Drug development targeting *Mycobacterium tuberculosis* signaling

Ph. D. thesis

Edina Rita Székely

Semmelweis University
School of Pharmaceutical Sciences

Supervisor: Dr. György Kéri, professor, D.Sc.

Referees: Dr. László Tóthfalusi, associate professor, Ph.D.
Dr. Zoltán Bártfai, professor, D.Sc.

Head of the Theoretical Exam Committee:

Dr. Tamás Török, professor, D.Sc.

Members of the Theoretical Exam Committee:

Dr. Éva Ádám, professor, D.Sc.
Dr. Rita Farkas Tömösköziné,
senior research fellow, Ph.D.

Budapest
2012

1 Introduction

Tuberculosis (TB) is a major health problem with one-third of the world's population being infected by *Mycobacterium tuberculosis*. A new individual is infected every second whilst another one dies from the disease every 15 s. The problem is that the disease is not always active. Pathogenic mycobacteria stay nondividing in a persisting, a dormant state and are reactivated only during physiological stresses, for example after HIV infection, in the course of treatment with anti-inflammatory agents, immunosuppressive drugs or in otherwise immunocompromised individuals.

The estimated number of TB infected people in 2010 was 8.8 million and estimated number of death was 1.45 million, mostly in countries of Asia and Africa. The TB infected cases is increasing in developed countries as well, due to the weakening of the immune system.

In Hungary in 1950, the Korányi National Institute of TB and Pulmonology counted more than 45,000 new cases until in 2009 only 1448. In 1950 there were 490 new cases per 100,000 inhabitants but in 2009 only 14,4. In 2010 the number of infected people increased a bit to 1811.

The normal tuberculosis has a treatment and can be cured. The treatment is based on the appropriate combination and dose of the drugs for the required period.

The elimination of *M. tuberculosis* form the tissue based on the combination of the four first line drugs (rifampicin, izoniazid, pyrazinamide, ethambutol) takes very long time (at least 6 months) and often challenges the patience of both the patient and the staff. Due to long treatment duration compliance is a major issue and noncompliance results in a large number of treatment failures and leads to the emergence of drug resistant *M. tuberculosis* strains. To avoid inappropriate treatment the WHO promoted the DOTS (Directly Observed Treatment with Short-course chemotherapy) strategy. DOTS consists of five components and is both practical and effective for diagnosis, treatment and monitoring of tuberculosis.

1.1 Some target proteins of *Mycobacterium tuberculosis*

1.1.1 Protein kinases (PknA, PknB, PknG)

Protein kinases play important role in the fight against infectious diseases. *M. tuberculosis* encodes 11 Ser/Thr protein kinases from which PknA and PknB are

transmembrane receptor kinases and play important role in the cell division and morphology and are essential for the survival of the bacteria. GarA is the physiological substrate of PknB with an FHA (forkhead-associated) domen on the C-terminus. The FHA domen is essential for the phosphorylation of the substrate at Thr22, at the N-terminus.

PknG is a soluble STPK playing important role in the glutamate metabolism of the mycobacterium and inhibits the phagosome-lysosome fusion in infected macrophages. PknG is non-essential for the growth of M. tuberculosis, but essential for the surviving in the host.

1.1.2 Nicotinamide adenine dinucleotide kinase (NADK) and Nicotinamide adenine dinucleotide sythetase (NADS)

NAD kinase catalyzes the phosphorylation of NAD in the presence of ATP in a magnesium-dependent reaction, representing the sole source of freshly synthesized NADP in all living organisms. The enzyme is essential for the growth even in multidrug-resistant *M. tuberculosis* strains. Consequently it is an attractive target for novel antitubercular agents.

NAD synthetase catalyzes the ATP-dependent conversion of nicotinic acid adenindinucleotide (deamido-NAD) to NAD, which represents the last step in NAD *de novo* biosynthesis.

2 Aim of the work

The aim of my work was the identification and characterization of new antituberculotic agents, which target different validated proteins (mainly kinases) of the *M. tuberculosis* pathogen and the survival and growth of the bacteria itself. Due to the fact that the work was done under the framework of the New Medicines for Tuberculosis (NM4TB, LSHP-CT-2005-019923) and the SME-STREP for Tuberculosis Drug Development (TB-DRUG, FP6-CT-2006-037217) projects, my Ph.D. thesis contains results which were obtained in collaboration with the project partners.

Thanks to the modern techniques of molecular biology several target proteins were identified which play important role in the physiological processes of the bacteria and with the proper target inhibition the pathogen can be killed and eliminated.

In the early phase of the drug discovery the aim is to find a successful lead molecule, which can be developed further and enter to preclinical, clinical phases. Due to the

increasingly strict rules the drug development become very complex and expensive, which can be optimized with improving efficiency and through this, decreasing the dose dependent side effects.

To select the proper lead molecule thousands of compounds need to be tested in biochemical assay system. Most frequently fluorescence based medium and high throughput screening (MTS/HTS) methods are used to select relatively fast the lead molecule.

During my work I optimized biochemical MTS/HTS methods and tested thousands of molecules for the following five, physiologically important proteins: PknA, PknB, PknG, NADK and NADS.

