1. Final Publishable Summary Report

1.1 Executive Summary

Despite the availability of highly efficacious treatment for decades, tuberculosis (TB) remains a major global health problem. TB is caused by the bacillus *Mycobacterium tuberculosis* (*Mtb*) and is the **second leading cause of death** from an infectious disease worldwide. It is easily spread in the air when infected people expel bacteria and is characterized by an extremely long incubation time. Individuals carrying dormant pathogens are **latently infected**, but can still develop full-blown TB once the host defence is weakened. Consequently, the increased probability of developing TB among people infected with HIV has led to the **deadly liaison of HIV/AIDS with TB**. TB affects mostly adults in the economically productive age groups and thus, has a **devastatingly disrupting effect on the societal and economical framework of above all the developing countries**. The situation is also worsening in parts of the European Union because of the increasing incidences of multidrug-resistant (**MDR**) and extensively drug-resistant (**XDR**) *Mtb* strains. Without treatment, TB mortality rates are high. Even though treatment using combinations of the available anti-TB drugs can dramatically reduce mortality rates, the prevalence of MDR- and XDR-TB urgently calls for **novel anti-TB drugs**. Additionally, novel drugs should also focus on killing of dormant bacteria as they can **shorten treatment time**. Continuous flow from dormant to active state *Mtb* could explain the need for prolonged TB drug treatment for up to 4-6 months.

The NATT collaborative project reflects the awareness of the need for new drugs to treat TB and brought together experts of different disciplines from Europe and India. The applied approach that led to the identification of **new lead compounds** for anti-TB drug development was twofold. First, considerable efforts were dedicated to the **discovery and biochemical characterisation of novel *Mtb* protein targets**. The systematic biochemical analysis and structural elucidation of mycobacterial **FACL13** and **FAAL28** proteins revealed the determinants of substrate specificity. Also, the existence of a third **novel ACCase complex** in *Mtb* has been determined and its structure elucidated. Further, a better insight into the nucleotide excision repair (NER) system of *Mtb* has been provided. It was found that the heterodimeric helicase-nuclease **AdnAB**, encoded by the UvrD homologues ***Rv3201c*** and ***Rv3202c***, plays a functional role in recombination and in the maintenance of genomic integrity. Finally, the role of **ThyX**, a mycobacterial flavin dependent thymidylate synthase, in TB pathogenesis was unravelled. Analyzing the expression levels of both *thyA* and *thyX* under different conditions indicated that both *Mtb* ThyA and ThyX may play an important role *in vivo*. Additionally, observations made with *ΔthyA* strains suggest that ThyX has another function next to its thymidylate synthase activity. Within this joint project, several inhibitors targeting these newly discovered proteins were identified. These efforts have revealed that these **novel targets are druggable** and thus, might yield a new and powerful armoury against MDR- and XDR-TB strains. More so because inhibitors acting on these targets employ novel mechanisms not utilized by currently available drugs. By systematically modifying different parts of the TMC-207 molecule, an **extensive Structure Activity Relationship (SAR) for DARQs** has been generated. This led to 5 lead compounds that show good inhibitory activity *in vitro* (MIC_{99} = 0.03-0.5 µg/mL) and cause 99% growth inhibition of three types of drug resistant MDR-TB strains at 3.125 µg/mL. The second approach of the NATT project focussed on restoring intracellular killing of *Mtb*. To assess this, a fully automated **multidisciplinary screening platform has been established** for a bacterial cell survival assay in primary human macrophages. Via multi-parametric image analysis, compounds were identified that release the phagosome maturation arrest. This multi-parametric assay will largely support drug discovery as well as other research areas in cell biology and might particularly facilitate the identification of toxic compounds at a very early stage. Further, it is expected that this approach will help to identify potential novel drug targets. Concluding, the NATT discoveries have provided valuable information for the development of novel **antibiotics against TB and other infectious diseases**. Additional information about the NATT consortium and the participating partners can be found on the **NATT website**: http://www.natt-tbc.com.

1.2 Summary Description of Project Context and Objectives

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is an ancient disease that has taken a deadly new turn with the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains along with the dangerous liaison with the human immunodeficiency virus (HIV). **One third of the world's population is currently infected with dormant *Mtb* germs**. 50% of MDR-TB cases worldwide are estimated to occur in China and India and, in several countries of the world, including eastern parts of Europe, high proportions (e.g. 28% in north western Russia) of MDR strains have been noted. More recently, the increasing emergence of XDR-TB has become a significant point of concern in the combat against TB. As of June 2008, 49 countries have confirmed cases of XDR-TB. By 2010, that number had risen to 58 (WHO, March 2010). XDR-TB is virtually untreatable, especially in patients with a compromised immune system and in patients living in developing countries where an accurate diagnosis and access to all six classes of second-line drugs might be problematic.

Non multidrug-resistant TB can be treated by chemotherapy. Normally, combinations of three to four first-line drugs are given over a period of 4 to 6 months. The complicated and long-lasting treatment regimen, together with numerous side effects of the anti-TB drugs, leads to a high proportion of non-compliance patients. This, and other reasons (prescribing the wrong treatment, drugs are of poor quality), has contributed to the **increasing emergence of MDR- and XDR-TB strains**.

Tuberculosis is characterized by an extremely long incubation time and only ca. 10% of infected individuals develop disease. However, the host fails to achieve sterile eradication and therefore the vast majority of *Mtb*-infected individuals carry dormant pathogens, i.e., they are latently infected. In the latently infected host, *Mtb* can persist in a **metabolically reduced stage of dormancy (latent infection)**, where it rarely replicates if at all and does not cause active TB. The mycobacteria do so by arresting phagosome maturation. Hence, *Mtb* resides in the early phagosome with neutral pH and access to iron and other essential molecules. Although the mechanisms by which *Mtb* interferes with phagosome maturation are incompletely understood, it has been shown that they actively interfere with the host cell. These persistent, dormant pathogens can however develop active TB once the host defence is weakened. During the standard drug therapy of patients with active TB, metabolically active bacteria are eliminated by chemotherapy and consequently dormant bacteria are resuscitated to become metabolically active. Continuous flow from dormant to active state could explain the need of prolonged TB drug treatment for up to 4-6 months.

The increasing incidences of MDR- and XDR-TB urgently demand **novel drugs** for curing tuberculosis. Additionally, novel drug treatment schemes should also focus on killing of dormant bacteria because they most likely use novel drug targets and secondly, can **shorten treatment time**. However, no new TB drugs have been brought to the market in the last 30 years. Owing to the low commercial interest in treating TB, it is clearly a disease that should be tackled using financial support from government, European Commission, WHO and philanthropic organizations. The Global Alliance for TB Drug Development is a driving force in the development of drugs against TB; however, their work primarily involves known targets.

The NATT collaborative project aimed to identify and validate novel targets with a special emphasis on the most critical aspects of TB: dormancy and MDR/XDR-TB. Our ultimate goal was to develop lead compounds, which will 1) act on **novel mechanisms** not utilized by currently available drugs and therefore are powerful against **MDR-** and **XDR-TB** strains and/or 2) act on **dormant *Mtb*** with low metabolic and replicative activity for treatment of latent TB. To achieve this ambitious goal a

competitive consortium was formed with leading scientists providing expert know-how and insight in chemistry, microbiology, molecular genetics, protein structure, cell biology and infection models. This truly multidisciplinary joint venture with the active contribution of **3 Indian partners** has directed substantial efforts to building an efficient pipeline from bench chemistry to *in vivo* evaluation.

The novel targets selected by the consortium make part of the most vulnerable cell processes that have proven their susceptibility to antimycobacterial drugs and antibiotics in general. Targeting processes involved in **cell wall and membrane synthesis** as well as **DNA replication and maintenance** have proven its merits in the development of antibiotics and are still successfully pursued in current (anti-TB) drug development campaigns. Within this context, also **cellular processes involved in the energy household** are considered as a target resource potentially exposed to potent *Mtb* growth inhibitors. Intervention at the level of pathogen-host cell interaction is a possible alternative to the classical strategy based on development of new "antibiotics" that block the pathogen replication cycle. In particular, in this proposal a major objective was to experimentally test the possibility that **chemical re-activation of the phagosome maturation machinery** may restore the intracellular killing of phagocytosed Mycobacteria. Within the consortium, *in vitro*, *ex vivo* and *in vivo* screening assays were set up to determine the tuberculostatic and/or tuberculocidal activities of novel compounds.

Targeting the mycobacterial cell membrane

Mycolic acids, prominent molecules of the robust cell wall of *Mtb*, are essential for the mycobacterium's survival, virulence and antibiotic resistance. To produce these complex lipids, mycobacteria exploit an assembly-line enzymatic machinery, wherein two fatty acid synthases (FAS I and FAS II) generate *n*-long chain fatty acid precursors, which are further extended by polyketide synthases (Pks) imparting complexity and structural diversity. The commonly known mechanism of *n*-fatty acid activation involves conversion of fatty acids into their corresponding CoA-derivatives (catalyzed by acyl-CoA synthetases (ACSs), also referred to as FadD proteins). Sequencing of the mycobacterial genome revealed 34 FadD homologues, some displaying a new enzymatic activity. These proteins, **fatty acyl-AMP ligases (FAAL)**, convert fatty acids into the corresponding acyl-adenylates. Since FAAL proteins feed into Pks to produce a number of crucial lipids, small molecules inhibitor of these proteins may have **tremendous therapeutic potential**. The main objectives relating to this part of the research can be summarized as follows:

1. Expression and purification of FAAL and Pks proteins and the establishment of *in vitro* assays
2. Determination of the three-dimensional structure of FAAL proteins and Pks proteins
3. Study of the active binding site in order to design putative inhibitors
4. Screening of FAAL and Pks inhibitors

Acyl-CoA carboxylase (ACCase) catalyzes the carboxylation of acyl-CoA substrates, the first committed step that provides the building blocks for the *de novo* fatty acid biosynthesis (by FAS I, FAS II and Pks). Sequence analysis of the *Mtb* genome reveals a total of six accD genes (*accD1–6*) and three accA genes (*accA1–3*). Comparative genomic analyses and overexpression as well as genetic deletion studies have indicated that the β-subunits AccD4 and AccD5 are the most likely candidates to provide extender units for cell envelope lipids and have identified these subunits as essential proteins for mycobacterial survival. We hypothesize that up-regulation of AccD5 under conditions that are eventually lethal for *Mtb* could be part of a self-protection mechanism to a hostile, phagolysosomal environment. This assumption is further supported by the failure to isolate any *accD5 Mtb* knock-out mutants, indicating that AccD5 activity is essential to *Mtb* survival. Consequently, ACCase components potentially play a role

in **latent TB** and are attractive targets to tackle **TB persistence**. The main objectives of this part can be summarized as follows:

1. Structural biology on acyl-CoA carboxylase
2. Functional and comparative studies on acyl-CoA carboxylase
3. Co-crystallization experiments for optimization/design of inhibitors

Targeting the mycobacterial genome

Thymidylate synthase catalyzes the synthesis of 2'-deoxythymidine-5'-monophosphate (dTMP) from 2'-deoxyuridine-5'-monophosphate (dUMP). Until recently, the only known pathway for *de novo* synthesis of thymidylate uses thymidylate synthase ThyA, a widely used target for inhibiting cellular proliferation. Recent genomic analysis has demonstrated that *Mtb* possesses genes for both ThyA as for a novel **thymidylate synthase**, called **ThyX**. This enzyme uses methylenetetrahydrofolate only as a one-carbon donor, whereas flavin adenine dinucleotide (FAD) fulfils the role of reductant. ThyX is present in a number of human pathogenic bacteria, absent in the human genome and shows no sequence similarity with ThyA. These features make ThyX a very attractive target to inhibit *Mtb* growth. Although already indicated by transposon site hybridization (TraSH) experiments, this project will study whether *thyX* is essential for optimal growth of the pathogen. Furthermore, *Mtb* might preferentially use either ThyA or ThyX under different growth conditions (i.e. activation from dormant state). Therefore, initiating a MedChem campaign in order to discover ThyX inhibitors is an important part of this joint project:

1. Rational design of *Mtb* ThyX inhibitors based on molecular modeling
2. Synthesis of the designed inhibitors (SAR)
3. Evaluation of the compounds *in vitro* and *in vivo* against *Mtb*

In *E. coli*, UvrD is involved in multiple DNA repair pathways: nucleotide excision repair, mismatch repair, recombination repair and replication restart. Interestingly, *Mtb* has four genes encoding proteins containing a domain homologous to UvrD: ***uvrD1*** (*Rv0949*), ***uvrD2*** (*Rv3198c*), ***Rv3201c*** and ***Rv3202c***. The expression of all four of these genes is increased following DNA damage and *uvrD1* and Rv3201c have been reported to be expressed at elevated levels during human infection, whilst Rv3202c is induced in macrophages. The four genes are not functionally redundant, but rather have specific roles, potentially determined by protein interaction partners. Furthermore, the *uvrD1* mutant appears to have a persistence defect in a mouse infection model, suggesting that UvrD1 might represent a novel target for the development of drugs active against **persistent or latent bacteria**. *Mtb* UvrD1 has been expressed in *E. coli* and purified, and its helicase activity characterised. Using a fluorescent helicase assay, a small library of compounds has been screened for inhibitors of UvrD1, yielding a number of compounds from four different chemical groups. These compounds thus provide a starting point for further development of inhibitors of the *Mtb* UvrD family helicases:

1. Synthesis and enzymatic screening of UvrD1 inhibitors
2. testing of compounds on whole bacteria (H37Rv and resistant clinical isolates)
3. Expression, purification and biochemical characterisation of Rv3201c and Rv3202c.
4. Construction of *Mtb* strains in which Rv3201c and Rv3202c are inactivated, and evaluation of their phenotypes

Targeting the mycobacterial energy pathway

Like all other organisms, mycobacteria utilize F_1F_0-ATP synthase to generate adenosine triphosphate (ATP), the biological currency of energy. **F_1F_0-ATP synthase** is a biological rotary motor made up of two major structural domains, F_0 and F_1, and uses the proton-motive force moving down an electrochemical gradient in order to generate ATP from ADP and phosphate. The F_0 domain contains three different subunits with (a, b_2, $c_{10\text{-}12}$) stoichiometry, spans the cytoplasmic membrane and is the proton channel of the complex, coupling the proton translocation with the actual ATP synthesis in F_1. The F_1 domain consists of five different subunits with (α_3, β_3, γ, δ, ε) stoichiometry and is located in the cytoplasm, where it generates and sets free ATP. Although found in most living organisms, including humans, there is very little sequence similarity between the mycobacterial and human b-subunit and c-subunit of the F_0 domain of F_1F_0-ATP synthase. This observation provides a solid basis to consider this mycobacterial enzyme as a potential therapeutic target.

