

Fighting Against Resistant Strains: The Case of Benzothiazinones and Dinitrobenzamides

Silvia Buroni, Giovanna Riccardi and Maria Rosalia Pasca
*Department of Genetics and Microbiology, University of Pavia
Italy*

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; Sasseti et al., 2003; Sasseti 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-dioxo-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).

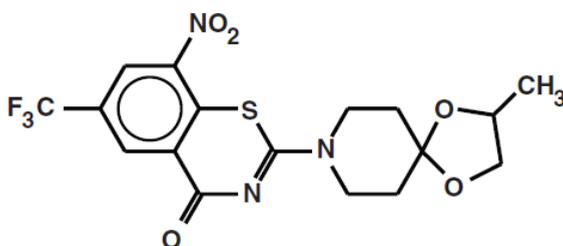


Fig. 1. Chemical structure of the lead compound BTZ038.

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).

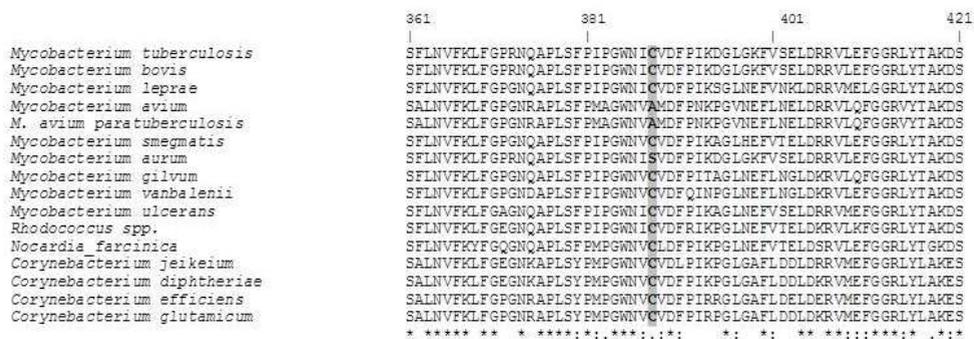


Fig. 2. Multiple alignment of the BTZ resistance-determining region in orthologs of *dprE1* from various actinobacteria by ClustalW. The cysteine (or correspondent amino acid) involved in the BTZ binding is highlighted in grey.

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).

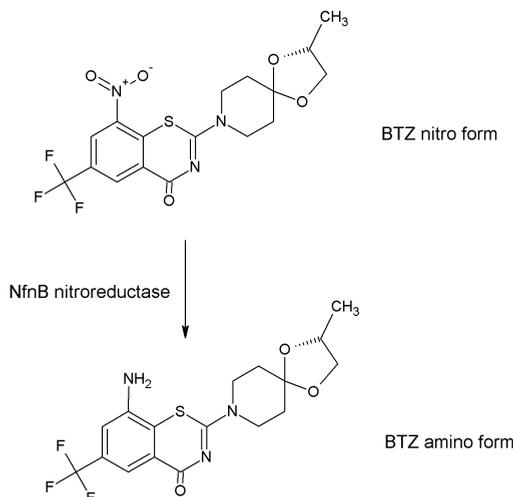


Fig. 3. Nitroreduction of BTZ nitro form in amino form by NfnB nitroreductase.

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 $\Delta nfnB$ 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 (Opera™) over 7 days (Christophe et al., 2009).

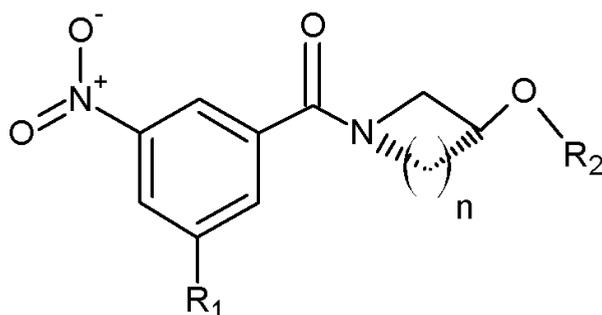


Fig. 4. **Chemical structure of dinitrobenzamide compounds.** R_1 can be either a nitro ($-\text{NO}_2$), an amino ($-\text{NH}_2$), a hydroxylamine ($-\text{NHOH}$) or a hydrogen ($-\text{H}$) group, whereas R_2 stands for different aromatic substituents.

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 (Araf) 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 Araf residues to the galactan domain, while lipoarabinogalactan originates from the addition of Araf 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 Araf 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).

