCT313 was requisite for the antibacterial activity, and *N*-acetylation is also required to show the activity. The substitution of dithiocarbamate lead to loss of antibacterial activity and dithiocarbamate exhibits the antibacterial activity. Thus, the main body of the activity is dithiocarbamate. The acetyl group at C-2 of OCT313 was substituted by, either propyl, butyl, benzyl or oleic acid groups. According to a length of the fatty acid chain, the antibacterial effect changes. So, acetyl group is optimum as a carbon chain at C-2 position. The meaning of *N*-acetylation

to the antibacterial activity of OCT313 is not still clear. It may be involved in the localization of the compound in the cell wall of the bacilli by changing the liquid phase of the compound from hydrophobic to hydrophilic.

In order to investigate the target of OCT313 on the bacilli, the drug resistant clones were made. During the production of the resistant clones, it was revealed that the production of drug resistance to OCT313 is very low, 10^{-7}. This character is very important to prevent from the emerging drug resistant clones during the therapy. The precise targets of OCT313 on the bacilli are now under investigation. OCT313 is effective to MDR-TB clinical isolates to the same extent as the drug susceptible TB clinical isolates. There is no cross resistance of OCT313 with other currently used TB drugs. As the TB regimen includes more than two drugs which have different modes of antibacterial actions, this character is very important when developing new drugs.

A dithiocarbamate is an analog of carbamate in which both oxygen atoms are replaced by sulfur atoms. The primary and secondary amines react with carbon disulfide to form dithiocarbamates. One of characters of dithiocarbamates is ligands for chelating metals (Jones et al., 1980). Dithiocarbamates readily forms complex with many metal salts such as copper, ferrous, ferric, cobaltous, and nickel salts. The diethyldithiocarbamate ion chelates to metals via the two sulfur atoms. Oxidation of sodium diethyldithiocarbamate gives the disulfide, also called a tetraethylthiuram disulfide (Dalvi, 1988), which is marketed as an anti-alcoholism drug labeled as Antabuse and Disulfiram (Barth & Malcolm, 2010). Other more complicated bonding modes of dithiocarbamates are known to be a unidentate ligand and a bridging ligand using one or both sulfur atoms. The carbon length of dithiocarbamate at C-1 position of OCT313 was changed, and then, the effect of these synthesized compounds on the antibacterial activity was investigated. Methyl group has most strong activity among them. The target of OCT313 is not yet clear, however, the activities of binding to enzymes and/or chelating metals could be critical to show the anti-mycobacterial activities.

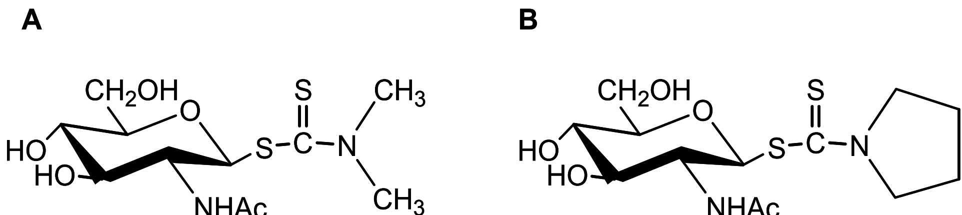

Fig. 1. Dithiocarbamate sugar derivatives. A: OCT313; 2-acetamide-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate, B: OCT313HK: 2-acetamido-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-carbodithioate.

The advantages of sugar conjugating dithiocarbamates are reducing their toxicity and making narrow anti-bacterial spectrum. We have previously analyzed the toxicity of dimethyldithiocarbamate (DMDC) and its sugar derivative, OCT313. The toxicity of OCT313 to human cell lines was 100 times lesser than that of DMDC. Furthermore, our unpublished data indicate that administration of OCT313 to mice was not toxic and tolerable to inhibition of cholinesterase, which is mostly known to be side effect of carbamate compounds (Thorn, 1967).

In summary, the derivative of *N*-acetyl glucosamine, OCT313, could be new drug target of MTB and low frequency of emerging drug resistance. The procedure of sugar conjugating compounds is useful to reduce their toxicity.

2.4 OCT313HK, 2-acetamido-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-carbodithioate

Dimethyldithiocarbamate (DMDC), a functional moiety of OCT313 at C-1 is revealed to be critical for the anti-mycobacterial activity (Horita et al, 2009). The bactericidal and fungicidal effects of dihiocarbamate and thiuram disulfide were patented by Tisdale and Williams in 1934 (Wilson & Fishbein, 1972). Many studies have revealed that the antimicrobial activities of DMDC and diethyldithiocarbamate (DDC) since 1942 (Gordon, 1942; van Raalte, 1952; Thorn & Ludwig, 1962). DMDC has the antifungal activity to *Fusarium roseum* by inhibiting oxidation pathway of α-keto-glutamic acid, because the augmentation of α-keto-glutamic acid in the fungus was observed by treatment of ferric dimethyldithiocarbamate (Ferbam) (Sisler & Marshall, 1957). The drug targets of DMDC or DDC in *Penicillium*, and *Aspergillus* were suggested to be the enzymes at oxidation pathway of α-keto-glutamic acid or pyruvate pathway coupling with α-lipo acids or acetyl-CoA, since the augmentation of pyruvate in the organisms was observed by the treatment of DMDC or DDC (Kaars & Van der Kerk, 1956). Intriguingly, zinc dimethyldithiocarbamate (Ziram) is able to inhibit the metabolic pathway of keto-acid, however, does not affect the synthesis or metabolic pathway of citric acid (Dimond et al., 1941). Furthermore, thiuram, tetramethylthirum disulfide, which is dimer of DMDC, has strong growth inhibitory activity to yeast under anaerobic environment (Manten, 1950). This mode of action of dithiocarbamates is considered to be an inhibition of respiratory chain in yeast. DMDC was also reported to have a growth inhibitory activity on *Saccharomyces cerevisiae* by inhibiting a synthesis of acetyl-CoA (GoksØyr, 1955). These data suggest that dithiocarbamates can inhibit different metabolic pathways in each organism. The precise target of dithiocarbamates on mycobacterium is not been revealed until the present.

Many studies of physiological activities of dithiocarbamates in mammals and human were reported (Taylor et al., 1987; Shah et al., 1997; Kang et al., 2008). And the effects of them against pathogenic bacteria, fungi and parasite were also reported (Erol et al., 1995; Cascio et al., 1996; Adachi et al., 1997; Nagano et al., 1997; Ohtake et al., 1998; Le Quellec et al., 1996; Weuffen et al., 1967a, 1967b). Majority of the predictable targets of dithiocarbamates against these organisms is considered to inhibit metal containing enzymes by their activities of chelating metals or enzymes by bind covalently to thiol group of cysteine residues (Taylor et al., 1987; Shah et al., 1997; Kang et al., 2008). Structure-activity-related studies of dithiocarbamate against bacteria were reported (Chabrier et al., 1956; Miller & Elson, 1949). When dithiocarbamates were written as $X(Y)NCS_2M$ (X=hydrogen or alkyl, Y=hydrogen, alkyl or aryl, M=metallic in nature), the strong order of antibacterial activity at X(Y)N position was piperizyl>$(CH_3)_2N$>morpholinyl. $(CH_3)_2N$ also had anti-fungal activity.

In the 1950's, the effect of dithiocarbamates on *Mycobacterium* was reported (Liebermeister, 1950; Schraufstätter, 1950; Garattini & Leonardi, 1955; Jeney & Zsolnai, 1956). Recently, Makarov *et al.* and Byrne *et al.* reported that DDC and pyridine dithiocarbamate (PDTC) had anti-tuberculosis activity against dormant stage of *M. tuberculosis* (Makarov et al., 2006; Byrne et al., 2007). In order to improve the activity of OCT313 (GlcNAc-DMDC), DMDC at C-1 position of OCT313 was substituted by PDTC, which was designated as OCT313HK (GlcNAc-PDTC) (Horita et al., 2011). The MIC of OCT313HK against *M. tuberculosis* became 4 times

lower than that of OCT313. Interestingly, antibacterial spectrum of PDTC became narrow by conjugating with GlcNAc. The antibacterial activity of OCT313HK is specific to slow growing *Mycobacterium*, such as *Mycobacterium bovis* and *M. tuberculosis*, but not to *Mycobacterium avium* and *Mycobacterium smegmatis*. OCT313HK is not effective to *Escherichia coli* and *Staphylococcus aureus*. Furthermore, those compounds have bactericidal and bacteriolytic activity to the bacilli. The analysis of the resistance clones of BCG for OCT313 and OCT313HK predict that the first mode of action of those compounds is a cell wall of the bacilli. Our preliminary experiment showed that OCT313 and OCT313HK can inhibit mycobacterial enzyme involved in the cell wall synthesis, however, DMDC and PDTC have different mechanisms. These data suggest that sugar conjugated dithiocarbamate have different targets of inhibition on cell wall consisting enzymes in mycobacteria from dithiocarbamate.

In summary, GlcNAc conjugated DMDC and PDTC would have novel drug targets in *Mycobacterium* species. It is desirable that the antibacterial spectrum of OCT313HK is specific to slow growing mycobacteria, because of the mal-effect of long term therapy with anti-tubercular drugs on an indigenous bacterial flora. Both OCT313 and OCT313HK are effective to multi drug resistance (MDR) including extremely multi drug resistance (XDR) of *M. tuberculosis*, thus, cross resistance with currently used anti-tubercular drugs. In the animal study using chronic infection model of tuberculosis, OCT313 reduced bacterial number in lung. The cytotoxicity of dithiocarbamates on human cell lines is reduced by conjugating to sugar. Therefore, sugar conjugated dithiocarbamates could be useful leading compounds to develop anti-mycobacterial drugs.

3. Future view of sugar conjugated dithiocarbamates

Nowadays, the proper usage of antibiotics and the drug dosage regimens following Pharmacokinetics / Pharmacodynamics (PK/PD) theory are emphasized on medication for infectious disease (Mouton et al., 2011; Vaddady et al., 2010). In the case of treatment of tuberculosis these consensuses are applicable. Furthermore, drug interaction with other medications should be a major concern at developing new drugs and usage of anti-TB drugs. As diarylquinoline TMC-207, an ATP synthase inhibitor, which was discovered by Tibotec, the drug metabolism catalyzed by CYP enzymes is also critical in the regimen (Matteelli, 2010). The excellent characters of the potential lung transfer, enhanced permeability and retention effect of drugs in lung are important factors for drugs late into anti-TB treatment. Dithiocarbamates have potentially inhibitory effect of both mammal and bacterial enzymes (Taylor et al., 1987; Shah et al., 1997; Kang et al., 2008; Erol et al., 1995; Cascio et al., 1996; Adachi et al., 1997; Nagano et al., 1997; Ohtake et al., 1998). Those are also effective to fungi (Le Quellec et al., 1996; Weuffen et al., 1967a, 1967b). It is very interesting to note that the drug target of DMDC or PDTC is changed when conjugated with GlcNAc. This is a very unique observation of sugar conjugated dithiocarbamates derivatives. Our preliminary studies indicated that OCT313 had post antibiotic effect on *Mycobacterium*, and the sufficient drug retention in the lung was observed. The effect of metabolic enzymes on OCT313 and OCT313HK is not known. The interaction of these drugs to other TB drugs including developing drugs should be investigated. Moreover, the route of administration is unclear. The innovation of rapid accumulation system to achieve the sufficient drug concentration in lung is also preferable.

PDTC is also reported to have anti-viral activity against human immunodeficiency virus through inhibiting NF-κB (Schreck et al. , 1992; Pande & Ramos , 2003; Bai et al., 2008).

OCT313HK has not been investigated for the anti-viral effect on HIV. Possibly, sugar conjugated PDTC will be leading compounds for the treatment of both TB and AIDS.

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12

Fighting Against Resistant Strains: The Case of Benzothiazinones and Dinitrobenzamides

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1. Introduction

The resurgence of tuberculosis is ascribed to several factors such as: large scale migration, co-infection with immunodeficiency virus, and the emergence of *Mycobacterium tuberculosis* drug-resistant strains.

Drug-resistant tuberculosis is a growing global health problem. This has led to an increased urgency to understand the molecular mechanisms of drug action and drug resistance, which could give significant insight into the development of new compounds. New molecules should be useful to fight both drug-susceptible as well as drug-resistant strains (Caminero et al., 2010).

It is noteworthy that the development of drug-resistant *M. tuberculosis* strains is essentially favored by an inappropriate drug prescription of physicians and irregular intake of the drugs by patients (Goldman et al., 2007). Bacterial resistance to antibiotics typically involves drug inactivation or modification, target alteration, diminished drug accumulation associated with decrease in permeability and/or increase in efflux. Other resistance mechanisms include inhibition of the activation of pro-drugs into active drugs. Bacterial resistance may be an intrinsic feature of an organism, or may result from spontaneous mutations or the acquisition of exogenous resistance genes. As *M. tuberculosis* does not possess plasmids and horizontal gene transfer is thought to be rare, all resistances appear to emerge through mutations in chromosomal genes (Gillespie, 2007). Chromosomal alterations that result in resistance to antitubercular drugs may be associated with a fitness cost. Based on work in experimental models and from observations in clinical drug resistant isolates, it has been observed that among the various resistance mutations that appear with similar rates, those associated with the least fitness cost are selected in the population (Böttger and Springer, 2008). Consequently, to reduce the rate of spread of resistant bacteria it is necessary to identify targets for which the resistance mechanisms have the most negative effects on bacterial fitness. *M. tuberculosis* shows a high degree of intrinsic resistance to several antibiotics and chemotherapeutic agents attributed to the low permeability of its cell wall, in particular because of its specific lipid-rich composition and structure (Jarlier and Nikaido, 1994). However, some reports have suggested that efflux pumps may also be involved (De Rossi et al., 2006). The balance between the drug transport into the cell and drug efflux is not yet clearly understood, and further studies are required in

mycobacteria (De Rossi et al., 2006). The development of mycobacterial resistance to antibiotics has co-evolved over time with the discovery of antitubercular drugs. Indeed, *M. tuberculosis* mutants resistant to any single drug were identified and most of them characterized.

Drugs utilized to treat tuberculosis are classified into first-line (isoniazid, rifampicin, pyrazinamide, and ethambutol) and second-line agents (quinolones, aminoglycosides, linezolid, ethionamide, and *D*-cycloserine). The commonly used antitubercular regimen is based on four first-line agents: isoniazid, rifampin, pyrazinamide, and ethambutol, for the first two months, followed by rifampin and isoniazid treatment for a further four months, as recommended by the World Health Organization.

Strains of *M. tuberculosis* that are resistant to both isoniazid and rifampicin, with or without resistance to other drugs, named multidrug-resistant strains (MDR-TB), require a further two years of treatment with second-line drugs such as: quinolones, aminoglycosides, ethionamide, *D*-cycloserine and basic peptides. The emergence of Extensively drug-resistant tuberculosis (XDR-TB), caused by MDR-strains also resistant to two major second-line agents (aminoglycosides and fluoroquinolones), greatly alarmed the World Health Organization. These resistant strains have been identified in all regions of the World, most often in Asia and countries of the former Soviet Union (Caminero et al., 2010).

Beside the problem of XDR-TB cases, which remains unresolved in many regions of the World, new totally drug-resistant strains (TDR), or super XDR-TB isolates, have been reported. *M. tuberculosis* isolates are defined TDR if they are resistant to all first-line (isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide) and second-line drugs (quinolones, aminoglycosides, linezolid, ethionamide, and *D*-cycloserine) (Velayati et al., 2009). Generally, the spectrum of resistance reflects the drugs that the patients have used and the way in which therapy was controlled.

The treatment of tuberculosis becomes more complicated as the antibiotic resistance profile of *M. tuberculosis* broadens. MDR and XDR tuberculosis are generally thought to have high mortality rates; for TDR-TB there are no drugs available. In fact, with the exception of the fluoroquinolones, no new antitubercular drug has been introduced in therapy in the past 45 years (Caminero et al., 2010).

In recent years, the increasing concern for drug resistance has hurried the need for the development of new control measures. At present, the identification of new drugs and new cellular targets is prioritised (Riccardi et al., 2009).

Presently, ten compounds with antitubercular activity have entered clinical trials (Lenaerts et al., 2008; Riccardi et al., 2009), while other promising ones are still in pre-clinical development (Lienhardt et al., 2010). This has been prompted since the availability of the *M. tuberculosis* genome sequence (Cole et al., 1998), when several Laboratories in the World have utilized the genomic data to identify and validate targets as starting point for the development of new antitubercular drugs. Genomic sequence information allows also the use of comparative genomic analysis to identify new potential targets, and provides some assurance against mammalian toxicity if homologous proteins are absent from mammalian sequence databases. Sequence similarities can also give useful hints on putative protein functions. Transposon mutagenesis and signature-tagged mutagenesis have been used to

identify essential *M. tuberculosis* genes (Lamichhane et al., 2005; Sassetti et al., 2003; Sassetti and Rubin, 2003). The functional categories to which these genes belong are: lipid metabolism; carbohydrate, amino acid, inorganic ion and nucleotide transport and metabolism; energy production and conversion; secretion; cell envelope biosynthesis; cell division; DNA replication, recombination and repair; transcription and translation; post-translational modification; chaperones; coenzyme metabolism; signal transduction. These essential mycobacterial genes could encode good targets for tuberculosis drug development. Another intriguing field of research concerns the metabolism of several molecules, important for bacterial survival.

The use of protein structure data to design molecules, that are most likely to interact with inhibitable proteins, constitute another field of research, aimed to create novel classes of *ad hoc* inhibitors able to interact with a bacterial protein and block its function. Thanks to the efforts of the Tuberculosis Structural Genomics, more than 260 X-ray crystal structures of interesting proteins have been completed (Chim et al., 2011). The availability of these structures provides the opportunity to carry out virtual screenings for drug discovery. Virtual screening can be used to identify compounds that are consistent with a pharmacophore model, without considering the target/s or to develop inhibitors of a protein, on a known three-dimensional structure. The pharmaceutical industry has favoured this target-based approach to drug discovery. While successful in some cases, this high throughput screening approach has failed miserably in the antibacterial discovery area (Payne et al., 2007; Fischbach and Walsh, 2009). Also in our own experience, target-based screens generate hits but these usually fail to show useful minimum inhibitory concentrations against *M. tuberculosis.* The reasons for this collective failure remain unclear but may include the inability of many synthetic compounds to enter bacteria and find their target, the presence of efflux systems or other innate resistance mechanisms.

For tuberculosis drug discovery the research process has often begun either *via* phenotypic screening of compound libraries against *M. tuberculosis* or a surrogate organism, such as *Mycobacterium smegmatis* or *Mycobacterium bovis* BCG growing *in vitro* culture, or against a target of interest. This effort has created the pipeline of new candidate drugs at various stages of preclinical and early clinical evaluations (Lenaerts et al., 2008; Lienhardt et al., 2010; Riccardi et al., 2009).

In the last five years, within the European Community network, the “New Medicines for Tuberculosis” consortium (to which our laboratory belongs) has been working to successfully develop new drugs for the treatment of tuberculosis through an integrated approach (http://www.sciprom.ch/nm4tb/). The group has just obtained new funding from the European Community with the project “More Medicines for Tuberculosis”. Development and implementation of novel enabling technologies required for drug development, target validation in well-established areas such as the central metabolism, cell wall and nucleic acid synthesis, in addition to more challenging yet highly innovative topics, are the major expected results of the network. Within this project, a new effective antimycobacterial agent, belonging to the nitrobenzothiazinone class and with a minimal inhibitory concentration of 1 ng/ml, that quickly kills *M. tuberculosis in vitro*, *ex vivo* and in murine models of tuberculosis, has been discovered (Makarov et al., 2009). The lead compound benzothiazin-4-one (BTZ038) has a single chiral center, and both enantiomers, BTZ043 (S) and BTZ044 (R), were found to be equipotent *in vitro*. This drug is also active

against extensively- and multi-drug resistant clinical isolates, and it is in the pre-clinical trial phase. A huge amount of genetic and biochemical data indicates that DprE1 is the target of benzothiazinones (Makarov et al., 2009). DprE1 enzyme works in concert with DprE2 to catalyse the epimerization of decaprenyl-D-ribose to decaprenyl-D-arabinose in the biosynthesis of arabinogalactan, a fundamental component of mycobacterial cell wall (Wolucka, 2008). The DprE1 enzyme thus represents a proven vulnerable antimycobacterial drug target that could turn out magic for tuberculosis treatment (Manina et al., 2010b). In order to monitor the potential development of benzothiazinone-resistance, a total of 240 sensitive and MDR clinical isolates from four European hospitals were surveyed for the presence of mutations in the *dprE1* gene and for benzothiazinone susceptibility. All 240 strains were susceptible, thus establishing the baseline prior to the introduction of BTZ043 in clinical trials (Pasca et al., 2010).

Moreover, it has been shown that DprE1 is the target also of another class of very promising drugs, the dinitrobenzamides (Christophe et al., 2009). These compounds are active against mycobacteria and non toxic for the host cell, and they have been identified through the screening of chemicals which interfere with *M. tuberculosis* replication within macrophages (Christophe *et al*., 2009). In particular, the dinitrobenzamide derivatives were found to be highly active against *M. tuberculosis*. Two compounds were selected, N-(2-(4-methoxyphenoxy) ethyl)-3,5-dinitrobenzamide and N-(2-(benzyloxy) ethyl)-3,5-dinitrobenzamide, for further studies and target identification. Analysis of the broad antimicrobial spectrum was undertaken and revealed that the effect of these dinitrobenzamide derivatives has the most potent activity observed against mycobacteria with a minimal inhibitory concentration of 75 ng/ml. Of particular importance, these compounds were also highly active against MDR- and XDR-TB clinical isolates. In order to gain insight into the possible targets of dinitrobenzamides, the effect of these chemicals on the lipid composition of the cell envelope of *M. tuberculosis* was investigated; results showed that they affected the synthesis of the arabinan domains of arabinogalactan and lipoarabinomannan. In particular, analyses revealed complete inhibition of decaprenyl-phospho-arabinose formation in the dinitrobenzamide-treated extract concurrent with the accumulation of decaprenyl-phospho-ribose, indicating that the target of both dinitrobenzamide inhibitors is probably the heteromeric decaprenyl-phospho-ribose 2′ epimerase.

Our group also identified and characterized a novel resistance mechanism to benzothiazinones in *M. smegmatis* (Manina et al., 2010a), and this mechanism was confirmed also for dinitrobenzamides (M.R. Pasca, personal communication). The over-expression of the nitroreductase NfnB led to the inactivation of the drug by reduction of a critical *nitro*-group to an *amino*-group. The direct involvement of NfnB in the inactivation of the lead compound BTZ043 was demonstrated by enzymology, microbiological assays and gene knockout experiments. The crystal structure of NfnB and docking analysis of NfnB-benzothiazinones have been performed in order to understand their interaction and the mechanism of nitroreduction (Manina et al., 2010a). Although *M. tuberculosis* seems to lack nitroreductases able to inactivate these drugs, our findings are valuable for the design of new benzothiazinones, which may be more effective *in vivo*.

In conclusion many approaches are utilized to fight tuberculosis. The two principal research routes to find out new antibacterial molecules and novel bacterial targets are from drug to

target and from target to drug. Until now the first one appears to be the most easily attainable, leading to the discovery of new molecules which are currently in clinical trials and the last published benzothiazinones and dinitrobenzamides. However, we should remember that there is a high attrition rate during clinical trials before a candidate is approved for human use and resistance to new compounds will eventually arise, so drug discovery efforts should be intensified.

2. Benzothiazinones

The class of Benzothiazinones (BTZs) comprises a series of sulfur-containing heterocycle compounds with antibacterial and antifungal activity (Makarov et al., 2006). One of BTZs, 2-[2-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-8-nitro-6-(trifluoromethyl)-4H-1,3 benzothiazin-4-one (BTZ038), was selected for further studies (Figure 1; Makarov et al., 2009).

Fig. 1. **Chemical structure of the lead compound BTZ038.**

This compound was synthesized in seven steps with a yield of 36%. Structure activity relationship work showed that the sulfur atom and the nitro group at positions 1 and 8, respectively, were critical for activity. BTZ038 has a single chiral center, and both enantiomers, BTZ043 (S) and BTZ044 (R), were found to be equipotent *in vitro*.

The minimal inhibitory concentrations (MICs) of a variety of BTZs against different mycobacteria were very low, ranging from ~0.1 to 80 ng/ml for fast growers and from 1 to 30 ng/ml for members of the *M. tuberculosis* complex. The MIC of BTZ043 against *M. tuberculosis* H37Rv and *M. smegmatis* were 1 ng/ml (2.3 nM) and 4 ng/ml (9.2 nM), respectively (Makarov et al., 2009), indicating that this compound is more potent than isoniazid (500 ng/ml). BTZ043 displayed similar activity against all clinical isolates of *M. tuberculosis* that were tested, including MDR and XDR strains (Makarov et al., 2009).

BTZ043 is bactericidal, reducing viability *in vitro* by more than 1000-fold in under 72 hours.

The uptake, intracellular killing, and potential cytotoxicity of BTZ compounds in an *ex vivo* model using a high-content screening approach (Fenistein et al., 2008), in order to monitor macrophages infected with *M. tuberculosis* expressing the green fluorescent protein, were determined. Macrophages treated with BTZ043 were protected as compared with those treated with the negative controls (Makarov et al., 2009).

The *in vivo* efficacy of BTZ043 was assessed 4 weeks after a low-dose aerosol infection of BALB/c mice in the chronic model of tuberculosis. Four weeks of treatment with BTZ043 reduced the bacterial burden in the lungs and spleens by 1 and 2 logs, respectively, at the

concentrations used. Additional results suggested that BTZ efficacy is time- rather than dose-dependent (Makarov et al., 2009).

To find the target for BTZ, two independent genetic approaches were employed. Firstly, cosmids bearing DNA from *M. smegmatis* that confer increased resistance on *M. smegmatis* were identified and the region responsible was pinpointed by subcloning. This approach revealed that the *MSMEG_6382* gene of *M. smegmatis* or its *M. tuberculosis* ortholog, *Rv3790*, mediated increased BTZ resistance. Secondly, *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* mutants with a high-level of BTZ resistance were isolated and characterized. All these resistant mutants harbored missense mutations in the same gene (Makarov et al., 2009).

Biochemical studies showed that *Rv3790* and the neighboring gene *Rv3791* code for proteins that act in concert to catalyze the epimerization of decaprenylphosphoryl-D-ribose (DPR) to decaprenylphosphoryl-D-arabinose (DPA) (Mikusova et al., 2005), a precursor for arabinan synthesis without which a complete mycobacterial cell wall cannot be produced (see the paragraph below).

In all of the drug-resistant mutants we examined, the same codon of Rv3790 (or DprE1) was affected: cysteine at position 387 was replaced by serine or glycine codons, respectively. The BTZ resistance–determining region of *dprE1* was highly conserved in orthologous genes from various Actinobacteria, except that in a few cases cysteine (387 codon) was replaced by serine or alanine. The corresponding bacteria, *Mycobacterium avium* and *Mycobacterium aurum*, were found to be naturally resistant to BTZ thus supporting the identification of DprE1 as the BTZ target (Makarov et al., 2009; Figure 2).

Further corroboration was obtained biochemically by using membrane preparations from *M. smegmatis* to catalyze the epimerization reaction from radiolabeled DPR precursor, which was produced *in situ* from 5-phosphoribose diphosphate (Mikusova et al., 2005), in the presence or absence of BTZ. Addition of BTZ abolished the production of DPA from DPR. The reaction requires both DprE1 and DprE2. Furthermore, when the highly BTZ-resistant mutant of *M. bovis* BCG, or *M. smegmatis* were used as sources of enzymes, epimerization was no longer subjected to inhibition, thereby confirming identification of the BTZ target (Makarov et al., 2009).

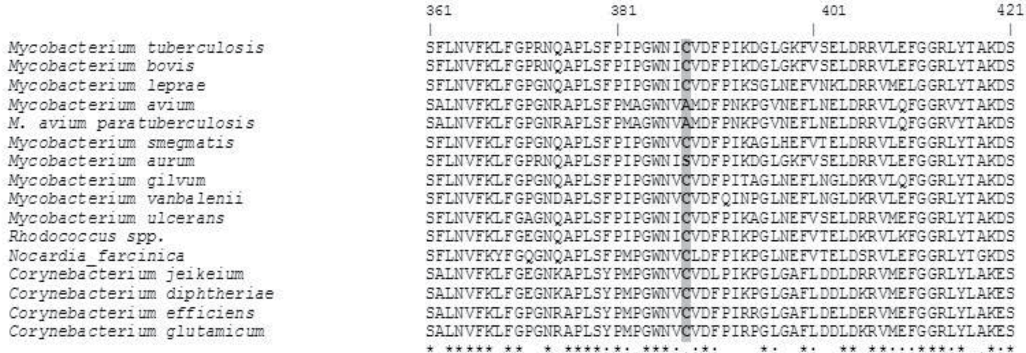

Fig. 2. **Multiple alignment of the BTZ resistance–determining region in orthologs of *dprE1* from various actinobacteria by ClustalW.** The cysteine (or correspondent amino acid) involved in the BTZ binding is highlighted in grey.