Targeting the **b-subunit of the ATP synthase F_0 domain** is till date poorly explored. Based on protein expression, it has been stated that the observed decrease in ATP synthesis in OSA (*n*-octanesulphonylacetamide) or DCCD (dicyclohexylcarbodiimide) treated mycobacterial cultures may be due to direct or indirect interaction of both compounds with the b-subunit. Therefore, this subunit could be considered as a promising new target. The **c-subunit of the F_0 domain** is a well-validated therapeutic target. In fact, diarylquinolines (DARQs) interact with the c-subunit and are a well-established class of potent inhibitors of *Mtb* F_1F_0-ATP synthase (e.g. TMC207). These observations will direct this project in the design and synthesis of inhibitors targeting the b- or the c-subunit; the objectives are:

1. Design and synthesis of novel ATP synthase b- and c-subunit inhibitors
2. Screening of new chemical entities (NCE)
3. Evaluation of biological data and establish the Structure Activity Relationship (SAR)

Intervention at the level of the pathogen-host cell interactions

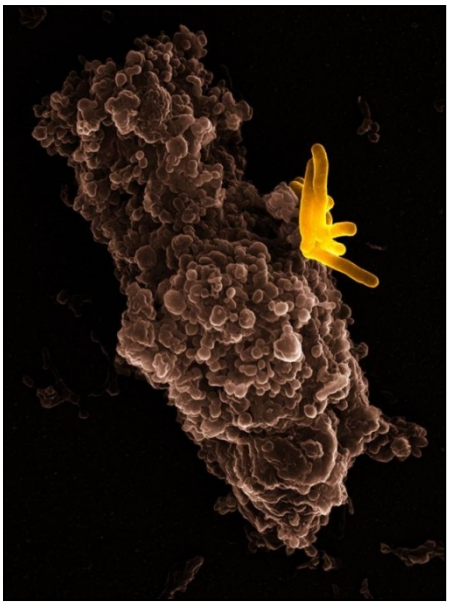
After infecting the host, pathogens are normally ingested by macrophages in a process called phagocytosis. During this process, phagosomes containing the pathogen progressively fuse with endosomes and lysosomes. As a result, the phagosomes mature into acidified degradative phagolysosomes by the gradual acquisition of lysosomal enzymes. However, *Mtb* can survive and multiply within the hostile intracellular environment of macrophages. In fact, engulfed *Mtb* is able to **block the phagosome maturation process** by preventing the formation of phagolysosomes. The phagosome maturation arrest is induced by mycobacterial products, which are capable of modulating intracellular trafficking and inhibiting the fusion with late endosomes and lysosomes. **Latent TB** and the development of persistent infections are tightly linked to the survival of *Mtb* in host macrophages.

The consortium will set up and use a robust quantitative multi-parametric image analysis system to launch a screening campaign in order to target at the same time both the **bacterial machinery and the cellular host** defence armamentarium. The goal is to identify chemical compounds that can fully restore the endocytic transport and killing of intracellular *Mtb*. Targeting the host cell machinery will allow rapid clearance of the pathogen, avoid the development of multi-drug resistant strains and this system can be applied to combat various types of viral and bacterial pathogens. The key objectives of this research part of the NATT project are:

1. Select small molecules that modulate phagosome maturation
2. Evaluate if chemical reactivation of phagosome maturation kills the intracellular mycobacteria

1.3. Description of the main S&T results/foregrounds

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is an ancient disease that has taken a deadly new turn with the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains along with the dangerous liaison with the human immunodeficiency virus (HIV). **One third of the world's population is currently infected with dormant *Mtb* germs**. 50% of MDR-TB cases worldwide are estimated to occur in China and India and, in several countries of the world, including eastern parts of Europe, high proportions (e.g. 28% in north western Russia) of MDR strains have been noted. More recently, the increasing emergence of XDR-TB has become a significant point of concern in the combat against TB. As of June 2008, 49 countries have confirmed cases of XDR-TB. By 2010, that number had risen to 58 (WHO, March 2010). XDR-TB is virtually untreatable, especially in patients with a compromised immune system and in patients living in developing countries where an accurate diagnosis and access to all six classes of second-line drugs might be problematic.



Non multidrug-resistant TB can be treated by chemotherapy. Normally, combinations of three to four first-line drugs are given over a period of 4 to 6 months. The complicated and long-lasting treatment regimen, together with numerous side effects of the anti-TB drugs, leads to a high proportion of non-compliance patients. This, and other reasons (prescribing the wrong treatment, drugs are of poor quality), has contributed to the **increasing emergence of MDR- and XDR-TB strains**.

Tuberculosis is characterized by an extremely long incubation time and only ca. 10% of infected individuals develop disease. However, the host fails to achieve sterile eradication and therefore the vast majority of *Mtb*-infected individuals carry dormant pathogens, i.e., they are latently infected. In the latently infected host, *Mtb* can persist in a **metabolically reduced stage of dormancy (latent infection)**, where it rarely replicates if at all and does not cause active TB. The mycobacteria do so by arresting phagosome maturation. Hence, *Mtb* resides in the early phagosome with neutral pH and access to iron and other essential molecules. Although the mechanisms by which *Mtb* interferes with phagosome maturation are incompletely understood, it has been shown that they actively interfere with the host cell. These persistent, dormant pathogens can however develop active TB once the host defence is weakened. During the standard drug therapy of patients with active TB, metabolically active bacteria are eliminated by chemotherapy and consequently dormant bacteria are resuscitated to become metabolically active. Continuous flow from dormant to active state could explain the need of prolonged TB drug treatment for up to 4-6 months.

The increasing incidences of MDR- and XDR-TB urgently demand **novel drugs** for curing TB. This is of importance as *Mtb* kills approximately two million people annually (WHO, 2009) and no novel anti-tubercular compound has been introduced since rifampcin in 1963. The MDR and XDR-TB associated mortality rates and the high cost of second line antibiotics heighten the urgent demand for compounds with **novel modes of action** to treat tuberculosis. Additionally, novel drug treatment schemes should also focus on killing of dormant bacteria because they most likely use novel drug targets and secondly, can **shorten the complicated treatment regimen**, a driving force for the emergence of drug-resistant tuberculosis.

Owing to the low commercial interest in treating TB, this is clearly a disease that should be tackled using financial support from government, European Commission, WHO and philanthropic organizations. The Global Alliance for TB Drug Development is a driving force in the development of drugs against TB; however, their work primarily involves known targets.

The NATT collaborative project aimed to identify and validate novel targets with a special emphasis on the most critical aspects of TB: dormancy and MDR/XDR-TB. Our ultimate goal was to identify compounds, which will 1) act on **novel mechanisms** not utilized by currently available drugs, and therefore are powerful against **MDR-** and **XDR-TB** strains and/or 2) act on **dormant *Mtb*** with low metabolic and replicative activity for treatment of latent TB. To achieve this ambitious goal a competitive consortium was formed with leading scientists providing expert know-how and insight in chemistry, microbiology, molecular genetics, protein structure, cell biology and infection models:

- **Prof. Dr. Piet Herdewyn**, Laboratory of Medicinal Chemistry (LMC), University of Leuven, Belgium (Partner 1, coordinator of the NATT project)
- **Prof. Dr. Stefan Kaufmann**, Max Planck Institute for Infection Biology (MPI-IB), Berlin, Germany (Partner 2a)
- **Prof. Dr. Marino Zerial**, Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany (Partner 2b)
- **Prof. Dr. Jyoti Chattopadhyaya**, Bioorganic Chemistry Department (BOC), University of Uppsala, Sweden (Partner 3)
- **Dr. Elaine Davis**, National Institute for Medical Research (NIMR), Medical Research Council (MRC), London, United Kingdom (Partner 4)
- **Dr. Matthias Wilmanns**, European Molecular Biological Laboratory (EMBL), Hamburg, Germany (Partner 5)
- **Dr. Rajesh S. Gokhale**, National Institute of Immunology (NII), New Delhi, India (Partner 6)
- **Prof. Dr. Prati Pal Singh**, National Institute of Pharmaceutical Education and Research (NIPER), Punjab, India (Partner 7)
- **Dr. Ram S. Upadhayaya**, Institute of Molecular Medicine (IMM), Pune, India (Partner 8)

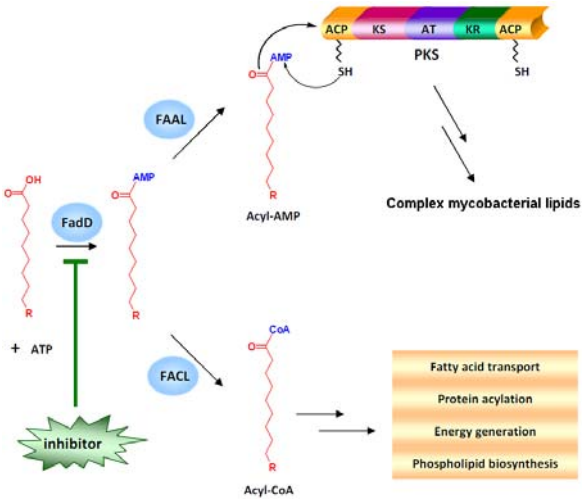
Within the NATT consortium a research plan to develop novel inhibitors, targeting unexplored as well as validated *Mtb* and host cell proteins, was set up. The novel targets selected make part of the most vulnerable cell processes that have proven their susceptibility to antimycobacterial drugs and antibiotics in general. Targeting processes involved in **cell wall and membrane synthesis** as well as **DNA replication and maintenance** has proven its merits in the development of antibiotics and is still successfully pursued in current (anti-TB) drug development campaigns. Within this context, also **cellular processes involved in the energy household** are considered as a target resource. Intervention at the level of pathogen-host cell interaction is a possible alternative to the classical strategy based on development of new antibiotics that block the pathogen replication cycle. In particular, a major objective was to experimentally test the possibility that **chemical re-activation of the phagosome maturation machinery** may restore the intracellular killing of phagocytosed Mycobacteria.

The group of S. Kaufmann, MPI-IB, Berlin (Partner 2a) has set up several *Mtb* whole cell screening assays to determine the tuberculostatic and/or tuberculocidal activities of novel compounds synthesized. To

test activities in broth culture, a whole cell based resazurin (or Alamar Blue) microplate assay as well as ^{3}H-uracil incorporation assays were performed. The group of P. P. Singh, NIPER, Punjab (Partner 7) made available the **BACTEC 460** and **MGIT 960 TB culture system**. Further, P. P. Singh offered access to **16 Indian clinical isolates of *Mtb*** collected from different parts of India. To specifically test activity against dormant *Mtb* cultures, CFU and ^{3}H-uracil incorporation assays were established in PBS treated nutrient starved and hypoxia induced non-replicating persistent cultures (the **Loebel starvation** and the **Wayne model**). The Kaufmann and Singh group made also available an assay assessing the inhibition by a given compound of the replication of *Mtb* engulfed by a host cell. Further assays included determination of bactericidal activity of test compounds as well as **efficacy studies in the TB mouse infection model** (S. Kaufmann). Gating criteria had to be applied to reduce the number of candidates to the most potent ones, since it was impossible to screen large numbers of compounds *in vivo*.

Mycobacterial fatty acyl-AMP ligases (FAAL)

Lipid metabolism is essentially important to all organisms, but even more so to mycobacteria which have many special lipids that are directly responsible for its survival in the host, its pathogenicity and antibiotic resistance. Mycolic acids are high molecular weight α-alkyl, β-hydroxy fatty acids and are prominent molecules of the robust cell wall of *Mtb*. To produce these complex fatty acids, mycobacteria exploit an assembly-line enzymatic machinery, wherein two fatty acid synthases (FAS I and FAS II) generate *n*-long chain fatty acid precursors, which are further extended by polyketide synthases (Pks) imparting complexity and structural diversity.