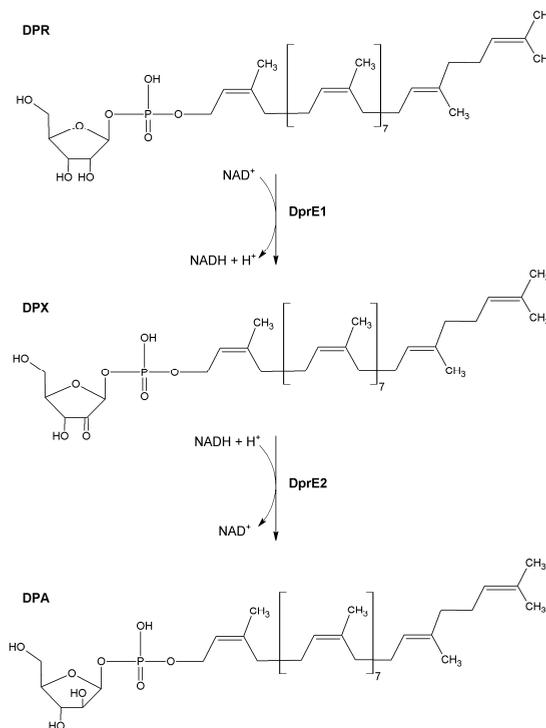


Fig. 5. The enzymatic reaction catalyzed by decaprenylphosphoryl- β -*D*-ribose 2' epimerase (composed by DprE1 and DprE2) is reported. DPR indicates decaprenylphosphoryl-*D*-ribose, while DPX is the intermediate decaprenylphospho-2-ketofuranose and DPA the decaprenylphosphoryl-*D*-arabinose.

The epimerization reaction is reported in Figure 5. It is catalyzed by the decaprenylphospho-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 (Sasseti 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 Araf 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 Araf 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 antimycobacterial 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.

6. Acknowledgment

We thank the European Commission for support (VI Framework, contract N. LSHP-CT 2005- 018923 and VII Framework, contract N. 260872).

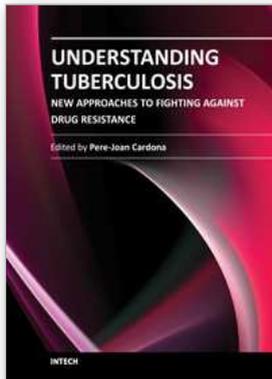
7. References

- Alderwick, L. J.; Birch, H. L.; Mishra, A. K.; Eggeling, L. & Besra, G. S. (2007). Structure, function and biosynthesis of the *Mycobacterium tuberculosis* cell wall: arabinogalactan and lipoarabinomannan assembly with a view to discovering new drug targets. *Biochemical Society Transactions*, Vol. 35, No. Pt 5, (November 2007), pp. 1325-1328, ISSN 0300-5127.
- Andersson, D. I. (2006). The biological cost of mutational antibiotic resistance: any practical conclusions? *Current opinion in microbiology*, Vol. 9, no. 5, (October 2006), pp. 461-465, ISSN 1369-5274.
- Ayers, B.; Long, H.; Sim, E.; Smellie, I. A.; Wilkinson, B. L. & Fairbanks, A. J. Stereoselective synthesis of beta-arabino glycosyl sulfones as potential inhibitors of mycobacterial cell wall biosynthesis. *Carbohydrate Research*, Vol. 344, No. 6, (April 2009), pp. 739-746, ISSN 0008-6215.
- Barry, C. E.; Crick, D. C. & McNeil, M. R. (2007). Targeting the formation of the cell wall core of *Mycobacterium tuberculosis*. *Infectious Disorders Drug Targets*, Vo. 7, No. 2, (June 2007), pp. 182-202, ISSN 1871-5265.