In order to monitor the potential development of BTZ resistance, a total of 240 sensitive and multidrug-resistant *M. tuberculosis* clinical isolates from four European hospitals were surveyed for the presence of mutations in the *dprE1* gene and for BTZ susceptibility (Pasca et al., 2010).

The pharmaceutical industry usually estimates the likelihood of the development of resistance against a new drug by focusing on the mutation resistance rate, on the assumption that this rate is a major determinant of resistance development in clinical settings (Andersson, 2006). Therefore, drug targets for which the resistance mechanisms have the most negative effect on fitness are expected to show a low resistance development rate (Andersson, 2006). It is noteworthy that *in vitro M. tuberculosis* BTZ-resistant mutants were rare, arising at a frequency of 10^{-8} (Makarov et al., 2009) and they have not a good fitness (M. R. Pasca, unpublished results).

The *M. tuberculosis* clinical isolates (including MDR- and XDR-TB strains) from four different European hospitals were screened for mutations in the Cys387 codon of *dprE1* and for BTZ sensitivity. Seventy-eight *M. tuberculosis* clinical isolates came from the National Institute for Infectious Diseases (INMI) "L. Spallanzani" hospital in Rome, Italy; 118 strains were isolated at the Sondalo Division of the Valtellina and Valchiavenna, Italy, hospital authority; 32 clinical isolates were from the "Ambroise Paré" Hospital in Boulogne-Billancourt, France; and 12 strains were isolated at the "Central Institute of Tuberculosis" in Moscow, Russia. All strains were isolated between 2003 and 2009 and 1 was an XDR strain and 38 (15.8%) had an MDR phenotype. Moreover, 35 MDR strains were resistant to other first- and second-line drugs and they were sensitive to only a few drugs. Some *M. tuberculosis* clinical isolates were resistant to one or more drugs but did not meet the MDR definition (24.6%). Seven *M. tuberculosis* clinical strains were isolated from HIV-positive patients. The *dprE1* gene was amplified by PCR and sequenced from all clinical isolates. None of the *M. tuberculosis* isolates had mutations in the *dprE1* gene, and hence, these isolates were presumably sensitive to BTZ (Pasca et al., 2010). This result confirms that BTZ resistance mutations are not present in the strains of *M. tuberculosis* currently circulating.

From these results, it is reasonable to hypothesize that the cysteine residue has a fundamental role in BTZ sensitivity, possibly in drug binding.

To confirm this hypothesis, we showed that all the *M. tuberculosis* clinical isolates were sensitive to BTZ043, with values ranging from 0.75 to 30 ng/ml. This result confirms that BTZ043 is very active against both sensitive and resistant strains of *M. tuberculosis*, including MDR and XDR strains (Pasca et al., 2010).

These results have very important implications for future clinical trials. Specifically, it will be possible to perform an easy and rapid diagnostic test for BTZ resistance in clinical isolates simply by sequencing the *dprE1* gene around the Cys387 codon or by using a real time-PCR assay, thus bypassing the need for systematic MIC determination.

Given the previous results, the BTZs have been shown to be a new class of potent antimycobacterial agents.

Other information about the binding between the BTZs and their target came from Trefzer and collaborators that demonstrated that BTZs are activated in the bacterium by reduction

of an essential nitro group to a nitroso derivative, which then specifically reacts with a cysteine residue in the active site of DprE1 (Trefzer et al., 2010).

As early metabolic studies with bacteria or mice indicated that the BTZ nitro group could be reduced to an amino group, and as many antitubercular drugs are prodrugs that require activation by *M. tuberculosis*, the S and R enantiomers of the amino derivatives and the likely hydroxylamine intermediate were synthesized and tested for antimycobacterial activity *in vitro*. The amino and hydroxylamine derivatives were substantially less active (500- to 5000-fold) respect to the nitro form (Makarov et al., 2009).

In this context, another novel resistance mechanism to BTZs was described in *M. smegmatis* (Manina et al., 2010a). The over-expression of the nitroreductase NfnB leads to the inactivation of the drug by reduction of a critical nitro group to an amino group (Figure 3).

Fig. 3. **Nitroreduction of BTZ nitro form in amino form by NfnB nitroreductase.**

The direct involvement of NfnB in the inactivation of the lead compound BTZ043 was demonstrated by enzymology, microbiological assays and gene knockout experiments.

M. smegmatis resistant mutants which showed a low level of BTZ resistance (16X-MIC) were isolated. One of these mutants harbored neither mutations in *MSMEG_6382* nor in *MSMEG_6385*, the genes orthologous to *M. tuberculosis dprE1* and *dprE2*, respectively. In order to identify the gene responsible for the BTZ resistance phenotype, this mutant was transformed with a wild-type *M. smegmatis* cosmid library, and a selection for sensitivity to BTZ043 was carried out. A transformant colony characterized by a resistance decrease from 16X-MIC to 2X-MIC was isolated, and the correspondent cosmid responsible for restoration of BTZ043 sensitivity, was isolated and partially sequenced. Subcloning experiments of the cosmid were then performed and this led to the identification of a fragment responsible for the resistance containing the *MSMEG_6503* gene, coding for a putative transcriptional regulator from the TetR family, and the *MSMEG_6505* gene, coding for NfnB enzyme due to its 35% sequence identity to the *E. coli* NfnB/NfsB nitroreductase. The sequence analysis revealed no mutations in the *nfnB* gene, but a point mutation in *MSMEG_6503*. This

mutation led to the substitution of leucine at position 137 by proline, leading to the hypothesis that the resistance of this mutant could be due to a defective repressor (MSMEG_6503), possibly causing over-expression of NfnB and, consequently, the reduction of the BTZ nitro-molecule to its less active amino-derivative (Manina et al., 2010a).

Consistent with this hypothesis, the MIC of BTZ045, the amino derivative of BTZ043, for *M. smegmatis* was already known to be 0.5 μg/ml *versus* 4–8 ng/ml of the original nitro compound (Makarov et al., 2009).

Moreover, 14 other spontaneous mutants, showing different levels of resistance (8–32X-MIC), were found to carry mutations in the *MSMEG_6503* gene, including deletions and insertions. All the mutant strains were predicted to produce a truncated form of the protein. One of these mutants presented a mutation in the hypothetical repressor binding site (Manina et al., 2010a).

In order to verify the role of MSMEG_6503 in *nfnB* regulation, MSMEG_6503 proteins from *M. smegmatis* wild-type and from one of the mutants were purified and used in DNA binding assays. MSMEG_6503 from *M. smegmatis* wild-type strain was able to bind and efficiently retard the *MSMEG_6503-nfnB* intergenic region, while the mutant protein bound this region less efficiently (Manina et al., 2010a).

High levels of *nfnB* expression were detected by Real-Time PCR in the *M. smegmatis* BTZ resistant mutants (Manina et al., 2010a).

To further confirm the direct role of NfnB in the BTZ resistance, an in-frame unmarked deletion was created in the *nfnB* gene and the Δ*nfnB* strain was sensitive to BTZ (Manina et al., 2010a).

Both wild-type *M. smegmatis* and one of the resistant mutants were evaluated for their ability to convert the nitro-compound to the amino-derivative, by high-pressure liquid chromatography (HPLC) analysis of culture media. The mutant transforms the nitro- to the amino-compound more efficiently and more rapidly compared with *M. smegmatis* wild-type strain (Manina et al., 2010a).

In order to assess the activity of the purified NfnB protein towards BTZ043, an indirect evaluation was initially performed, using an assay in which BTZ043 blocks the epimerization of DPR to DPA (Makarov et al., 2009). Recombinant NfnB enzyme was thus added to the assay mixtures to monitor DPA synthesis. When BTZ043 was pretreated with purified NfnB prior to addition to the reaction mixture, DPA was still formed, most likely due to the conversion of the active drug (BTZ043) to its inactive amino form (Manina et al., 2010a).

M. tuberculosis most probably lacks enzymes able to inactivate BTZ043 either in aerobic or anaerobic environment, consistent with the low MIC values of BTZ043 in *M. tuberculosis* and the fact that all the BTZ-resistant mutants isolated so far in this species harbored mutations in the target gene *dprE1* (Manina et al., 2010a).

The crystal structure of NfnB was determined at 1.75 Å resolution. The structure of NfnB in complex with NADPH was obtained by the prior addition of the cofactor during crystallization, and was refined in the same crystallographic space group at 1.80 Å resolution (Manina et al., 2010a).

It was shown that a common amino acid stretch between NfnB and DprE1 is likely to be essential for the interaction with BTZ. An amino acid sequence alignment shows a common amino acid stretch of 30 amino acids between NfnB (residues 86–115) and DprE1 (residues 386–417), displaying 46% sequence identity (59% similarity). It is noteworthy that this amino acid stretch is located at the C-terminal end of DprE1, the same region in which spontaneous mutations conferring resistance to BTZ were identified (Makarov et al., 2009), suggesting that this polypeptide portion might also play a key role in defining the relative specificity of NfnB towards the nitromolecules from the BTZ class. Docking analysis of NfnB-BTZ was performed in order to understand their interaction and the mechanism of nitroreduction (Manina et al., 2010a).

However, it has also been observed that the nitro-BTZ compounds are transformed into the corresponding amino-derivatives not only in *M. smegmatis* cultures overexpressing NfnB, but also in blood and urine from treated mice (V. Makarov, unpublished data), strongly suggesting that one or more nitroreductases, either mammalian or from the intestinal microbial flora, could carry out such a conversion. Indeed, it is well known that nitroaromatic compounds can be converted into their metabolites in the intestine, by the action of several microbial nitroreductases (Roldán et al., 2008).

Although *M. tuberculosis* seems to lack nitroreductases able to inactivate these drugs, this finding is useful for the design of new BTZ molecules or new antitubercular drugs, which may be more effective *in vivo*.

3. Dinitrobenzamides

Another new class of potent antitubercular drugs, the dinitrobenzamide derivatives (DNBs) (Figure 4), was identified through a screening of chemicals which interfere with *M. tuberculosis* replication within macrophages (Christophe et al., 2009). The most active compounds among DNBs exhibited substitutions of the benzene moiety with a nitro group at positions 3 and 5. The two major compounds from this series, [N-(2-(4-methoxyphenoxy) ethyl)-3,5-dinitrobenzamide] and [N-(2-(benzyloxy) ethyl)-3,5-dinitrobenzamide], named DNB1 and DNB2 respectively, were also highly active against *M. tuberculosis* MDR and XDR strains (Christophe et al., 2009; Figure 4).

To find the DNB derivatives, a new phenotypic cell-based assay for high throughput screening of chemical compounds that interfere with the replication of *M. tuberculosis* within macrophages was developed. This assay is based on the use of automated confocal fluorescent microscopy to monitor intracellular growth of green fluorescent protein-expressing *M. tuberculosis* H37Rv in Raw264.7 macrophages (Christophe et al., 2009).

A screening of a library of 56,984 small molecules led to the identification of 135 active compounds with potent intracellular anti-mycobacterial efficacy and no host cell toxicity. Among these, the DNB derivatives showed high activity against *M. tuberculosis*. The assay was set-up for the high throughput screening of large chemical libraries in 384-well format. To set up the optimal conditions of *M. tuberculosis* infection, Raw264.7 macrophages were first infected with mycobacteria that constitutively express green fluorescent protein using different multiplicities of infection followed by kinetic analysis of intracellular bacterial growth. Confocal images of live samples were acquired using an automated confocal microscope (OperaTM) over 7 days (Christophe et al., 2009).

Fig. 4. **Chemical structure of dinitrobenzamide compounds.** R_1 can be either a nitro (-NO_2), an amino (-NH_2), a hydroxylamine (-NHOH) or a hydrogen (-H) group, whereas R_2 stands for different aromatic substituents.

This chemical library was first screened at a single concentration. 486 fully active hits were then confirmed by means of serial dilution experiments. More than one quarter of the hits (135 hits) had an MIC less than 5 mM, and 8% had a MIC below 1 mM, which is equivalent to that of isoniazid (Christophe et al., 2009).

The largest cluster had 69 members with an isonicotinohydrazide moiety similar to that of isoniazid, used as a positive reference in the assay. The second largest cluster of 24 derivatives shares a common benzamide scaffold. A series of related derivatives was synthesized for further studies. To identify the chemical substituents necessary for benzamide antibacterial activity, over 155 additional derivatives were synthesized and their structure-activity relationship was analyzed using both intracellular assay and the *in vitro* growth assay. The most potent compounds exhibited substitutions of the benzene moiety with a nitro group at positions 3 and 5. The reduction of one nitro- to hydroxylamine and amino groups led to totally inactive compounds. In contrast, derivatives with an N-substitution by benzyloxy-ethyl or by phenoxy-ethyl showed enhanced activity with an MIC below 0.2 mM. More importantly, cyclic-benzamides had an MIC below 80 nM in the *in vitro* assay. Moreover, substitution of the benzyloxy moiety by a chlorine- or fluorine atoms at position 3 led to increased potency in both assays in contrast to carboxyl substitutions. Two compounds, N-(2-(4-methoxyphenoxy) ethyl)-3,5-dinitrobenzamide (DNB1) and N-(2-(benzyloxy) ethyl)-3,5-dinitrobenzamide (DNB2) were selected for the characterization. No cell toxicity was noted for these compounds using conventional cytotoxicity assays of uninfected cells, indicating that this assay can predict the cytotoxicity. Analysis of the broad antimicrobial spectrum revealed that the effect of these dinitrobenzamide derivatives was mainly restricted to Actinomycetes. Of particular importance, DNB1 and DNB2 were also highly active against MDR and XDR clinical isolates. The bactericidal effect on *M. tuberculosis* of DNB1 and DNB2 was found to be time-dependent and to require several days to reach bacterial clearance, implying that they could interfere with *de novo* mycobacterial component biosynthesis. This is further corroborated by the fact that the DNB compounds lost their activity in a non-replicating *M. tuberculosis* system (Christophe et al., 2009).

In a preliminary experiment using the acute mouse model of *M. tuberculosis*, a one log reduction of the colony forming unit in the lungs of DNB treated animals compared to non-

treated controls was observed after a three week daily treatment with 30 mg/kg/day following an intranasal infection.

To gain insight into the possible targets of dinitrobenzamides, the effect of DNB1 and DNB2 on the lipid composition of the cell envelope of *M. tuberculosis* was investigated; no effects on the biosynthesis of fatty acids, mycolic acids and/or other lipids were noted. By contrast, DNB1 and DNB2 showed a clear-cut effect on the synthesis of the arabinan domains of arabinogalactan and lipoarabinomannan. DPA is the only known arabinofuranose (Ara*f*) donor in the biogenesis of arabinogalactan and lipoarabinomannan in mycobacteria and is thus an essential precursor (Wolucka, 2008). The effects of DNB in the inhibition of the synthesis of DPA were tested. Analyses revealed complete inhibition of DPA formation in the DNB-treated extracts, concurrent with the accumulation of DPR, indicating that the target of DNB inhibitors could be the heteromeric decaprenylphospho-ribose epimerase encoded by the *dprE1*/*dprE2* genes in *M. tuberculosis* H37Rv, the same target of benzothiazinones (Christophe et al., 2009; Makarov et al., 2009). Moreover BTZ resistant mutants of *M. smegmatis* and *M. bovis* BCG having a mutation in *dprE1* gene, were also resistant to DNB inhibitors.

Recently it has been demonstrated that the DNB and the BTZ have not only the same target, but also the same mechanisms of resistance (M.R. Pasca, unpublished data).

4. The decaprenyl-phosphoribose 2'-epimerase

Mycobacteria cell envelope is a peculiar characteristic of these microorganisms. It forms an efficient permeability barrier, playing a crucial role in intrinsic drug resistance as well as in macrophage survival under stress conditions. Several fundamental antitubercular drugs, such as isoniazid and ethambutol, target enzymes involved in some specific cell-wall biosynthetic pathways (Barry et al., 2007). Furthermore the mycobacterial cell envelope is still considered an outstanding cellular source for target discovery.

The mycobacterial cell envelope is composed of three main portions. From the external side towards the cytoplasmic membrane we find: a highly impermeable layer of mycolic acids (long-chain (C70–C90) α-branched, β-hydroxy fatty acids), the complex polysaccharide arabinogalactan, and a peptidoglycan layer. Arabinogalactan is covalently attached to peptidoglycan by a phosphodiester linkage and is esterified by mycolic acids. The entire complex is named mycolyl-arabinogalactan–peptidoglycan (Alderwick et al., 2007), and represents a strong protective barrier for the pathogen. This complex also keeps a dynamic trait, useful for facing different environmental conditions. Chemical analysis of the cell envelope composition has also revealed the presence of diverse noncovalently bound lipids (such as phosphatidyl-myo-inositol mannosides), lipopolysaccharides (such as lipoarabinomannans), mannose-capped lipoarabinomannans, poly-L-glutamate–glutamine polymers, enzymes and other proteins. In slow-growing mycobacterial species, such as *M. tuberculosis*, proteins and polysaccharides are present in the outermost stratum known as the capsule (Umesiri et al., 2010).

Synthesis of arabinan domain of arabinogalactan derives from sequential additions of Ara*f* residues to the galactan domain, while lipoarabinogalactan originates from the addition of Ara*f* units on the mannan domain by specialized arabinosyltransferases (Wolucka, 2008).

These enzymes utilize the unusual sugar decaprenylphosphoryl-*D*-arabinose (DPA, Figure 5) as the only donor of Ara*f* residues in both mycobacteria and corynebacteria (Meniche et al., 2008). It is noteworthy that without DPA, a complete mycobacterial cell wall cannot be produced. DPA is synthesized from phosphoribose diphosphate through a series of three successive reactions (Meniche et al., 2008): (i) transfer of phosphoribose diphosphate to decaprenyl phosphate to form decaprenylphosphoryl-5-phosphoribose, (ii) removal of the 5′ phosphate in order to give decaprenylphosphoryl-*D*-ribose (DPR, Figure 5), and (iii) epimerization of DPR into DPA, which likely occurs *via* a sequential oxidation-reduction mechanism involving an intermediate (DPX, Figure 5), which is a product of DPR oxidation and a precursor of DPA. Although not unambiguously identified, DPX is most probably a decaprenylphosphoryl-2-keto-β-D-erythro-pentofuranose (Figure 5).

Fig. 5. **The enzymatic reaction catalyzed by decaprenylphosphoryl-β-D-ribose 2′ epimerase (composed by DprE1 and DprE2) is reported.** DPR indicates decaprenylphosphoryl-*D*-ribose, while DPX is the intermediate decaprenylphospho-2-ketofuranose and DPA the decaprenylphosphoryl-*D*-arabinose.

The epimerization reaction is reported in Figure 5. It is catalyzed by the decaprenyl-phospho-ribose 2′-epimerase, a heteromeric enzyme composed of two types of polypeptides encoded by the *Rv3790* (*dprE1*) and *Rv3791* (*dprE2*) genes, respectively (Wolucka, 2008; Mikusova et al., 2005). The membrane-associated enzymes DprE1 and DprE2 have been suggested to act as decaprenylphosphoryl-β-D-ribose oxidase and decaprenylphosphoryl-D-2-keto erythro pentose reductase, respectively (Makarov et al., 2009). It is noteworthy that

for epimerase activity, a simultaneous expression of both polypeptides is required (Mikusova et al., 2005).

Little is known about the decaprenyl-phosphoribose 2′-epimerase. The DprE1 protein contains a FAD-binding N-terminal and a C-terminal D-arabinono-1,4-lactone oxidase-like enzyme domains (Wolucka, 2008). What is evident now, however, is that the decaprenyl-phosphoribose 2′-epimerase enzyme is a very important and authentic validated target for antitubercular drugs, being the target for at least three different classes of antitubercular drugs, namely benzothiazinones, dinitrobenzamides, and benzoquinoxalines whose lead compound is VI-9376, a molecule structurally related to benzothiazinones (Makarov et al., 2009; Christophe et al., 2009; Magnet et al., 2010).

Both the *dprE1* and *dprE2* genes were predicted to be essential by Himar1-based transposon mutagenesis in *M. tuberculosis* H37Rv (Sassetti et al., 2003), thus validating both enzymes as targets for drug development. Moreover, recently the construction of a conditional gene knockout strain targeting the ortholog of *dprE1* in *M. smegmatis*, *MSMEG_6382*, has been reported (Crellin et al., 2011). Disruption of the chromosomal copy of *MSMEG_6382* was only possible in the presence of a plasmid-encoded copy of *MSMEG_6382*. Curing of this ''rescue'' plasmid from the bacterial population resulted in a cessation of growth, further demonstrating gene essentiality. This study provides the first direct experimental evidence for the essentiality of DprE1 in mycobacteria. Moreover, the essentiality of DprE1 in *M. smegmatis*, combined with its conservation in all sequenced mycobacterial genomes, suggests that decaprenylphosphoryl-*D*-arabinose synthesis is essential in all mycobacteria (Crellin et al., 2011). Overall, this study further validates DprE1 as a promising target for new anti-mycobacterial drugs.

Although many potential drug targets have been already identified, greater efforts are required in target validation to properly show their essentiality for bacterial growth and survival (Williams and Duncan, 2007). A rational antibiotic design strategy should aim to identify targets for which the resistance mechanism has the most negative effect on fitness, as in the case of the DprE1 enzyme. In fact, our data suggest that DprE1 is an optimal target also for MDR- and XDR-TB strains. The treatment of these strains is difficult if not impossible nowadays and could instead be defeated by a drug targeting *dprE1* gene product. The disruption of this essential ring in the construction of the mycobacterial cell wall makes the pathogen weak and completely unable to survive in a hostile environment, such as the macrophage. Moreover, decaprenylphosphoryl-*D*-arabinose is the only known Ara*f* donor in the biogenesis of arabinogalactan and lipoarabinomannan in mycobacteria and is thus an essential precursor.

It is worth mentioning that decaprenylphosphoryl-*D*-arabinose is a lead molecule for the design of substrates′ analogs for mycobacterial arabinosyltransferases and of new inhibitors (Wolucka, 2008). Since 2004, the synthesis of decaprenylphosphoryl-*D*-arabinose analogs as antitubercular agents has been performed. Even if these molecules inhibit mycobacterial growth, their effect was not satisfactory (Centrone and Lowary, 2004). Other efforts have been made by researchers in order to interfere with arabinogalactan and lipoarabinomannan biosynthesis. Indeed, in 2009 Pathak and co-workers reported the synthesis of modified Ara*f* disaccharides as substrates and inhibitors of *M. tuberculosis* arabinosyltransferases (Pathak et al., 2009). Furthermore, Ayers and collaborators synthesized β-arabino glycosyl sulfones,

mimicking decaprenylphosphoryl-*D*-arabinose, as possible inhibitors of cell wall biosynthesis in mycobacteria (Ayers et al., 2009). They demonstrated a low to moderate anti-mycobacterial activity, which is strongly dependent on alkyl chain length.

In summary, DprE1, as well as its orthologs, are not only optimal targets for benzothiazinones, dinitrobenzamides and the lastly described benzoquinoxalines (Makarov et al., 2009; Christophe et al., 2009; Magnet et al., 2010), but also promising targets for the discovery of new molecules in treating infections caused by *M. tuberculosis*, *Mycobacterium leprae*, *Nocardia* spp., *Rhodococcus* spp., and *Corynebacterium* spp.

At present, a fundamental goal is the production of large amounts of soluble DprE1 protein in order to develop an enzymatic assay suitable for high throughput screenings and to solve the DprE1 structure to design new molecules affecting the arabinogalactan biosynthesis.

5. Conclusion

The inexorable rise in cases of tuberculosis worldwide highlights the need for new drugs and, in particular, for those that can shorten the duration of treatment. Recently, two new promising antitubercular drugs were developed (Makarov et al., 2009; Christophe et al., 2009). Both these drugs have the same cellular target, the DprE1 of *M. tuberculosis* (Manina et al., 2010b). The preclinical development program of benzothiazinone lead (BTZ043) has already begun and this class of drugs is very promising.

Moreover, another class of antitubercular drugs has been shown to have DprE1 as target, the benzoquinoxalines (Magnet et al., 2010).

This highlights the vulnerability of DprE1 and its importance as an antitubercular target.

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Quinolone Resistance in Tuberculosis Treatment: A Structural Overview

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1. Introduction

1.1 Fluoroquinolones in tuberculosis treatment

Fluoroquinolones are key antibiotics for the second-line treatment of multidrug-resistant tuberculosis (MDR-TB), i.e. resistant to the two most powerful antituberculous drugs currently available, isoniazid and rifampicin (WHO, 2001; 2011). Unfortunately, extensively drug resistant strains (XDR-TB), defined as MDR-TB with bacillary resistant to any fluoroquinolones and to one of the three injectable second line anti-TB drugs (amikacin, kanamycin or capreomycin), are emerging worldwide (Center for Disease Control and Prevention, 2006). XDR-TB strains constitute a major concern for public health since they are virtually untreatable and responsible for up to 100% of deaths. Besides being increasingly popular in treatment of tuberculosis complicated by intolerance or by relative contraindication for first-line drugs, fluoroquinolones are under studies to shorten the duration of treatment or replace first line drugs in susceptible tuberculosis. Newer fluoroquinolones such as moxifloxacin have already demonstrated potential for shortening treatment duration (Rustomjee et al., 2008; Conde et al., 2009; Dorman et al., 2009).

Fluoroquinolones are also one of the most widely prescribed antibiotics as they are generally well tolerated with high oral bioavailability plus broad-spectrum antibacterial activities against genitourinary infections and against common respiratory tract pathogens. Structurally, these drugs contain a quinoline ring system and hence are given the name quinolones (Fig. 1). After the first generation of drugs were found to be active, it was noted that a fluorine atom at the 6-position of the quinoline ring imparted greater potency, and hence the second generation of drugs was called the fluoroquinolones (Fig. 1).

Fig. 1. Chemical structures of a fluoroquinolone (left) and moxifloxacin (right). The fluorine atom, signature of fluoroquinolones, is at position 6 in the quinoline ring. In the moxifloxacin structure, oxygen atoms are coloured in red, nitrogen atoms in blue, hydrogen atoms in green and the fluor atom in purple. R1 is a cyclopropyl group, R5 is a hydrogen atom, R7 is an azabicyclo group and X8 a methyl ether group.

2. Bacterial type II topoisomerases are the target of fluoroquinolones

Topoisomerases are ubiquitous nucleic acid-dependant nanomachines essential to cell life and solve the topological problems of DNA that occur as a result of DNA manipulations during replication, transcription, recombination, chromosome segregation and condensation, maintenance of nuclear architecture and apoptosis (Champoux, 2001). They have been divided into two classes, type I and type II, according to their basic mechanism of action (Champoux, 2001; Forterre et al., 2007). Type I DNA topoisomerases introduce transient single-stranded breaks to force the passage of one DNA strand through the other, whereas type II DNA topoisomerases introduce transient double-stranded breaks to force the passage of a second DNA duplex through this broken duplex. All organisms contain at least one type I and one type II topoisomerases. Type I and type II topoisomerases can sometimes perform similar function *in vivo*, for example efficient separation of interlocked chromosomes at the end of DNA replication via their decatenation activity (Nurse et al., 2003). All type II topoisomerases can also relax both positive and negative superturns that accumulate during transcription and replication. Topoisomerases thus play an essential role for the preservation of genome stability in all life forms (Forterre et al., 2007).

Type II topoisomerases have been further sub-classified into two families, type IIA and IIB, based on evolutionary considerations (Champoux, 2001; Forterre et al., 2007). The type IIA includes bacterial DNA gyrase and topoisomerase IV, eukaryal and viral topoisomerases, whereas the type IIB includes archaeal topoisomerase VI, and their homologues in plants, a few protists, and a few bacteria (Forterre et al., 2007; Forterre & Gadelle, 2009). Type IIA and IIB topoisomerases are both formed by the association of two subunits, sharing homologous sequences and domain organization (Fig. 2). Bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, consist of two subunits, GyrA or ParC and GyrB or ParE, which form the catalytic active heterotetrameric (A_2B_2/C_2E_2) complex. Subunit A consists of two domains, the N-terminal breakage-reunion domain and a carboxy-terminal domain, termed BRD and CTD, respectively (represented in blue and green respectively in Fig. 2 and 3). Subunit B consists of the ATPase domain followed by the Toprim domain (represented in yellow and red, respectively, in Fig. 2 and Fig. 3). The GyrB Toprim domain and GyrA BRD come from separate subunits and cooperatively form the enzyme catalytic core (see section

5). The BRD contains the catalytic tyrosine responsible for the cleavage and religation of the DNA double helix.