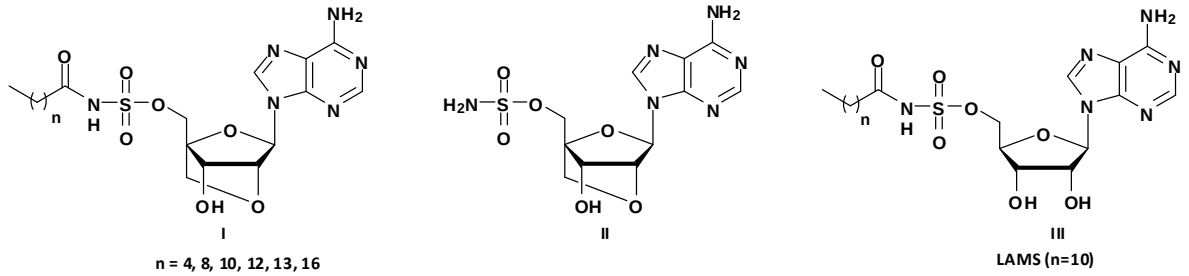


The commonly known mechanism of *n*-fatty acid activation involves conversion of fatty acids into their corresponding CoA-derivatives. This reaction is catalyzed by acyl-CoA synthetases (ACSs or fatty acyl-CoA ligase, FACL), also referred to as FadD proteins. Sequencing of the mycobacterial genome revealed 34 FadD homologues and many of the homologues are found adjacent to the *pks* genes in the *Mtb* genome. Systematic investigations of a number of these FadD proteins revealed new enzymatic activity. These proteins, **fatty acyl-AMP ligases (FAAL)**, convert fatty acids into the corresponding acyl-adenylates and do not catalyze formation of CoA thioesters. Cell-free reconstitution studies of FAAL proteins with their cognate Pks have indeed demonstrated involvement of these enzymes in biosynthesis of mycolic acids, dimycocerosate esters (DIMs) and siderophore mycobactin. Many of the lipids biosynthesized by the combination of FAAL and Pks are unique to mycobacteria and are essential for their survival, infecton and virulence. Since FAAL proteins feed into Pks to produce a number of crucial lipids, small molecules inhibitor of these proteins may have **tremendous therapeutic potential**. At the same time polyketide enzymes are also excellent targets for drug discovery.

The group of R. Gokhale, NII, New Delhi (Partner 6) has performed the systematic biochemical analysis and structural elucidation of mycobacterial **FACL13** and **FAAL28 proteins** which has revealed the determinants of substrate specificity in this family of fatty acid activating enzymes. The study highlights a **conserved hydrophobic anchorage** that arrests acyl-CoA catalysis in FAALs. Based on a unique

insertion motif the Gokhale group has demonstrated that the FAAL homologues could be identified in various genomes and established the role of the insertion in mediating dichotomy of fatty acid activation. Thus, providing a fascinating example of how nature has used the same structural scaffold for varying substrate specificities by fine tuning the residues occupying the binding pocket. Also **FadD9** protein has been successfully cloned from *Mtb* using an *E. coli* expression system and the catalytic function of both the adenylation and reductase domain has been characterized biochemically. It was revealed that FadD9 reduces carboxylic acids primarily to the corresponding aldehydes. For **Pks2** plays a role in the sulpholipid biosynthesis, the *pks2* gene was cloned and expressed in *E. coli*. Biochemical characterization has shown that Pks2 catalyses an iterative, stereoselective condensation of methylmalonate extender units leading to multi-methylated fatty acids. In this regard also **Pks7** and the reductase domain of **Rv0101 protein** have been cloned and expressed in *E. coli*, and post translational modification of phosphopantathenylation was achieved by the co-expression of surfactin synthase. Finally, **FadD23** has been characterized demonstrating its role in the acyl AMP transfer onto the ketosynthase domain of Pks2.

To target *Mtb* FAAL and FACL, **locked nucleic acid (LNA) phosphoramidites and sulphamoyl analogues of acyl adenylate** (**I**, **II**) as well as the corresponding **unlocked derivatives** (**III**) were synthesized by the group of Jyoti Chattopadhyaya, BOC, Uppsala (Partner 3). LNA is a conformationally restricted, bicyclic nucleic acid where a ribonucleoside is linked between the 2'-oxygen and the 4'-carbon atoms with a methylene unit. As the catalytic mechanism of FAAL proteins involves the synthesis of an acyl-adenylate intermediate from a fatty acid and ATP, the development of non-hydrolysable LNA analogues of this intermediate was anticipated to be a potentially fruitful strategy. Therefore, it was decided to introduce the sulphamoyl ester (as a substitute for the phosphate ester bond) into the LNA analogues of acyl adenylate. Noteworthy, two naturally occurring nucleoside antibiotics, nucleocidin and ascamycin, contain a similar sulphamoyl linkage.



To screen the synthesized compounds for their inhibitory activity, three enzymatic assays (**FACL19**, **FACL6** and **FAAL28**) using ^{14}C labelled fatty acid, ATP and CoA-SH were set up by the Gokhale group. The residual activity of FACL19 in the presence of the several synthesized compounds (**I** and **II**) and lauroyl sulphamoyl adenosine (LAMS) was compared (Figure 1). Several inhibitors could be identified and the binding affinity of the compounds varies by altering the length of the acyl group bond to the sulphamoyl moiety. On the other hand, it was noticed that a locked inhibitor (**I**) does not show any appreciable advantage over the corresponding unlocked derivative (**III**); for the longer acyl chains, the unlocked derivatives are superior over the locked counterparts (Figure 2). Since the results obtained from the enzymatic screening assays were encouraging, these molecules were selected to be screened in a *Mtb* whole cell based assay by the Singh Group. Unfortunately the whole cell based screening, performed at a fixed concentration of 6.25 mg/mL (BACTEC 460 radiometric method), revealed that none of the

compounds show significant activity against *Mtb* H37Rv. The divergence between these results clearly suggests that the compounds may indeed be active in the purified enzymatic assay, but fail to pass the 'real-life activity test' modelled by the whole cell based assay.

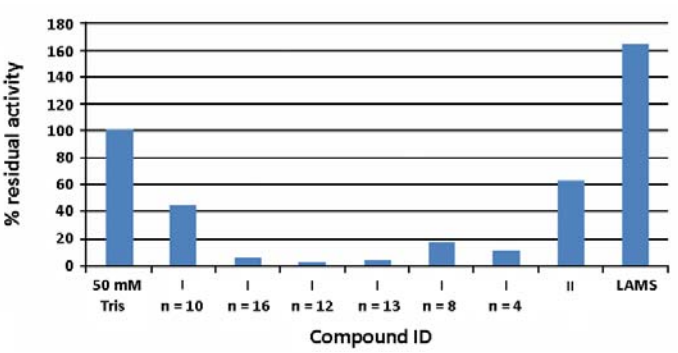


Figure 1 inhibition of FACL19 by inhibitors **I** and **II** at 100µM

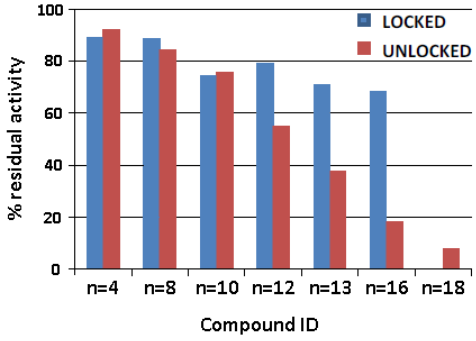


Figure 2 inhibition of FAAL28 by **I** and **III** at 10µM

Mycobacterial acyl-CoA carboxylase (ACCase)

The ACCase is a large multi-functional enzyme complex having two distinct active sites and a molecular mass approaching 1 million Dalton, making it one of the cell's large molecular machines. The complex catalyzes the **carboxylation of acyl-CoA substrates**, the first committed step that provides the building blocks for the *de novo* fatty acid biosynthesis (by FAS I, FAS II and Pks). Sequence analysis of the *Mtb* genome reveals a total of six *accD* genes (*accD1–6*; encoding CT) and three *accA* genes (*accA1–3*; ; encoding BC, BCCP) (Figure 3). Biotin, which is covalently bound to the biotin carboxyl-carrier protein (BCCP), is carboxylated in an ATP-dependent manner by biotin carboxylase (BC) to carboxybiotin. BCCP then translocates the carboxylated biotin to the carboxyltransferase (CT), which transfers the carboxyl group from biotin to the acyl-CoA substrate. Within the NATT project, the group of M. Wilmanns, EMBL, Hamburg (Partner 5) aimed to identify novel ACCase complexes and determine the structure of such a complex in order to better understand its activity. The latter would lead to better understanding of fatty acid metabolism in general, not just that of mycobacteria. The data collected during this study will prove valuable in attempts to develop new drugs against the tubercle bacillus, especially in structure-based approaches to drug design and discovery that have historically and successfully targeted mycobacterial lipid metabolism.

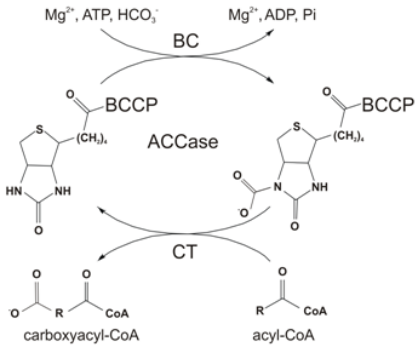


Figure 3 the ACCase complex

The existence of a **third novel ACCase** complex in *Mtb* capable of **carboxylating short-chain acyl-CoAs** was determined. The *Mtb* ACCase complex composed of the AccA1 α-subunit protomer and AccD1 β-subunit protomer, which assemble into a **high-molecular weight complex**, was successfully co-expressed in an engineered strain of *M. smegmatis*, created to improve the yield of TB proteins. The Wilmanns group has determined the low-resolution structure of the novel AccA1-AccD1 holo-complex using electron microscopy (Figure 4) which revealed a **symmetric dodecamer**

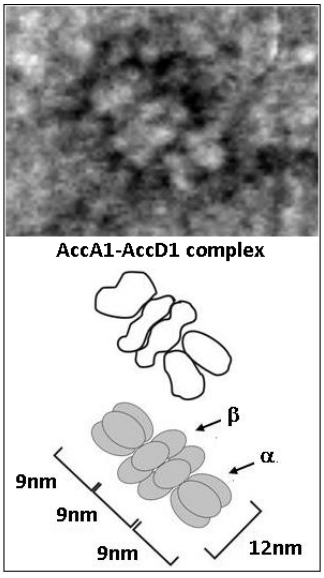
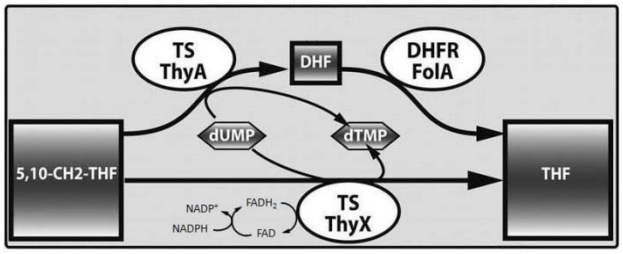


Figure 4 AccA1-AccD1 complex

composed of 6 α- and 6 β-subunit protomers. The hexameric β-subunit forms a central core flanked on either side by three α-subunits that are correctly post-translationally biotinylated. The structural data implies that a **conformational change in the α-subunit protomers** is necessary to allow the translocation of the carboxylated biotin to the substrate-carboxylating active sites of the β-subunit. Currently, protein crystals have been grown using different conditions, both with and without substrate. Unfortunately, these crystals have diffracted poorly and the sought-after high-resolution structure has remained elusive. Nevertheless, using the existing data the Wilmanns group can confidently place the subunits in their correct structural context. So, having identified another TB-specific ACCase, the **repertoire of potential drug targets in this family of genes has been extended**. In addition, other ACCase components, AccD4, AccD3, AccA3, and the AccE (epsilon subunit) have been cloned and trial expression of these individual protomers has been executed. While these different proteins could be purified to homogeneity, crystal growth screens for each of these have failed. Further, it has been determined that the closely related *Mtb* paralogs AccD2 and AccA2 directly interact to form a high-molecular weight complex. It therefore has similar biochemical properties to AccD1-AccA1, but due to low expression levels not enough material could be produced to further characterize this complex.