- Böttger, E. C. & Springer, B. (2008). Tuberculosis: drug resistance, fitness, and strategies for global control. *European Journal of Pediatrics*, Vol. 167, No. 2, (February 2008), pp. 141-148, ISSN 0340-6199.
- Caminero, J. A.; Sotgiu, G.; Zumla, A. & Migliori, G. B. (2010). Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *The Lancet infectious diseases*, Vol. 10, No 9, (September 2010), pp. 621-629, ISSN 1473-3099.
- Centrone, C. A. & Lowary, T.L. (2004). Sulfone and phosphinic acid analogs of decaprenolphosphoarabinose as potential anti-tuberculosis agents. *Bioorganic & Medicinal Chemistry*, Vol. 12, No. 21, (November 2004), pp. 5495-5503, ISSN 0968-0896.
- Chim, N.; Habel, J. E.; Johnston, J. M.; Krieger, I.; Miallau, L.; Sankaranarayanan, R.; Morse, R. P.; Bruning, J.; Swanson, S.; Kim, H.; Kim, C. Y.; Li, H.; Bulloch, E. M.; Payne, R. J.; Manos-Turvey, A.; Hung, L. W.; Baker, E. N.; Lott, J. S.; James, M. N.; Terwilliger, T. C.; Eisenberg, D. S.; Sacchettini, J. C. & Goulding, C. W. (2011). The TB Structural Genomics Consortium: a decade of progress. *Tuberculosis (Edinburg, Scotland)*, Vol. 91, No. 2, (March 2011), pp. 155-172, ISSN 1472-9792.
- Christophe, T.; Jackson, M.; Jeon, H. K.; Fenistein, D.; Contreras-Dominguez, M.; Kim, J.; Genovesio, A.; Carralot, J. P.; Ewann, F.; Kim, E. H.; Lee, S. Y.; Kang, S.; Seo, M. J.; Park, E. J.; Skovierová, H.; Pham, H.; Riccardi, G.; Nam, J. Y.; Marsollier, L.; Kempf, M.; Joly-Guillou, M. L.; Oh, T.; Shin, W. K.; No, Z.; Nehrbass, U.; Brosch, R.; Cole, S. T. & Brodin, P. (2009) High content screening identifies decaprenyl-phosphoribose 2' epimerase as a target for intracellular antimycobacterial inhibitors. *PLoS Pathogens*, Vol. 5, No. 10, (October 2009), p. e1000645, ISSN 1553-7366.
- Cole, S. T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S. V.; Eiglmeier, K.; Gas, S.; Barry, C. E. 3rd; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M. A.; Rajandream, M. A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J. E.; Taylor, K.; Whitehead, S. & Barrell, B. G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, Vol. 393, No. 6685, (June 1998), pp. 537-544, ISSN 0028-0836.
- Crellin, P. K.; Brammananth, R. & Coppel R. L. (2011). Decaprenylphosphoryl-*b*-D-ribose 2'-epimerase, the target of benzothiazinones and dinitrobenzamides, is an essential enzyme in *Mycobacterium smegmatis*. *PLoS ONE*, Vol. 6, No. 2, (February 2011), p. e16869, ISSN 1932-6203.
- De Rossi, E.; Ainsa, J. A. & Riccardi, G. (2006). Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiology Review*, Vol. 30, No. 1, (January 2006), pp. 36-52, ISSN 0168-6445.
- Fenistein, D.; Lenseigne, B., Christophe, T.; Brodin, P. & Genovesio A. (2008). A fast, fully automated cell segmentation algorithm for high-throughput and high-content screening. *Cytometry Part A: the journal of the International Society for Analytical Cytology*, Vol. 73, No. 10, (October 2008), pp. 958-964, ISSN 1552-4922.
- Fischbach, M. A. & Walsh, C. T. (2009). Antibiotics for emerging pathogens. *Science*, Vol. 325, No. 5944, (August 2009), pp. 1089-1093, ISSN 0036-8075.
- Gillespie, SH. (2007). Tuberculosis: evolution in millennia and minutes. *Biochemical Society Transactions*, Vol. 35, No. Pt 5, (November 2007), pp. 1317-1320, ISSN 0300-5127.
- Goldman, R. C.; Plumley, K. V. & Laughon, B. E. (2007). The evolution of extensively drug resistant tuberculosis (XDR-TB): history, status and issues for global control. *Infectious Disorders Drug Targets*, Vol. 7, No. 2, (June 2007), pp. 73-91, ISSN 1871-5265.