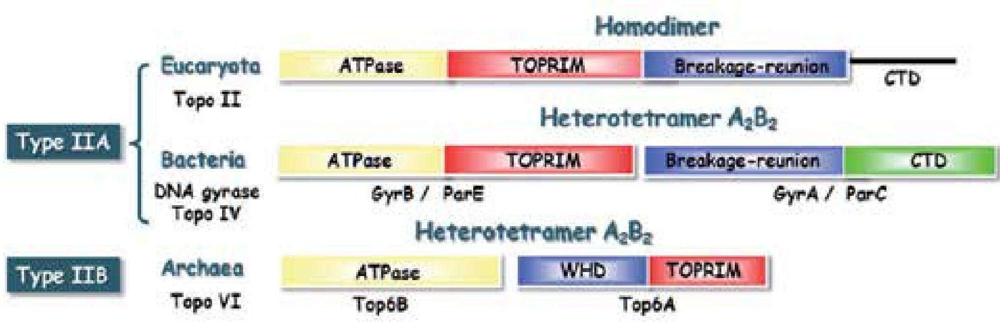

Fig. 2. Schematic representation of the sequence and domain organization of type II topoisomerases found in eukaryotes, bacteria, virus and archaea. The type IIA topoisomerase family includes bacterial DNA gyrase and topoisomerase IV (Topo IV), eucaryal and viral topoisomerases (Topo II), whereas the type IIB topoisomerase family includes archaeal topoisomerase VI (Topo VI), and their homologues in plants and a few protists and bacteria. Topo IIA and Topo IIB are both formed by the association of two subunits A and B. Bacterial type IIA and type IIB topoisomerases are A_2B_2 heterotetramers, whereas the eukaryotic Topo II is a heterodimer, with the A and B subunits fused in a single polypeptide (Nter-B-A-Cter). The names of the four conserved domains are indicated. The winged helix domain (WHD) in the type IIB topoisomerase corresponds to the breakage-reunion domain (BRD) of type IIA topoisomerase.

Crystallographic studies of individual domains show that the structure of the BRD is a heart-shaped arrangement with two dimer interfaces (Morais-Cabral et al., 1997), that the CTD displays a spiral six-bladed β-pinwheel structure (Corbett et al., 2004) and that the ATPase domain belongs to the GHKL ATPases, a broad family of enzymes with a common fold unrelated to other canonical ATP-binding folds (Brino et al., 2000) (Fig. 3). The Toprim domain interacts with the A subunit, possesses a magnesium-binding site and is essential for DNA binding (Fig. 3). Although no structure for a fully intact active type IIA topoisomerase has been determined yet, combination of structural and biochemical studies of the individual domains has lead several authors to propose a global quaternary structure model and a catalytic mechanism of the holoenzyme (Schoeffler & Berger, 2008). The BRD binds a DNA segment termed the 'gate-' or G-segment at the DNA-gate (Fig. 3). The N-terminal ATPase domains dimerize upon ATP binding, capturing the DNA duplex to be transported (T-segment). The T-segment is then passed through a transient break in the G-segment opened by the breakage-reunion domains, the DNA is resealed and the T-segment released through a protein gate, the C-gate, prior to resetting of the enzyme to the open clamp form (Fig. 3).

Quinolones, which target the two bacterial type II topoisomerases, exert their powerful antibacterial activity by interfering with the enzymatic reaction cycle. Specifically, they bind to the enzyme-DNA binary complex, thereby stabilizing the covalent enzyme tyrosyl-DNA phosphate ester (see also section 6 and Fig. 7). The resulting ternary complexes block DNA

replication and lead to cell death (Hooper, 2003). In addition, hydrolysis of this linkage leads to the accumulation of double-stranded DNA fragments, which accounts for the bactericidal activity.

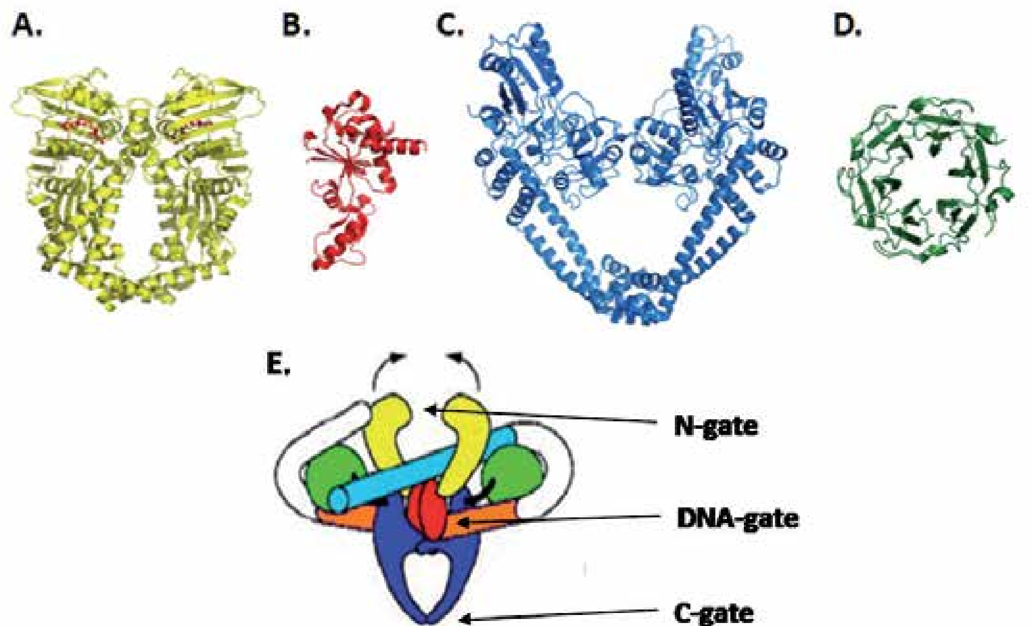

Fig. 3. Crystal structures of the individual domains of type II topoisomerases, as defined in Fig. 1. Structures of the GHKL domain (A), Toprim (B), BRD (C) and DNA gyrase CTD (D) domains. Two ATP molecules are represented in red in (A). (E) Global architecture of type IIA topoisomerases proposed based on the structures of the isolated domains adapted from Corbett & Berger (2004). Color code is the same than in Fig. 1. The dimerization interfaces of the heterotretramer constitute the three gates (N-, DNA- and C-gate), through which the DNA double helix (T-segment, represented in cyan) will be transported. The G-segment that binds the DNA-gate is represented in orange.

3. *M. tuberculosis* DNA gyrase as the sole target of fluoroquinolones

Bacterial genomes usually encode two type IIA enzymes, DNA gyrase and topoisomerase IV, that together help manage chromosome integrity and topology (Champoux, 2001). DNA gyrase is unique in introducing negative supercoils into DNA, an activity mediated by the CTD of its DNA binding subunit (GyrA) and is therefore responsible for the DNA unwinding at replication forks. Although closely related to DNA gyrase, topoisomerase IV has a specialized function in mediating the decatenation of interlocked daughter chromosomes (Levine et al., 1998) and relaxes positive supercoils. Particularly intriguing is that some bacteria possess in their genome only one type II topoisomerase, DNA gyrase.

Given the important role of DNA supercoiling in DNA replication, transcription, and chromosome dynamics, it is not surprising that DNA gyrase genes have been found in all bacterial genomes sequenced to date. In contrast, the topoisomerase IV gene is absent in a

few bacteria such as *Treponema pallidum*, *Helicobacter pylori*, and notably *Mycobacterium tuberculosis*, the intracellular pathogen that causes tuberculosis (Cole et al., 1998) (Fig. 4). Consequently, these bacteria are unusual in producing a "single" type II topoisomerase. DNA gyrase is therefore the unique target of fluoroquinolones in these organisms, as it has been demonstrated for *M. tuberculosis* (Mdluli & Ma, 2007).

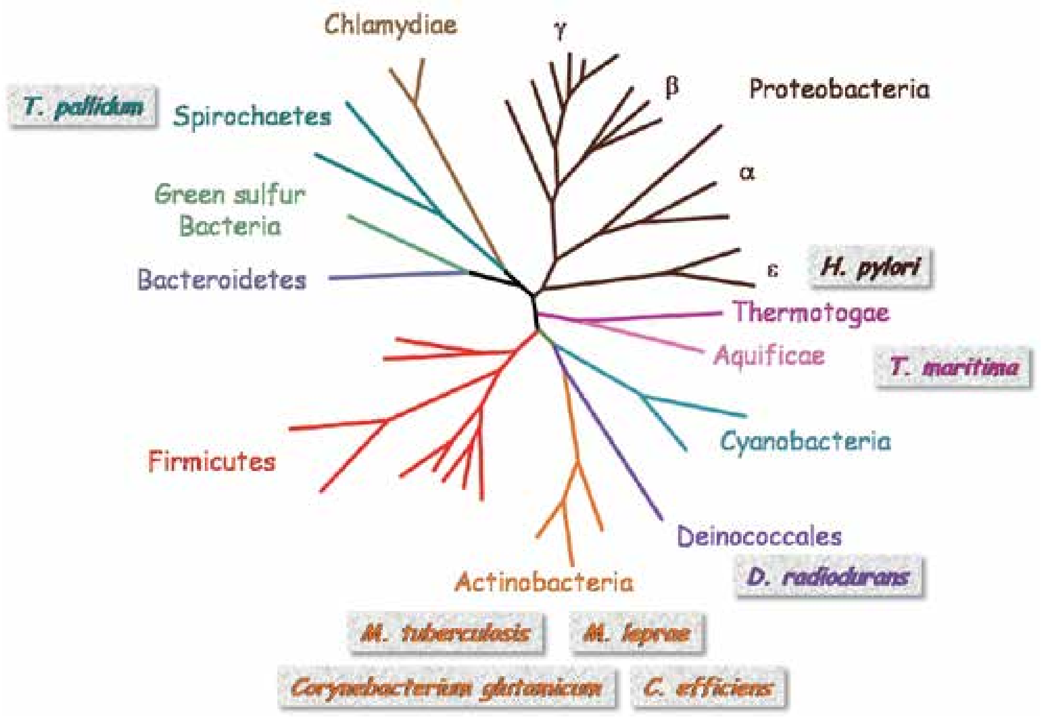

Fig. 4. Bacterial phylogenetic tree showing the distribution of species known to possess only one type II topoisomerase gene in their genome.

The direct consequence of the presence of a unique type II topoisomerase in *M. tuberculosis* has been investigated and it has been shown that *M. tuberculosis* DNA gyrase presents two specificities. First, this enzyme possesses an "hybrid activity". In addition to its normal supercoiling activity (e.g. comparable to that of *E. coli* DNA gyrase), it possesses a decatenation activity superior to that of DNA gyrase from species harboring two type II topoisomerases (Aubry et al., 2006). In addition, it shows an enhanced DNA relaxation and cleavage activities. However, the unique type II topoisomerase of *M. tuberculosis* decatenates kDNA less efficiently than a genuine topoisomerase IV, e.g., that of *S. pneumoniae*. The second specificity concerns the susceptibility to fluoroquinolones. The *M. tuberculosis* DNA gyrase is indeed naturally less susceptible to fluoroquinolones than other bacterial DNA gyrases. We have shown that three residues of the *M. tuberculosis* sequence play an important role in this natural resistance mechanism (Matrat et al., 2006). Replacing M74 in GyrA, A83 in GyrA, and R447 in GyrB of *M. tuberculosis* gyrase by their *E. coli* homologs (Ile, Ser and Lys, respectively, see residues pink underlined in Fig. 5) resulted in active enzymes as quinolone susceptible as the *E. coli* DNA gyrase. However, the question whether the

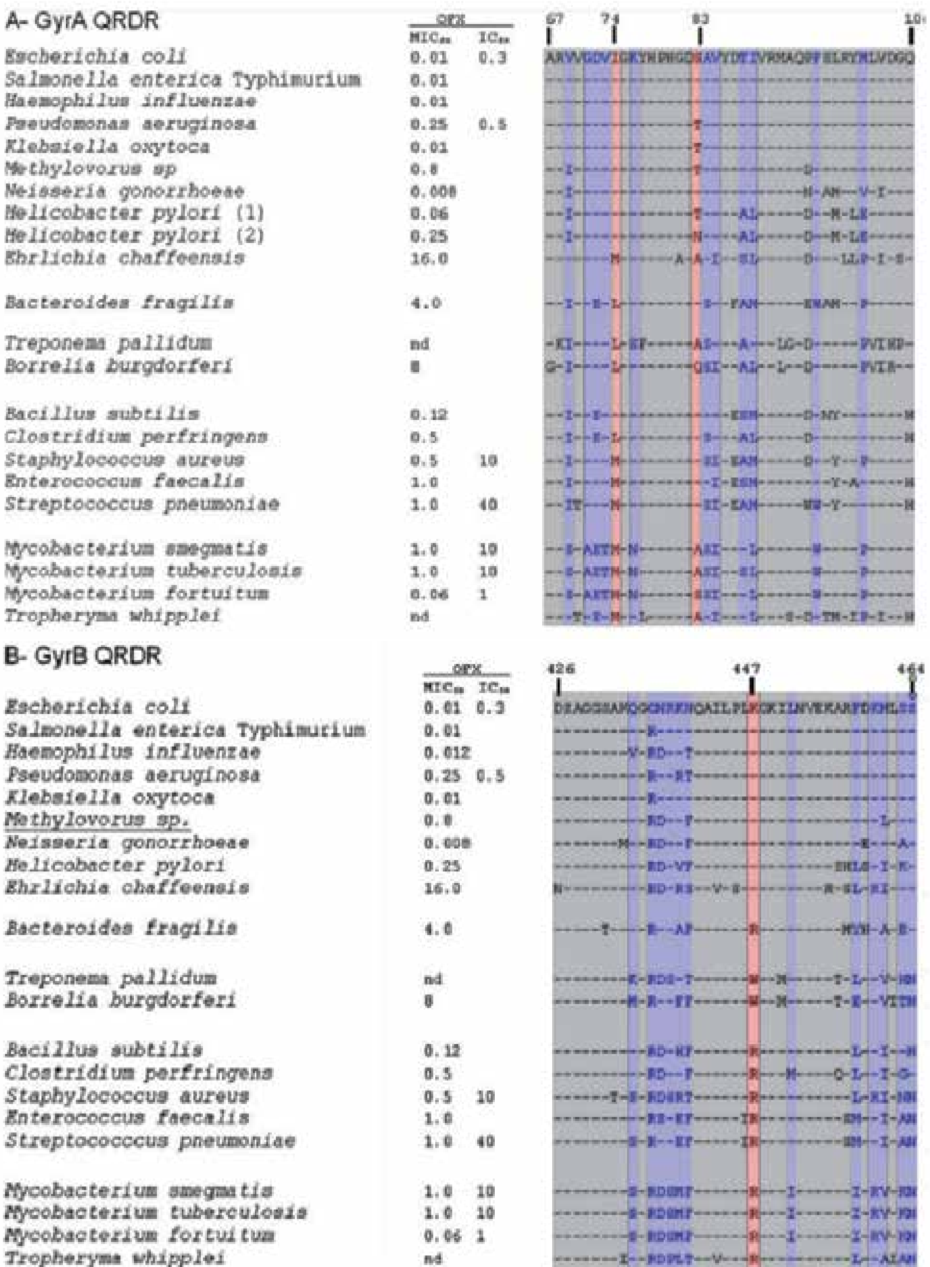

Fig. 5. Two parts of the multiple alignment of subunit A and B, respectively, centred on the quinolone resistance determining regions (QRDR, see section 4 for definition), after Matrat et al. (2008). Residues highlighted in blue underline the most important residues shown to be involved in acquired resistance to fluoroquinolones. Residues highlighted in pink underline residues of the *M. tuberculosis* DNA gyrase sequence involved in the natural resistance mechanism. The numbering at the top of the alignments corresponds to the *E. coli* DNA gyrase numbering. The values of MIC_{50} and IC_{50} are given for ofloxacin when known.

"single" DNA gyrases of *Treponema pallidum* or *Helicobacter pylori* display similar specificities, like increased decatenation activity or lower susceptibility to quinolones, is still open.

4. The quinolone resistance determining regions and the quinolone binding pocket

Bacterial resistance to antibiotics typically involves drug inactivation or modification, target alteration, or decrease in drug accumulation associated with decrease in permeability and increase in efflux (Li & Nikaido, 2004). Fluoroquinolone resistance mechanisms can involve two of these three mechanisms, target alterations and overexpression of intrinsic multidrug resistance (MDR) efflux pumps (Hooper, 2002). However, resistance to fluoroquinolone is mainly due to target modification, e.g. mutations in the DNA gyrase and/or topoisomerase IV genes. Mutations conferring bacterial resistance to quinolones occur in two short discrete segments termed the quinolone resistance-determining regions (QRDR) in the breakage-reunion domain of subunit A (QRDR-A) and more rarely in the Toprim domain of subunit B (QRDR-B) (Hooper, 1999) (Fig. 6).

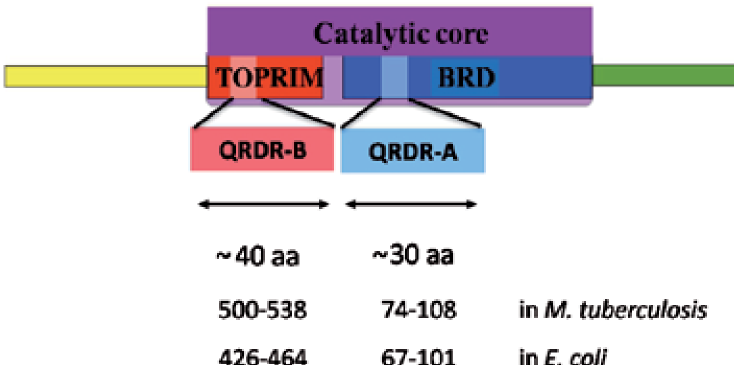

Fig. 6. Schematic representation of the *M. tuberculosis* DNA gyrase sequence. Subunit A contains the breakage-reunion domain (BRD, blue) and the C-terminal domain (CTD, green). The subunit B contains the ATPase domain (yellow) and the Toprim domain (red). Localization of the QRDR is indicated in pink and light blue. The color code is the same than in Fig. 2 and 3. The approximate lengths of the QRDRs are indicated (30 and 40 aa for the QRDR-A and -B, respectively). The residue ranges of the QRDR-A and -B are indicated for the *M. tuberculosis* and the *E. coli* DNA gyrase sequences.

Whereas residues of the QRDR-A have been known for a while that they are spatially close to the catalytic tyrosine responsible for the double-stranded DNA cleavage, residues of the QRDR-B were only hypothetically thought to be involved in the quinolone-binding pocket (Heddle & Maxwell, 2002). This ambiguity was brought about by the two first structures of the catalytic core (an homodimer formed by two single polypeptides composed of the Toprim domain and the BRD) of the yeast topoisomerase II (Fass et al., 1999; Berger & Gamblin, 1996). In these structures, which correspond to two different conformational arrangements of the catalytic core, the two QRDRs are too far from each other to form a unique binding pocket for the quinolone molecule (Fig. 7).

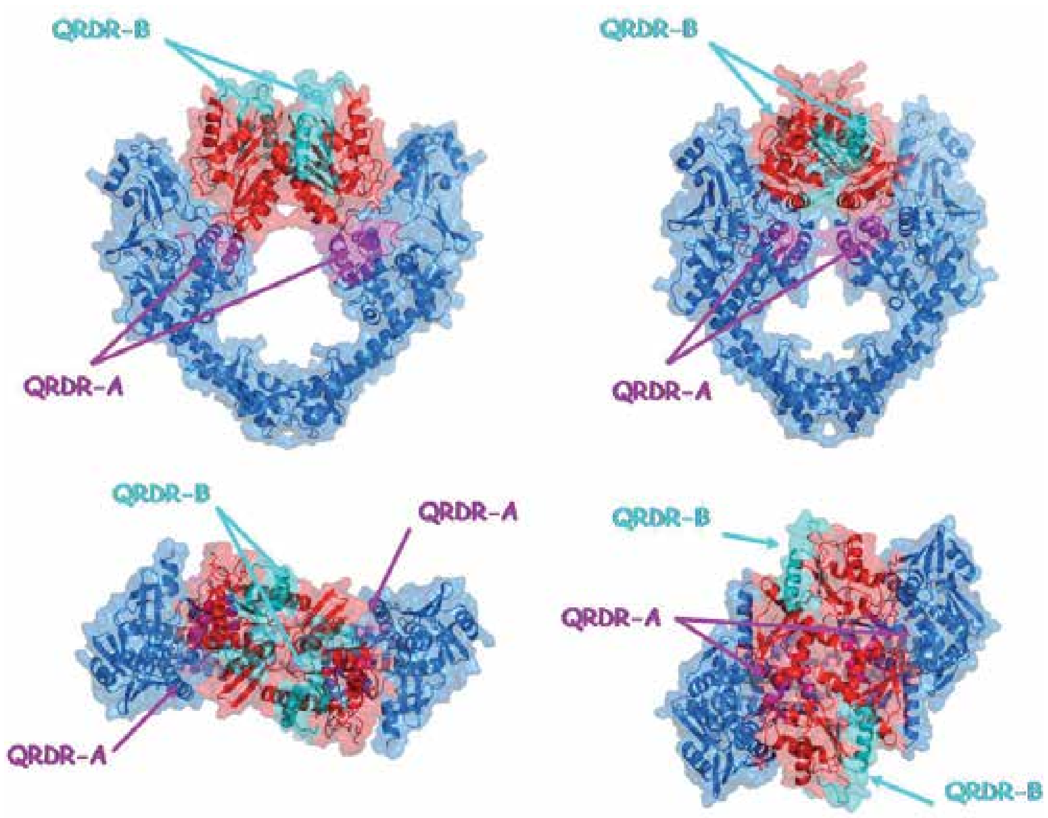

Fig. 7. Side view (top left and right) and top view (bottom left and right) of the catalytic core of the yeast topoisomerase II, composed of the Toprim domain (represented in red) and the breakage-reunion domain (BRD, represented in blue). (Left) "Open" conformation of the catalytic core (PDB code 1BGW, Berger et al., 1996). The DNA-gate is open and the C-gate is closed (for definition of DNA- and C-gate, see Fig. 3). The QRDR-B (represented in cyan) are close together but distant from the QRDR-A (represented in purple). The QRDR-A includes the two DNA-binding helices, H3 and H4. (Right) "Intermediate" conformation of the catalytic core (PDB code 1BJT, Fass et al., 1999). The DNA-gate is slightly open and the C-gate is closed. The QRDR-B are more distant from each other and are closer to the QRDR-A, but still do not form a unique quinolone-binding pocket.

Involvement of QRDR-B residues in the quinolone-binding pocket was definitely confirmed in 2009 with the first structure of the *S. pneumoniae* topoisomerase IV Toprim and breakage-reunion domains in complex with DNA and moxifloxacin (Lapogonov et al., 2009). This structure highlighted the fact that the GyrA BRD and the GyrB-Toprim domain form the catalytic core complex, where the double-stranded DNA interacts at the interface of the two domains (Fig. 8). This structure brought for the first time the structural evidence of the covalent link formed between the DNA and the enzyme, a phosphodiester bond between

the oxygen of the tyrosine and the phosphor atom of a phosphate group (Fig. 8). In addition, this structure also confirmed that the QRDR-A corresponds to the DNA-binding domain of the subunit A often referred to as a CAP-like domain constituted by a helix-turn-helix motif (H3 and H4). The QRDR-B corresponds to the DNA-binding region of subunit B that involves a helix and a long loop (see section 5). However, the main conclusion of this work was that the conformational arrangement observed in this structure brings together the two QRDRs to form a unique quinolone-binding pocket (see section 6) at the interface formed by the DNA, the BRD and the Toprim domain (Fig. 8).

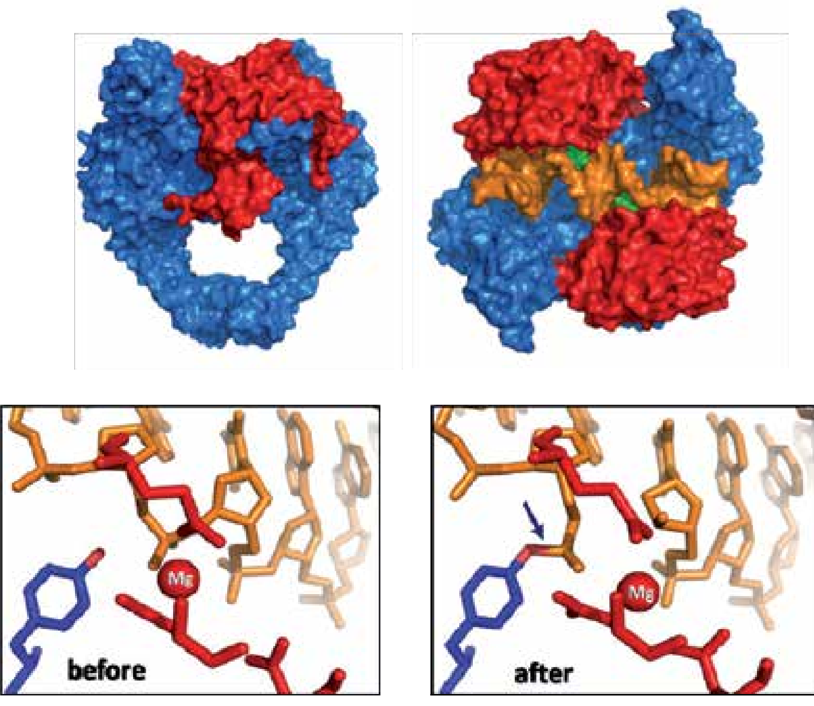

Fig. 8. Crystal structure of the *S. pneumoniae* topoisomerase IV catalytic core that comprises the GyrB Toprim domain and GyrA BRD in complex with DNA and moxifloxacin. The Toprim domain is represented in red, the BRD in blue, the 35 base pairs DNA oligonucleotide in orange and the moxifloxacin in green. (Top) Side and top views of the molecular surface of the catalytic core. (Bottom) Close view of the catalytic tyrosine (represented in blue sticks) before and after formation of the covalent link between the enzyme and DNA (the phosphodiester bond is indicated by a black arrow). Residues of the Toprim domain chelating the magnesium ion are represented in red sticks.

5. Structural studies of the *M. tuberculosis* DNA gyrase catalytic core

We recently solved the crystal structures of two domains of the *M. tuberculosis* DNA gyrase, the GyrB Toprim (TopBK) and the GyrA breakage-reunion (GA57BK) domains (Piton et al., 2009; 2010). These two domains form the DNA gyrase catalytic core that corresponds to the smallest entity able to catalyse DNA cleavage (Piton et al., 2010).

We solved two high resolution structures of the Toprim domain to 2.1 and 1.95 Å resolution, respectively. These two structures display two different conformations of the magnesium binding site. The QRDR-B is constituted by residues 500 to 538 (461-499 in the PDB numbering). Part of this region is disordered and corresponds to a loop that has been named DNA-binding loop (DBL) because it is folded in the presence of DNA (Fig. 9). We obtained four crystal forms of the breakage-reunion domain, diffracting from 4.2 to 2.7 Å resolution depending on the crystal form (Piton et al., 2009). This was the first structure determination report of the BRD of a DNA gyrase from a species containing one unique type II topoisomerase. The structure of this domain is unexpectedly very similar to other structures of BRD solved for bacterial type II topoisomerases, DNA gyrase and topoisomerase IV (Morais-Cabral et al., 1997; Carr et al., 2007). It displays the typical heart-like shaped structure with a hole of 30 Å diameter at the center allowing the passage of the DNA from the DNA-gate to the C-gate (Fig. 9). The QRDR-A is localised at the DNA-gate (shown in purple in Fig. 9).

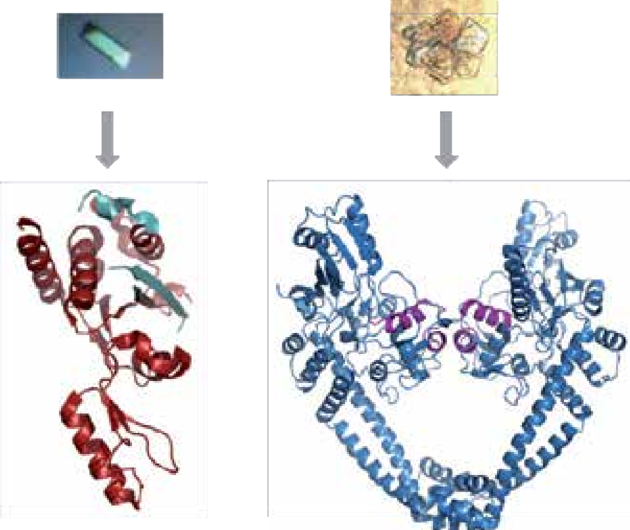

Fig. 9. Crystals (top) and crystal structures (down) of the GyrB Toprim (TopBK, PDB code 3IG0, 242 residues) domain represented in red (left) and the GyrA breakage-reunion (BRD, GA57BK, PDB code 3IFZ, 508 residues per monomer) represented in blue (right). The QRDR-B is represented in cyan in the TopBK structure (residues 500-538), the QRDR-A is represented in purple in the GA57BK structure (residues 74-101).