Mycobacterial thymidylate synthase ThyX

Thymidylate synthase (TS) enzymes catalyse the reductive methylation of deoxyuridine monophosphate (dUMP) to synthesise deoxythymidine monophosphate (dTMP or thymidylate), and so are important for **DNA replication and repair**. Two different types of TS proteins have been described (ThyA and ThyX), which have different enzymatic mechanisms and unrelated structures. **ThyA**, known as the conventional TS, uses *(R)*-*N*-5,*N*-10-methylene-5,6,7,8-tetrahydrofolate (CH_2-THF) as methyl donor and as a hydride donor yielding dTMP and dihydrofolate (DHF) as a byproduct. As reduced folate derivatives are essential for a variety of biological processes, DHF is converted back to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR; *folA* in *E. coli* and *dfrA* in *Mtb*). The more recently discovered **ThyX**, known as the alternative or flavin dependent TS (FDTS), applies CH_2-THF only as a one-carbon donor, whereas reduced flavin adenine dinucleotide ($FADH_2$) fulfills the role of reductant. ThyX enzymes are **NADPH-oxidases**, since the continuous replenishment of $FADH_2$ requires the presence of sufficient amounts of NADPH. In both cases THF is converted back to mTHF by serine hydroxymethyltransferase.



Most organisms contain either *thyA* or *thyX* and this TS encoding gene is usually essential (or its deletion creates a thymidine auxotroph if the organism also encodes thymidine kinase). Mycobacteria are unusual as they **encode both *thyA* and *thyX*** and the biological significance of this is not yet understood. Both *Mtb* ThyA and ThyX proteins have been shown to have TS activity *in vitro*, with ThyA being substantially more efficient than ThyX; however, *thyA* is not thought to be essential. On the other hand, *thyX* was identified in a screen for essential genes in *Mtb*. An interesting explanation for this duality is that *Mtb* might preferentially use either *thyX* or *thyA* under different growth conditions (i.e. activation from the dormant state). *Mtb* ThyX (and ThyX from other pathogenic microorganisms) is an **attractive target for drug design** as *thyX* is absent from the human genome, it lacks sequence similarity with ThyA and its enzymatic mechanism is different from ThyA.

To establish whether either or both of these enzymes are plausible drug targets, the group of E. Davis, MRC, London (Partner 4) investigated the biological roles of *Mtb thyA* and *thyX* by studying their **expression levels**, **essentiality** and **roles in pathogenesis**. The *in vitro* and *in vivo* expression levels of *thyA* and *thyX* were firstly analysed by quantitative reverse transcription PCR (Figure 5). Under all conditions tested, *thyA* expression exceeds that of *thyX;* no condition could be identified where *thyX* expression alone is increased. However, expression of both genes significantly increased upon *in vitro* acid exposure (pH 5.4) and when grown within naive and activated murine macrophages. Thus, **both ThyA and ThyX may play an important role *in vivo***.

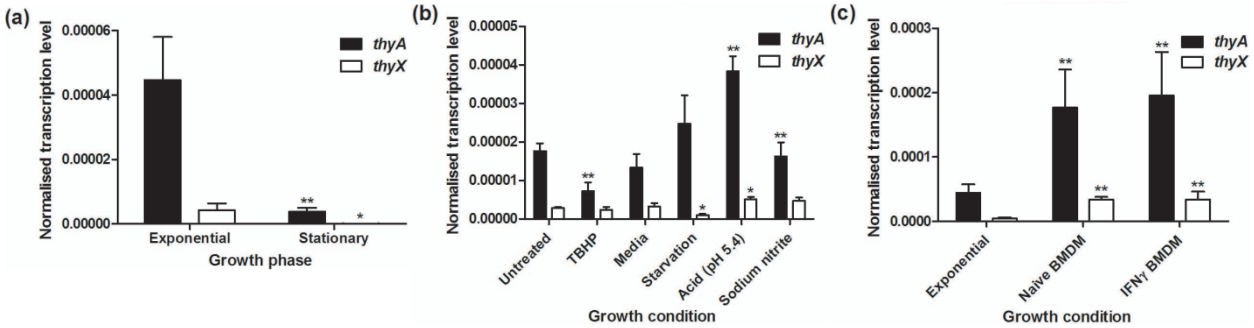


Figure 5 RNA extracts of *Mtb* H37Rv (a) grown to exponential and stationary phases, (b) after 24 hour exposure to different stress conditions (TBHP = *tert*-butyl hydroperoxide), (c) after 48 hour intracellular growth within murine bone marrow derived macrophages (BMDM)

All attempts to isolate *thyX* deletion strains under normal conditions or in the presence of thymidine were unsuccessful, indicating that **thyX is essential *in vitro***. This observation is in agreement with the results of TraSH experiments performed by Sassetti *et al*. (2003) and confirms that the ThyX enzyme is a plausible drug target. The requirement for *Mtb thyX* in the presence of *thyA* seems to imply that the **essential function of ThyX is something other than TS**. The Davis group successfully deleted *thyA* from the *Mtb* genome, and this deletion conferred an *in vitro* growth defect that was not observed *in vivo* (Figure 6). It was shown that the *thyX* expression *in vitro* is not up-regulated to compensate for the lack of *thyA* expression in the Δ*thyA* strain. So, presumably **ThyX performs TS activity within *Mtb* Δ*thyA*** at a sufficient rate *in vivo* for normal growth, but the rate *in vitro* is less than optimal. This could be due to a change in *thyX* transcription rate and/or ThyX TS activity. The Davis group has shown that *Mtb thyX* mRNA levels are higher *in vivo* than *in vitro*. Also, *C. jejuni* ThyX is known to be inhibited by oxygen, consequently *Mtb* ThyX may be more active *in vivo* under conditions where oxygen availability is limited, than *in vitro*. Hence, it would be interesting to further investigate whether *thyX* is actually essential for *in vivo* growth.

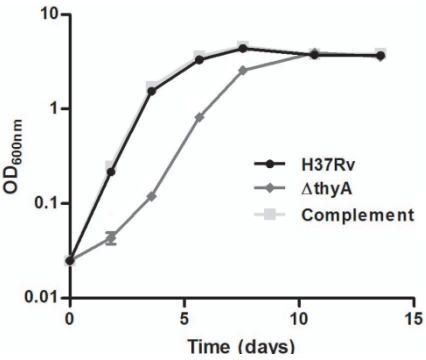
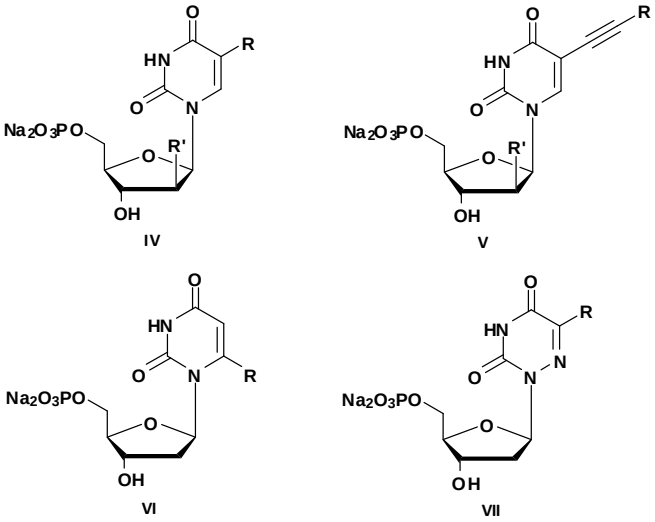


Figure 6 *In vitro* growth of *Mtb* H37Rv, ΔthyA and the complement strain

For ThyX shares no structural and sequence homology with ThyA and differs considerably in its biochemical reaction mechanism, it was anticipated that selective inhibitors of either of the two enzymes are accessible. Up to now, no selective inhibitors of mycobacterial ThyX are known in the literature. Theoretically, in analogy to 5-F-dUMP, a mechanism-based inhibitor of classical TS, any 5-substituted dUMP derivative can mediate inhibition of ThyX. Substitution of the proton at *C*-5 for another substituent abolishes proton abstraction from *C*-5 during the catalytic cycle, resulting in a "dead-end complex" of the inhibitor with CH_2H_4folate. Besides, a number of long-chain 5-alkynyl

2'-deoxyuridine (dU) and 1-(β-*D*-arabinofuranosyl)uracil (araU) nucleosides have been disclosed as antimycobacterial agents in an *in vitro Mtb* whole cell assay (e.g. **Va**, Table 1). These structural and mechanistic considerations prompted the group of P. Herdewyn, KUL, Leuven (Partner 1) to synthesize and evaluate various 5-alkynyl dUMP and araUMP analogues as selective inhibitors of mycobacterial ThyX vs. ThyA. The inhibition of ThyX-catalyzed dTMP-synthesis was measured in a radioactive assay using purified *Mtb* ThyX and [5-^{3}H]-dUMP as a radio-labelled substrate. The inhibition of mycobacterial ThyA was assayed through a photometric assay employing purified ThyA and measuring the decrease in absorption by DHF.

First, numerous **5-substituted 2'-deoxyuridine monophosphate derivatives** (**IV** and **V**, R'=H) and the corresponding **araUMP analogues** (**IV** and **V**, R'=OH) were synthesized and screened for their inhibitory activity of ThyX and ThyA. The introduction of a long-chain acetylenic moiety at *C*-5 of dUMP (**V**, R'=H) leads to selective mycobacterial ThyX inhibitors with **IC_{50} values ranging from 0.9 to 28 µM** (Table 1). The chain length was gradually amended and different functional groups were introduced at different positions into the alkyl tail. Other compounds of this series (**IV**, R'=H) have an aryl or a cyanovinyl group on the *C*-5 position. The most potent congeners display IC_{50} values of about 10µM. On the other hand, the corresponding araUMP analogues (**IV** and **V**, R'=OH) completely lack inhibitory activity against ThyX and so, any further synthetic efforts towards araUMP analogues were discontinued. Interestingly, none of the synthesized derivatives exhibit significant levels of ThyA inhibition, thereby highlighting their **selectivity for mycobacterial ThyX** (Table 1). Making use of the ThyX-BrdUMP-FAD crystal structure, the most potent compound **Va** was docked into the active site of ThyX (Figure 7). It was revealed that the propargylamide side chain points towards the outside of the catalytic site and the **carbamoyl oxygen is engaged in an additional hydrogen bond** with Lys165. Further, the model indicates that there is only limited space in the ThyX pocket for *C*-6 substituents. However, this substitution pattern might hamper the hydride transfer from $FADH_2$ to the *C*-6 position of dUMP. So, analogues with an aryl or alkyl group on the *C*-6 position were synthesized (**VI**); however, none of these compounds inhibit ThyX or ThyA. Finally, 6-aza derivatives (**VII**) were synthesized. We hypothesized that the hydride transfer from $FADH_2$ is abolished because of electrostatic repulsion due to the nitrogen atom. Of the synthesized 6-aza dUMP analogues, only the parent compound 6-aza dUMP (**VII**, R=H) and the long-chain propargylamide (**VII**, Table 1) display weak ThyX inhibitory activity, whereas the introduction of small alkyl groups leads to compounds totally devoid of ThyX inhibitory activity.



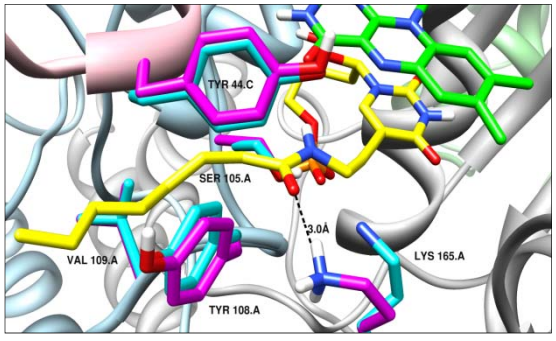


Figure 7 Derivative **Va** (yellow) bound to the BrdUMP binding site. Residues from the x-ray structure are shown in cyan colour. The residues in magenta have moved slightly to accommodate the inhibitor tail.

Table 1 Inhibition of *Mtb* ThyX and *Mtb* ThyA by compounds **IV-VII**

Compound	R'	R	ThyX inhibition IC_{50} (µM)	ThyA inhibition IC_{50} (µM)
5-FdUMP	/	/	0.29	0.57
Va[1]	H	$C_{10}H_{21}$	27.8	>50
IV	H	4-F-C_6H_4	10.0	>50
Vb	H	$CH_2NHCOC_7H_{15}$	0.91	>50
Vc	H	$CH_2NHCOCH_2Ph$	7.3	>50
Vd	H	$CH_2NHCONHC_6H_{13}$	4.84	>50
VII	/	$CH_2NHCOC_7H_{15}$	41%inh@50µM	>50

[1] M. Johar, T. Manning, C. Tse, N. Desroches, B. Agrawal, D.Y. Kunimoto, R. Kumar *J. Med. Chem.* **2007**, *50*, 3696-3705.

The most potent ThyX inhibitors prepared have been tested in a cellular *Mtb* screening assay by the group of S. Kaufmann. None of these compounds did inhibit *Mtb* growth, most likely because of poor transport of these highly polar, negatively charged compounds across the mycobacterial cell envelope. Therefore, non-phosphorylated congeners of compounds **IV**-**VII** were screened. Again, no growth inhibition of *Mtb in vitro* could be detected. The negative results might be explained by 1) impeded penetration of the pathogen, 2) *Mtb* ThyA activity and/or the remaining activity of ThyX are sufficient for survival, 3) degradation or inactivation of the compounds by the pathogen. Nevertheless, these ThyX inhibitors might serve as a good starting point towards the development of ThyX inhibitory compounds with improved physicochemical characteristics.