- Jarlier, V. & Nikaido, H. (1994). Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiology Letters*, Vol. 123, No. 1-2, (October 1994), pp. 11-18, ISSN 0378-1097.
- Lamichhane, G.; Tyagi, S. & Bishai, W. R. (2005). Designer arrays for defined mutant analysis to detect genes essential for survival of *Mycobacterium tuberculosis* in mouse lungs. *Infection and Immunity*, Vol. 73, No. 4, (April 2005), pp. 2533-2540, ISSN 0019-9567.
- Lenaerts, A. J.; Degroote, M. A. & Orme, I. M. (2008). Preclinical testing of new drugs for tuberculosis: current challenges. *Trends in Microbiology*, Vol. 16, No. 2, (February 2008), pp. 48-54, ISSN 0966-842X.
- Lienhardt, C.; Vernon, A. & Raviglione, M. C. (2010). New drugs and new regimens for the treatment of tuberculosis: review of the drug development pipeline and implications for national programmes. *Current Opinion in Pulmonary Medicine*, Vol. 16, No. 3, (May 2010), pp. 186-193, ISSN 1070-5287.
- Magnet, S.; Hartkoorn, R. C.; Székely, R.; Pató, J.; Triccas, J.A.; Schneider, P.; Szántai-Kis, C.; Orfi, L.; Chambon, M.; Banfi, D.; Bueno, M.; Turcatti, G.; Kéri, G. & Cole ST. Leads for antitubercular compounds from kinase inhibitor library screens. *Tuberculosis (Edinburgh, Scotland)*, Vol. 90, No. 6, (November 2010), pp. 354-360, ISSN1472-9792.
- Makarov, V.; Manina, G.; Mikušová, K.; Möllmann, U.; Ryabova, O.; Saint-Joanis, B.; Dhar, N.; Pasca, M. R.; Buroni, S.; Lucarelli, A. P.; Milano, A.; De Rossi, E.; Belanova, M.; Bobovska, A.; Dianišková, P.; Kordulakova, J.; Sala, C.; Fullam, E.; Schneider, P.; McKinney, J. D.; Brodin, P.; Christophe, T.; Waddell, S.; Butcher, P.; Albrethsen, J.; Rosenkrands, I.; Brosch, R.; Nandi, V.; Bharath, S.; Gaonkar, S.; Shandil, R. K.; Balasubramanian, V.; Balganes, T.; Tyagi, S.; Grosset, J.; Riccardi, G. & Cole, S. T. (2009). Benzothiazinones kill *Mycobacterium tuberculosis* by blocking arabinan synthesis. *Science*, Vol. 324, No. 5928, (May 2009), pp. 801-804, ISSN 0193-4511.
- Makarov, V.; Riabova, O. B.; Yuschenko, A.; Urlyapova, N.; Daudova, A.; Zipfel, P. F. & Möllmann, U. (2006). Synthesis and antileprosy activity of some dialkylidithiocarbamates. *The Journal of antimicrobial chemotherapy*, Vol. 57, no. 6 (June 2006), pp. 1134-1138, ISSN 0305-7453.
- Manina, G.; Bellinzoni, M.; Pasca, M. R.; Neres, J.; Milano, A.; Ribeiro, A. L.; Buroni, S.; Skovierová, H.; Dianišková, P.; Mikušová, K.; Marák, J.; Makarov, V.; Giganti, D.; Haouz, A.; Lucarelli, A. P.; Degiacomi, G.; Piazza, A.; Chiarelli, L. R.; De Rossi, E.; Salina, E.; Cole, S. T.; Alzari, P. M. & Riccardi, G. (2010a) Biological and structural characterization of the *Mycobacterium smegmatis* nitroreductase NfnB, and its role in benzothiazinone resistance. *Molecular Microbiology*, Vol. 77, No. 5, (September 2010), pp. 1172-1185, ISSN 0950-382X.
- Manina, G.; Pasca, M. R.; Buroni, S.; De Rossi, E. & Riccardi, G. (2010b). Decaprenylphosphoryl- β -D-ribose 2'-epimerase from *Mycobacterium tuberculosis* is a magic drug target. *Current Medicinal Chemistry*, Vol. 17, No. 27, (2010), pp. 3099-3108, ISSN 0929-8673.
- Meniche, X.; de Sousa-d'Auria, C.; Van-der-Rest, B.; Bhamidi, S.; Huc, E.; Huang, H.; De Paepe, D.; Tropis, M.; McNeil, M.; Daffé, M. & Houssin, C. (2008). Partial redundancy in the synthesis of the D-arabinose incorporated in the cell wall arabinan of *Corynebacterineae*. *Microbiology*, Vol. 154, No. Pt 8, (August 2008), pp. 2315-2326, ISSN 1350-0872.
- Mikusova, K.; Huang, H.; Yagi, T.; Holsters, M.; Vereecke, D.; D'Haese, W.; Scherman, M. S.; Brennan, P. J.; McNeil, M. R. & Crick, D.C. (2005). Decaprenylphosphoryl arabinofuranose, the donor of the D-arabinofuranosyl residues of mycobacterial