Using the two crystal structures we determined, the GyrA BRD (GA57BK) and the GyrB Toprim (TopBK) domains, we performed the modeling of the *M. tuberculosis* catalytic core in complex with a 35 base pairs DNA oligonucleotide and the most used fluoroquinolone,

moxifloxacin. In both the crystal structure of the *S. pneumoniae* topoisomerase IV and the modeled *M. tuberculosis* DNA gyrase catalytic cores, the fluoroquinolone molecules are bound at the ternary interface formed by the DNA, the BRD and the Toprim domain (Fig. 8 and 10). The general mode of action known for quinolones, common to DNA gyrase and topoisomerase IV, was the inhibition of the broken DNA religation. The way how these molecules are responsible for this inhibition has been clarified by the structure. Whereas quinolones are not DNA intercalators, the fluoroquinolone molecule is intercalated in the ternary complex between the dinucleotide step where the covalent bond between the DNA and the enzyme (with the catalytic tyrosine) is formed (Fig. 8 and 10). As the catalytic core is dimeric, two fluoroquinolones molecules are bound spaced by four base pairs (Fig. 10). The consequence of this intercalation is that the O3'-P religation is structurally impossible because both atoms are too far away from each other (more than 9 Å) (Fig. 10).

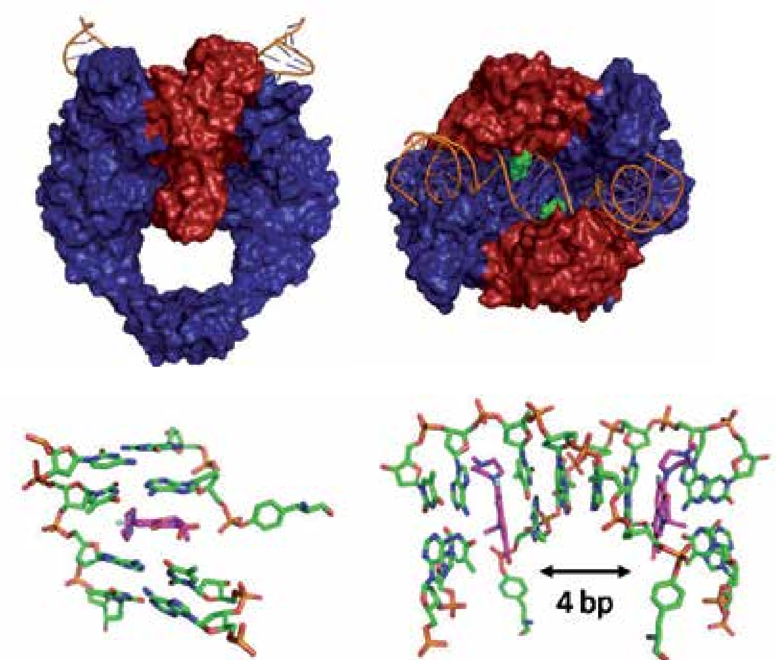

Fig. 10. Structure of the *M. tuberculosis* DNA gyrase catalytic core in complex with DNA and moxifloxacin. Side view (top left) and top view (top right) of the molecular surface of the catalytic core. The Toprim domain is represented in red, the BRD in blue, the 35 base pairs DNA oligonucleotide in orange and the moxifloxacin in green. (Bottom left) Close view of the structure of the intercalated moxifloxacin (magenta) in the broken DNA double helix (green). The catalytic tyrosine (Y129 in the *M. tuberculosis* DNA gyrase sequence) is shown in green outside the DNA helix. (Bottom right) Close view of both moxifloxacin molecules in the broken DNA showing the four base pairs in between the two bound fluoroquinolones. Both catalytic tyrosines of each monomer are shown in green in the DNA major groove.

6. Fluoroquinolone resistance in *M. tuberculosis*

Fluoroquinolone resistance in *M. tuberculosis* is defined by resistance to ofloxacin (ability of the bacilli to grow on medium containing 2 mg/l ofloxacin (WHO, 2001) and is mainly due to mutations in DNA gyrase in the QRDRs (see section 4) (Takiff et al., 1994). Mutations within the QRDRs are responsible of at least 75% of fluoroquinolone resistance in *M. tuberculosis*, affecting most commonly the GyrA subunit (G88A, A90V, S91P, D94G, H, N, Y) but also the GyrB subunit (Aubry et al., 2006b; Veziris et al., 2007; Siddiqi et al., 2002; Feuerriegel et al., 2009). Modeling of the *M. tuberculosis* DNA gyrase catalytic core allowed to clearly establish that QRDR residues of both subunits are spatially close and form the quinolone-binding pocket (QBP) (Fig. 11). In this pocket, the fluoroquinolone is maintained on one side by three residues of the QRDR-A, G88, D89 and A90, that are in close contact with the quinolone conserved carboxylic function (R3 group, Fig. 1 and 11), and on the other side by five residues of the QRDR-B, D500, R521, N538, T539 and E540 in close contact with the R1, R7 and R8 groups of the quinolone (Fig. 1 and 11). Almost all these residues have been shown to be directly involved in the level of resistance to fluoroquinolone when they are modified (Aubry et al., 2004; 2006; Matrat et al., 2006; 2008; Mokrousov et al., 2008; Von Groll et al., 2009; Kim et al., 2011). The model shows that the geometry of the QBP is crucial for the recognition and the stability of fluoroquinolone in the pocket. Any modification of the amino acids belonging to the QBP leads to either a direct or indirect change of the geometry of the pocket (Fig. 11). The pocket becomes too large to stabilize the fluoroquinolone, when the side chain of the modified amino acid becomes smaller. In contrast, the pocket becomes too small to fix the fluoroquinolone, when the side chain of the modified amino acid is bulkier. An indirect effect on the geometry of the pocket is observed when amino acid substitutions are localised in the H4 helix that interacts with the major groove of the DNA. As DNA is also part of the QBP (Fig. 11), modification of the structure of the DNA will modify the geometry of the pocket, and can lead to destabilisation of the fluoroquinolones in the pocket. This mechanism could explain why substitutions of the amino acid D94 (position mostly found in *M. tuberculosis* strains resistant to quinolones) have paradoxical effects, e.g. the substitution by an amino acid with a smaller side chain, such as alanine or glycine, increases the resistance to the same level than the substitutions by amino acids with bulky side chains.

Resistance to fluoroquinolones of *M. tuberculosis* DNA gyrase results from two mechanisms. On one hand, the lower natural affinity of the *M. tuberculosis* DNA gyrase catalytic core for fluoroquinolones is responsible for "intrinsic resistance" and is attributable to the amino acid nature at three positions, 81 and 90 in the QRDR-A and 521 in the QRDR-B (see section 3). On the other hand, DNA gyrase modifications found in the QRDRs of DNA gyrases of clinical *M. tuberculosis* strains are responsible for "acquired resistance". Interestingly, amino acid at two positions in the QBP play a crucial role in both natural and acquired resistance, position 90 in the QRDR-A, which is an alanine in the *M. tuberculosis* sequence and position 521, an arginine in the *M. tuberculosis* QRDR-B. Both amino acid are essential for the shape of the QBP and, as a result, for the binding of quinolone. For example, the substitution of residue A90 in the wild-type *M. tuberculosis* DNA gyrase to S90 generates a pocket better adapted to the size of moxifloxacin (Fig. 12), increasing the susceptibility to this inhibitor. In contrast, the A90V substitution, found in some strains resistant to moxifloxacin, generates a

pocket too small to bind moxifloxacin (Fig. 12). In the QRDR-B, the side chain of residue R521, an amino acid located in the Toprim DBL, points towards the DNA minor groove and forms a "flap" that blocks the fluoroquinolone in the pocket through contact with the R7 group (Fig. 11). The increase of susceptibility to fluoroquinolones by the substitution of R521 to lysine can be explained by the lower energy cost when moving an arginine than a lysine in the DNA minor groove (Rohs et al., 2010). This means that the "flap" created by this amino acid, would find it easier to open up and destabilize the fluoroquinolone, when this residue is an arginine rather than a lysine.

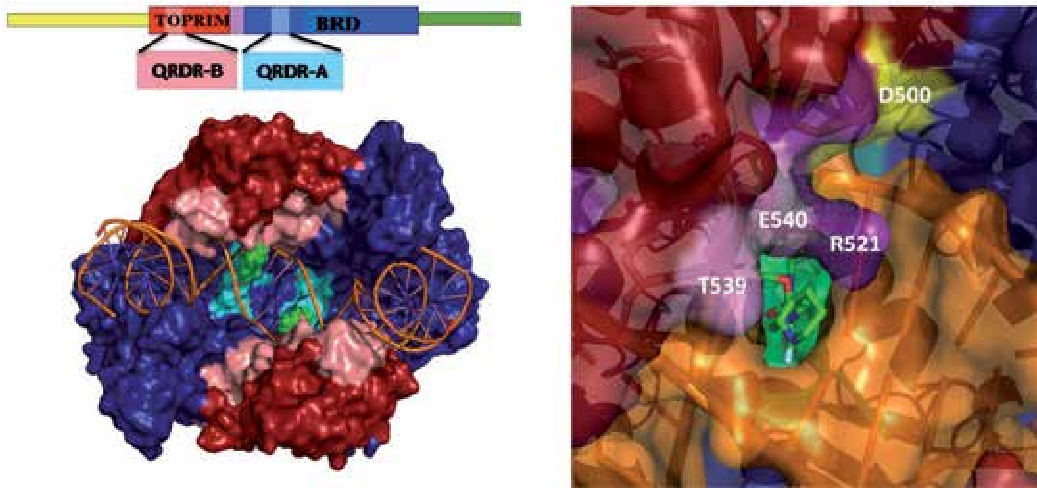

Fig. 11. (Top) Schematic representation of the *M. tuberculosis* DNA gyrase sequence. Subunit A contains the breakage-reunion domain (BRD, blue) and the C-terminal domain (CTD, green). The subunit B contains the ATPase domain (yellow) and the Toprim domain (red). *End of legend*. Localization of the QRDR is indicated in pink and light blue. (Bottom left) Top view of the molecular surface representation of the M. *tuberculosis* DNA gyrase catalytic core in complex with DNA and moxifloxacin showing the QBP (color code is the same than in Fig. 10). The QRDR-A and -B are represented in pink and light blue, respectively. (Right) Close view of the quinolone-binding pocket. The DNA-protein complex is represented in transparent molecular surface and moxifloxacin in sticks (color code is the same than in Fig. 10). The residues of the QRDR-B (Toprim) belonging to the QBP are indicated in pink, purple and yellow. Resides A90 of the QRDR-B is represented in light green in the background of the pocket.

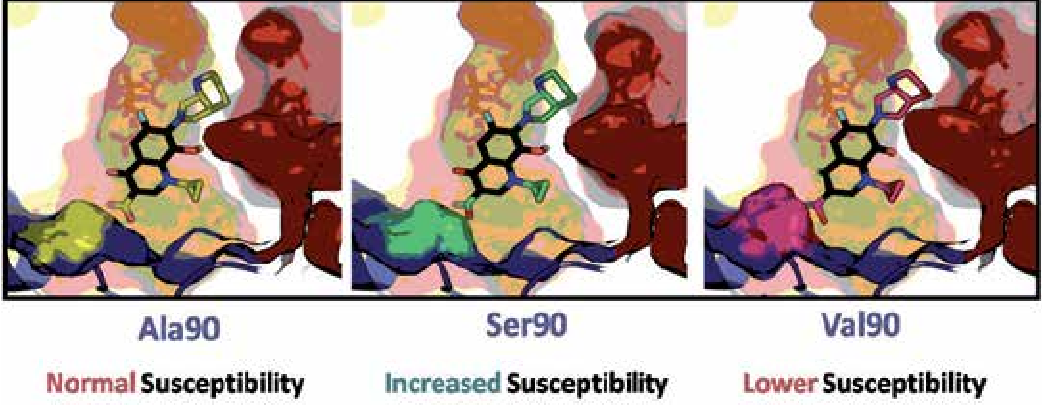

Fig. 12. Effect of the substitution of A90 (QRDR-A) on the geometry of the quinolone-binding pocket. (Left) Quinolone-binding pocket of the wild-type *M. tuberculosis* DNA gyrase. The A90 is coloured in yellow. (Middle) Substitution of A90 to serine (S90 is represented in green). (Right) Substitution of A90 to valine (V90 is represented in magenta).

7. Conclusion

This chapter has described the structural insights into the bacterial type II topoisomerases, the targets of quinolones and into the quinolone resistance mechanism, discussed in the context of the enzyme modifications, e.g. amino acid substitutions, described in the literature that are implicated in quinolone resistance. It has shown that the tridimensional structure of the *M. tuberculosis* catalytic core in complex with DNA and a quinolone molecule is a powerful tool to better understand the relationships between the sequence, the structure and the resistance phenotype in *M. tuberculosis* DNA gyrase.

Knowledge of structural data has applications both in the diagnosis of resistance in *M. tuberculosis* but also in tuberculosis treatment. This powerfulness consists in four essential aspects. It strongly contributes to (1) the analyses of the results obtained by molecular studies since the variety of new mutations, especially in the GyrB subunit, is increasing, (2) the discrimination between substitutions identified in fluoroquinolone-resistant clinical strains that are implicated or not in fluoroquinolone resistance (Pantel et al., 2011), (3) the prediction of the effect on susceptibility to quinolones of new undescribed mutations, and finally (4) to the design of new quinolones, as quinolones used nowadays for tuberculosis treatment have been designed before structural data on the *M. tuberculosis* DNA gyrase catalytic core were available.

8. Acknowledgment

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14

Antimycobacterial Activity Some Different Lamiaceae Plant Extracts Containing Flavonoids and Other Phenolic Compounds

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1. Introduction

Mycobacterium tuberculosis is a pathogenic bacteria species of the genus *Mycobacterium*, first discovered in 1882 by Robert Koch, which causes tuberculosis (TB) (Ryan & Ray, 2004). The disease is characterized by symptoms such as sepsis, septic shock, multiple organ failure (Muckart & Bhagwanjee, 1997). It may spread to the central nervous system and cause TB meningitis, intracranial tuberculomas, or abscesses (Harisinghani et al., 2000; Hwang et al., 2010).

After the late 1980s, tuberculosis morbidity and mortality rates became a major health problem for industrialized countries (Raviglione et al., 1995; Heym & Cole, 1997). Multidrug-resistant tuberculosis (MDR TB) and extensively drug resistant tuberculosis (XDR TB) has become a common phenomenon, which cause drugs to be ineffective. MDR-TB results from either primary infection or may develop in the course of a patient's treatment. MDR TB is resistant to at least two first-line anti-TB drugs, isoniazid (INH) and rifampicin (RIF), which are most powerful anti-TB drugs; XDR TB is resistant to INH and RIF, plus fluoroquinolone and at least one of three injectable second-line drugs such as capreomycin, kanamycin, and amikacin. Treatment of XDR-TB is not possible by first-line anti-TB drugs, which are less effective, expensive and toxic; in addition treatment takes two years or more (WHO, 2011a; WHO, 2011b).

Mycobacteria are resistant to most common antibiotics and chemotherapeutic agents due to the mycobacterial cell wall composition of bacterial peptidoglycans (Slayden & Barry, 2000; Lee et al., 1996; Brennan et al., 1995), a lipophilic layer of long-chain fatty acids, and mycolic acids (Barry et al., 1998). The rich lipids of the cell wall has an important role in their virulence (Murray, Rosenthal and Pfaller, 2005). This structure provides a highly hydrophobic and efficient barrier to antibiotics and chemotherapeutic agents (Jarlier & Nikaido 1994). Thus, this cell wall composition restricts the choice of drug treatment. Compounds capable of blocking efflux pumps so that antibiotics can gain access to their targets are of obvious importance (Viveiros et al, 2003). Increased activity of existing efflux

pumps were caused by ineffective therapy of TB patients, which is develops bacterial resistancy to one or more drug. Recent researches showed that mycobacteria have multiple putative efflux pumps which is a key factor for gaining resistance (Braibant, 2000; De Rossi et al., 2002). In addition to, chromosomal gene mutation and then accumulation of these mutations also one of the origine of multidrug-resistant (Ramaswamy & Musser, 1998; Gillespie, 2002; Viveiros et al., 2003).

Some well-known drugs and their mechanism of actions affect bacteria in different ways. Streptomycin (STR) has been used to treat tuberculosis patients since the 1940s; INH was used to treat tuberculosis in the 1960s; RIF was first used at the beginning of the 1970s (Toungoussova et al., 2006); and ethambutol (EMB) was introduced in 1961 as a bacteriostatic first-line drug (Perdigão et al., 2009). RIF inhibits transcription to RNA and translation to proteins by binding its' beta subunit of RNA polymerase in bacteria; however, if bacteria produce a different beta subunit, they are not affected by the drug (O'Sullivan et al., 2005). STR is a protein synthesis inhibitor. STR interacts with a 30S subunit of ribosome and disrupts protein synthesis (Sharma et al., 2007; Springer et al., 2001). Its mechanism of action starts with binding tightly to the phosphate backbone of 16S rRNA in different domains and making contact with the S12 ribosomal protein; finally it causes misreading of the bacterial genetic code during translation (Carter et al., 2000; Hosaka et al., 2006). INH is activated by an enzyme, catalase-peroxidase, called KatG in *M. tuberculosis*. KatG, isonicotinic acyl and NADH form a complex that binds enoyl-acyl carrier protein reductase (InhA) and affects fatty acid synthase. The identification of an enoyl-acyl carrier protein (ACP) reductase plays a role in INH resistance named InhA. In this way, mycolic acid synthesis and cell wall development are inhibited (van Veen & Konings, 1998; Slayden & Barry, 2000; Suarez et al., 2009). As a result, when exposed to INH, Mycobacteria lose their acid-fastness and viability. Changes in the catalase-peroxidase gene (katG) and the inhA genes have been defined as one of the mechanisms of drug resistance in *M. tuberculosis* (Morris et al., 1995; Heym et al., 1995; Mohamad et al., 2004). EMB is a potent synthetic antimycobacterial agent that may cause optic neuropathy in patients (Kozak et al., 1998).

EMB has a bacteriostatic effect and interferes with mycolic acid synthesis, phospholipid metabolism, and arabinogalactan synthesis (Kilburn et al., 1977; Takayama & Kilburn, 1989) and affects nucleic acid metabolism (Forbes et al.,, 1965). EMB has synergistic actions, when combined with other agents, against *Mycobacterium avium* (Inderlied and Salfinger, 1995). TB is currently one of the most serious infectious diseases all over the world. Antimycobacterial drugs cause unpleasant side effects and trigger changes in the antibiotic target, thereby reducing the efficacy of drug therapies. Mycobacteria have recently increased their virulence and tuberculosis (TB) is the most lethal infection in the world. Between 1980 and 2005, 90 million cases of TB worldwide were reported to the WHO (World Health Organization) and over three in every thousand people die of TB, which is the highest rate in the world (Lall and Meyer, 1999). Yang et al. (2010) also reported that the prevalence of MDR-TB among the Chinese people has increased since 1985. The WHO stated, ‘‘The global incidence of TB was estimated to be 136 cases per 100,000 population per year in 2005. In addition, the WHO region of the Americas and the WHO African region represent a total of 8.8 million new cases of TB and 1.6 million deaths from TB every year” (World Health Organization, 2008a). There were 9.5 million TB-related child deaths globally in 2006 (World Health Organization,

2008b). Today, one of the most important global health problems is changes in behavior of TB, such as resistance to anti-TB drugs and the influence of the HIV epidemic (World Health Organization, 2008a). WHO Global TB Control (2009) reported that there were approximately 0.5 million cases of MDR-TB in 2007. The World Health Organization (2010) reported that there were 9.4 million new TB cases globally and approximately 1.7 million people died from TB. The organization also reported that 1.2 million people were living with HIV and 76% of these people were residing in the African region while 14% were living in the South East Asian region in 2009 (World Health Organization, 2010). In South Africa, TB is the most commonly notified disease and the fifth largest cause of death among the black population. The prevalence of TB continues to increase all over the world. Although the main reasons are known to be the human immunodeficiency virus (HIV) and the emergence of drug-resistant strains of TB (WHO, 2009), the other factors include poverty, drug addiction, inadequate health conditions and migration (Antunes et al., 2000; Merza et al., 2011). WHO reports (2011a) estimated that the risk of developing tuberculosis (TB) is between 20 and 37 times greater in people living with HIV than among the general population. In addition, infection with Human immunodeficiency virus type 1 (HIV-1) disrupts immunological control of *Mycobacterium* infections due to the loss of CD4+ T cells. Salte et al. (2011) reported that *Mycobacterium avium* is one of the most common opportunistic infections among AIDS patients. Snider et al. (1985) examined the transmission of MDR-TB strains from adult to child contacts and confirmed the progression of the disease by DNA fingerprint studies. INH-resistant strains caused much infection in children who were in contact with adults.

Mycobacteria are Gram-resistant non-motile pleomorphic rods with a waxy cell wall. These bacteria include high lipid content within the cell wall (Wilbur et al., 2009; Jackson et al., 2007), the complex lipids esterified with long-chain fatty acids. Myobacteria are referred to as acid fast Gram-positive due to their resistance to dilute acid and ethanol-based de-colorization procedures and their lack of an outer cell membrane. When they are stained using concentrated dyes, combined with heat, they do not give up the color by the dilute acid and ethanol-based de-colorization procedures (Ryan & Ray, 2004).

Some medicinal plants have been used to treat the symptoms of TB including *Acacia nilotica*, *Cassine papillosa*, C*henopodium ambrosioides*, *Combretum molle*, and *Euclea natalensis* from Africa (Watt and Breyer-Brandwijk, 1962; Pujol, 1990; Lall & Meyer, 2001; Bryant, 1966).

Natural products are an important source of new chemical compounds and, hopefully, therapeutic agents for many bacterial diseases. Lall and Meyer (1999) reported antimycobacterial activity of *Euclea natalensis* (Ebenaceae), which is rich in naphthoquinones, against drug-sensitive and drug-resistant strains of *M. tuberculosis.* Gordien et al. (2009) studied two terpenes, sesquiterpene and longifolene; and two diterpenes, totarol and trans-communic acid, obtained from the aerial parts and roots of *Juniperus communis*. They reported that totarol showed the highest activity against *Mycobacterium tuberculosis* H37Rv and that longifolene and totarol exhibited the most activity against rifampicin-resistant variants. Phenolic compounds have some effects on microbial metabolism and growth, depending on their concentration and active compounds (Alberto et al., 2001; Reguant et al., 2000).

Many studies have shown that phenolic compounds inhibit the growth of a wide range of Gram-positive and Gram-negative bacteria (Davidson et al., 2005; Estevinho et al., 2008)

Flavonoids are the most common group of polyphenolic compounds. Flavonoids are plant secondary metabolites with a fused ring system, which are found as glycosides in plants. Of the well-known flavonoids, apigenin has a calming effect, while quercetin and kaempferol have a sedative effect (Jäger & Saaby, 2011).

In previous studies, flavonoids were reported to show antimicrobial (Cushnie & Lamb, 2006, 2011), anti-allergic (Chen et al., 2010), anti-inflammatory (Seo et al., 2000), and anti-carcinogenic (Lee et al., 2008) activities. Until 2004, it was suggested (Cushnie and Lamb, 2005, 2011) that their antibacterial efficacy was dependent upon cytoplasmic membrane damage by perforation (Ikigai et al., 1993), inhibition of nucleic acid synthesis (Mori et al., 1987) and disruption of energy metabolism due to NADH-cytochrome c reductase inhibition (Haraguchi et al., 1998). Currently, some other supporting mechanisms have emerged to indicate the role of flavonoids in antibacterial activity; these mechanisms include damage to the cytoplasmic membrane by generating hydrogen peroxide (Tamba et al., 2007; Kusuda et al., 2006; Sirk et al., 2008), inhibition of nucleic acid synthesis (Gradisar et al., 2007; Wang et al., 2010) and inhibition of ATP synthase (Chinnam et al., 2010). While Puupponen-Pimiä et al. (2001) reported that catechin, rutin and quercetin did not affect the growth of *E. coli*, Vaquero et al., (2007) reported that quercetin was the strongest inhibitor active against bacteria, dependent on concentration.

Lamiaceae, also known as mint, is a family of flowering plants that includes 250 to 258 genera and approximately 6,000 to 6,970 species across the world (Zomlefer, 1994; Mabberley, 1997). The family has a cosmopolitan distribution and contains many plant species with culinary and medicinal purposes; examples of the former are basil, mint, rosemary, sage, savory, marjoram, oregano, thyme, lavender, and perilla (Naghibi et al., 2005). The Lamiaceae family of plants have been used since ancient times as folk remedies for various health problems such as common cold, throat infections, acaricidal, psoriasis, seborrheic eczema, hemorrhage, menstrual disorders, miscarriage, ulcer, spasm and stomach problems (Takayama et al., 2011; Loizzo et al., 2010;. Ribeiro et al., 2010). Their constituents, particularly diterpenoids and triterpenoids, have been found to have antiseptic, antibacterial, anti-inflammatory, cytotoxic, cardio-active and other properties (Ulubelen, 2003).

In our previous studies, we tested more than 100 plant extracts, some of which showed antimycobacterial activity against *Mycobacterium tuberculosis*. In this study, in the light of our past experiences, we present a continuation of the testing of some of the plant extracts and the efficacy of their antimycobacterial properties.

2. Materials & methods

2.1 Plant materials

Aerial parts (herbs in the flowering stage) of plants, *Origanum acutidens* (Hand.-Mazz.) Ietswaart, *Origanum sipyleum* L., *Salvia viridis* L., *Salvia microstegia* Boiss&Bal., *Satureja boissieri* Hausskn. ex Boiss., *Stachys byzantina* C.Koch., *Stachys cretica* L., *Stachys cretica* subsp. *smyrnaea* Rech. fil., *Thymus syriacus* Boiss., and *Thymus cilicicus* Boiss&Bal.*(endemic)* were collected from different parts of Turkey between 2009 and 2010. The plants were identified by Assoc. Prof. Dr. F. Satil at Balıkesir University, Turkey. Voucher specimens were deposited in the herbarium of Balikesir University Department of Biology. Herbarium plant data, such as locality, altitude, and collection time and identification number of species are given in Table 1.

2.2 Preparation of plant extracts

The plants [*O. acutidens* (60 g), *O. sipyleum* (66 g), *Salvia viridis* (12 g), *S. microstegia* (100 g), *Satureja boissieri* (101 g), *Stachys byzantina* (65 g), *S. cretica* (37 g), *S. cretica* subsp *smyrnaea* (71 g), *T. syriacus* (44 g), and *T. cilicicus* (85 g) (endemic)], were air-dried at room temperature. Extracts of dried plants were prepared by the sequential extraction method (Chan et al., 2008) using 1 L of chloroform (CL), ethyl acetate (EA) and methanol (ME) at room temperature over a period of fifteen days. Finally, three extract fractions were obtained from each plants. The extracts were filtered through filter paper concentrated using a rotary evaporator and dried in vacuo at 40 ºC. They were stored at −20◦C until use. The total yields from chloroform (CL), ethyl acetate (EA) and methanol (ME) extracts were *O. acutidens* (0.57, 0.74, 4.88g), *O. sipyleum* (2.14, 1.61, 5.50g), *Salvia viridis* (0.22, 0.15, 1.56 g), *S. microstegia* (8.17, 0.77, 6.70g), *Satureja boissieri* (4.05, 1.08, 8.82g), *Stachys byzantina* (7.69, 1.10, 5.15g), *S. cretica* (1.16, 0.59, 3.47g), *S. cretica* subsp. *smyrnaea* (1.92, 1.43, 6.92g), *T. syriacus* (1.80, 1.08, 2.85g), and *T. cilicicus* (2.37, 3.18, 5.35g) respectively. All stocks were stored at -20 ºC. To conduct antimicrobial activity tests, samples were dissolved in dimethyl sulfoxide (DMSO) and prepared at a concentration of 100 mg/mL. All the extracts used were sterilized by passing through a syringe filter (Sartorius, Ø 0.22 µm.) before use.

2.3 Chemicals and samples

Gradient grade MeOH and acetonitrile were purchased from MERCK. Gradient grade water (18m) was prepared using a Purelab Option-Q elga dv25 system. All standard stock solutions (1 mg/mL) were prepared by dissolving each compound in MeOH. Standards, rosmarinic acid, trans cinnamic acid, and ferulic acid were purchased from Aldrich, caffeic acid and gallic acid from Sigma-Aldrich and all other chemicals used were obtained from Sigma. All solutions were filtered through a membrane filters (Sartorius, Ø 0.22 µm.) before injection into the capillary.

2.4 LC-MS conditions

Analyses were performed with Agilent LC-MS system (1200 LC with a single quadrupole) with ESI source negative mode. Source parameters were optimized to provide highest sensitivity. The source parameters are: Gas temperature 350 °C, drying gas flow 12 l/min, nebulizer pressure 50 psi, capillary voltage 3500 V., seperation was carried by a C-18 column (EC-C18 4,6x50mm 2.7um). Mobile phases are A: Water (5 mM ammonium formate+ 0.5 % formic acid) and B (acetonitrile). The gradient program is: 5 % B for starting condition and increased up to 45 % B in 1 min, hold 2 min, increase % B to 95 from 3 to 6 min, hold 1 min and decrease % B to 5% at final step. Total run time is 12 min. Injection volume is 5 µl. The detection was accomplished using MS SIM mode. Scan mode is also used. The LC–MS analysis was based in a method described by Pérez-Magariño et al. (1999).