Mycobacterial helicases UvrD1 and AdnAB

The success of *Mtb* as a human pathogen lies to some extent in its ability to **survive and replicate in macrophages**. It is currently unknown how it manages to overcome the assault on its DNA by macrophage generated reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), which represent an otherwise very effective defence against intracellular pathogens. Pathways involved in maintenance of genomic integrity appear to be important for ***Mtb* pathogenesis and persistence in the host**. Nucleotide excision repair (NER) plays a pivotal role in the DNA repair machineries and has been extensively studied in *E. coli*. This repair mechanism is mediated by the UvrABC excinuclease enzyme complex and the helicase UvrD, a system capable of dealing with a broad range of bulky and/or helix-distorting lesions formed through exposure to DNA-modifying agents. Except for NER, *E. coli* UvrD is involved as well in mismatch repair and is implicated in recombination and replication. Consistent with these multiple roles, UvrD deficient *E. coli* cells are more susceptible to DNA damaging agents, exhibit elevated mutation rates, and are hyper-recombinogenic.

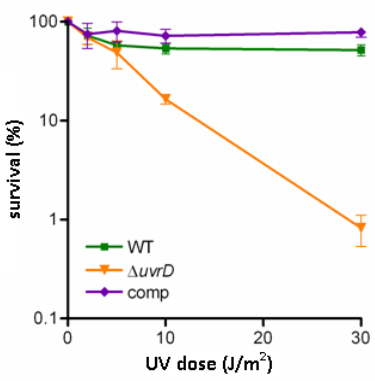


Figure 8 Survival of the wild-type (WT), *ΔuvrD1* and complement strain upon UV irradiation

Interestingly, *Mtb* has four genes encoding proteins containing a domain homologous to UvrD: ***uvrD1***, ***uvrD2***, ***Rv3201c*** and ***Rv3202c***. The expression of all four of these genes is increased following DNA damage and *uvrD1* and *Rv3201c* have been reported to be expressed at elevated levels during human infection, whilst Rv3202c is induced in macrophages. The group of E. Davis (Partner 4) has expressed (in *E. coli*) and purified UvrD1 and has characterized its helicase activity. This revealed that *Mtb* UvrD1 has an ATPase activity which is strictly DNA dependent, and exhibits helicase activity with 3'→5' polarity of unwinding. Further, it was found that *Mtb* UvrD1 unwinds *in vitro* duplex DNAs containing a nick or fork structures as well as in clearing stalled replication forks extremely efficiently, consistent with a role for *Mtb* UvrD1 in the NER pathway. The Davis group has constructed a

strain of *Mtb* in which *uvrD1* is inactivated (*ΔuvrD1*), and this strain exhibits **increased sensitivity to DNA damage *in vitro***, a phenotype that is restored to wild-type upon complementation (Figure 8). This suggests that the four genes are not functionally redundant, but rather have specific roles. Supported by the response of the mutant strain to a variety of DNA damaging agents, it was thus demonstrated that NER is functional in mycobacteria and that **UvrD1 plays an important role in DNA repair** and mutation prevention. Furthermore, the *uvrD1* mutant appears to have a **persistence defect in a mouse infection model**. These observations suggest that UvrD1 might represent a novel target for the development of drugs active against persistent or latent *Mtb*, more so because no identifiable homologues of *uvrD* can be identified in human or mammalian sequence databases.

Within the scope of the NATT project, *Δrv3201c* and *Δrv3202c* mutant strains were isolated indicating that these genes are not essential for *in vitro* growth. However, during the course of the joint project, it was shown by Sinha *et al.* (2009) that both genes function as a complex (AdnAB), which acts as a heterodimeric helicase-nuclease (AdnA=Rv3202c, AdnB=Rv3201c; *M. smegmatis*). Therefore, it was investigated whether the AdnAB complex plays a role in DNA repair. First, a double knockout of *adnA* and *adnB* (*ΔadnAB*) was created in *Mtb* confirming that the complex is not required for survival. Comparing the recombination rates of the wild-type, the *ΔadnAB* and the complement strain, it is concluded that **AdnAB plays a functional role in recombination** during stationary late growth phases (Figure 9). Also, the mutation rate of the *ΔadnAB* mutant is enhanced when assayed for spontaneous mutants against rifampin. The *ΔadnAB* mutant displays **enhanced sensitivity to a range of DNA damaging agents** including mitomycin C, ciprofloxacin, ofloxacin, bleomycin and sodium nitrite, whilst no effect following treatment with *tert*-butyl hydroperoxide or exposure to UV was observed. Although these observations indicate that AdnAB plays a role in DNA repair, it was shown that the *ΔadnAB* mutant has **no reduced virulence in both the macrophage infection and aerosol mouse infection model**. In fact, the group of S. Kaufmann observed that the double knockout mutant strain was indistinguishable from wild-type or complemented controls regarding the progress of the disease (bacterial burden in lung and spleen of animals infected at a low dose of approximately 50 bacilli/mouse) or lung-histopathology (Figure 10).

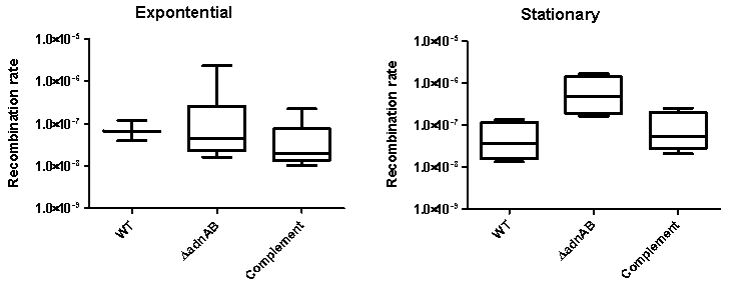


Figure 9 Recombination rates of the wild-type (WT), Δ*adnAB* and complement strain during the exponential and stationary growth phase

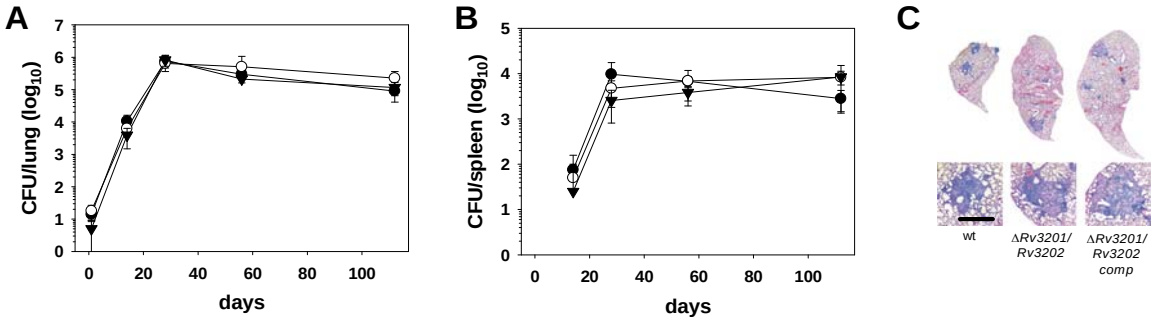


Figure 10 Mice were infected with wild-type (WT) (closed circles), *ΔadnAB* (open circles), or complement strains (closed triangle). Bacterial burden of lungs (A) and spleens (B) were collected at days 1, 14, 28, 56 and 112 (n=4). (C) Thin sections of the left lung lobe (day 112) were Giemsa stained and histopathologically examined by bright field microscopy (scale bar is 0.5 mm).

Secondly, **AdnAB wild type and a mutant protein were expressed and purified** from *E. coli*. The mutant protein was generated by introducing point mutations within the nuclease domain of AdnB rendering a **nuclease inactivated enzyme complex**. The isolated and purified *Mtb* UvrD1 and UvrD2 proteins, as well as the AdnAB nuclease mutant protein were used to set up enzymatic assays to identify helicase inhibitors. Before the start of the NATT project, the group of E. Davis used a high throughput fluorescence helicase assay to screen a library of 18.880 compounds against *Mtb* UvrD1. This screen identified four potent inhibitors with different chemical scaffolds and IC_{50}'s < 10µM. On further testing of the four best hits, their ability to inhibit the helicase activity of UvrD1 was confirmed; however none of these four compounds proved to be active against UvrD2.

Two hit compounds belonging to two different scaffold classes, namely a benzothiazole (inhibitor **VIII**) and a 5-cyanopyrimidine (inhibitor **IX**), were selected for a medicinal chemistry campaign. In order to test the activity of these hit compounds on whole *Mtb* and to gain an indication as to whether any effect was actually mediated via UvrD1, an **assay based on enhanced sensitivity to mitomycin C** (MitC) was developed by the Davis group (Figure 11). This assay indicated that inhibitor **IX** only causes growth inhibition in the presence of MitC suggesting that this compound likely acts via UvrD1. Inhibitor **VIII** inhibits bacterial growth equally in the presence or absence of MitC indicating that it acts on a different target in the cell. Still, this compound was particularly potent.

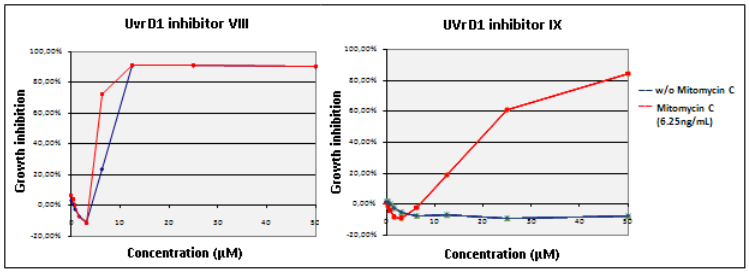
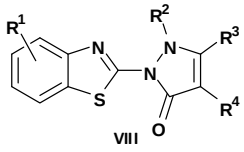


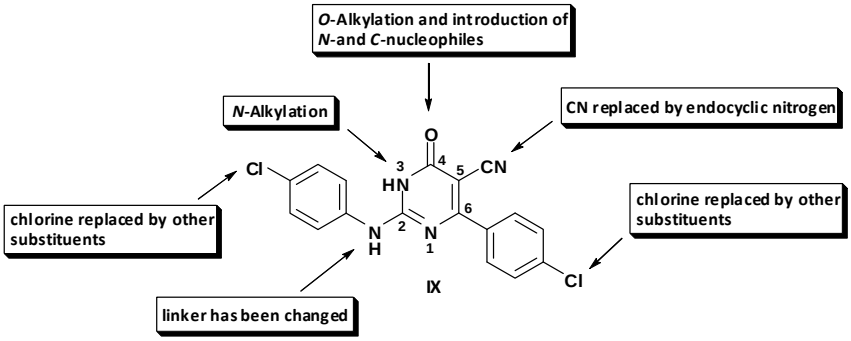
Figure 11 Growth inhibition of *Mtb* in the presence of inhibitor **VIII** or **IX**

The Herdewyn group structurally modified both selected hit compounds and the corresponding Structure Activity Relationship (SAR) was generated. Keeping the benzothiazole scaffold in place, the substituents of the hit compound were altered and additional substituents were introduced at four different parts of the scaffold (**VIII**, R^{1-4}). Over 100 derivatives synthesized were tested at 50µM against UvrD1, UvrD2 and the nuclease inactivated AdnAB enzyme. **Several derivatives with modest inhibitory activity** against UvrD1 (20-100% inhibition at 50µM) could be identified; none of the compounds displayed activity in the UvrD2 assay. Within this series, several compounds inhibit as well the helicase activity of AdnAB (40-100% inhibition at 50µM). Further, some analogues **inhibit the growth of *Mtb*** in the presence (IC_{50} < 20µM) as well as in the absence (IC_{50} = 25-50µM) of MitC. This observation indicates that these benzothiazole derivatives probably also interact with protein targets other than UvrD1 within *Mtb*.



VIII

The medicinal chemistry approach towards the 5-cyano-pyrimidine (**IX**) scaffold was twofold. First, the scaffold itself was modified leading to the synthesis of triazine and purine congeners. Regretfully, these derivatives did not cause inhibition of the helicase enzymes. Secondly, the substituents and functional groups were changed at several sites of the original scaffold (**IX**). Only one compound could be identified that displays a weak inhibitory activity in the UvrD1

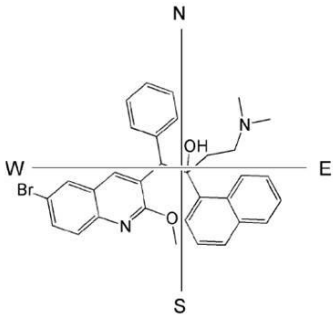


assay (30% inhibition at 50µM; inactive in UvrD2 and AdnAB). However, several analogues in this series **modestly inhibit the growth of *Mtb*** (+MitC, IC_{50}=100µM). Several compounds displaying inhibitory activity in a helicase assay (UvrD1, AdnAB) and/or the cellular *Mtb* assay (±MitC) were assessed in bone marrow derived macrophages infected with *Mtb.* Six compounds led to significant reduced CFU in macrophages following infection. One compound that inhibits UvrD1 and AdnAB (100% at 50µM) as well as *Mtb* growth *in vitro* (IC_{50} (+MitC) = 19µM, IC_{50} (-MitC) = 34µM) induces a **3 log drop in the macrophage infection experiment**. The synthesized UvrD1 and mutant AdnAB inhibitors were judged not being sufficiently potent for *in vivo* testing in a mouse infection model.