- arabinan, is formed via a two-step epimerization of decaprenylphosphoryl ribose. *Journal of Bacteriology*, Vol. 187, No. 23, (December 2005), pp. 8020-8025, ISSN 0021-9193.
- Pasca, M. R.; Degiacomi, G.; Ribeiro, A. L.; Zara, F.; De Mori, P.; Heym, B.; Mirrione, M.; Berra, R.; Pagani, L.; Pucillo, L.; Troupioti, P.; Makarov, V.; Cole, S. T. & Riccardi, G. (2010). Clinical isolates of *Mycobacterium tuberculosis* in four European hospitals are uniformly susceptible to benzothiazinones. *Antimicrobial Agents and Chemotherapy*, Vol. 54, No. 4, (April 2010), pp. 1616-1618, ISSN 0066-4804.
- Pathak, A. K.; Pathak, V.; Suling, W. J.; Riordan, J. R.; Gurcha, S. S.; Besra, G. S. & Reynolds, R. C. Synthesis of deoxygenated alpha(1→5)-linked arabinofuranose disaccharides as substrates and inhibitors of arabinosyltransferases of *Mycobacterium tuberculosis*. *Bioorganic & Medicinal Chemistry*, Vol. 17, No. 2, (January 2009), pp. 872-881, ISSN 0968-0896.
- Payne, D. J.; Gwynn, M. N.; Holmes, D. J. & Pompliano, D. L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews. Drug Discovery*, Vol. 6, No. 1, (January 2007), pp. 29-40, ISSN 1474-1776.
- Riccardi, G.; Pasca, M. R. & Buroni, S. (2009). *Mycobacterium tuberculosis*: drug resistance and future perspectives. *Future Microbiology*, Vol. 4, No. 5, (June 2009), pp. 597-614, ISSN 1746-0913.
- Roldán, M. D.; Pérez-Reinado, E.; Castillo, F. & Moreno-Vivián, C. (2008). Reduction of polynitroaromatic compounds: the bacterial nitroreductases. *FEMS Microbiology Reviews*, Vol. 32, no. 3, (May 2008), pp. 474-500, ISSN 0168-6445.
- Sassetti, C. M., Boyd, D. H. & Rubin E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Molecular Microbiology*, Vol. 48, No. 1, (April 2003), pp. 77-84, ISSN 0950-382X.
- Sassetti, C. M. & Rubin E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proceedings of the National Academy of Science of the United States of America*, Vol. 100, No. 22, (October 2003), pp. 12989-12994, ISSN 0027-8424.
- Trefzer, C.; Rengifo-Gonzalez, M.; Hinner, M. J.; Schneider, P.; Makarov, V.; Cole, S. T. & Johnsson, K. (2010). Benzothiazinones: prodrugs that covalently modify the decaprenylphosphoryl-β-D-ribose 2'-epimerase DprE1 of *Mycobacterium tuberculosis*. *Journal of the American Chemical Society*, Vol. 132, no. 39, (October 2010), pp. 13663-13665, ISSN 0002-7863.
- Umesiri, F. E.; Sanki, A. K.; Boucau, J.; Ronning, D. R. & Sucheck, S. J. (2010). Recent advances toward the inhibition of mAG and LAM synthesis in *Mycobacterium tuberculosis*. *Medicinal Research Review*, Vol. 30, No. 2, (March 2010), pp. 290-326, ISSN 0198-6325.
- Velayati, A. A.; Masjedi, M. R.; Farnia, P.; Tabarsi, P.; Ghanavi, J.; Ziazarifi, A. H. & Hoffner, S. E. (2009). Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest*. Vol. 136, No 2 (April 2009), pp. 420-425, ISSN 0012-3692.
- Williams, K.J & Duncan, K. (2007). Current strategies for identifying and validating targets for new treatment-shortening drugs for TB. *Current. Molecular Medicine*, Vol. 7, No. 3, (May 2007), pp. 297-307, ISSN 1566-5240.
- Wolucka, B. A. (2008). Biosynthesis of D-arabinose in mycobacteria - a novel bacterial pathway with implications for antimycobacterial therapy. *The FEBS Journal*, Vol. 275, No. 11, (June 2008), pp. 2691-2711, ISSN 1742-464X.



Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-948-6

Hard cover, 376 pages

Publisher InTech

Published online 15, February, 2012

Published in print edition February, 2012

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

How to reference

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

Silvia Buroni, Giovanna Riccardi and Maria Rosalia Pasca (2012). Fighting Against Resistant Strains: The Case of Benzothiazinones and Dinitrobenzamides, *Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance*, Dr. Pere-Joan Cardona (Ed.), ISBN: 978-953-307-948-6, InTech, Available from: [http://www.intechopen.com/books/understanding-tuberculosis-new-approaches-to-fighting-against-drug-resistance/fighting-against-resistant-strains-the-case-of-benzothiazinones-and-dinitrobenzamides-](http://www.intechopen.com/books/understanding-tuberculosis-new-approaches-to-fighting-against-drug-resistance/fighting-against-resistant-strains-the-case-of-benzothiazinones-and-dinitrobenzamides)

INTECH

open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.