2.5 Preparation of standards

Twenty standards were used for quantitative and qualitative determination: trans-cinnamic acid [(R_t) 4.98 min], ρ-coumeric acid (R_t 3.95 min), vanillic acid (R_t 3.79 min), gallic acid (R_t 1.89 min), caffeic acid (R_t 3.72 min), ferulic acid (R_t 3.99 min),), apigenin (R_t 4.83 min), naringenin (R_t 4.85 min), luteolin (R_t 4.43 min), epicatechin (R_t 3.67 min), quercetin (R_t 4.42

min), carnosic acid (R_t. 8.55 min), chlorogenic acid (R_t 3.59 min), rosmarinic acid (R_t 3.97 min), apigenin 7-glucoside (R_t 3.89 min), oleuropein (R_t 3.969 min), amentoflavone (R_t 5.16 min), naringin (R_t 3.83 min), rutin hydrate (R_t 3.69 min), hesperidin (R_t 3.85 min). Calibration concentrations were 1,4,5 and 20 ppm except one, apigenin 7-glucoside, was 0.9, 1.8, 4.5, 9, and 18 ppm and injection volume was 5 µL for all standards.

2.6 Organisms

The extracts were screened against four strain, *M. tuberculosis* H37Ra (ATCC 25177), *M. tuberculosis* H37Rv (ATCC 25618) and two-positive *M. tuberculosis* isolates obtained from patient from hospital, for antibacterial activity.

2.7 Preparation of *Mycobacterium tuberculosis* inocula

Bacterial suspensions of *M. tuberculosis* were prepared either from Lowenstein–Jensen slants or from complete 7H9 broth cultures. To prepare an inoculum that was less than 15 days old from a culture grown on Lowenstein-Jensen medium, a suspension was prepared in Middlebrook 7H9 broth. The turbidity of the suspension was adjusted to a 1.0 McFarland standard. The suspension was vortexed for several minutes and was allowed to stand for 20 min for the initial settling of larger particles. The supernatant was transferred to an empty sterile tube and was allowed to stand for an additional 15 min. After being transferred to a new sterile tube, the suspension was adjusted to a 0.5 McFarland turbidity standard by visual comparison. One mL of the adjusted suspension was diluted in 4 mL of sterile saline solution.

No	Genus species authority (Lamiaceae)	Locality	Altitude (m)	Collection Time	Herbarium Number
1	*Origanum acutidens* (Hand.-Mazz.) Ietswaart.	Between Elazig-Erzincan	1230	15.Jul.2009	FS 1605
2	*Origanum sipyleum* L.	Between Balıkesir-Savastepe	200	02.Jul.2009	FS1561
3	*Salvia viridis* L.	Balikesir-Cagis	160	02. Jun.2010	FS1560
4	*Salvia microstegia* Boiss&Bal.	Van, Gurpinar	1100	26.Jun.2009	FS 1559
5	*Satureja boissieri* Hausskn. ex Boiss.	Adiyaman-Yazibaşı village	980	20.Sep.2010	FS1562
6	*Stachys byzantine* C.Koch.	Bursa, Mezitler	860	08.Jul.2009	FS1602
7	*Stachys cretica* L.	Balikesir-Edremit, Kazdagi,	350	23.Jun.2009	FS1603
8	*Stachys cretica* subsp. *smyrnaea* Rech.fil.	Balikesir-Edremit, Kazdagi,	1260	17.Jul.2009	FS1604
9	*Thymus syriacus* Boiss.	Gaziantep-Burc forest	850	03.Aug.2009	FS1558
10	*Thymus cilicicus* Boiss&Bal.*(endemic)*	Antalya, Belek	1000	12.Jul.2010	FS1556

Table 1. Herbarium data of plants

To prepare *M. tuberculosis* inoculum using a BACTEC MGIT tube with positive growth, the positive tubes were used beginning from the day after the sample first became positive (day-1 positive), up to and including the fifth day (day-5 positive). The positive tubes that were older than five days were subcultured into fresh growth medium. Tubes that were day-1 and day-2 positive were used in the inoculation procedure for the susceptibility tests. The tubes that were between day-3 and day-5 positive were diluted using 1 mL of the positive broth and 4 mL of sterile saline solution; the 5 mL diluted suspension samples were used for the inoculation procedures.

2.8 Antimycobacterial activity test

Antimycobacterial bioassay was performed using the Microplate Alamar Blue Assay (MABA) method (Collins and Franzblau, 1997). MIC was recorded as the lowest drug concentration that prevented to turn blue to pink colour by adding Alamar blue. MBC was also recorded the minimum extract concentration that do not cause any color changing in cultures reincubated in fresh medium.

2.8.1 Determination of Minimal Inhibitory Concentrations (MICs) for *Mycobacterium tuberculosis*

Microplates were inoculated with the bacterial suspension (20 µL per well except for the negative control wells) and incubated at 37 °C for 6 days. Alamar blue (15 µL, Trek Diagnostic system) was then added to the bacterial growth control wells (without extract) and the microplates were incubated at 37 °C for an additional 24 hours. If the dye turned from blue to pink, (indicating positive bacterial growth) then Alamar blue solution was added to the other wells to determine the MIC values. All tests were performed in triplicate.

2.8.2 Determination of mycobactericidal activity

All the extracts prepared from aerial parts of plants, the herbarium data of these species shown in Table 1, were analyzed by LC-MS. The quantity of chemicals in the methanol extracts are given in Table 2. Chromatograms of phenols in all extracts were compared to chromatograms of standards (Figs. 1–3).

The plant extracts described above were used in mycobactericidal activity tests. Two-fold dilution series in triplicate sets of parallel microplate wells were used for each extract. To determine the minimum bactericide concentrations (MBCs), fresh Middlebrook 7H9 culture broth (185 µL) was transferred to each well. A fifteen microliter of an Mycobacterial suspension, from MIC concentration and higher concentration wells obtained from the MIC test described above was added to each well, in order to determine the minimum bactericide concentration (MBC).

Two microplate wells were used as positive and negative controls, and each test was repeated in triplicate. For the negative controls, 200 mL of fresh broth (Middlebrook 7H9 culture medium and OADC) was used. For positive controls, including 185 µL and inoculums from former positive control wells (15 µL) was used. After 24 hours of incubation and colour development using the Alamar blue solution, MBCs were recorded as the minimum extract concentration that did not cause any colour change in cultures when reincubated in fresh medium.

3. Results

3.1 Phenolics determined by LC-MS analyses

The ten samples and selected standards were analyzed by MS in ESI negative ion mode. Scan mode is also used. In this method, trans-cinnamic acid, p-coumaric acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, apigenin, naringenin, luteolin, epicatechin, quercetin, carnosic acid, chologenic acid, rosmarinic acid, apigenin 7-glucoside, amentoflavone, oleuropein, naringin, rutin hydrate and hesperidin were chosen as standard phenolics to determine the phenolic structures of the samples according to ionization response in ESI mass spectrometry and chromatographic retention time.

Ion profile of negative ion electrospray LC/MS analysis experimental conditions are given above, from plants CL, EA and ME extracts is shown Fig. 1-2 and Table 2. Phenolics of samples were identified by comparing standard phenolic data such as retention times, main ions observed under fragmentation voltage of 80 Volt.

No	Phenolics	R_t min	$[M-H]^-$	Fragment ions
1	*trans*-Cinnamic acid	4,984	147	80
2	ρ-Coumaric acid	3,95	163	80
3	4-Hydroxy-3-metoxybenzoic acid (vanillic acid)	3,747	167	80
4	Gallic acid monohyrate	1,893	169	80
5	Caffeic acid	3,724	179	80
6	Ferulic acid	3,991	193	80
7	Apigenin	4,831	269	80
8	(±)-Naringenin	4,859	271	80
9	Luteolin	4,433	285	80
10	(-)-Epicatechin	3,675	289	80
11	Quercetin	4,427	301	80
12	Carnosic acid	8,555	331	80
13	Chlorogenic acid	3,597	353	80
14	Rosmarinic acid	3,971	359	80
15	Apigenin 7-glucoside	3,896	431	80
16	Amentoflavone	5,169	537	80
17	Oleuropein	3,969	539	80
18	Naringin	3,834	579	80
19	Rutin hydrate	3,699	609	80
20	Hesperidin	3,853	609	80

Table 2. LS-MS characteristics of phenolic compounds

The major phenolic compounds of *T. cilicicus* CL extract were rutin hydrate and naringenin; for EA extract, rosmarinic acid and apigenin; and for ME extract, rosmarinic acid, oleropein, and apigenin.

The highest rosmarinic acid level within all plants were determined in *S. viridis* for CL extracts; in *S. boissieri* and *T. cilicicus* for EA extracts; *O. sipyleum S. byzantine* and *S. boissieri* for ME extracts.

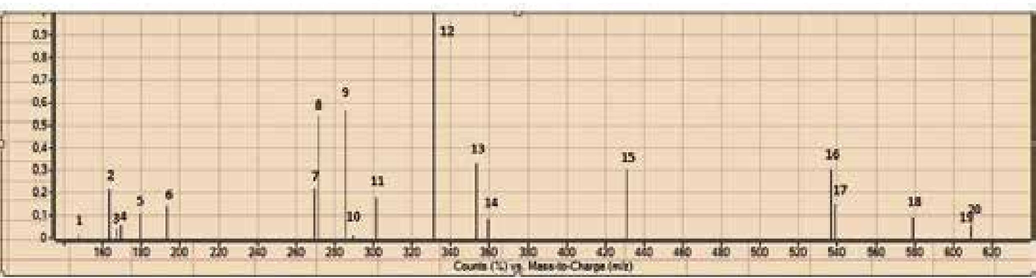


Fig. 1. ESI-MS Spectra of standard phenolics, 1; trans-cinnamic acid 2; p-coumaric acid 3; vanillic acid 4; gallic acid 5; caffeic acid 6; ferulic acid 7; apigenin 8; naringenin 9; luteolin 10; epicatechin 11; quercetin 12; carnosic acid 13; chlorogenic acid 14; rosmarinic acid 15; apigenin 7-glucoside 16; amentoflavone 17; oleuropein 18; naringin 19; rutin hydrate 20; hesperidin

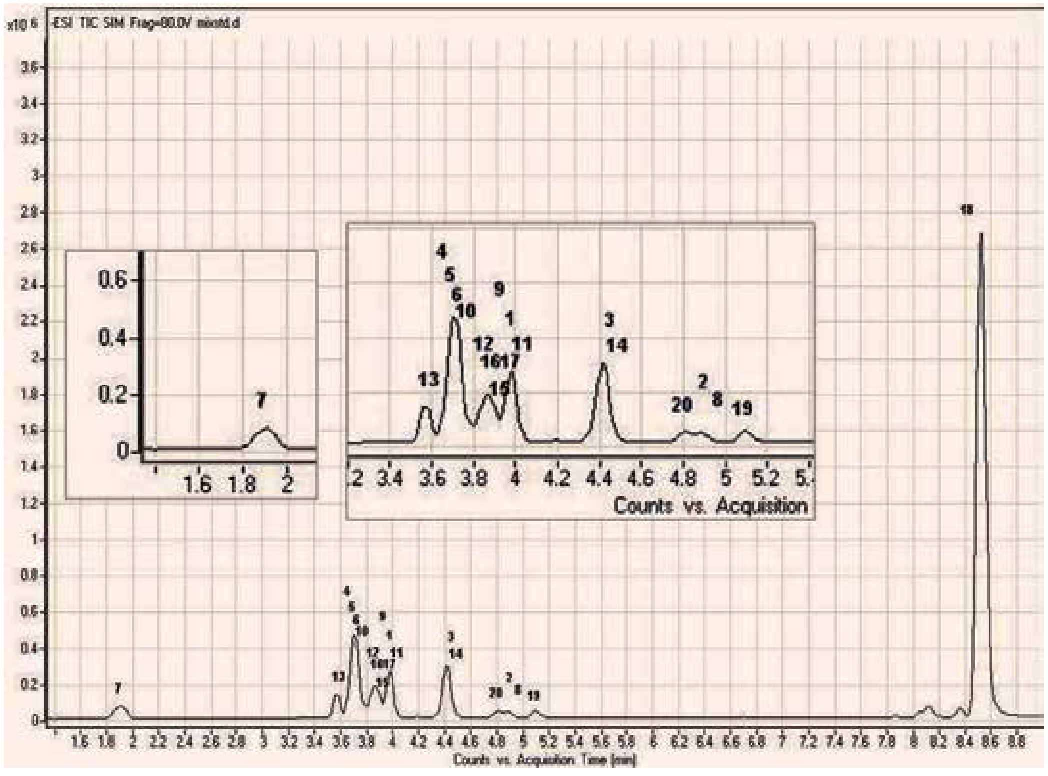
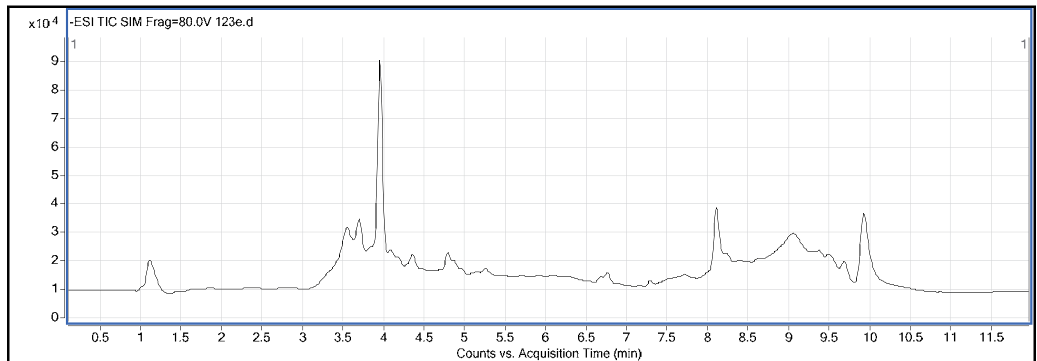
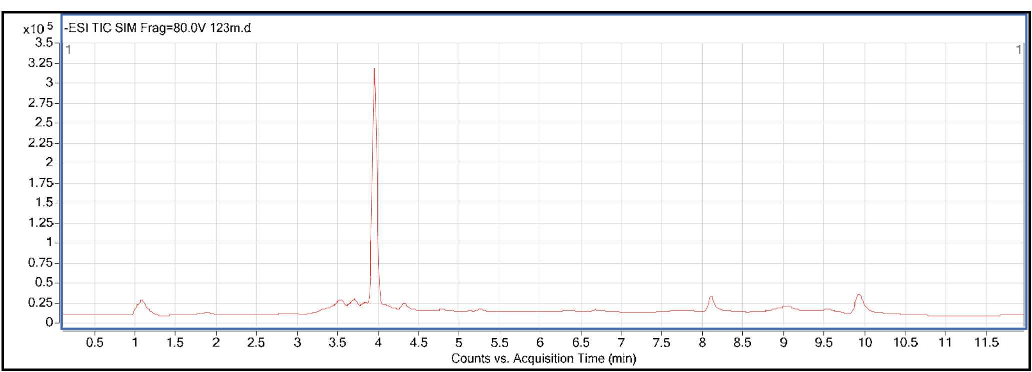


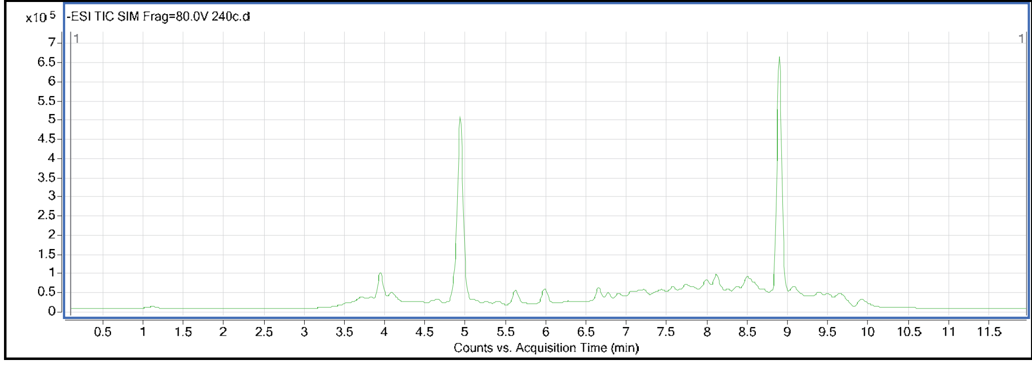
Fig. 2. ESI-TIC SIM chromatogram of standard phenolics, 1; trans-cinnamic acid 2; p-coumaric acid 3; vanillic acid 4; gallic acid 5; caffeic acid 6; ferulic acid 7; apigenin 8; naringenin 9; luteolin 10; epicatechin 11; quercetin 12; carnosic acid 13; chlorogenic acid 14; rosmarinic acid 15; apigenin 7-glucoside 16; amentoflavone 17; oleuropein 18; naringin 19; rutin hydrate 20; hesperidin



A



B



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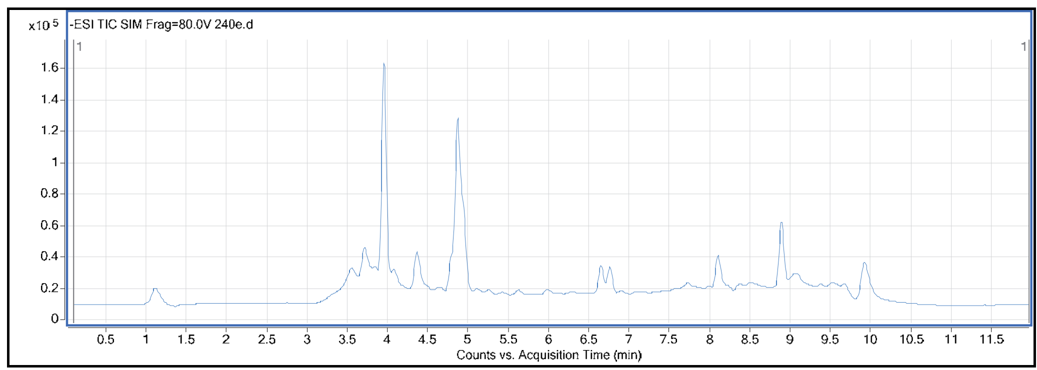
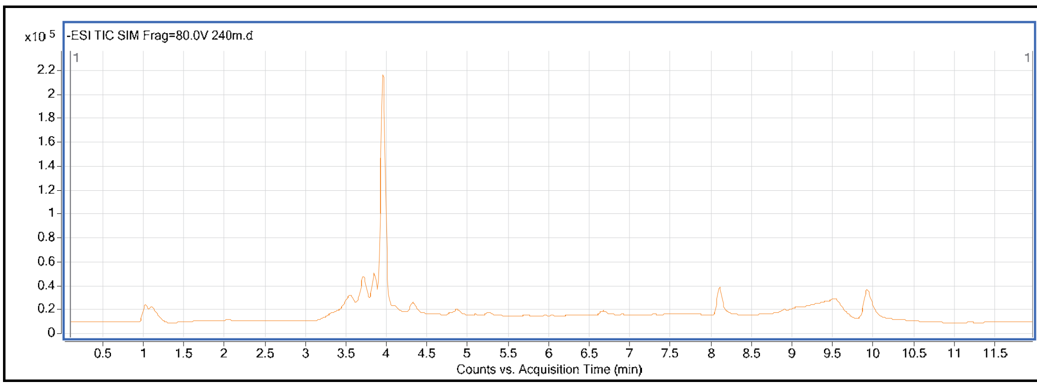


D



E

Fig. 3. ESI-TIC SIM chromatogram of *O.sipyleum* A) EA extract, B) ME extract; *S. boissieri* C) CL extract D) EA etract and E) ME extract. (Not all chromatograms are included).

The major phenolic compounds for *T. syriacus* were rutin hydrate and naringenin for CL extract; rosmarinic acid, apigenin naringenin, and vanillic acid for EA extract; rosmarinic acid, apigenin, luteolin, and oleropein for ME extract.

The major phenolic compounds of *O. acutidens* determined by LC-MS analyses were rutin hydrate for the CL extracts; rosmarinic acid and oleuropein for the EA extracts, rosmarinic acid; and vanillic acid for the ME extracts. The major phenolics of *O. sipyleum* were rutin hydrate for CL extracts; rosmarinic acid and vanillic acid for EA and ME extracts. The major phenolics of CL extracts of *S. viridis* were rosmarinic acid and rutin hydrate; for EA extracts, oleuropein followed by rosmarinic acid; for ME extracts, rosmarinic acid, chlorogenic acid and hesperidin.

The major phenolic compounds for *S. microstegia* were rutin hydrate for CL extracts; apigenin, luteolin, and rosmarinic acid for EA extract; and rosmarinic acid, apigenin and luteolin for ME extracts. In *S. boissieri*, the major phenolics for CL extracts were apigenin and naringenin; for EA extracts, rosmarinic acid, naringenin and hesperidin; for ME extracts, rosmarinic acid and hesperidin (Fig 3). The major phenolic compounds in the CL extracts of

Pt	Ex	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
O. acutidens	CI	52,87	*	*	*	104,13	*	*	10,32	*	51,28	*	*	*	*	*	*	*	*	*	*
	Ea	1029,88	*	*	60,54	106,20	34,67	*	47,17	223,42	435,04	61,65	354,02	*	*	*	23,35	723,56	*	*	329,69
	M	27306,44	130,50	*	85,19	208,98	230,09	*	*	345,49	589,89	*	*	280,85	*	150,24	94,88	95,31	*	*	182,66
O. sipyleum	CL	141,64	93,63	*	175,89	606,25	*	*	*	*	77,30	*	*	*	*	*	*	*	*	*	*
	EA	3431,07	131,42	*	293,80	569,54	*	*	*	*	784,83	*	*	*	*	*	*	263,22	*	*	82,75
	ME	58648,70	345,47	*	41,24	25,92	178,81	*	*	13,13	929,00	76,12	*	64,40	66,57	37,05	403,60	352,15	*	*	177,79
S. viridis	CL	409,20	24,37	*	122,01	153,62	*	*	*	*	81,76	5,55	*	*	*	*	*	51,39	*	*	*
	EA	898,43	284,22	*	218,39	607,4	*	*	*	*	76,22	*	*	*	*	*	585,95	1375,02	*	*	*
	ME	7978,30	393,66	*	237,32	246,56	32,53	*6,92	*	15,89	159,48	66,11	*	2316,58	809,02	120,00	1206,49	52,38	*	*	181,48
S. microstegia	CL	50,09	*	*	71,10	121,27	*	*	*	*	15,18	29,92	*	*	*	*	*	50,28	*	*	10,82
	EA	2927,63	111,29	*	194,532	551,48	*	*	*	1019,87	689,43	86,98	376,94	126,95	4883,57	*	96,67	180,79	*	*	5507,09
	ME	20759,50	119,37	*	425,61	167,26	255,86	*	*	318,971	217,88	25,93	*	96,10	1210,50	162,47	775,78	27,62	*	*	1315,47
S. boissieri	CL	52,39	30,28	*	*	122,80	313,40	*	*	*	165,17	364,24	429,47	*	*	*	*	53,72	38,11	*	1423,20
	EA	6380,13	153,53	*	*	575,62	*	*	*	*	465,64	635,25	3778,51	452,64	*	*	1692,63	*	*	*	789,85
	ME	34416,56	239,78	*	225,05	230,25	156,12	*	*	*	247,21	245,99	480,27	411,78	119,31	238,50	15878,94	648,55	*	*	139,60

Pt	Ex	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
S. byzantina	CL	75,69	*	*	58,31	165,10	*	*	*	*	*	17,71	*	*	*	*	*	81,71	*	*	85,21
	EA	1675,88	76,60	*	*	475,972	*	*	*	*	318,83	*	*	*	2130,60	107,53	354,41	312,75	*	*	5352,95
	ME	42665,98	132,21	*	115,22	309,00	176,08	*	20,66	79,42	127,44	16,16	*	78,23	958,49	1477,40	2187,10	239,67	*	*	2116,40
S. cretica	CL	69,61	22,81	*	*	126,01	*	*	249,72	*	209,24	52,39	*	*	*	*	*	31,50	*	*	*
	EA	115,74	24,26	*	*	109,84	*	*	*	*	121,07	45,85	*	26,12	*	*	29,81	350,88	*	*	81,67
	ME	598,99	117,17	*	94,41	274,14	*	*	*	*	160,79	20,18	19,44	8090,32	144,33	80,71	232,07	45,19	*	*	398,86
S. cretica subsp. smyrnaea	CL	117,24	*	*	*	111,94	*	*	38,58	*	255,04	14,99	*	*	*	*	*	48,86	*	*	*
	EA	144,56	23,26	*	35,22	145,60	*	*	61,36	208,65	509,90	52,02	14,63	481,18	23,51	*	123,65	171,18	*	*	148,91
	ME	114,23	142,70	*	61,72	397,43	*	*	*	25,53	225,07	15,59	*	13986,78	*	285,12	1066,13	56,18	*	*	175,43
T. syriacus	CL	48,34	52,92	*	89,55	112,60	*	*	*	*	210,73	133,71	137,15	*	*	*	17,33	53,27	*	*	97,82
	EA	2783,2	113,76	*	*	228,36	*	*	*	*	891,03	220,39	986,57	*	385,80	*	39,73	131,63	*	*	2732,39
	ME	21952,28	333,75	*	40,87	301,74	166,01	*	*	13,56	352,18	109,07	202,50	210,00	1082,13	*	587,01	780,81	*	*	1713,63
T. cilicicus	CL	60,70	42,24	*	99,72	116,22	*	*	*	*	84,40	34,64	103,27	*	*	*	*	33,85	*	*	57,10
	EA	5120,55	258,34	*	*	1038,34	*	*	*	*	450,38	*	797,61	*	*	*	*	*	*	*	4201,30
	ME	36529,24	301,38	*	41,87	260,59	853,26	*	*	38,55	334,22	15,11	200,71	15,32	1678,39	*	1025,33	8870,71	*	*	1945,58

Pt: Plants; Exts: Extracts; CL: Chloroform; EA: Ethyl Acetat; ME: Methanol; 1; Rosmarinic Acid 2; Naringin 3; Quercetin 4; Epicatechin 5; Rutin Hydrate 6; Caffeic Acid 7; Gallic Acid 8; Trans-cinnamic Acid 9; ρ-coumaric acid 10; Vanillic Acid 11; Ferulic Acid 12; Naringenin 13; Chlorogenic Acid 14; Luteolin 15; Apigenin 7-glucoside 16; Hesperidin 17; Oleuropein 18; Carnosic Acid 19; Amentoflavone 20; Apigenin

Table 3. Chemical concentrations in chloroform, ethyl acetat and methanol extracts of Lamiaceae species.

S. byzantina were rutin hydrate; in the EA extract, apigenin, luteolin and rosmarinic acid; and in the ME extract, rosmarinic acid, hesperidin and apigenin.

In *S. cretica*, the major phenolics in the CL extracts were trans-cinnamic acid and vanillic acid; oleuropein, vanillic acid and rosmarinic acid for EA extract; and chlorogenic acid and rosmarinic acid for ME extract. In *S. smyrnaea*, the major phenolics were rosmarinic acid and rutin hydrate for CL extracts; vanillic acid and chlorogenic acid for EA extract; chlorogenic acid and hesperidin for ME extracts.

The highest rutin hydrate contents were determined in *O. sipyleum* and *S. viridis* for CL extracts; *T. cilicicus, S. viridis, and S. boissieri* for EA extracts; *S. cretica* subsp. *smyrnaea, S. byzantina*, and *T. syriacus* for ME extracts.

Gallic acid was determined only in methanol extracts of *S. viridis*. Carnosic acid was also found in CL extract of *S. boissieri*. Only the EA extracts of *S. microstegia*, S. *byzantina, T. cilicicus* and *T. striacus* included the highest level of apigenin.

Trans-cinnamic acid was found in extracts of four plants (*O. acutidens*, S. *byzantina*, and *S. cretica* subsp. *smyrnaea*). Quercetin and amentoflavone were not found. The highest level of chlorogenic acid was found in ME extracts of *S. cretica* subsp. *smyrnaea*, *S. cretica*, and *S. viridis*. Luteolin occurred mostly in EA and ME extracts in *S. microstegia*, S. *byzantina*, and *T. cilicicus*. The highest hesperidin level was found in *S. boissieri* ME extract and it follows *S. byzantina* ME extracts; In addition, ME extracts of *S. viridis* and *T. cilicicus* also included high levels of hesperidin. The highest oleuropein content was determined in ME extracts of *T. cilicicus*, followed by *T. syriacus* and *S. boissieri*. Within EA extracts, *S. viridis* and *O. acutidens* had the highest level of oleuropein.