Mycobacterial F_1F_0-ATP synthase

Like all other organisms, mycobacteria utilize F_1F_0-ATP synthase to generate adenosine triphosphate (ATP), the biological currency of energy. Upon hydrolysis of ATP, a high-energy phosphoanhydride bond is ruptured which generates adenosine diphosphate (ADP) and phosphate, and liberates a large amount of free energy. **F_1F_0-ATP synthase** is a biological rotary motor made up of two major structural domains, F_0 and F_1, and uses the proton-motive force moving down an electrochemical gradient in order to generate ATP from ADP and phosphate. The F_0 domain contains three different subunits with (a, b_2, $c_{10\text{-}12}$) stoichiometry, spans the cytoplasmic membrane and is the proton channel of the complex coupling the proton translocation with the actual ATP synthesis in F_1. The F_1 domain consists of five different subunits with (α_3, β_3, γ, δ, ε) stoichiometry and is located in the cytoplasm, where it generates and sets free ATP. Although found in most living organisms, including humans, there is very little sequence similarity between the mycobacterial and human b-subunit (*atpF* gene encoding protein) and c-subunit (*atpE* gene encoding protein) of the F_0 domain of F_1F_0-ATP synthase. This observation provides a solid basis to consider this mycobacterial enzyme a potential therapeutic target.

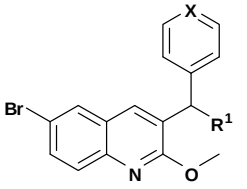
Targeting the **b-subunit of the *Mtb* ATP synthase F_0 domain** is till date poorly explored. Based on *atpF* (encoding the b-subunit) overexpression, it has been proposed that the observed decrease in ATP synthesis in *n*-octanesulphonylacetamide (OSA) or dicyclohexylcarbodiimide (DCCD) treated mycobacterial cultures may be due to direct or indirect interaction of both compounds with the b-subunit. Therefore, this subunit could be considered as a promising new target. The **c-subunit of the F_0 domain** is a well-validated therapeutic target. In fact, biochemical studies and binding assays using diarylquinoline (DARQ) resistant strains with mutations in *atpE* (encoding the c-subunit) suggested that the c-subunit of the ATP synthase is the target for DARQs. These quinoline derivatives have proven to be safe in a phase I study in human volunteers. Presently, TMC-207 (MIC=0.060 µg/mL (0.1µM)) is being evaluated in a phase II clinical trial.



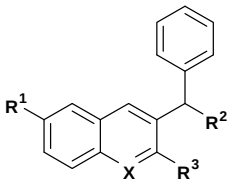
TMC207

Within the NATT project, new compounds have been designed and synthesized to inhibit the ATP synthase of *Mtb* by the group of R. S. Upadhayaya, IMM, Pune (Partner 8) and the Chattopadhyaya group. This approach is considered of great importance as the synthesis and isolation of the active diastereomer of TMC-207 is tedious which creates a big hurdle to provide the drug at an affordable price for the poor third world patients. Secondly, TMC-207 has shown drug-drug interactions with rifampicin hampering its use in a combination therapy with this important front-line anti-TB drug. In order to elaborate on the SAR and to develop synthetically more accessible analogues of TMC-207, the functional groups in the **four hemispheres (NE, NW, SE, SW)** of the TMC-207 molecule were systematically altered. First, the hydroxyl and naphthalene group of respectively the NE and SE part were modified upon introduction

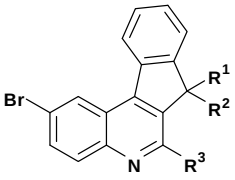
of different amines and amides leading to compounds (**X**). The main reason behind the change of the SE naphthalene moiety was to reduce the bulk of the molecule. Among these derivatives, four compounds display a minimum inhibitory concentration (MIC) of 6.25 µg/mL in a *Mtb* whole cell assay (performed by the groups of P. P. Singh and S. Kaufmann). Molecular modelling and docking studies with TMC-207 were applied to spot critical binding sites of the ATP synthase target. The results suggest a possible role for functional groups other than the hydroxyl and *N*,*N*-dimethylamino group in ligand-protein interactions. Consequently, the NW phenyl moiety as well as the bromine on the SW quinoline ring was subjected to structural modifications leading to the identification of seven derivatives (**XI**, **XII** and **XIII**) with MICs of 3.12-6.25 µg/mL. Eventually, several **conformationally constrained quinolines** (**XIV**) were synthesized. It was anticipated that the entropic penalty for complex formation with the target protein could be reduced upon restriction of the rotationally freedom of the NW phenyl ring, potentially enhancing the binding affinity. Among the constrained analogues, numerous inhibitors of *Mtb* growth were identified, the most potent congeners displaying a MIC < 0.39 µg/mL. Several **compounds inhibit the growth of an isoniazid-resistant *Mtb* strain** at 6.25 µg/mL. For two compounds, the growth inhibition on intracellular *Mtb* could be confirmed at an inhibitor concentration of 5 µM. These active compounds have been screened for their cytotoxic effect on human monocytic cells. The results show that the human cell survival is almost unperturbed (100% survival).



X (X=CH; R^2=OMe)
XI (X=N; R^2=Me)

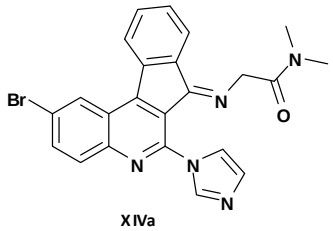


XII (X=N; R^2=H; R^3=OR)
XIII (X=CH; R^2=OR; R^3=H)

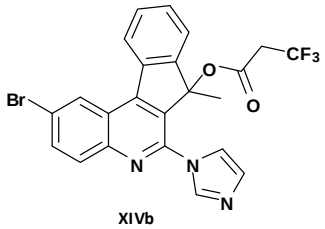


XIV

The conformationally constrained analogues have been found to suffer from intense solubility problems. To improve their bioavailability, a prodrugs approach was employed. Accordingly, esters were synthesized which exhibit good inhibitory activities (MICs ranging from 0.2 to 6.0 µg/mL). Eventually, Partner 8 and 3 have generated a **library of approximately 500 compounds**, all built around the quinoline scaffold, out of which compounds (for example XIVa-b) were selected to be subjected to an *in vivo* efficacy test in a mouse infection model. First, the maximum tolerance dose (MTD) was determined and found to be in the range of 450-500 mg/kg. Regretfully, the mouse model tests revealed that none of the compounds display *in vivo* efficacy. Analysis of sacrificed mice has demonstrated that the test compounds accumulate in the abdomen which strongly suggests their non-bioavailability. Therefore, more research is required towards a strong medicinal chemistry approach and the correct formulation in order to develop water soluble analogues of these lead compounds.



XIVa
MIC < 0.07µg/ml
MTD > 450mg



XIVb
MIC = 0.5µg/ml
MTD > 450mg

Intervention at the level of the pathogen-host cell interactions

After infecting the host, pathogens are normally ingested by macrophages in a process called phagocytosis. During this process, phagosomes containing the pathogen progressively fuse with endosomes and lysosomes, and mature into acidified degradative phagolysosomes by the gradual acquisition of lysosomal enzymes. However, *Mtb* can survive and multiply within the hostile intracellular environment of macrophages because it is able to **block the phagosome maturation process** by preventing the formation of phagolysosomes. As a result, mycobacteria significantly reduce the capability of the lysosomal system of the host macrophage cell to degrade and thereby eliminate the pathogen. During this process, there is a mutual interaction between pathogen and host cell wich modulates intracellular trafficking and inhibits the fusion with late endosomes and lysosomes. The past 30 years of TB research has focused on identifying drugs directly eliminating the pathogen from the infected cell and largely neglected the host cell as a potential target for drug discovery. Interference with the dynamic pathogen-host cell interactions opens the way for innovative therapeutic approaches against TB infection.

In the frame of the NATT project, the group of M. Zerial, MPI-CBG, Dresden (Partner 2b) has developed a pipeline of **high content cell-based image analysis screens**. A fully automated multidisciplinary screening platform has been established for a bacterial cell survival assay (employing *M. bovis* BCG expressing the fluorescent marker GFP) in primary human macrophages to assess compound effects. The proprietary software for quantitative multiparametric image analysis (QMPIA, Motion Tracking) has been further developed for the specificities of the macrophages and the segmentation of mycobacteria (Figure 12). Over **40 parameters** have been quantified describing **bacteria, host cell and toxic effects** to host cells. Accordingly, it will be possible to filter out those compounds exerting host cellular toxicity at the very early phase of drug discovery. 85 compounds of the chemistry groups of the NATT project have been screened and several hit compounds on bacterial survival in the host cell were identified in this assay (Figure 13). To gain more insight into the mode of action of compounds, data from a chemical (using chemical compounds) and RNAi endocytosis screen (using a genome wide siRNA library) have been integrated. It is expected, that this approach will help to identify potential drug targets and consequently the bio-inspired design of more suitable compounds for combating TB. Furthermore, a panel of cell-based image analysis assays has been developed and tested using selected compounds and the high content imaging platform with its MotionTracking software. These assays include: 1) large scale assays for autophagy, 2) assay for phagosome maturation, and 3) endocytic trafficking (both large and small scale assay as well as live cell imaging). As a proof-of-principle, selected chemical compounds have been analysed in further details that release the mycobacteria-mediated phagosome maturation arrest ultimately leading to the destruction of the pathogen. Also electron microscopy analysis has been performed to quantify phagosomal

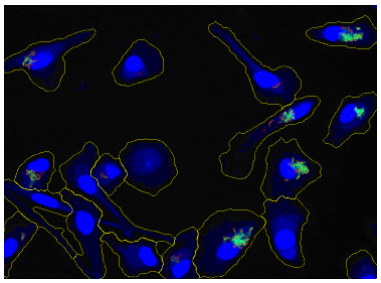


Figure 12 A microscopy image showing several infected macrophages; host cell nuclei - blue, mycobacteria - green.

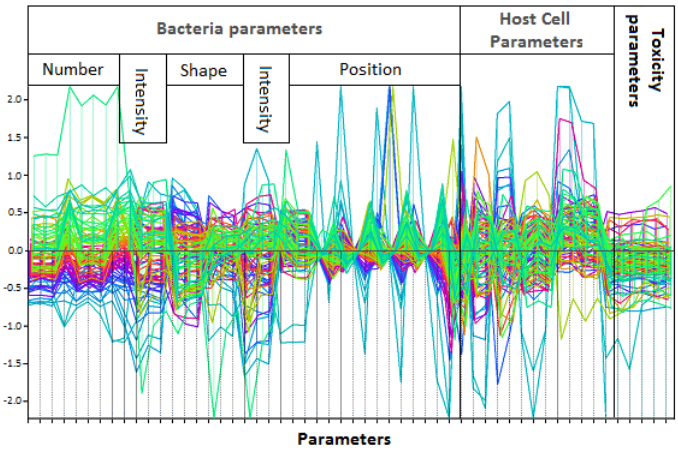


Figure 13 Multiparametric profiles of all NATT compounds tested

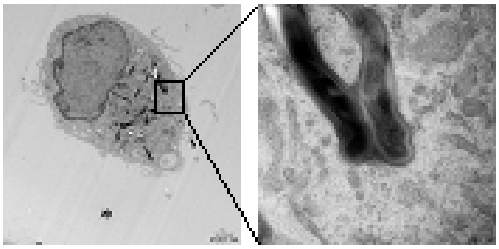


Figure 14 High-resolution electron microscope images of a *M. bovis* BCG infected human primary macrophage

structures in the infected host cells upon compound addition as further validation of the results (Figure 14). In summary, the obtained data suggest that **compounds release the phagosome maturation arrest** either by inducing autophagy, a well characterized anti-mycobacterial host mechanism, or by accelerating endocytic trafficking in the host cell. The NATT project has led to the establishment and evaluation of an **unconventional assay system capable of identifying cellular pathways as potential targets**, traditionally not investigated by drug discovery in the battle against TB. The generated knowledge from the comparative chemico-genomic screens will provide alternative pathways for further investigations in diverse fields of cell biology as well as a solid background for system understanding of the disease.

Additional information about the NATT consortium and the participating partners can be found on the **NATT website**: http://www.natt-tbc.com. The website offers an insight on tuberculosis as a major health problem and provides an overview of the project's research. Detailed information on each beneficiary of the NATT consortium can be found, including direct links to the partner's homepages, their email addresses and an overview of relevant papers published by each partner. Further, the EC contribution, the duration of the project as well as the starting and end date can be found on the website.

Project Coordinator:
Prof. Dr. Piet Herdewyn
Laboratory of Medicinal Chemistry, Rega Institute,
University of Leuven, Belgium
Minderbroedersstraat 10 blok x - bus 1030,
3000 Leuven, Belgium
Tel: +32 (0)16 337 387
Piet.Herdewijn@rega.kuleuven.be

1.4. Potential Impact, Dissemination and Exploitation of Foreground

Despite the availability of highly efficacious treatment for decades, tuberculosis (TB) remains a major global health problem. TB is an ancient disease, caused by the bacillus *Mycobacterium tuberculosis* (*Mtb*) and usually affects the lungs (pulmonary TB); however, other sites can be affected as well (extra-pulmonary TB). The disease is easily spread in the air when infected people expel bacteria, and is characterized by an extremely long incubation time. Indeed, most people do not show any symptoms at all and only ca. 10% of infected individuals develop active disease. Nonetheless, the host fails to achieve sterile eradication and therefore the vast majority of *Mtb*-infected individuals carry dormant pathogens, that is, they are **latently infected**. These persistent, dormant pathogens rarely replicate if at all, but can still develop full-blown TB once the host defence is weakened. Consequently, the increased probability of developing TB among people infected with the Human Immunodeficiency Virus (HIV) has led to the **deadly liaison of HIV/AIDS with TB**.

TB is the **second leading cause of death** from an infectious disease worldwide (after HIV, which caused an estimated 1.8 million deaths in 2008). It affects mostly adults in the economically productive age groups; around two-thirds of cases are estimated to occur among people aged 15–59 years. Hence, this disease has a **devastatingly disrupting effect on the social and economical framework of above all the developing countries**. One third of the world's population is currently infected with dormant *Mtb* germs. In 2010, there were an estimated 8.5-9.2 million new cases and 1.2-1.5 million deaths worldwide. Compared with 1993, when the World Health Organization (WHO) declared TB a global public health emergency, this estimated number of incident cases increased with 21%. The majority of cases in 2010 occurred in Asia (59%) and Africa (26%); the five countries with the largest number of incident cases in 2010 were India (2.0 million–2.5 million), China (0.9 million–1.2 million), South Africa (0.40 million–0.59 million), Indonesia (0.37 million–0.54 million) and Pakistan (0.33 million–0.48 million). India alone accounted for an estimated one quarter (26%) of all TB cases worldwide, and China and India combined accounted for 38% (WHO, 2011).

The situation is **also worsening in parts of the European Union** because of the increasing incidences of multidrug-resistant (**MDR**) and extensively drug-resistant (**XDR**) *Mtb* strains. In several countries of the world, including eastern parts of Europe, high proportions (e.g. 28% in north western Russia) of MDR strains have been noted. Despite a substantial progress in the coverage of continuous surveillance and surveys of drug resistance, the WHO evaluated the progress to not be sufficient yet in order to provide a definitive assessment of global and regional trends in MDR-TB (WHO, 2011). As of June 2008, 49 countries have confirmed cases of XDR-TB. By 2010, that number had risen to 58 (WHO, March 2010). XDR-TB has been notified in numerous EU member states including the Czech Republic, France, Germany, Italy, Latvia, Portugal, Spain, Sweden and the UK. **XDR-TB is virtually untreatable**, especially in patients with a compromised immune system and in patients living in developing countries where an accurate diagnosis and access to all classes of second-line drugs might be problematic.

Without treatment, TB mortality rates are high. Even though treatment using combinations of anti-TB drugs mainly developed in the 1940s and 1950s can dramatically reduce mortality rates, the prevalence of MDR- and XDR-TB urgently calls for **novel anti-TB drugs**. Additionally, novel drug treatment schemes should also focus on killing of dormant bacteria because they most likely use novel drug targets and secondly, can **shorten treatment time**. Indeed, the long-lasting treatment regimen leads to a high proportion of non-compliance patients and is one of the driving forces for the emergence of drug-resistant TB.

Hence, it is plain obvious that research projects, which invest in the discovery of original and innovative strategies to fight this global burden of TB, **warrant the identification of novel anti-TB drugs**. Ultimately, it will promote drug development and provision of drugs, **affordable for those who are most in need**.

The NATT collaborative project reflects the awareness of the need for new drugs to treat TB and brought together experts of different disciplines from Europe and India. The results collected during the course of this study will prove **valuable in attempts to develop new drugs against the tubercle bacillus**, especially in structure-based approaches to drug design and discovery. The applied approach that led to the identification of **new lead compounds** for anti-TB drug development was twofold. First, considerable efforts were dedicated to the **discovery and biochemical characterisation of novel *Mtb* protein targets**. Several of these proteins were employed in medicinal chemistry campaigns affording new enzyme and *Mtb* growth inhibitors. These efforts have revealed that these **novel targets are druggable** and thus, might yield a new and powerful armoury against MDR- and XDR-TB strains. More so because inhibitors acting on these targets employ novel mechanisms not utilized by currently available drugs. Furthermore, emphasis was placed on identifying drugs that interfere with host–pathogen interactions and can potentially tackle dormant TB. Intervention at the level of pathogen-host cell interaction, in particular the chemical reactivation of the phagosome maturation machinery, is a possible alternative to the classical anti-TB strategy. To assess the restoring of intracellular killing of *Mtb*, a fully automated **multidisciplinary screening platform has been established** for a bacterial cell survival assay in primary human macrophages. Via multi-parametric image analysis, **compounds were identified that release the phagosome maturation arrest**.

The findings generated in this consortium will provide valuable information for the design of novel **antibiotics beyond this disease**. Antibiotic development in general has almost come to a standstill while the increasing incidences of resistant strains such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) urgently demand for a profound strategy towards the discovery and development of novel antibiotics. Information gained with drug candidates targeting host rather than pathogen structures promises to **open a new field of anti-infectives, particularly to treat chronic infections** where pathogens manipulate the host to their benefit.

Within this joint project, several partners have contributed to the generation of novel and potentially highly impacting findings. Below a more detailed overview is given, emphasizing on the important research results obtained during the course of the NATT collaborative project.

Intervention at the level of the pathogen-host cell interactions

The standard approach of TB research for identifying drugs which directly kill bacteria (antibiotics) has been without any major breakthrough for the last 30 years. In the frame of the NATT project, the Zerial group has focused on an unconventional target for drug discovery. Their approach used the typical feature of TB infected cells; they down regulate their intrinsic defence mechanism for eliminating bacteria (the lysosomal pathway). The basic principle of the NATT approach was accordingly to screen for compounds significantly restoring the cellular lysosomal activity. To this end, a **fully automated multidisciplinary screening platform** has been established for a bacterial cell survival assay to assess compound effects using primary human macrophages.

Several technology developments were necessary to establish this high throughput and high content imaging screen. The technology for culturing primary human macrophages and performing the various treatment steps as well as the imaging including image analysis had to be adapted and fully automated.

The proprietary software for quantitative multi-parametric image analysis (QMPIA, MotionTracking) has been further developed for the specificities of the macrophages and the segmentation of mycobacteria. **The automated segmentation is a significant achievement of the project that will largely support drug discovery as well as other research areas in cell biology** as it can be used for other pathogens or cellular components. Over 40 parameters have been used to quantify bacteria, host cell, and toxic effects to host cells.

A major impact of the screening platform is the **identification of toxic compounds at the very early phase of drug discovery**. This leads to significant cost reductions in the pipeline. The established assay and the platform can be used for screening large libraries of compounds that can result in the discovery of long-awaited novel TB drugs, also potentially active on multi-resistant strains. This has a large socio-economic impact in the light of the accelerated spread of the disease around the globe.

To gain more insight into the mode of action of compounds, data from chemical and RNAi endocytosis screens have been integrated. It is expected, that this approach will **help to identify potential drug targets** and consequently **the bio-inspired design of more suitable compounds** for combating the disease. This approach itself is a major breakthrough that will impact the field of drug discovery.

Exploring the pharmacophore of diarylquinolines (DARQs)

TMC-207 (bedaquiline; *Mtb* F_1F_0-ATP synthase inhibitor) is an anti-TB drug candidate presently being tested in Phase IIb trials for the treatment of newly-diagnosed MDR-TB patients. Final results are expected in 2012 (WHO, 2011). However, this potential new drug against TB displays a severe problem. First, the overall yield of the active isomer is only approximately 6%, involving the separation of the active diastereomer from the inactive one, which creates a big hurdle to provide the drug at an affordable price for the poor third world patients. With the diarylquinoline scaffold of TMC-207 in mind, the Chattopadhyaya and Upadhayaya group of the NATT consortium undertook a synthetic challenge to devise new lead molecules that are active against *Mtb*.

Both medicinal chemistry groups have synthesized over 500 molecules by systematically modifying different parts of the TMC-207 molecule. As a result, an **extensive Structure Activity Relationship (SAR) for DARQs** has been generated and additional critical binding sites of the *Mtb* F_1F_0-ATP synthase have been identified with molecular modeling and docking studies. These new classes of molecules were found active in the *Mtb* whole cell based assay (performed by the Kaufmann and Singh groups (NATT), and by scientific collaborators in Sweden, USA and India) and proved to be non-cytotoxic to human cells. **5 lead compounds** that show good inhibitory activity *in vitro* (MIC_{99} = 0.03-0.5 µg/mL; H37Rv) also display 99% growth inhibition of three types of drug resistant MDR-TB strains at 3.125 µg/mL. Unfortunately, it was observed that none of the compounds show significant *in vivo* efficacy in a mouse infection model. Therefore, more research is required towards a strong medicinal chemistry approach and correct formulation in order to develop water soluble analogues of these lead compounds. However, the ending of the NATT project resulted into lack of research funding that would allow further investigation towards the development water-solvable drug candidates.

It is hard to overestimate the importance of finding lead compounds for further scientific and industrial efforts in development of antimycobacterials against drug resistant TB. Due to similarities in the target protein structure and the target's crucial role in the energy household of microorganisms, the discovered lead compounds have **potential against other infectious disease and hospital pathogens**.

Intervention at novel Mtb protein targets

At the start of the collaborative project, the targets to be studied were selected based on their individual roles in the **most vulnerable cell processes**. These processes studied involve cell wall and membrane synthesis as well as DNA replication and maintenance. The fact that these processes have successfully proven their merits in the development of antibiotics in the past increases the potential impact of the findings and inhibitors originating from this study in terms of the development of new antimycobacterial drugs and antibiotics in general.

Driven by the observation that lipid metabolism of *Mtb* is crucial both for the infectious as well as latent stages of the bacillus, the Gokhale group embarked on a systematic biochemical analysis and structural elucidation of mycobacterial **FACL13** and **FAAL28** proteins. Their efforts reveal the determinants of substrate specificity in FACL and FAAL proteins and delineate the evolutionary relationship between this family of fatty acid activating enzymes. Acyl sulphamoyl analogues, resembling the common reaction intermediate, were found to inhibit both FAAL and FACL proteins. Therefore, this type of inhibitors is likely to **target multiple lipid biosynthetic as well as degradative pathways**. The Chattopadhyaya group designed and synthesized locked nucleic acid (LNA) phosphoramidites and sulphamoyl analogues of acyl adenylate as well as the corresponding unlocked derivatives. *In vitro* screening (FACL19, FACL6, FAAL28) of these derivatives affords a **better definition of the pharmacophore** for the acyl sulphamoyl analogues. This strategy of simultaneously inhibiting multiple enzymes has **tremendous potential as new generation multi-target drugs**. Since FACL enzymes in *Mtb* are being implicated in providing alternate energy sources during dormancy stage of infection, this strategy also suggests a **possible means to target different stages of *Mtb* infection**.

The Wilmanns group has determined the existence of **a third novel ACCase complex in *Mtb*** capable of carboxylating short-chain acyl-CoAs and supplied scientifically relevant information on the fundamentally important set of ACCase genes. This knowledge will have to be considered in drug development efforts that aim to produce drugs targeting the ACCase carboxylase activity. Moreover, it yields better understanding of fatty acid metabolism in general, not just that of mycobacteria. The existence of multiple complexes with similar activity may have **consequences for the efficacy of any ACCase-targeting drugs currently under development**. As such, the data would be of interest to those engaged in developing such drug leads. Furthermore, the structure of the novel ACCase complex has been revealed, which will prove **valuable in structure-based attempts to develop novel anti-TB drugs**.

During the NATT project, the Davis group has provided a better insight into the nucleotide excision repair (NER) system of *Mtb*. Having characterized earlier the helicase activity and cellular role of UvrD1, this group addressed two other proteins encoded by domains homologous UvrD: **Rv3201c** and **Rv3202c**. Both genes function as a complex (AdnAB), which acts as a heterodimeric helicase-nuclease. For it was revealed that AdnAB plays a functional role in recombination and in the maintenance of genomic integrity, it might be expected that targeting UvrD1 and AdnAB will **affect the *Mtb* pathogenesis and its persistence in the host**. It was noted by the Davis group before that the *uvrD1* mutant appears to have a persistence defect in a mouse infection model. However, the group of Kaufmann showed that the *ΔadnAB* mutant has no reduced virulence in both the macrophage infection and aerosol mouse infection model. Nevertheless, the helicase activity of UvrD homologues might still represent a **novel target for the development of drugs active against persistent or latent *Mtb***, more so because no identifiable homologues of UvrD can be identified in human or mammalian sequence databases.