3.2 Antimycobacterial activities, MICs & MBCs

The results were evaluated according to the literature. Extracts were tested against four mycobacteria strains (*M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv, and two-positive *M. tuberculosis* isolates) obtained from hospital patients, to determine the MIC and MBC using the micro dilution method (MABA) against reference strains.

All plant extracts showed antimycobacterial activity (Table 4). Within all CL extracts, *O. acutidens, S. microstegia,* and *T. syriacus* exhibited the lowest MIC value of 0.4 mg/mL against *M. tuberculosis* H37 Ra. The lowest MBC value was 6.3 mg/mL for *O. acutidens* and *S. boissieri.* The MBC value for the rest of species was 12.5 mg/mL.

The MIC value of CL extracts against *M. tuberculosis* H37 Rv was 0.4 mg/mL for *S. boissieri*, followed by *S. cretica, T. syriacus,* and *T. cilicus* at MIC 6.3 mg/mL. Although all CL extracts showed bactericidal activity against *M. tuberculosis* H37 Rv, the prominent MBC values are 0.8 mg/mL for *S. boissieri* and 3.1 mg/mL for *T. syriacus*. For TB-positive isolates1, the featured results were 0.8 mg/mL MIC and MBC for *S. boissieri* and 3.1 mg/mL MIC and MBC for *S. cretica* subsp. *smyrnaea. S. boissieri* was also effective at the concentration 0.8 mg/mL as MBC.

In the EA extracts, the most prominent efficacy was observed for *T. syriacus* at MIC 0.8 mg/mL; MBC 1.6 mg/mL for *T. syriacus* against *M. tuberculosis* H37 Ra. *S. boissieri* is also

effective at MIC and MBC 6.3 mg/mL against two TB-positive isolates; *T. cilicus* showed the same effect at MIC and MBC 3.1 on *M. tuberculosis* H37 Rv.

Of the ME extracts, the most effective against *M. tuberculosis* H37 Ra was *T. syriacus* (MIC and MBC 3.1 mg/mL). *Stachys byzantine* also showed considerable efficacy at MIC 3.1 mg/mL against TB-positive isolates1. Among the other extracts, MIC and MBC values ranged between 6.3-12.5 and 6.3-25 mg/mL, respectively (Table 4).

Plants	Extracts	**H37Ra**		**H37*Rv***		**Isolate1**		**Isolate2**	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Origanum acutidens	Cl	0.4	6.3	12.5	12.5	12.5	25	6.3	6.3
	Ea	6.3	6.3	6.3	6.3	3.1	6.3	3.1	6.3
	Me	6.3	6.3	25	25	3.1	12.5	6.3	12.5
O. sipyleum	Cl	3.1	12.5	25	25	12.5	25	6.3	12.5
	Ea	1.6	3.1	25	25	6.3	25	6.3	25
	Me	12.5	12.5	25	25	6.3	12.5	6.3	6.3
Salvia viridis	Cl	6.3	12.5	12.5	12.5	n.t	n.t	n.t	n.t
	Ea	1.6	3.1	12.5	12.5	6.3	12.5	6.3	12.5
	Me	12.5	12.5	25	25	6.3	25	6.3	25
S. microstegia	Cl	0.4	12.5	12.5	12.5	12.5	25	6.3	12.5
	Ea	6.3	12.5	25	25	3.1	12.5	3.1	12.5
	Me	12.5	12.5	12.5	25	6.3	25	6.3	25
S. boissieri	Cl	0.8	6.3	0.4	0.8	0.8	0.8	0.8	0.8
	Ea	1.6	3.1	12.5	12.5	3.1	3.1	3.1	3.1
	Me	12.5	12.5	12.5	12.5	6.3	12.5	6.3	12.5
Stachys byzantine	Cl	0.8	12.5	25	25	12.5	25	6.3	6.3
	Ea	12.5	12.5	12.5	12.5	3.1	6.3	6.3	12.5
	Me	12.5	12.5	25	25	3.1	12.5	6.3	25
S. cretica	Cl	0.8	12.5	6.3	12.5	12.5	12.5	6.3	12.5
	Ea	1.6	12.5	6.3	12.5	3.1	12.5	3.1	12.5
	Me	12.5	12.5	12.5	12.5	6.3	25	6.3	25
S. cretica subsp. *smyrnaea*	Cl	6.3	12.5	12.5	12.5	3.1	3.1	6.3	12.5
	Ea	6.3	12.5	12.5	12.5	3.1	12.5	3.1	12.5
	Me	25	25	12.5	12.5	3.1	25	3.1	25
Thymus syriacus	Cl	0.4	12.5	6.3	12.5	6.3	6.3	3.1	6.3
	Ea	0.8	1.6	3.1	3.1	3.1	12.5	3.1	12.5
	Me	3.1	3.1	50	50	6.3	12.5	6.3	25
T. cilicicus	Cl	0.8	12.5	6.3	25	6.3	25	6.3	12.5
	Ea	3.1	6.3	3.1	6.3	3.1	25	3.1	25
	Me	12.5	12.5	12.5	12.5	6.3	25	6.3	25

MIC:(mg/mL); MBC: (mg/mL;). n.t: not tested.

Table 4. Antibacterial activity of extracts of the plants as MIC (mg/mL) and MBC susceptibility test results against M. tuberculosis H37Ra (ATCC 25177) and *M. tuberculosis* H37Rv (ATCC 25618) obtained by MABA (*Microplate Alamar blue* assay) method.

4. Discussion

Lamiaceae plant extracts prepared by using different plant parts such as bark, stem, root, leaves, and fruits used in many biological activity studies. The extracts have been found to have antibacterial activity (Alma et al., 2003; Amanlou et al., 2004; Digrak et al., 2001; Bozin et al., 2006; Karaman et al., 2001), antifungal activity (Bouchra et al., 2003; Askun et al., 2008; Gulluce et al., 2003; Guynot et al., 2003; Souza et al., 2005), antimycobacterial activity (Ulubelen et al., 1997; Askun et al., 2009), antioxidant activity (Alma et al., 2003; Bozin et al., 2006; Mosaffa et al., 2006; Gulluce et al., 2003) and anti-inflammatory activity (Alcar´az et al., 1989; Jim´enez et al., 1986). Inhibitory effects of oregano components on some foodborne fungi were reported (Akgul & Kivanc, 1988). Askun et al. (2009) indicated that *Origanum minutiflorum* and *Thymbra spicata* methanol extracts showed antimycobacterial activity against *M. tuberculosis. T. spicata* var. *spicata* showed greater antimycobacterial efficacy (at MIC 196 µg/ml) than *O. minutiflorum* (MIC 392 µg/ml). They stated that a high quantity of rosmarinic acid might be responsible for antimycobacterial activity.

Recently, investigations of plant extracts are attracting great attentions due to their antibacterial properties (Payne et al., 2007; Rukayadi et al., 2009; Guzman et al., 2010). Previous studies showed that some plant extracts were conciderably effective against *M. tuberculosis*. Lall and Meyer (1999) reported that growth of *M. tuberculosis* is inhibited by acetone and water extracts of *Cryptocarya latifolia*, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala* and *Thymus* v*ulgaris.* They screened these active acetone extracts against H37Rv and a TB strain that was resistant to the drugs isoniazid and rifampicin. They reported that, while some plants (*Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *Nidorella anomala* and *Polygala myrtifolia)* exhibited MIC at 0.1 mg/mL against H37Rv, others (*Chenopodium ambrosioides*, *Ekebergia capensis*, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala* and *Polygala myrtifolia)* inhibited the resistant strain at the same MIC value.

Many natural products have attracted much attention as potential antimycobacterial agents (Kinghorn, 2001; Gupta et al., 2010; Guzman et al., 2010). In recent years, there are pleny of researches on phenolics and their biological activities involved in the literature. Phenolic compounds obtained from plant extracts show great variety, with at least 8000 different structures (Bravo, 1998). Estevinho et al. (2008) showed that differences in the profiles of phenolic compounds are dependent of the flora predominance. Chun et al. (2005) reported that high phenolic and antioxidant activity was related to high antimicrobial activity against ulcer-associated *H. pylori*. Cinnamic acid is a naturally occurring phenolic compound that shows antimicrobial activity. Chen et al. (2011) showed that cis-cinnamic acid that was transformed from trans-cinnamic acid showed higher synergistic effect with INH or RIF against tuberculosis than trans-cinnamic acid.

Siedel & Taylor (2004) investigated plants, *Pelargonium reniforme* and *P. sidoides* (Geraniaceae) fractionation of n-hexane extracts against *M. aurum, M. smegmatis, M. fortuitum, M. abscessus* and *M. phlei*. They reported that linoleic acid was the most potent compound (MIC of 2 mg/l) against *M. aurum*. Koysomboon et al. (2006) isolated flavonoids from the stems and roots of the mangrove plant *Derris indica*. They reported antimycobacterial activity at MIC values between 6.25 and 200 µg/mL, except in two of ten known compounds. Askun et al. (2009) indicated that *Origanum minutiflorum* and *Thymbra spicata* var. *spicata* methanol extracts have antimycobacterial activity against *M. tuberculosis.*

T. spicata var. *spicata* was more effective (MIC 196 μg/ml) than *O. minutiflorum* (MIC 392 μg/ml). They suggested that a high quantity of rosmarinic acid might be one of the responsible constituent for the observed antimycobacterial activity. Gordien et al. (2009) studied two terpenes, sesquiterpene and longifolene; and two diterpenes, totarol and trans-communic acid, obtained from the aerial parts and roots of *Juniperus communis*. They reported that totarol showed the highest activity against *Mycobacterium tuberculosis* H37Rv and that longifolene and totarol exhibited the most activity against rifampicin-resistant variants. These results supported the ethnomedicinal use of this species as a traditional anti-TB remedy. Kuete et al. (2010) investigated the antimycobacterial activity of five flavonoids (isobachalcone, kanzanol C, 4-hydroxylonchocarpin, stipulin, amentoflavone) and determined their effects on preventing the growth of mycobacteria with MIC < 10 μg/ml on *M. tuberculosis*. In addition, isobachalcone and stipulin showed total inhibition of *M. tuberculosis* strain H37Rv. Bernard et al. (1997) mentioned that rutin showed antibacterial activity on *E. coli* by inhibited topoisomerase IV-dependent decatenation activity and caused *E. coli* topoisomerase IV which is essential for cell survival, dependent DNA cleavage (Bernard et al., 1997; Normark et al., 1969; Cushnie& Lamb., 2005). Huang et al. (2008) indicated that evidence that vanillic acid might be helpful to prevent of the development of the development of diabetic neuropathy by blocking the methylglyoxal-mediated glycation system.

Mandalari et al. (2007) also reported that, pair-wise combinations of eriodictyol, naringenin and hesperidin showed both synergistic and indifferent interactions that were dependent on the test indicator organism and their cell wall structure. Parekh and Chanda (2007) reported that the crude methanol extract of *Woodfordia fruticosa* contains certain constituents, such as tannins, with significant antibacterial properties, which enables the extract to overcome the Gram-negative cell wall barrier.

Kamatou et al. (2007) studied 16 South African *Salvia* species that are used in traditional medicine to treat microbial infection. They identified three species, *S. verbenaca, S. radula* and *S. dolomitica*, which exhibited MIC value at 0.10 mg/mL and which also showed antibacterial activity. Green et al. (2010) reported on the activities of acetone extracts of four plants, while *Berchemia discolor* showed efficacy at MIC 12. 5μg/mL, on H37Ra and 10.5μg/mL on the clinical isolate; the others (*Bridelia micrantha*, *Warbugia salutaris*, and *Terminalia sericea*) showed efficacy at 25μg/mL on both H37Ra and clinical isolate. The authors validated that these plants include mycobactericidal compounds that are effective against multidrug-resistant *M. tuberculosis.* Graham et al. (2003) presented an antimycobacterial evaluation of 216 species of Peruvian plants (in 63 families). Dichloromethane extracts from slightly more than half of the samples tested showed MIC value at 50 μg/ml concentration against *M. tuberculosis.* Billo et al. (2005) reported that methanolic extract of *Amborella trichopoda* fruits shows MIC value between 1 and 2.5 μg/ml, which was better than pyrazynamide and ethambutol in the same conditions.

Fabryet et al. (1998) reported that solvent extracts of plants with MIC values less than 8 mg/mL may be considered as antimicrobially effective. Gautam et al., (2007), shows that extracts of plant species from wide range of families and genera have exhibited significant in vitro antimycobacterial activities and this efficacy is interestingly compatible with the ethnomedicinal knowledge on plants.

Lechner et al. (2008) showed that myricetin was the most efficient intensifier of INH susceptibility in all tested strains by decreasing the MIC value of INH by as much as 64-fold; the second most effective compound was quercetin. Huang et al. (1980) tested two benzenoid compounds isolated from *Ardisia japonica* in-vivo on 201 patients infected with *M. tuberculosis* (Okunade et al., 2004). They reported that both compounds showed over 80% efficacy.

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6. Conclusion

In order to test the plant extracts, a potential drug resistant *M. tuberculosis* isolates was obtained from pulmonary tuberculosis hospital patients. The strains and isolates were then treated with plant extracts that are used for ethnopharmacological purposes. The level of the phenolic compounds and some flavonoids extracts were determined by liquid chromatography–mass spectrometry (LC-MS). The evaluation of results included the plants efficacy, their major phenolics, flavonoids and antimycobacterial activities. All plants extract showed antimycobacterial activity.

O. acutidens, S. microstegia, and *T. syriacus* were exhibited the lowest MIC value at 0.4 mg/mL against *M. tuberculosis* H37 Ra. *S. boissieri* and *T. syriacus* showed activity at MIC 0,4 mg/mL against *M. tuberculosis* H37 Rv. The prominent MIC and MBC values against *M. tuberculosis* H37 Rv were determined at 0,8 mg/mL for *S. boissieri* and 3,1 mg/mL for *S. cretica* subsp. *smyrnaea. S. boissieri* and *T. cilicicus* were effective against two TB-positive isolates.

The present work provides a preliminary insight into the effects of phenolics against *M. tuberculosis.* Plants of the Lamiaceae family have been shown to include new and effective constituents against *Mycobacterium tuberculosis*. Examination of these species, reported above, shows that rutin hydrate and vanillic acid were plentiful in all three extracts for these genera in Lamiaceae. All extracts of the *Origanum* species*, Salvia*, *Satureja, Stachys* and *Thymus* genera were rich in rosmarinic acid. With the exception of *S. viridis*, these species did not contain gallic acid.

We suggest that phenolics and naturally occurring flavonoids (polyphenols) are mainly responsible for antimycobacterial, cytotoxicological and mutagenic activity against *M. tuberculosis*. In some plants, (*O. acutidens*, *O. sipyleum, S. microstegia,* and *Stachys byzantine)* MIC and MBC values of CL extracts were in the same concentrations. These results might be due to several factors, such as a toxic effect caused by some compounds in the extracts. Liu et al. (2010) showed that a high concentration of cinnamic acid has toxic effects on soil bacteria. The other reason might be that the primary targets of the flavonoids have not been studied as widely in bacteria as in eukaryotes. While flavonoids affect enzyme systems such as prostaglandin, cyclooxygenase and lipoxygenase in eukaryotic cells, the bacteriocidal effect of the flavonoids might have caused the metabolic disorders on metalloenzymes by which their heavy metal atoms combine with flavonoids as ligand complexes in bacteria.

These strong complexes might disrupt the metabolism of organism (Havsteen, 2002). Flavonoids are also known to have mutagenic and antitumor activities (Hodec et al., 2002; Havsteen, 2002). Quercetin affects bacteria by inhibiting the catalytic activity of DNA topoisomerase I and II (Constantinou et al., 1995; Hodec et al., 2002). Quercetin was also reported by Xu et al. (2000) and Spedding et al. (1989) to have inhibitory effects on HIV1-protease and reverse transcriptase.

It is imperative to investigate the use of new, cheaper and efficient compounds to control *Mycobacteria tuberculosis*. Recent studies have examined plants and the effectiveness of their different types of extracts on *M. tuberculosis*. Advanced research into the structure and activity relationships among naturally occurring flavonoids will yield greater understanding of their pharmacokinetics and effects on mycobacteria metabolism according to their structure. It is of great importance to determine the mechanisms of action of flavonoids on *M. tuberculosis* .

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Cinnamic Derivatives in Tuberculosis

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1. Introduction

Tuberculosis (TB) is a threat to worldwide public health, mainly caused by *Mycobacterium tuberculosis* (*M.tb.*) bacteria species. Despite the availability of effective treatment, tuberculosis is responsible for more than three million deaths annually worldwide. The high susceptibility of human immunodeficiency virus-infected persons to the disease (Nunn et al., 2005), the emergence of multi-drug-resistant (MDR-TB) strains (Rastogi et al., 1992, Kochi et al., 1993; Bloch et al., 1994) and extensively drug-resistant (XDR-TB) ones have brought this infectious disease into the focus of urgent scientific interest. For this reason, there is a growing need and urgency to discover new classes of chemical compounds acting with different mechanisms from those currently used. Cinnamic acid (**1; Fig. 1**) and derivatives have a century-old history as antituberculosis agents. For example, gradual improvement was observed when the TB-patients were treated with cinnamic acid (**1**) prepared from storax (Warbasse, 1894). Furthermore, in 1920s, ethylcinnamate (**2**) (Jacobson, 1919), sodium cinnamate (**3**) (Corper et al., 1920) and benzylcinnamate (**4**) (Gainsborough, 1928) were reported to be efficacious in the treatment of TB (Fig. 1). Nevertheless, we feel that this class of molecules remained underutilized until recent years. Particularly in the last two decades, there has been huge attention towards various natural and unnatural cinnamic derivatives and their antituberculosis efficacy. This chapter provides a comprehensive literature compilation concerning the synthesis so as the antituberculosis potency of various cinnamic acid, cinnamaldehyde and chalcone derivatives. We envisage that our effort in this chapter contributes a much needed and timely addition to the literature of medicinal research.

Fig. 1. Cinnamic acid, ethylcinnamate, sodium cinnamate and benzylcinnamate

2. Cinnamic acid derivatives as anti-TB agents

trans-Cinnamic acid (1) has a long history of human use as a component of plant-derived scents and flavourings (Hoskins, 1984). It belongs to the class of auxin, which is recognized as plant hormones regulating cell growth and differentiation (Thimann, 1969). The cinnamoyl functionality is also present in a variety of secondary metabolites of phenylpropanoid biosynthetic origin. Those containing a sesquiterpenyl, monoterpenyl and isopentenyl chain attached to a 4-hydroxy group represent quite a rare group of natural products (Epifano et al., 2007).

p-Coumaric acid (5) Caffeic acid (6) Ferulic acid (7) Sinapic acid (8)

Fig. 2. Cinnamic acid and its natural phenolic-analogues

The hydroxyl cinnamic acids such as *p*-coumaric acid (**5**), caffeic acid (**6**), ferulic acid (**7**), sinapic acid (**8**) (**Fig. 2**) are natural products arising from the deamination of the phenyl alanine (**9**) (**Scheme 1**) (Kroon & Williamson, 1999). Besides, they are important constituents in the biochemical pathway in plants leading to the lignin (Humphreys & Chapple, 2002; Boerjan et al., 2003), the second most abundant biopolymer after cellulose (Whetten et al., 1998) resulting mainly from the oxidative polymerization (Freudenberg, 1959) of the three hydroxycinnamyl alcohols, namely coumaryl (**10a**), coniferyl (**10b**) and sinapyl alcohols (**10c**). These key cinnamyl alcohols are produced through two successive enzyme-catalyzed reductions starting from the corresponding cinnamyl SCoA-esters. In recent years, *trans*-cinnamic acid derivatives have also attracted much attention due to their antioxidative (Chung & Shin, 2007), antitumor (De et al., 2011a) and antimicrobial (Naz et al., 2006; Carvalho et al., 2008) properties.

Phenylalanine (9)
CCR: Cinnamoyl CoA oxidoreductase
CAD: Cinnamyl alcohol dehydrogenase
R = R' = H; **Coumaryl alcohol (10a)**
R = H, R' = OMe; **Coniferyl alcohol (10b)**
R = R' = OMe; **Sinapyl alcohol (10c)**

Scheme 1. Lignin biosynthesis pathway

2.1 Cinnamic acid

In an attempt to develop a new strategy to circumvent MDR-TB by augmenting the potential of the existing drugs (Rastogi et al. 1994), *trans*-cinnamic acid (**1**) was used along with known antituberculous drugs such as isoniazid (**11**), rifampin (**12**), ofloxacine (**13**) or clofazimine (**14**) (**Fig. 3**). Interestingly, a synergistic increase was observed in the activity of various drugs against *Mycobacterium avium*. The synergistic activity of **1** (Rastogi *et al.* 1994) with a variety of drugs was manifested even with drug resistant isolates. Importantly, it was

proved that **1** is not bactericidal. The dose-dependent effect of cerulenin (**16**) and *trans*-cinnamic acid on *M.tb.* viability showed (Rastogi *et al.* 1996) that cinnamic acid was not bactericidal even at concentrations as high as 200 µg/mL, whereas cerulenin was bactericidal only at concentrations above 50 µg/mL.

Isoniazid (11) Rifampin (12) Ofloxacine enantiomers (13) Clofazimine (14)

Fig. 3. Known antituberculosis drugs

Amikacine (15) Cerulenin (16) Ethambutol (17)

Fig. 4. Known antibiotics used in synergy studies

Thus, the sub minimum inhibitory concentrations (sub-MIC) of both **16** and **1** used in synergy experiments (fixed concentrations of only 1 µg/mL) were not due to any direct effect of these two inhibitors on *tubercle bacilli* (TB). Out of the various drug combinations screened, those with **1** gave the best results. For example, enhancement of drug activity was even observed with the drug-resistant strain 92-0492 (resistant to isoniazid (**11**) & rifampin (**12**)) when **1** at sub-MIC concentrations was used in combination with antibiotics such as ofloxacine (**13**), clofazimine (**14**) and amikacine (**15**). Cerulenin (**16**) is a known antifungal antibiotic that inhibits fatty acid and steroid biosynthesis. In fatty acid synthesis, **1** proved to bind in equimolar ratio to β-keto-acyl-ACP synthase, one of the seven moieties of fatty acid synthase, blocking the interaction of malonyl-CoA (Nomura et al., 1972; Omura, 1976). It is therefore likely that the inhibitory effects that were observed in the synergy study resulted from the inhibition of fatty acid synthesis. However, the mode of action for **1** is still unknown. In a previous study with *M. avium* (Rastogi et al., 1994), it was suspected that **1** might have inhibitory effects because of its structural similarity to phenylalanine. Because of that similarity, **1** would inhibit glycopeptidelipid (GPL) biosynthesis, therefore increasing cell wall permeability and enhancing the inhibitory effect of antimycobacterial drugs. As *M.tb.* does not synthesize GPL antigens, this reasoning does not fully fit with this bacterial species. Apparently other sites are also

being affected by **1**, which in turn enhance the susceptibility of the organism to the effects of the antimycobacterial drugs. Although, *trans*-cinnamic acid (**1**) was used to treat tuberculosis before antimycobacterial chemotherapy was used (Ryan, 1992), this was the first example of MDR-TB activity in synergy with other drugs but the mechanism of action still remains unknown.

2.2 Natural compounds

In another context, a cinnamoyl ester was identified to be important in glycoside extracts of a native North American prairie plant named *Ipomoea leptophylla*. In fact, the organic soluble extracts from its leaves showed (Barnes et al. 2003) *in vitro* activity against *M.tb.* Through a bioassay-guided fractionation of these extracts, the authors isolated leptophyllin A (**18**), a resin glycoside bearing a *trans*-cinnamic residue attached to one rhamnose moiety (**Fig. 5**). This compound showed 13% inhibition at 6.25 μg/mL against *M. tb.* in the *in vitro* anti-TB assay. Furthermore, the bioassay results indicated that the cinnamic acid residue is required for the observed antimicrobial activity as an analogous compound bearing no cinnamic acid residue, leptophyllin B (**19**), also isolated from the same source, showed no *in vitro* activity. Recently, some bioactive styryllactones and alkaloids were isolated from flowers of *Goniothalamus laoticus* (Lekphrom et al. 2009). In particular, the authors isolated a styryllactone derivative, namely howiinin A (**20**), by fractionation of the ethyl acetate and methanol extracts from the flowers of this species (**Fig**. **6**). While inactive against *Plasmodium falciparum*, this compound possessing a cinnamoyl ester moiety showed an interesting anti-TB activity (MIC = 6.25 µg/mL) when tested against *M.tb.* strain $H_{37}Ra$.

Leptophyllin A (18)

Leptophyllin B (19)

Fig. 5. Resin glycosides from *I. Leptophylla*

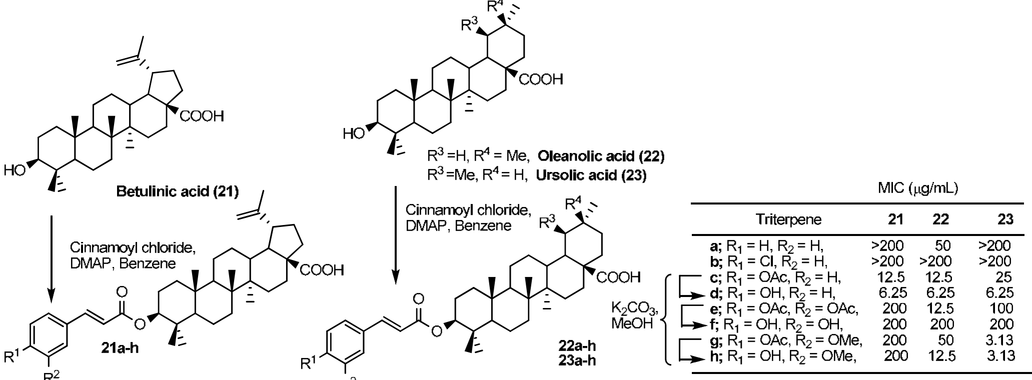
Howiinin A (20)

Fig. 6. Structure of Howiinin A

2.3 Synthetic compounds

2.3.1 Cinnamic ester derivatives

It is well known that triterpenes exhibit moderate to high *in vitro* antimycobacterial activity against *M. tb*. (Copp & Pearce, 2007; Okunade, Elvin-Lewis & Lewis, 2004). The modification of natural triterpenes such as betulinic acid (**21**), oleanolic acid (**22**) and ursolic acid (**23**) through introduction of cinnamoyl frames at the C-3 position has been reported (**Scheme 2**) (Tanachatchairatana et al., 2008). Different cinnamoyl derivatives such as cinnamate, *p*-coumarate, ferulate, caffeate and *p*-chlorocinnamate esters of the above mentioned triterpenes were synthesized by reacting with the suitable cinnamoyl chlorides in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP) in benzene. All the hydroxyl-cinnamic acids were acetylated to protect the phenolic group before generating the corresponding acid chlorides followed by coupling with the triterpenes. The hydroxycinnamate derivatives of the triterpenes (**21d,f,h**; **22d,f,h**; **23d,f,h**) were easily obtained by deacetylation of the acetylated derivatives (**21c,e,g**; **22c,e,g**; **23c,e,g**) using K_2CO_3 in methanol. The biological results indicated that the introduction of unsubstituted or *p*-chlorinated cinnamate ester functionality (**21a,b**; **22a,b**; **23a,b**) led to inactive compounds (MIC>200 µg/mL) or without any improvement in the antimycobacterial activity of the native triterpenes. Interestingly, the results also indicated that introduction of the *p*-coumarate moiety at the C-3 position of the triterpenes (**21d**, **22d**, **23d**) resulted in an 8-fold increase in antimycobacterial activity of the parent triterpenes **21** (MIC = 50 µg/mL) and **22** (MIC = 50 µg/mL), and a 2-fold increase in the activity of the triterpene **23** (MIC = 12.5 µg/mL). Introduction of a ferulate moiety (**21h**, **22h**, **23h**) resulted in a 4-fold increase in activity only in case of **23**. However, the presence of a *p*-hydroxy group plays a crucial role on the high antimycobacterial activity because its methylation and acetylation proved to decrease antimycobacterial activity in a significant manner, caffeate esters **21e,g** and **22e,g** being the exceptions.