In collaboration with the Herdewyn group, a medicinal chemistry campaign was set up to identify helicase inhibitors. Enzymatic assays employing purified UvrD1 and AdnAB nuclease mutant proteins were set up by the Davis group. A lead compound could be identified, which inhibits the helicase activity of UvrD1 and AdnAB, as well as *Mtb* growth *in vitro*. Moreover, this compound induces a 3 log drop in the macrophage infection experiment. These findings suggest that the **UvrD homologues are druggable and can represent a novel drug development approach in fighting TB**.

The Herdewyn group has selected the mycobacterial flavin dependent thymidylate synthase ThyX as a promising protein target. *Mtb* is unusual as it encodes both ThyA (the 'classical' thymidylate synthase) and ThyX. Within the framework of NATT, the **first selective ThyX inhibitors** have been synthesized. For *thyX* is absent from the human genome, these findings consolidate the fact that *Mtb* ThyX is an **attractive target for anti-TB drug design**. Moreover, other clinically relevant bacteria lack *thyA* and rely solely on the ThyX protein for their *de novo* thymidylate synthesis. Hence, the identified inhibitors might find application as tool or lead compound in the **development of antibiotics against other infectious diseases**. In parallel, the Davis group has further unravelled the role of ThyX in pathogenesis and has unambiguously proven that **ThyX is essential** for *Mtb* growth *in vitro*. Additionally, observations made with *thyA* deletion strains (*Mtb thyA* is not essential) suggest that **ThyX has another function** next to its thymidylate synthase activity. It is anticipated that these findings will prompt further research into this interesting and intriguing *Mtb* target. In particular, efforts should be dedicated to demonstrating whether *thyX* is essential for *in vivo* growth, and to revealing all functions of this enzyme. The Herdewyn group could identify by docking studies an additional binding site in the ThyX active site. Together with co-crystallization experiments, the **discovered compounds will have a significant impact on the rational design of ThyX inhibitors** and thus, will support drug development programmes focusing on the ThyX protein of pathogens.

Dissemination and exploitation of results

During the general assembly meetings, which were organised every 6 months and each time were combined with the steering board meeting, all project results were communicated to the whole group of the NATT consortium. This ensured that every partner was, and is, aware of all scientific progress realized within the consortium. Of each NATT meeting minutes were redacted which were subsequently distributed among all NATT partners. This allowed that all partners were able to disseminate the project's research to third parties. All partners were encouraged to seek contact with European companies when judged appropriate, of course under a confidentiality agreement.

Prompted by a call of proposals by the European Commission (FP7-HEALTH-2010-single-stage, HEALTH.2010.2.3.2-1), the members of the NATT joint project **initiated the setup of a competitive consortium** (LEAD4TB) to contend for this call. The NATT consortium approached 11 research groups outside NATT, out of which 3 SMEs, and found them prepared to step into the construction of this new research endeavour. Building on some preliminary results obtained during the ongoing NATT project an original and innovative research proposal was constructed. This newly built consortium **sought contact with the pharmaceutical industry** for scientific and practical support and was successful in generating interest with Eli Lilly and Company. In fact, the Lilly TB Drug Discovery Initiative was interested to get actively involved in the LEAD4TB project. Moreover, we were brought in contact with the Infectious Disease Research Institute (IDRI), a not-for-profit organization committed to applying innovative science to the research and development of products to prevent, detect and treat infectious diseases of poverty, and one of the founding members of the Lilly TB Drug Discovery Initiative. The IDRI as well as the Academia Sinica, Taiwan were willing to actively support our research project. Although the LEAD4TB

project was ultimately not selected for funding, its construction offered nevertheless a dissemination platform for the ongoing research and the potential of the NATT project.

On April 20th 2011, the NATT consortium organized in Uppsala a small **conference** open to researchers outside NATT. The conference was entitled '*New Approaches to Target Tuberculosis and Infectious Diseases'* and focused on the progress in development of drugs against resistant forms of *Mtb* and other infectious disease. This venue created an opportunity for the NATT project to disseminate its important scientific findings and promoted a scientific crosstalk with specialists in medicinal chemistry, microbiology and medicine. At the conference, students, PhD students and post-docs were given the opportunity to present results of their ongoing research (poster session). The consortium invited to this conference two speakers with expertise in the field:

- Dr. Ian Gilbert, professor of medicinal chemistry, College of Life Sciences, University of Dundee, Dundee
- Dr. Sven Hoffner, professor of clinical microbiology, Swedish Institute for Infectious Diseases Control, Stockholm

A **website** has been created in close collaboration with the Davis group, NIMR, London. The NATT website (http://www.natt-tbc.com/) offers a concise summary for anyone who is looking for more information on tuberculosis as a major global health problem. The emphasis is laid on dormant tuberculosis and on the increasing emergence of MDR- and XDR-TB strains. An overview of the project's research is provided focussing on the scientific strategies the consortium has applied, the selected *Mtb* targets characterised, and the *Mtb* growth inhibitors identified. Furthermore, the obtained results, the impact and potential applications are listed. Links to scientific publications referring to the NATT foreground are provided. Also, detailed information can be found on each beneficiary of the NATT consortium, including direct links to the partner's homepages, their email addresses and an overview of relevant literature published by each partner in the past. An overview of the NATT meetings and the conference organized by the consortium is also available. Finally, the EC contribution, the duration of the project as well as the start and end date can be found on this website. During the course of the NATT project the website was updated on a regular base in close collaboration with the Davis group.

The consortium members have published the scientific data and technological advances generated during the course of the project in high-ranking, peer-reviewed journals. In total **20 scientific papers** have originated from the NATT research. This foreground was presented as well at several workshops and conference across Europe and beyond. At **20 scientific venues** NATT results have been presented in front of the research community, both from universities and industry. The Kaufmann group was activity involved in a training workshop in TB immunology organized in Uganda. Furthermore, the NATT project has been acknowledged and presented to **the general public, media and policy makers** on 7 different occasions. The Zerial group participated in Junior Doktor Programme and the Dresden Science Night (http://www.dresden-wissenschaft.de/index.php?id=veranstalter).

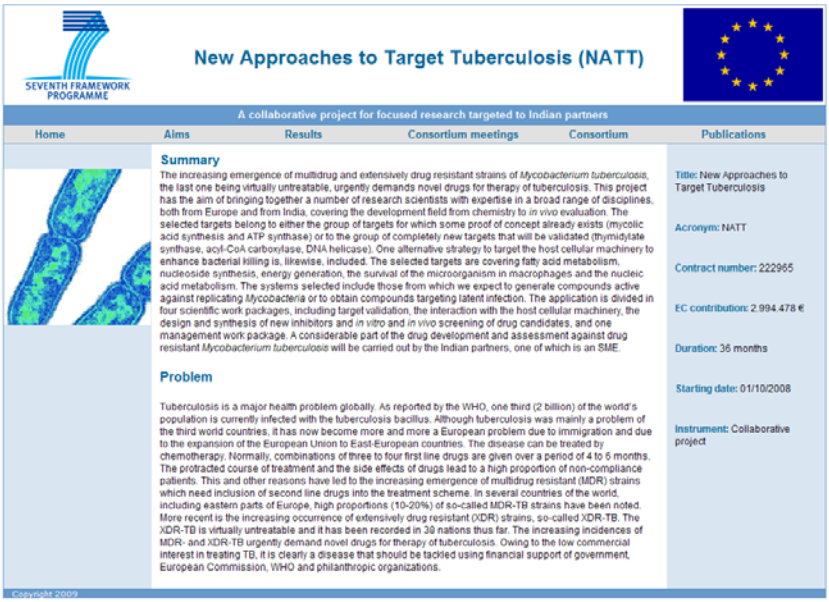
For the valorisation of the discoveries, the consortium had available most technologies within the framework of the collaboration. The medicinal chemistry groups were able to validate the purity of synthesized compounds and to validate schemes for the larger scale production. The validation at the biological level could be done within the consortium by determining the *in vitro* and *in vivo* antibacterial activity of synthesized compounds, including assaying the inhibitory activity against a series of multi-drug resistant strains. However, assistance will be needed for the validation of the pharmaco-kinetics, bio-informatics and animal toxicity. These validations are normally done according to well-established

procedures in pharmaceutical and biotechnological companies. For the protection and further development of substantial and relevant knowledge generated during this project, the following steps have been or will be followed: a) protection of intellectual property by the institutions that have contributed to the finding by filing patent applications; b) identifying potential licensees (pharmaceutical companies) that might be interested to further develop the knowledge. The management of intellectual property (IP) arising from the consortium achievements will be the responsibility of each partner that contributed to the said IP.

The Chattopadhyaya and Upadhayaya group **have filled a patent application** dealing with quinoline and naphthalene derivatives as anti-mycobacterial agents (WO2009091324; US2011059948). The Zerial group is planning the exploitation of the automated platform in the form of patenting and has initiated discussions about **possible partnerships with the pharmaceutical industry**. The major part of the project has been performed at the Technology Development Studio (TDS) at Max Planck Institute, Molecular Cell Biology and Genetics (MPI-CBG) that is a specialized unit focusing on high throughput and high performance screens. The TDS has been established with the support of the EU FP6 Project Endotrack and now the NATT project provided supplementary support for the further development of the technologies. The TDS **offers and performs workshops and training for dedicated graduates and postgraduates** to further develop their skills. MPI-CBG and the TDS also actively participate in open door activities to present the cutting-edge scientific technologies to the lay public.

1.5. The NATT Website and Contact Details

Additional information about the NATT consortium and the participating partners can be found on the **NATT website**: http://www.natt-tbc.com. The website offers a concise summary for anyone who is looking for more information on tuberculosis as a major global health problem. The emphasis is laid on dormant tuberculosis and on the increasing emergence of MDR- and XDR-TB strains. An overview of the project's research is provided focussing on the scientific strategies the consortium has applied, the selected *Mtb* targets characterised, and the *Mtb* growth inhibitors identified. Furthermore, the obtained results, the impact and potential applications are listed. Links to scientific publications referring to the NATT foreground are provided. Also, detailed information can be found on each beneficiary of the NATT consortium, including direct links to the partner's homepages, their email addresses and an overview of relevant literature published by each partner in the past. An overview of the NATT meetings and the conference organized by the consortium is also available. Finally, the EC contribution, the duration of the project as well as the start and end date can be found on this website.



Project coordinator

Prof. Piet Herdewijn, Ph.D.

Katholieke Universiteit Leuven
Laboratory for Medicinal Chemistry
Minderbroedersstraat 10, bl X bus1030
3000 Leuven
Belgium

website: http://www.kuleuven.be/research/researchdatabase/researchteam/50000720.html
email: Piet.Herdewijn@rega.kuleuven.be

Partners

Prof. Stefan H. E. Kaufmann, Ph.D.

Max Planck Gesellschaft zur Förderung der Wissenschaften E.V.
Max-Planck-Institute for Infection Biology, Department of Immunology
Charitéplatz 1
10117 Berlin
Germany

website: http://www.mpiib-berlin.mpg.de/research/immunology.html
email: kaufmann@mpiib-berlin.mpg.de

Prof. Marino Zerial, Ph.D.

Max Planck Gesellschaft zur Förderung der Wissenschaften E.V.
Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden,
Pfotenhauerstrasse 108
01307 Dresden
Germany

website: http://www.imprs-mcbb.de/groupleader/zerial.html
email: zerial@mpi-cbg.de

Prof. Jyoti Chattopadhyaya, Ph.D.

Uppsala Universitet
Institute of Cell and Molecular Biology
Bioorganic chemistry
Husargatan 3, Box 581
75123 Uppsala
Sweden

website: http://www.boc.uu.se/boc14www/res_proj/Res_general_info.html
email: jyoti@boc.uu.se

Douglas Young, Ph.D.
Elaine O. Davis, Ph.D. (left the project on March 31st 2011)

Medical Research Council
MRC National Institute for Medical Research
The Ridgeway, Mill Hill
London NW7 1AA
United Kingdom

website: http://www.nimr.mrc.ac.uk/research/douglas-young/
website: http://www.nimr.mrc.ac.uk/mycobact/davis/
email: dyoung@nimr.mrc.ac.uk

Matthias Wilmanns, Ph.D.

Europaisches Laboratorium für Molekularbiologie
EMBL Hamburg Outstation
Notkestrasse 85
22607 Hamburg
Germany

website: http://www.embl-hamburg.de/research/unit/wilmanns/index.html
email: wilmanns@embl-hamburg.de

Rajesh S. Gokhale, Ph.D.

Institute of Genomics and Integrative Biology
University campus
Mall Road
Delhi
India

website: http://igib.res.in/Sprofile/director.html
email: rsg@igib.res.in

Prof. Prati Pal Singh, Ph.D.

National Institute of Pharmaceutical Education and Research
Department of Pharmacology and Toxicology
Sector-67, Phase-X, S.A.S. Nagar
160 062, Punjab
India

website: http://www.niper.gov.in/
email: drppsingh2005@gmail.com

Ram S. Upadhayaya, Ph.D.

Institute of Molecular Medicine
New Drug Discovery Research
International Biotech Park
Genesis Campus, Phase-II, Opp Infosys
Tal Mulshi Hinjewadi,
411057 Pune
India

Email: shankarram@immindia.org