Triterpene	MIC (µg/mL) 21	22	23
a; R_1 = H, R_2 = H,	>200	50	>200
b; R_1 = Cl, R_2 = H,	>200	>200	>200
c; R_1 = OAc, R_2 = H,	12.5	12.5	25
d; R_1 = OH, R_2 = H,	6.25	6.25	6.25
e; R_1 = OAc, R_2 = OAc,	200	12.5	100
f; R_1 = OH, R_2 = OH,	200	200	200
g; R_1 = OAc, R_2 = OMe,	200	50	3.13
h; R_1 = OH, R_2 = OMe,	200	12.5	3.13

Scheme 2. Cinnamate-based triterpenes and their biological activities

2.3.2 Cinnamic amide derivatives

Rifampin (RIF; **12; Fig. 3**) is one of the most important drugs in TB treatment. In search for new compounds with structural modifications of existing lead drugs, the presence of a cinnamoyl moiety on rifampin's piperazinyl framework instead of a methyl group,

furnishing 3-(4-cinnamylpiperazinyl-iminomethyl)rifamycin derivative (**24**; rifamycin SV (T9); **Fig. 7**), resulted in enhanced antimycobacterial activity (Reddy *et al*. 1995; Velichka et al. 2010). The antimycobacterial activities of **24** on 20 susceptible and MDR-strains of *M.tb.* and 20 *M. avium* complex (MAC) strains were investigated (Dimova *et al.* 2010). The radiometric MICs of T9 for *M.tb.* were significantly lower than those of RIF. The MICs of T9 and RIF at which 90% of the RIF-susceptible strains were inhibited were <0.25 and <0.5 μg/mL, respectively. Compound **24** had better activity against MAC strains, and the MIC at which 90% of the MAC strains were inhibited was <0.125 μg/mL, while that of RIF was <2.0 μg/mL. Compound **24** also showed high *in vitro* bactericidal and intracellular activities which were significantly superior to those of RIF against both *M.tb.* and MAC strains. More importantly, **24** showed excellent *in vivo* activity against *M.tb.* $H_{37}Rv$ compared to RIF in both the lungs and spleens of C57BL/6 mice, indicating the potential therapeutic value of **24** in the treatment of mycobacterial infections.

Fig. 7. Structure of Rifamycin SV(T9)

In an attempt to find novel compounds active against TB, a series of phenylacrylamides designed by molecular hybridization of *trans*-cinnamic acids and guanylhydrazones were synthesized and antiTB efficacy were evaluated (**Scheme 3**) (Bairwa et al., 2010). While cinnamic acids are already known for their antituberculosis efficacy, guanylhydrazones have been shown to have antimicrobial activity including an interesting gram-negative bacterial endotoxin lipopolysaccharide (LPS) sequestering activity (Gadad et al. 2000; Wu et al. 2009). *M. tb.* contains lipoarabinomannan (LAM), a complex lipid glycoprotein anchored to the cell membrane by phosphatidylinositol which has structural and functional similarity to LPS, including the presence of anionic phosphate groups (Zhang et al., 1994). Biosynthesis of LAM is known to be a target for several antituberculosis agents, including the first line antitubercular agent, ethambutol (**17**; **Fig. 4**) (Scherman et al. 1995; Heijenoort, 2001). For the synthesis of the most active phenylacrylamide derivative (**28**; **Scheme 3**), the required guanylhydrazone (**25a**) was prepared by the microwave-assisted reaction of 3,4-dimethoxy benzaldehyde (**25**) with guanylhydrazine hydrochloride (**26**). In parallel, the phenyl 4-methoxycinnamate (**27a**) was prepared by esterification of 4-methoxycinnamic acid (**27**) via its treatment by phenol and thionyl chloride. Finally, the coupling of equimolar quantities of guanylhydrazone (**25a**) with phenylcinnamate (**27a**), was performed under microwave irradiation in the presence of triethylamine and ethanol as solvent to afford the target derivative (*E*)-*N*-(((*E*)-2-(3,4-dimethoxybenzylidene)hydrazinyl)(imino)methyl)-3-(4-methoxyphenyl)acrylamide (**28**). Compound **28** was found to be active when tested on resazurin microtiter plate assay

(REMA) against *M. tb.* $H_{37}Rv$ (MIC = 6.5 µM) along with good safety profile (CC_{50} = 340 µM) in VERO cell line. Importantly, analysis of structure–activity relationships revealed that both steric and electronic parameters play major role in the activity of this series of compounds.

Scheme 3. Synthetic route for the synthesis of phenylacrylamide derivatives

A series of 1-[(2,3-dichloroanilinomalonyl)-3-(*N*-2′-cyanoethyl)-2-(*N*-cinnamoyl) 2,3-Di-chloroanilino)]-5-phenyl pyrazolines (**38a-e**) have been synthesized from *N*-cinnamoyl-*N*-2′-cyanoethyl-2,3-dichloroaniline (**37**) in the presence of 2-[(*N*-cinnamoyl) 2,3-dichloroanilido] acetohydrazides (**33a-e**) and acetic acid in dioxane (**Scheme 4**) (Sharma et al., 2010, 2011). These compounds have been tested for *in vitro* antitubercular activity on *M.tb***.** $H_{37}Rv$ strains.

The compounds (**38a-e**) inhibited the growth of *M.tb.* at 100 µg/mL concentration. The acylhydrazide (**33a**) was also coupled with some aromatic aldehydes (**29a-c**) in the presence of catalytic H_2SO_4 in ethanol. The resulting hydrazones (**34a-c**) were also found to have anti-TB activity.

Using a molecular hybridization approach, a series of cinnamide derivatives (**39a**-**42a**) was designed as potential antimycobacterial agents (Kakwani et al. 2011). The diamine moiety of ethambutol (**17; Fig. 4**) and its other analogs proved to be a key feature. Various diamines (**39-42**) were coupled with **1** using ethylchloroformate and triethylamine to obtain cinnamide derivatives (**39a**-**42a**) (**Scheme 5**). The MICs of all synthesized compounds were determined against *M.tb.* $H_{37}Rv$ using Resazurin Microtitre plate Assay (REMA) method. The synthesized molecules (**39a**-**42a**) showed good to moderate activities with MIC in the range of 5–150 µM and good safety profiles. The most potent compound **39a**, having MIC of 5.1 µM (cytotoxicity measured on VERO cells line C1008 (CC_{50}) = 618 µM) exhibited synergy with rifampin (**12**). Under similar conditions ethambutol (**17**) showed MIC of 15.3 µM with a CC_{50} value of 1470 µM.

Scheme 4. Synthesis of cinnamoyl pyrazolines and analogs

Scheme 5. Synthesis of cinnamides from diamines

2.3.3 Cinnamic oxadiazole derivatives

The synthesis of some 1,3,4-oxadiazoles and oxo-imidazolines compounds as potent biologically active agents has been reported (Joshi et al., 1997). The synthetic routes are presented in **Scheme 6**. The common precursor **44** was obtained through condensation of 5-nitro-*o*-benzoylene-2,1-benzimidazole (**43**) with hydrazine hydrate. The cyclocondensation reaction of different aromatic acids with **44** in the presence of $POCl_3$ afforded 1,3,4-

oxadiazoles **45a-e**. Compounds **45a-d** were found to be more active against *M.tb.* $H_{37}Rv$ than the cinnamic derivative **45e** at 12.5 μg/mL.

Scheme 6. Synthesis of various 1,3,4-oxadiazoles and 5-oxo-imidazolines

2.3.4 Cinnamic benzimidazole derivatives

Benzimidazole scaffold being an important pharmacophore and privileged structure in medicinal chemistry (Khalafi-Nezhad et al., 2005; Evans et al. 1988), a new series of 5-(nitro/bromo)-styryl-2-benzimidazoles (**49a-f, 50a-f**; **Scheme 7**) was synthesized (Shingalapur et al., 2009) by simple condensation of 5-(nitro/bromo)-*O*-phenylenediamine (**46, 47**) with *trans*-cinnamic acids (**48a-f**) in ethylene glycol for 6 h at around 200°C (**Scheme 7**). The *in vitro* anti-TB activities of compounds **49a-f** and **50a-f** on the *M.tb.* $H_{37}Rv$ were determined at 7.25 μg/mL concentration. Interestingly, the bromo-substituted benzimidazole derivatives (**50a-f**) exhibited the best results with 63-83% inhibition.

Scheme 7. Synthesis of styryl-2-benzimidazoles series

2.3.5 Cinnamic acid hydroxamic derivatives

The sequestration of iron is a part of the non-specific mammalian immune response and thus, metal uptake and regulation of metal-ion concentrations are the key features of host-pathogen interactions (Agranoff & Krishna, 2004). Siderophores are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses (Neilands, 1995; Cornelis & Andrews, 2010). Siderophores are considered amongst the strongest soluble Fe(III)-binding agents known. Siderophores, produced by mycobacteria itself, were used (Guo et al., 2002) to target iron transport processes essential for the growth and survival of *M. tb.* Targeting the iron transport processes of *M.tb.* is challenging for several reasons. The complexity of the mycobactin architecture itself poses a daunting synthetic challenge, which hampers the generation of conjugates (Xu & Miller, 1998). Further, the iron transport mechanism involves an "iron-handoff" between two siderophore families, the exochelins and the mycobactins. In low iron environments, *M.tb.* biosynthesizes and secretes hydrophilic exochelins (e.g., Mycobactin J (**51**); **Scheme 8**) to bind exogenous ferric ion. The iron-complex is then transferred to intracellular siderophores (*i.e.* the mycobactins) which are lipophilic chelators associated with the

cytoplasmic membrane (Roosenberg et al., 2000). The mycobactin so-associated with iron either remains in the cell wall as an iron storage pool or is released into the cell by a mycobactin reductase. Therefore, the sequestration of the available iron into a form, which cannot be processed by *M.tb.* may be an alternative therapeutic way. The success of this approach relies on the understanding of the molecular recognition events involved in mycobacterial iron transport. In that context, the authors synthesized different iron chelators containing α,β-unsaturated hydroxamic acid motifs appended to a citric acid platform such as Nannochelin A (**56b**) and compared their activities with the corresponding *trans*-octenoyl derivative (**56a**). As shown in **Scheme 8**, the synthesis starts from diamines (**52a,b**) with a three-steps sequence to afford, after monoprotection, *N*-benzoylation and *N*-acylation with *trans*-cinnamoyl chloride, the dissymetric amino-compounds **53a,b**. **53a,b** were then treated with a 10% NH_4OH solution in methanol solution at 0 °C to deprotect the hydroxamic acid and the resulting derivatives were treated with trifluoroacetic acid (TFA) to produce the TFA salts **54a,b**. Finally, the condensation of **54a,b** with the activated Boc-protected citric acid (**55**) in 1,4-dioxane followed by TFA treatment gave the desired chelators **56a,b.** Notably, molecules that provided significantly higher growth index (GI) values than the native chelator **51** were identified as superior growth stimulants and more efficacious iron delivery agents. The systems containing longer tethers gave higher GI values (e.g., GI = 0.76 for **56a** *vs* 1.5 for **56b**). It was envisaged by the authors that the longer tether allows for a more conformationally flexible ligand to properly coordinate to iron thus providing an increase in hydrophobicity. However, the authors identified **56b** as a superior growth stimulant and a more efficacious iron delivery agent. Such ligands, which offer regulation of the initial iron delivery step, provide the opportunity to compare the iron transport mechanisms of both native and genetically modified mycobacteria.

Scheme 8. Synthesis of Nannochelin A

2.3.6 Cinnamic acid hydrazide, thioester and other derivatives

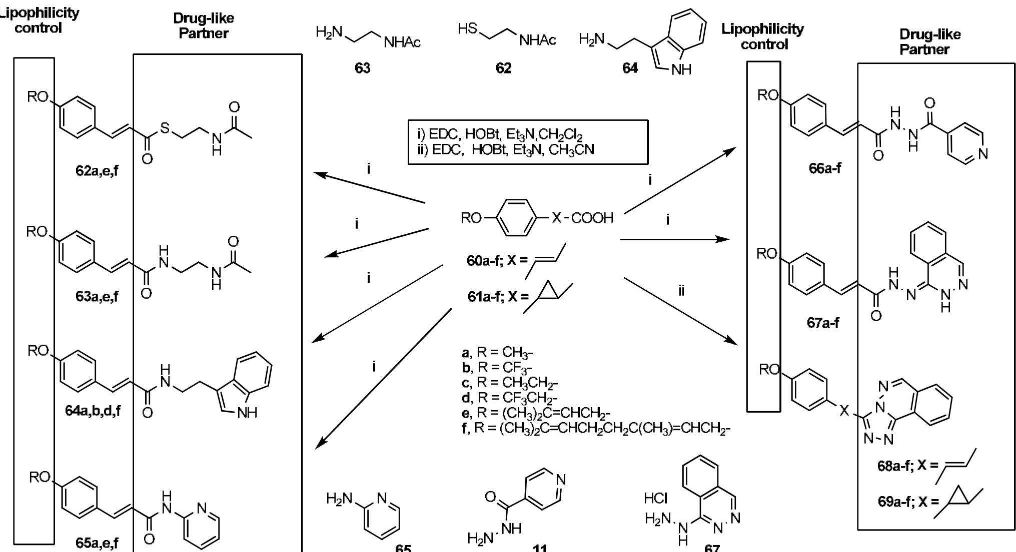
trans-Cinnamic acid hydrazide derivatives were presented as potential antituberculosis agents (Carvalho et al. 2008). The authors designed and explored the introduction of the *trans*-cinnamic moiety into isoniazid (**11**) core structure to ameliorate its activity. Isosteric substitution of the pyridine ring of **11** was also investigated by these authors. The synthetic route (**Scheme 9**) used for the preparation of the target compounds is rapid and relies on the formation/use of *p*-nitrophenyl esters (**58a-d**) as activated forms of cinnamic acid derivatives (**Scheme 9**). These stable intermediates (**58a-d**) were prepared by treating the appropriate cinnamic acid (**57a-d**) with thionyl chloride in the presence of 4-nitro-phenol. The target hydrazides (**11a-d**, **59a-d**; **Scheme 9**) were then obtained in good yields by coupling the so-formed activated acids (**58a-d**) with either acylhydrazide **11** or **59**. The anti-TB activities of these compounds were assessed against *M.tb.* Almost all of the isonicotinic derivatives **11a-d** were sensitive in the minimum concentration tested (MIC = 3.12 µg/mL). Nevertheless, all benzoic acid derivatives **59a-d** were much less active, thus reinforcing the pharmacophoric contribution of the isonicotinic moiety. Importantly, the authors identified that the 4-methoxycinnamic derivatives promote the better activity.

a, R^1 = H, R^2 = H
b, R^1 = H, R^2 = OMe
c, R^1 = H, R^2 = NO_2
d, R^1, R^2 =-OCH_2O-
57a-d
$SOCl_2$, 4-NO_2-phenol, $PhCH_3$
58a-d
pyridine
11, X = N
59, X = C
11a-d, X = N
59a-d, X = C

Scheme 9. Synthetic route for the preparation of the cinnamoyl hydrazides

In our recent effort, we have synthesized some 4-alkoxycinnamic acid thioesters, amides, hydrazides and triazolophthalazine derivatives (Yoya et al., 2009; De et al., 2011b) and evaluated their anti-TB efficacy **(Scheme 10)**. While 4-alkoxy substitutions were introduced to control the required lipophilicity following Lipinski's rules (Lipinski et al., 1997), their coupling partners were suitably chosen either to mimic biological intermediates or to modify any existing drug. Accordingly, various 4-alkoxycinnamic acids were coupled with *N*-acetylcysteamine (**62**) to afford the corresponding thioesters (**62a,e,f**) thereby mimicking the enoylacyl-ACP intermediate involved in the *M.tb.* fatty acid synthase II (FASII) cycle, an essential step towards mycolic acid (C_{26}-C_{56} fatty acids) biosynthesis. Mycolic acids are essential components of bacterial cell wall and notably, isoniazid is known to inhibit InhA (enoylacylreductase A; involved in FAS II cycle) thereby inhibiting the mycolic acid biosynthesis. The major advantage of FAS II-cycle as drug target is that it is an exclusive feature of prokaryotes. However, the synthesized thioesters (**62a,e,f**) showed poor anti-TB activities against *M.tb.* $H_{37}Rv$, possibly due to the weak C-S bond energy which makes these molecules labile under physiological conditions. No amide derivatives (**63a,e, 64a,b,d,f; 65a,e,f**) showed good biological activity except (*E*)-*N*-(2-acetamidoethyl)-3-(4-geranyloxyphenyl)acrylamide (**63f**) (MIC = 0.24 µM, *vs* INH; MIC = 0.6 µM). Unfortunately, they (**63a,e, 64a,b,d,f; 65a,e,f**) have poor cytotoxicity profile. To alleviate the concern for the proteolytic instability, we thus prepared a series of cinnamoylhydrazides (**66a-f**). All six 4-alkoxycinnamoyl isonicotinyl hydrazides (**66a-f**) showed good MIC and their cytotoxicity profile (IC_{50} ranging between 43-256 µM on THP-1 cell line) were very much encouraging.

Further, the radio-thin-layer chromatography analysis of **52e**, when introduced to the broth culture of *M.tb.,* revealed that this class of molecules inhibit the mycolic acid biosynthesis. Two representative INH-derivatives **66a** (same as **11b** in **Scheme 9**) and **66e** were tested on MYC5165, a *M.tb.* strain mutated in InhA and 1400 a *M.tb.* strain mutated in *katG*. The inhibitory activities of **66a** (MIC = 16 μM: MYC5165; 320 μM: 1400) and **66e** (MIC = 27 μM: MYC5165; 68 μM: 1400) were found to follow similar trends as that of INH (MIC = 18 μM: MYC5165; 729 μM: 1400) itself**,** thus not allowing at the moment to propose these compounds as isoniazid prodrugs or not. In order to explore the influence of other hydrazides, 1-hydrazinophthalazine hydrochloride **67**, an antihypertensive drug (Schroeder, 1952; Silas et al., 1982) of moderate potency, was coupled with acids **60a-f** in the presence of EDC.HCl, HOBt and triethylamine to afford phthalazinohydrazides (**67a-f**). For the family of 1-phthalazinohydrazides (**67a-f**), MIC results were moderate but the trend of cytotoxic behaviour was not acceptable. Under different experimental conditions, coupling of acids (**60a-f**) with **67** in acetonitrile under reflux furnished the corresponding 3-(4-alkoxystyryl)-[1,2,4]triazolo[3,4-α]phthalazines (**68a-f**). Interestingly, the combination of isopentenyl-side chain as 4-alkoxy substituent with triazolophthalazine (**68e**), showed excellent antitubercular potency (MIC = 1.4 μM), in comparison with other derivatives in the series (**68a-f**), and more importantly, with good cytotoxicity (IC_{50} = 449 μM on THP-1 cell line) and selectivity index (SI = 320). Finally, to our great delight, compound **68e** showed 100-fold better *in vitro* activity against MYC5165 strain (**68e**; MIC = 0.2 μM) and 1800-fold better activity against 1400 strain (**68e**; MIC= 0.4 μM) compared to INH.



Scheme 10. Synthetic route for the preparation of different cinnamoyl derivatives

Further, the radio-thin-layer chromatography analysis revealed that compound **68e** does not inhibit mycolic acid biosynthesis signifying a different mode of action than INH. In order to explore the importance of the enoyl-acyl backbone, the double bond was replaced by bioisosteric cyclopropyl moiety. Thus, 3-[2-(4-alkoxyphenyl)cyclopropyl]-[1,2,4]triazolo[3,4-α]phthalazine (**69a-f**; racemates) were synthesized and their *in-vitro* anti-TB activities were

also evaluated. Significantly, the MICs of the compounds (**69a-f**) were found to be poorer compared to **68a-f**. In regard to the difference in activities between the enoyl and cyclopropyl series, a plausible explanation could be the respective Michael acceptor ability. From a chemical point of view, 4-OCF_3 derivatives are expected to show better inhibitory activities compared to their 4-OCH_3 analogues. However, this is not the case as **66a** has a 4-fold better activity (MIC = 0.3 μM) compared to **66b** (MIC = 1.1 μM) and similarly **68a** (MIC = 53 μM) exhibits approximately 15-fold activity better than **68b** (702 μM). In view of these results, it was suggested that the Michael addition may not be the mode of action of these compounds. This view was also supported by the fact that mycobacterial lip B prefers to form thioester intermediate with deca-2-enoic acid during mycolic acid biosynthesis unlike *E.coli* lipB which forms a thioether *via* Michael addition (Ma et al.; 2006).

3. Cinnamaldehyde derivatives as anti-TB agents

Cinnamaldehyde (**70**), also biosynthesized starting from phenylalanine (**9**) in the process of lignin biosynthesis, occurs naturally in the bark of cinnamon trees and other species of the genus *Cinnamomum*. Owing to its typical odor and low toxicity to human exposure, cinnamaldehyde is used as food flavoring agent. It is also used as a fungicide, insecticide for mosquito larvae (Cheng et al., 2004) and has shown inhibitory activities towards proliferation, invasion and tumor growth in a murin A375 model of human melanoma (Cabello et al., 2009). Importantly, **70** and its derivatives have shown enormous potential as antimicrobial agents. For example, cinnamaldehyde is known to inhibit *E. coli* and *Salmonella typhimurium* growth (Helander et al., 1998). Its carbonyl group has affinity for proteins, preventing the action of decarboxylase amino acids on *E. aerogenes* (Wendakoon & Sakaguchi, 1995). From a chemical standpoint, worth precising is that **70** so as its 3-phenylacrylaldehydic congeners offer three main reactive sites: substitution on the phenyl ring, addition on the α,β-unsaturation and reactions of the aldehyde functionality. The α,β-unsaturated carbonyl moiety can be considered as a Michael acceptor (Chew et al., 2010), which is often employed in the design of drugs (Ahn & Sok, 1996).

3.1 Cinnamaldehyde

The growth of *M. avium* subsp. paratuberculosis is inhibited by cinnamaldehyde (**70**) with a MIC of 25.9 μg/mL (Wong et al., 2008). Importantly, the authors suggest that the mechanism of antimicrobial activity of naturally occurring compounds such as cinnamaldehyde is specific rather than nonspecific since it is concentration dependent (Friedman et al., 2002). Possible modes of action include disruption of cell membranes, inhibition of essential enzymes, chelation of essential trace elements (such as iron), and targeting of cell membranes. Cinnamaldehyde (**70**) is also known to inhibit the bacterial cell division protein FtsZ (Domadia et al., 2007). FtsZ, a prokaryotic homolog of tubulin, regulates cell by assembling into the macromolecular structure called Z-ring at the site of cell division (Romberg & Levin, 2003). While cinnamaldehyde (**70**) proves to decrease the *in vitro* assembly reaction and bundling of FtsZ, **70** was also found to perturb the Z-ring morphology *in vivo* and to reduce the frequency of the Z ring per unit cell length of *Escherichia coli*. In addition, GTP-dependent FtsZ polymerization is inhibited by **70**, cinnamaldehyde (**70**) inhibiting the rate of GTP hydrolysis and binding FtsZ with an affinity constant of $1.0 \pm 0.2\ \mu M^{-1}$. Isothermal titration calorimetry revealed that the binding of **70** to

FtsZ is driven by favorable enthalpic interactions. This signifies that **70** binds FtsZ, perturbs the cytokinetic Z-ring formation and inhibits its assembly dynamics. The authors suggested that **70**, a small molecule of plant origin, is a potential lead compound that can be developed as an anti-FtsZ agent towards drug design.

3.2 Cinnamaldehyde-derived hydrazones

The synthesis and antimycobacterial efficacy of a new class of styryl derivatives (**74a-c**) were reported (**Scheme 11**) (Biava et al., 1997). The desired styryl derivatives (**74a-c**) were prepared in a three-steps sequence that begins by the reduction of the starting nitro compounds (**71a-c**) furnishing *ortho-*, *meta-* or *para*-aminotoluidines possessing either an imidazole, pyrazine or morpholine frame (**72a-c**). The so-obtained compounds (**72a-c**) were then coupled under reductive amination conditions (**Scheme 11**) with cinnamaldehyde (**70**) to afford the toluidine-styryl derivatives (**74a-c**). Among all synthetized compounds, derivatives **73c** (R=Imidazole) and **74a-c** (R=Imidazole) were the most active against five different *M.tb.* strains with MIC values ranging between 1 to 64 µg/mL.

R = ; **a** : ortho; **b** : meta; **c** : para
NO_2 ; H_2/Pd-C ; EtOAc ; RH_2C ; **71a-c** ; NH_2 ; **72a-c** ; CHO ; **70** ; EtOH, H^+ ; N ; **73a-c** ; $NaBH_4$, EtOH ; NH ; **74a-c**

Scheme 11. Synthesis of toluidine derivatives

In 2010, a series of 5-(4-isopropylthiazol-2-yl)-4-((*E*)-((*E*)-3-phenylallylidene)amino)-4*H*-1,2,4-triazole-3-thiol (**78**; **Scheme 12**) was synthesized (Kumar *et al.* 2010). 4-Isopropylthiazol-2-carbahydrazide **76** was converted into the corresponding dithiocarbazinate, which upon cyclization with hydrazine hydrate yields 4-amino-5-(4-isopropyl-1,3-thiazol-2-yl)-4*H*-1,2,4-triazole-3-thiol (**77**). The triazole (**77**) was condensed with **70** in the presence of catalytic amount of H_2SO_4 in refluxing ethanol to afford **78**. Synthesized compound **78** was evaluated (Shiradkar et al. 2007; Joshi et al. 2008) for its preliminary cytotoxicity and antitubercular activity against *M.tb.* $H_{37}Rv$ strain by broth dilution assay method and showed a promising activity (MIC = 4 µg/mL).

75 ; $NH_2NH_2.H_2O$; OEt ; **76** ; NHNH$_2$; CS_2, $NH_2NH_2.H_2O$; EtOH, KOH ; **77** ; NH_2 ; SH ; **70** ; H_2SO_4 ; **78**

Scheme 12. Synthesis of 2-substituted -5-[isopropylthiazole] clubbed 1,2,4-triazole

Scheme 13. Different hydrazone derivatives synthesized from cinnamaldehyde

Several other hydrazone derivatives (**79a-e**) of cinnamaldehyde were made and their antiTB efficacy was also tested(**Scheme 13**) (Abdel-Aal et al. 2009). Among them, compound **79d**, arising from cinnamaldehyde (**70**) and isoniazid (**11**), showed maximum anti-TB activity (at 50 µg/mL) with the same MIC of the reference drug isoniazid (INH, 12.5 µg/mL).The synthetic hydrazone (**86**), exhibiting anti-TB activity (MIC = 25 µg/mL), was recently reported by Rao *et al.* (**Scheme 14**) (Rao et al., 2010). The authors synthesized **86** through 3-methyl-2-oxoquinoxalin (**82**) that is easily obtainable from *ortho*-diaminobenzene (**80**) by refluxing in ethanol along with acetylacetic acid (**81**). Treatment of **82** with 2-chloro ethylacetate (**83**) in the presence of K_2CO_3 in DMF gave the corresponding ethyl 2-(3-methyl-2-oxoquinoxalin-1(2*H*)-yl)acetate (**84**) which was converted to 2-(3-methyl-2-oxoquinoxalin-1(2*H*)-yl)acetohydrazide (**85**) by refluxing in ethanol in the presence of hydrazine hydrate (**Scheme 14**). Compound **85** was then coupled with **70** in DMF in the presence of acetic acid to furnish the desired hydrazone (**86**) which showed anti-TB activity (MIC = 25 µg/mL).

Scheme 14. Synthesis of a quinaxoline-derived cinnamaldehyde hydrazone

3.3 Cinnamaldehyde-derived metal complexes

A new series of copper(II) and zinc(II) complexes has been designed and synthesized using a new type of Schiff base (**89a-h**) derived from the reaction of 3-(3-phenyl-allylidene)-pentane-2,4-dione (**88**) with *para*-substituted aniline and benzene-1,2-dithiol (**90**) (**Scheme 15**) (Raman et al., 2010). The intermediate **88** was first obtained by aldol condensation between **70** and acetylacetone (**87**) using piperidine as base. The minimum inhibitory concentrations of the complexes have also been investigated against *M.tb.* strain $H_{37}Rv$. The lowest MIC values were obtained for -NO_2 group containing complexes (**91g**; MIC = 2.9

μg/mL, **91h**; MIC = 3.8 μg/mL) which are more active against $H_{37}Rv$ strain than the other complexes.

Scheme 15. Preparation of Cu(II) and Zn(II) complexes starting from cinnamaldehyde

4. Chalcone derivatives as anti-TB agents

Chalcones, one of the major classes of natural products with widespread distribution in fruits, vegetables, spices, tea and soya based foodstuffs, are of great interest for their interesting pharmacological activities (Di Carlo et al., 1999). Chalcones, or 1,3-diaryl-2-propen-1-ones, belong to the flavonoid family (Dimmock et al., 1999; Ni et al., 2004). From a structural standpoint, they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl unit (**Fig. 8**). Interestingly, a vast number of naturally occurring chalcones are polyhydroxylated onto both aryl rings conferring them significant radical-quenching properties which have raised interest in using chalcones or chalcone-rich plant extracts as drugs or food preservatives (Nowakowska, 2007). Besides, Chalcones have been reported to possess many useful properties, including anti-inflammatory, antimicrobial, antifungal, antioxidant, cytotoxic, antitumor and anticancer activities (Dimmock et al., 1999; Go, Wu & Liu, 2005).

4.1 Natural and synthesized chalcones

M.tb. , *M. bovis*, *M. kansasii*, *M. xenophii* and *M. marinum* were inhibited by licochalcone A (**92**; MIC = 20 mg /mL), extracted and purified from Chinese licorice roots (**Fig. 8**) (Friis-Møller et al. 2002). Besides, the presence of a halogen substituent on A-ring of 2'-hydroxychalcones proved to play a crucial role on anti-TB activity. It has indeed been found that chalcones substituted by a halogen atom at the 3-position demonstrate stronger anti-TB activity than those substituted by a halogen atom at the 2- or 4-position (Lin et al., 2002). In that manner, chalcones **93** and **94** with a 2'-hydroxyl group on B-ring and a 3-chloro- or 3-iodo-group on A-ring showed the strongest activity, with 90-92% inhibition against *M.tb.* $H_{37}Rv$ at a drug concentration of 12.5 mg/mL. The activity of 2'-hydroxychalcone (61% inhibition) was further enhanced by introducing a chloro (89%) or a methoxy group (78%) at the 4'-position of B-ring. Nevertheless, introduction of an additional substituent, such as a methoxy, amino, bromo or carboxyl group on B-ring led to a dramatic decrease or a complete loss of activity (Lin et al., 2002). Recently, the activities of some synthetic chalcones were also assayed against *M.tb.* protein tyrosine phosphatase A (PtpA) which is an enzyme associated with *M.tb.* infectivity (Chiaradia et al., 2008). Note that tyrosine phosphatases are secreted by pathogenic bacteria, and MPtpA is an example that was shown to be required

for growth of *M.tb.* in human macrophages (Bach et al., 2008). In the search for lead compounds, a series of 38 chalcones were prepared by aldol condensation between aldehydes and acetophenones and five of the so-prepared compounds (**95-99**) presented moderate to good activities (**Scheme 16**). The structure–activity analysis revealed that the predominant factor for the activity is the molecular planarity and/or hydrophobicity and the nature of the substituents. Later on, the molecular recognition of these inhibitors on PtpA was investigated through molecular modeling, these investigations revealing that the binding and the inhibitory activity of the chalcones are predominantly governed by the positions of the two methoxyl groups at the B-ring (Mascarello et al., 2010). Besides, the –OMe groups proved to establish key hydrogen bonds with the amino acid residues Arg17, His49 and Thr12 in the active site of PtpA while the 2-naphthyl A-ring undergoes л-stacking interaction with the Trp48 residue. Interestingly, reduction of mycobacterial survival in human macrophages upon inhibitor treatment suggests their potential use as novel therapeutics.

Fig. 8. Chalcones with antiTB activities

Scheme 16. General strategy for chalcone synthesis

4.2 Chalcone hydrazone derivatives

A series of *N*'-nicotinoyl-3-(4'-hydroxy-3'-methylphenyl)-5-(substituted phenyl)-2-pyrazolines (**102a-d**) were synthesized by the reaction between isoniazid (INH; **11**) and chalcones (**101a-d**) and were tested for their antimycobacterial activity *in vitro* against *M.tb.* $H_{37}Rv$ and INH-resistant *M.tb.* (INHR- *M.tb.*) strains using the agar dilution method(**Scheme 17**) (Shaharyar et al. 2006). Among the synthesized compounds, *N*'-nicotinyl-3-(4'-hydroxy-3'-methyl phenyl)-5-(1''-chlorophenyl)-2-pyrazoline (**102d**) was found to be the most active agent against *M.tb.* and INHR- *M.tb.*, with minimum inhibitory concentration of 0.26 μM. When compared to INH-compound **102d** was found to be 2.8- and 43.7-fold more active against *M.tb.* $H_{37}Rv$ and INHR-*M.tb.*, respectively.

Scheme 17. Synthesis and anti-TB activities of chalcone-derived pyrazoline compounds

4.3 Chalcones with substitutions at the aromatic ring

A series of acetylenic chalcones were evaluated for antituberculosis activity (**Scheme 18**) (Hans et al., 2010). The acetylenic functionality not only serves as a site for further chemical diversification but is also of great interest in medicinal chemistry and the pharmaceutical industry. Moreover, it functions as a key pharmacophoric unit in acetylenic antibiotics (Maretina & Trofimov, 2006) and its presence in anticancer (Siddiq & Dembitsky, 2008) and antitubercular (Deng et al. 2008) agents is noteworthy. From a synthetic standpoint, hydroxyacetophenones (**103a,b**) were treated with propargyl bromide (**104**) in the presence of K_2CO_3 in DMF to afford the respective propargyloxyacetophenones (**105a,b**) that were then treated with methoxybenzaldehydes (**106,107**) under basic conditions to provide the chalcones (**108a,b**) featuring the desired propargyloxy moiety. Most compounds were more active against non-replicating (MABA) than replicating (LORA) cultures of *M.tb.* $H_{37}Rv$, an unusual pattern with respect to existing anti-TB agents.

Scheme 18. Synthesis and anti-TB activities of acetylenic chalcones

The introduction of a quinoline moiety to chalcones as aromatic substituent was envisaged as an interesting way of designing new anti-TB agents. In that manner, a series of substituted quinolinyl chalcones (**113**, **114**) was synthesized under basic conditions and evaluated for their *in vitro* anti-TB activity against *M.tb.* $H_{37}Rv$ (**Scheme 19**) (Sharma et al., 2009). The structure–activity relationship analysis revealed that different physicochemical and structural requirements are needed for anti-TB activity. Two compounds **113** and **114** have shown anti-TB activity at MIC 3.12 μg/mL. By comparison, pyrazinamide (**115**), a

known antituberculosis drug, showed a MIC value of 50 μg/mL under similar assay. Moreover, these molecules were nontoxic against VERO and MBMDM cell lines.

Scheme 19. Synthesis of substituted quinolinyl chalcones

4.4 Physico-chemical study

Importantly, a quantitative structure activity relationship (QSARs) methodology has been developed (Sivakumar et al. 2007) for the reported anti-TB activity of chalcones, chalcone-like compounds, flavones and flavanones using a statistical technique called genetic function approximation (GFA). The generated equations in each model were analyzed, for both the goodness of fit and predictive capability. The analysis also points out to the importance of the hydrogen, PMI-mag and HOMO bond donors. The study indicated that the reported compounds are more lipophilic in nature and hence, as expected, exhibit good activity since *M.tb.* has a high concentration of lipid layer. These theoretical models deserve to be explored further to design potent, newer compounds having better anti-TB activity.

5. Conclusion

Cinnamic acids, cinnamaldehydes and chalcones are unique as drug candidates in tuberculosis. Natural cinnamic-based substances such as ethyl- (**2**) and benzyl- (**4**) cinnamates not only have anti-TB activities, they are traditional medicines for hypertension as well. Cinnamaldehyde and Licochalcone A also have good potentials as anti-TB agents. Sharing a common α,β-unsaturated carbonyl functionality, these molecules offer Michael-acceptor properties, particularly to the glutathione (GSH) and cystine residues. Although, mycobacteria, unlike *E.coli*, do not prefer the formation of Michael-adduct, the presence of the cinnamic moiety certainly increased the anti-TB efficacy on several occasions. Importantly, the replacement of the double bond with an isosteric cyclopropyl ring decreased the anti-TB efficiency of the triazolophthalazines. On the other hand, introduction of cinnamoyl moiety to isoniazid did not significantly alter the trend of biological activity or the mode of action. These observations indicate that the anti-TB activity depends not only on the α,β-unsaturation but also on the functionalization of the carbonyl part of the cinnamoyl derivatives. Several hydrazone derivatives of cinnamaldehyde and chalcones have notable anti-TB activities. Importantly, substituents at the benzene ring of the cinnamic acids also play a crucial role in the biological activities. Notably, Isoprenyloxy cinnamoyl-triazolophthalazine derivative (**68e**) and chalcone derived pyrazoline derivative (**102d**)

showed excellent anti-TB activity against INH-resistant strains. In summary, cinnamic acid, cinnamaldehyde and chalcone derivatives are regarded as potent anti-TB agents. Surprisingly, in spite of their relevant anti-TB activities, little is still known about the mode of action and understanding of the implicated molecular mechanisms. We hope to learn more about these versatile molecules and its derivatives in addition to the synthesis of new, useful biologically important compounds in the near future.

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16

Potential Use of *I. suffruticosa* in Treatment of Tuberculosis with Immune System Activation

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1. Introduction

1.1 Tuberculosis and immune system

Mycobacterium tuberculosis is a serious threat to humankind, with over 8 million cases of tuberculosis (TB) annually, killing almost 3 millions of people per year around the world (WHO, 2008). Moreover, side effects from first-line anti-TB drugs can cause significant morbidity, and compromise treatment regimens for TB (Yee et al., 2003). Most healthy individuals are able to control TB infection with a vigorous immune response, halting the progression of the disease, but not necessarily eradicating the microorganism (McKinney, 2000).

The bacterium resides within macrophages, allowing them to resist the antimicrobial effector mechanisms of the host (Raupach & Kaufmann 2001). Macrophages constitute one of the main phagocyte cells of the immunological system and they are the first cells involved in an immunological response. Part of their effectiveness is due to the production of nitric oxide (NO), hydrogen peroxide (H_2O_2) and cytokines, as well as phagocytosis of strange particles (Allavena et al., 2008; Carlos et al., 2004; Keil, 1999). Thus, the elimination of tuberculosis bacillus is involved in the production of these effectors molecules from immune system.

The hydrogen peroxide, generated by macrophages in a reaction catalyzed by an NADPH oxidase, was the first identified effector molecule that mediated mycobacteriocidal effects of mononuclear phagocytes (Lopes et al., 2005; Walker & Lowrie, 1981). In spite of several studies have indicated significant *M. tuberculosis* resistance to oxidative stress *in vitro* and *in vivo,* a recent study showed that H_2O_2 induced the complete sterilization of the cultures of *M. tuberculosis* by 24 h, after the exposition to 50mM of H_2O_2 (Volskuill et al., 2011).

NO formed by the action of the inducible form of nitric oxide synthase (iNOS) reacts with oxygen radical forming RNI. NO and related RNI have been reported to possess antimycobacterial activity (Chan et al., 2004; Kwon, 1997). Although the role of NO in human tuberculosis remains unsettled evidence supporting its importance has come from a variety of areas (Nathan & Shiloh, 2000) including the demonstration that human granulomas contain iNOS, endothelial-NOS and nitrotyrosine, a compound whose accumulation indicates production of NO (Nathan, 2002). Additionally, the ability of human alveolar macrophages to kill *M. tuberculosis* is dependent on the activity of iNOS and the human macrophages taken from healthy subjects latently infected with *M. tuberculosis* produce NO controlling the growth of the bacteria (Yang et al., 2009). The presence of NO within human granulomas could contribute to host resistance since *in vitro* experiments demonstrate direct RNS-mediated bacteriostatic (Firmani & Riley, 2002; Ouellet et al., 2002; Voskuil et al., 2003) and bactericidal activity (Nathan, 2002). Mice deficient in both *phox* and *iNOS* are much more susceptible to *M. tuberculosis* infection than either mutant alone which would indicate that RNS and ROS protect the host in a partially redundant fashion (Shiloh & Nathan, 2000; Volskuill et al., 2011).

TNF-α is a cytokine that plays multiple roles in immune and pathologic responses in tuberculosis, also required for acute infection control (Babbar et al., 2006; Flynn et al., 1995; Palladino et al., 2003). The pro-inflammatory cytokine TNF-α produced by activated macrophages is a central contributor to the immune response against *M. tuberculosis* (Flynn, 1986; Marino et al., 2007). The role of TNF is of clinical interest due to the association of anti-inflammatory TNF-α blocking drugs with reactivation of latent TB in humans (Keane et al., 2001; Wintrop,2006). This cytokine has multiple immunological functions during infection with *M. tuberculosis*: It has a direct role in immune cell recruitment via up-regulation of endothelial adhesion molecules (Zhou, et al., 2007) facilitating transendothelial migration of immune cells to the site of infection. TNF-α regulates production of chemokines by macrophages (Algood et al., 2006; Roach et al., 2002); chemokines can further induce transendothelial migration and coordinate recruitment of immune cells within the tissues. TNF-α activates macrophages in conjunction with the cytokine IFN-γ (Flesch & Kaufmann 1986; Roock et al., 1986; Carlos et al., 2009) such activated macrophages can kill intracellular mycobacteria. TNF-α can also induce necrotic or apoptotic cell death in macrophages (Laster et al., 1988) that is promoted by TB infection (Keane, et al., 2001).

1.2 Plant with antimycobacterial and immunostimulating activity

With proposal to stimulate the immune system, some plants can be used in collaboration with the standards drugs for the treatment of tuberculosis. Moreover, there are a lot of plants that can be able to presenting an antimycobacterial activity.

It is possible to assign this effect to the substances contained in its structure which are responsible for protecting the plant structure from aggressive agents in what concerns the active ingredients of plants with an antimicrobial character. Most of these substances are part of the secondary metabolites which consist of substances produced by plants which are not vital and involved in metabolic mechanisms. Flavonoids, tannins, terpenes, alkaloids, phenolic compounds, etc are examples of secondary metabolites. Thereby, many of these compounds protect the vegetal structure against external aggression such as insects, solar radiation, fungi, bacteria and viruses (Heldt, 1997).

Terpenoids are known as natural insecticides. This class also includes limonoids, limonene and myrcene which plays an important role in the protection of the plants against insects. Some terpenoids have already been tested and have manifested an activity against mycobacterium (Cantrell et al., 2001). Terpenes are composed by basic units of active isoprene isopentenilpirofosfatou, and originate triterpenes and sesquiterpenes previously mentioned in literature as substances with bacterial features (Januario et al., 2002; Pietro et al., 2000).

Essential oils such as geraniol, citronellol, cineole and other genus *Eucalyptus L'Herit*, are recognized as bactericide (Hinou et al. 1989; Leite et al., 1998). The alkaloid obtained from extracts of leaves of *A. Vasic*, vasicine acetate and 2-acetyl benzyl amine showed promising antimycobacterial activities in several strains of *M. tuberculosis* (Gupta et al., 2010).

The endophytic fungi are microorganisms capable of producing potentially bioactive metabolites. These molecules may have hormonal, antibiotic or antitumor activities and other biological functions of enormous industrial and biotechnology interest. Tan and Zou (2001) examined the diversity of metabolites from isolated endophytic fungi and reported the isolation of substances belonging to different structural groups such as steroids, xanthones, phenols, isocoumarins, alkaloids, quinones, furandionas, terpenoids, peptides, cytochalasins and aliphatic compounds. 3-D citosporona, fomopsolida and the acid "*coletótricose*" stand out for their antibacterial activity shown in several studies (Brady et al. 2000; Zou et al., 2000).

Considering the importance of immunomodulation in the treatment of tuberculosis, the activation of some components of the immune system is a great advantage when it is associated with the bacterial/bacteriostatic activity of the plants. As examples of substances which have immunostimulant and antimicrobial actvities associated, the lectin derived from *Synadenium carinatum* has an important stimulatory activity of granulocytes and NK cells. It is also able to stimulate the expression of TNF-α, IL-1β and iNOS in murine peritoneal macrophages (Cardoso, 2006). This activity is due, partially, to the presence of tannins. This class of secondary metabolites can stimulate the production of IL-1β and TNF-α in macrophages as well as having a significant antimicrobial activity with MIC <100μg/mL against *M. tuberculosis* (Lawal et al., 2011; Miyamoto et al. 1993).

Scutellaria baicalensis has also an immunostimulant action combined with antibacterial activity. In addition to the activity against *M. tuberculosis, S. baicalensis* employs a toxic activity against cholera, typhoid, streptococcus, *E. coli, Pneumococcus, Klebsiella pneumoniae, Proteus vulgaris Mycobacterium tuberculosis, Pseudomonas aeruginosa* and *Corynebacterium diphtheria*. This plant induces the production of TNF-α possibly due to the effect of flavonoid wogonin (Chang et al. 1986; Franzblav & Cross 1986; Huang, 1993; Jen et al., 2002). This mediator was also investigated as TNF-α acts in the production of nitric oxide. The results showed that low concentrations of wogonin induce the production of nitric oxide and high concentrations inhibit the production (Jen et al., 2002).

Similarly, *Glycyrrhiza glabra* evinced antimicrobacterial activity in the concentration of 0.5mg/mL. After a phytochemical analysis, the tuberculosis which is toxic to the bacilli at concentrations of 0.029 mg / mL (Gupta et al., 2008) was attributed to the activity against *M. glabridin*. Regarding the immunomodulatory activity, it has been observed that the extract of *Glycyrrhiza glabra* intensely activated granulocytes and NK cells (Cheell et al., 2010).

Our research group studied *Indigofera suffruticosa* Miller (Fabacesae) with the aim to collaborate with the discovery of alternatives treatments for tuberculosis. Since at this moment there is no new drug generation able to eliminate the bacillus and simultaneously stimulate the immune system we investigated the antimycobacterial and immunological activity of methanol (METH) and dichloromethane (DCM) extracts of *I. suffruticosa*.

I. suffruticosa is found in tropical and subtropical areas and is well adapted to growth in semi-arid regions and soils of low fertility (Paiva, 1987). A chemical investigation of extracts of leaves of *I. suffruticosa* in Natural Products Alert (NAPRALERT) and Chemical Abstracts databases has revealed the presence of alkaloids, flavanoids, steroids, proteins, carbohydrates and indigo. Some recent reports have demonstrated the in vitro bioassay activity of plant-derived terpenoids against *M. tuberculosis* (Cantrell et al., 2001; Higuchi et al., 2008). The literature also reports the antimycobacterial activity of many classes of natural products: such as alkanes, phenolics, acetogenic quinines, flavonoids, triterpenes, flavonones and chalcones (Copp, 2003; Higuchi et al., 2008; Pavan et al., 2009). Previous results demonstrated that indigotin alkaloid can enhance macrophage functions and therefore contribute to the host defense against pathogens and tumors (Lopes et al., 2006).

2. Materials and methods

2.1 Plant material and samples

Aerial parts of *I. suffruticosa* were collected in Rubião Junior, Botucatu city, São Paulo State, Brazil, and identified by Prof. Dr. Jorge Yoshio Tamashiro. The immunological assays were performed as soon as the plant was collected. A voucher specimen (HUEC 129598) was deposited at the Herbarium of the Universidade Estadual de Campinas (Unicamp), Campinas, SP, Brazil. Aerial parts of *I. suffruticosa* (1.1 Kg) were dried Activity of the *I. suffruticosa* (40°C), powdered and extracted exhaustively at room temperature with dichloromethane and methanol, successively. Solvents were evaporated at 40°C under reduced pressure to afford the DCM (15.2 g) and METH (30.0 g) extracts. Each extract was first solubilized in dimethyl sulfoxide (DMSO) and then diluted in an appropriated culture medium, RPMI-1640 for the immunological assays and Middlebrook 7H9 for the determination of antimycobacterial activity (62.5-4000 µg/mL).

2.2 Peritoneal macrophages

Peritoneal macrophages Thioglycollate-elicited PEC were harvested from Swiss mice using 5.0 mL of sterile PBS, pH 7.4. The cells were washed twice by centrifugation at 200 g for 5 min at 4ºC and re-suspended in RPMI-1640 medium (Sigma). The adherent cells were obtained by incubation for 1 h at 37ºC in an atmosphere of air/CO2 (95:5, v/v) (Forma Scientific) and, incubated with LPS or RPMI-1640 medium. This protocol was in agree with the regulations of Research Ethics Committee (# 01/2005).

2.3 MTT assay for cell viability

PEC ($5x10^6$ cells/mL) was re-suspended in RPMI- 1640 medium. The suspension (100 µL) and the extracts (100 µL) were added to each well of a 96-well tissue culture plate and they were incubated for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

(MTT) colorimetric assay was performed as described by Mosmann (1983) . Only cells and culture medium (RPMI- 1640) were used as a control that corresponds to 100% of macrophages viability.

2.4 Measurement of H_2O_2 production

Hydrogen peroxide measurement the adherent cells of PEC ($2x10^6$cells/mL) was measured using the horseradish peroxidase-dependent phenol red oxidation microassay (Pick & Mizel, 1981). Phorbol myristate acetate (PMA, Sigma, St. Louis, MO) were used as a positive control.

2.5 Measurement of NO production

NO measurement the adherent cells of PEC ($5x10^6$cells/mL) was mensured using Griess reagent (Green et al., 1982). E. coli O111B lipopolysaccharide (LPS – 1 µg/mL) solution were used as positive control.

2.6 Measurement of TNF-α production

The determination of TNF-α in the supernatants was based in its property to destroy L929 tumoral cell line (mouse tumour fibroblast) Carlos et al. (1994). LPS (1 µg/mL) was used as a positive control.

2.7 Determination of antimycobacterial activity by MABA

The minimum inhibitory concentration (MIC) of DECE was determined against M. tuberculosis H37Rv (American Type Culture Collection 27294) in Middlebrook 7H9 medium using the Microplate Alamar Blue Assay - MABA (Collins & Franzblau, 1997). For standard test, the MIC value of Isoniazid (Sigma) was determined each time. The acceptable MIC of Isoniazid ranged from 0.015 to 0.05 µg/mL.

2.8 Statistical analysis

The results are expressed as means ± SD of five experiments. One-way ANOVA with Dunnett's post test was performed using GraphPad InStat (San Diego, California, US) with the level of significance set at $p < 0.05$.

3. Results and discussion

Actually, TB multiple drug resistance has become a major threat worldwide and thus calls for an urgent search for new and effective treatments for this deadly disease. Naturally occurring compounds as extracts from plants have indicated that inhibitory activity against *M. tuberculosis* is widespread in nature (Okunade et al., 2004).

The cytotoxicity effect of the extract was evaluated by the determination of MTT (a tetrazolium salt: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Mosmann, 1983) (Table 1). The index of cytotoxicity 50 (IC_{50}) found was in the concentration of 200µg/mL.

Extracts	Viability (%)
Control	100 ± 0,00
600[a] 400[a] 200[a] 100[a]	15,73 ± 1,90 30,29 ± 4,32 55, 29 ± 1,36 71, 57 ± 1, 82

Table 1. Effect of methanolic and dichloromethane extracts of *Indigofera suffruticosa* on the viability of peritoneal macrophages. [a]- μg/mL

This study evaluated the antimycobacterial activity the extracts of *I. suffruticosa* and its action in innate immune system. The antimycobacterial activity of METH and DCM is presented in table 2. Gu et al. (2004) considered active plant extracts with MIC value ≤128 μg/mL. Thus we considered a promising result the MIC of 125 μg/mL found in METH crude extract.

	Methanolic	Dichloromethane	Isoniazida
MIC	125[b]	1000[b]	0,05[b]

Table 2. Minimal inhibitory concentration (MIC) in presence of methanolic and dichloromethane extracts of the plant *Indogofera suffruticosa*. [a]Standard drug, [b]μg/mL.

The extracts presented a high production of nitric oxide with statistically significant values compared to the negative control ($p<0,001$). The amount of NO produced by the METH extract (105, 99 μmol/5.10^5 cells) was larger than the production of DCM extract (58, 9 μmol/5.10^5 cells) (Fig. 1).

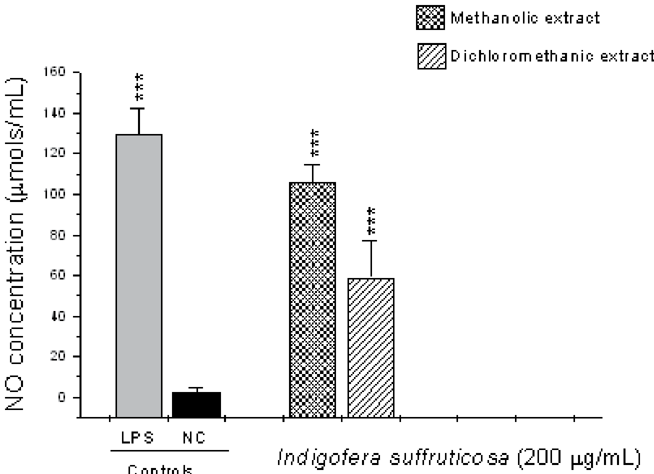

Fig. 1. Induction of nitric oxide.

The results regarding of TNF-α confirmed a significant production of this cytokine at levels near the positive control (252,7 and 234,6 units/mL, METH and DCM extracts, respectively) confirming a correlation between the synthesis of TNF-α and NO (Fig. 3) (Bogdan et al., 1991; Carli et al., 2009).

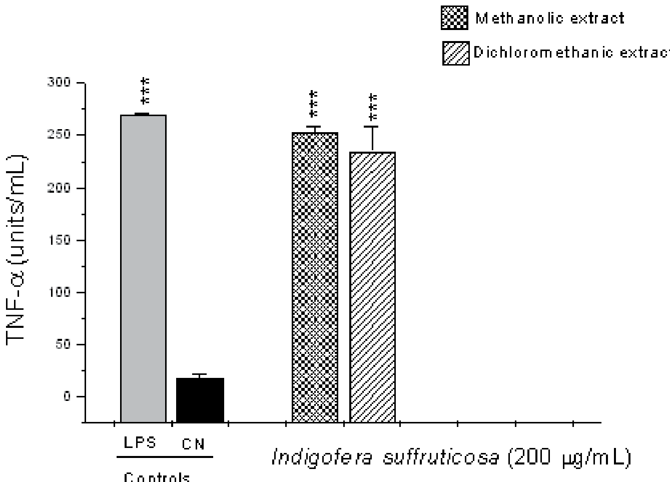

Fig. 2. Induction of tumor necrosis factor-α.

I. suffruticosa did not produce significant amounts of H_2O_2 when compared with control negative (p>0,05), METH (0,59 nmols/2.10^{5}cells) and DCM (3,3 nmols/2.105cells) (Fig. 3). This fact can be justified by the presence of tannins, such as gallic acid in extracts of *I. suffruticosa*. This class of substances has been showed an antioxidant potential being responsible for the scavenger of free radicals such as hydrogen peroxide. (Akira et al., 2002).

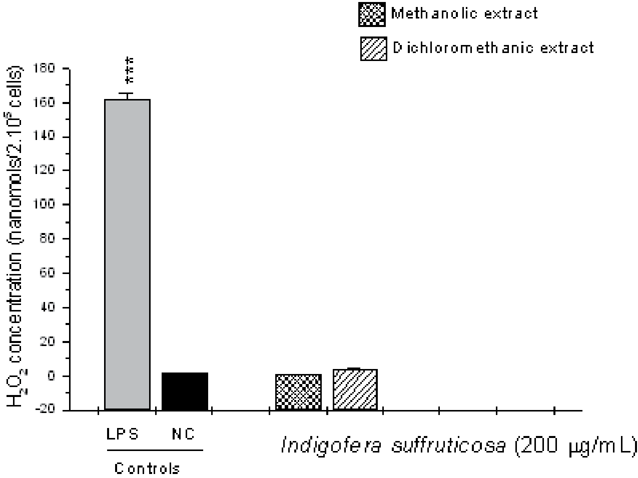

Fig. 3. Induction of hydrogen peroxide.

Thus, this screening suggests that both extracts of the *I. suffruticosa* promoted the activation of the macrophages. The significant production of studied mediators (NO and TNF-α) by activated macrophages in presence of *I. suffruticosa* is very important, since macrophages produces several effector molecules that can enhance or restore the ability of the innate immune system to fight against TB infection.

Nitric oxide (NO) formed by the action of the inducible form of nitric oxide synthase (iNOS) reacts with oxygen radical forming RNI. NO and related RNI have been reported to possess antimycobacterial activity (Kwon, 1997). Phagocytes kill intracellular organisms during an initial oxidative phase dependent on NADPH oxidase followed by a prolonged nitrosative phase during which bacterial growth is inhibited by iNOS (Nathan & Shiloh, 2000).There are several potential mechanisms that can explain how NO may affect microbial life-cycle. NO and other RNI can modify bacterial DNA, protein and lipids at both the microbial surface and intracellularly. They can alter cytokine production and induce or prevent apoptosis of host cells by controlling caspase activity (Raupach & Kaufmann, 2001).

M. tuberculosis strongly induces the release of several cytokines during infection. Tumor necrosis factor-α (TNF-α) is a cytokine that plays multiple roles in immune and pathologic responses in tuberculosis, also required for acute infection control (Flynn et al., 1995). It plays a major role in the recruitment of inflammatory cells to the site of infection and in the formation and maintenance of granulomas (Gaemperli et al., 2006). This cytokine is necessary for optimal co-ordination of both the differentiation of specific T cells to secrete the appropriate T helper 1 cytokines and the development of granulomas in which activated macrophages restrict mycobacterial growth (Ehlers, 2003). TNF-α is required for control of latent TB and it is also a key element for activating macrophages to produce iNOS and thus in maintaining the pathway for generating NO and preventing reactivation of the disease (Adams et al., 1995).

4. Conclusion

We suggest that the extract may be an important bactericidal source against *M. tuberculosis* once the same has natural origin and do not present the toxic effects provoked by the drugs current used in the treatment of tuberculosis. Moreover, a possible association with traditional drugs can be suggested considering that the most of standard drugs do not present the same simultaneous effect microbiological and immunological of the extract here tested. These results described here highlight the importance of conducting an in-depth study of the species of the Brazilian biome, and show the great potential of it's biodiversity in the treatment of infection diseases.

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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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