Unfortunately the target based drug development also has disadvantages as often happens that the compound effective in biochemical assay does not have good inhibitory effect *in vitro* or *in vivo.*

To avoid this failure the whole cell-based drug development could be a good solution but the target need to be identified later.

3 Materials and methods

3.1 Compounds

Test compounds were selected from Vichem's Nested Chemical Library™ (NCL) of kinase inhibitors, which contains more than 12,000 molecules based on 108 core structures. Small focused sub-libraries were built around the leads using a unique, proprietary kinase inhibitory chemistry. These molecules are mostly small molecular ATP binding site inhibitors and represent a broad spectrum of the potentially active chemical structures.

The EVL (Extended Validation Library) contains around 1,000 molecules based on diverse structures and can be considered as the base-library of the NCL.

3.2 Radiometric kinase assay

The radiometric PknB kinase assay was carried out in 20 µl final volume on 96-well plates. The reaction started with the addition of the ATP solution (20 µM ATP; 1.5 µCi of [γ-^{33}P]ATP; 500 µM $MnCl_2$) into the kinase solution (10 µM GarA; 2.5 nM PknB; 50 mM Hepes pH=7.0; 0.01% Brij35; 1mM DTT; 5% glycerol). The reactions were conducted at room temperature for 30 min and stopped with 5 µl 150 mM H_3PO_4. 4 µl

of each well were spotted onto P81 cellulose paper, which was thoroughly washed with 1% H_3PO_4 five times for 10 min to wash away any unreacted labelled ATP. The dry P81 papers were visualized and quantified with PhosphorImager system (Storm, Molecular Dynamics). All test compounds were initially added at 50 µM concentration. The actives from the primary screen were subsequently tested at 5 µM concentration and IC_{50} values were determined using eight concentration steps. K252-a and staurosporine were used as the reference inhibitors.

3.3 Transcreener technology

3.3.1 HTRF® Transcreener ADP technology

The HTRF® Transcreener® ADP assay (Cisbio) is a competitive immunoassay in which native ADP and d2-labeled ADP compete for binding to a monoclonal anti-ADP labeled with Eu^{3+} cryptate. The assay consists of two steps: an enzymatic step followed by a universal detection step.

PknA: 20 mM HEPES pH=7.5; 1 mM DTT; 1 mM $MnCl_2$; 5 % glycerol; 0.01 % Brij35; 45 µM ATP; 40 µM GarA; 1.5 µM PknA

3.3.2 Transcreener® ADP^2 FP technology

The Transcreener® ADP^2 FP assay is a far-red, competitive fluorescence polarization (FP) assay based on the detection of ADP. The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to an ADP^2 Antibody. The tracer is displaced by ADP, the invariant product generated during the enzyme reaction. The displaced tracer freely rotates leading to a decrease in fluorescence polarization. The assay uses a far red tracer to minimize interference from fluorescent compounds.

PknB: 50 mM HEPES pH=7.0; 1 mM DTT; 0.5 mM $MnCl_2$; 0.01 % Brij35. 5 % glycerol; 2.25 µM ATP; 0.1 mg/ml GarA; 1 nM PknB

PknG: 20 mM MOPS pH=7.5; 1 mM DTT; 10 mM $MnCl_2$; 0.01 % Brij35; 5 % glycerol; 10 µM ATP; 0.1 mg/ml GarA; 15 nM PknG

NADK: 40 mM Tris pH=8.0; 1 mM DTT; 10 mM $MgCl_2$; 100 mM NaCl; 100 mM KCl; 0.01 % NP-40; 5 % glycerol; 400 µM ATP; 1,200 µM NAD; 800 nM NADK

3.3.3 Transcreener™ AMP/GMP technology

The Transcreener™ AMP/GMP assay is a far-red, competitive fluorescence polarization (FP) assay based on the detection of AMP/GMP. The principle is the same as in case of the Transcreener® ADP[2] FP assay except that AMP/GMP is present instead of ADP.
NADS: 20 mM HEPES pH=7.5; 1 mM DTT; 10 mM $MgCl_2$; 22 mM KCl; 10 mM NH_4Cl; 100 µM ATP; 200 µM NaAD; 140 nM NADS

3.4 Virtual screening

3.4.1 Structure-activity relationship

To generate pharmacophore model we used our in-house developed software. We built the quantitative structure–activity relationship (QSAR) model based on the experimental data using the ADABoost method, where we calculated thousands of molecular descriptors and correlated them with the biological data.

3.4.2 Docking

The published crystal structures of the proteins were used for docking and the Vichem, Tripos and WDI compound libraries (19,033 molecules) were screened virtually. The docking was performed based on the interaction energies (H-bridge, Van der Waals, Coulomb interaction). Docking calculations were performed by means of the docking program FlexX 1.20.1. with default settings. Thirty docking poses were saved per ligand. Docking poses were scored by the own scoring function of FlexX.

3.5 Antibacterial assay

The in vitro activity of compounds (10 µM) against *Corynebacterium glutamicum* (ATCC 13032) and
M. tuberculosis H37Rv was determined using the resazurin reduction microtiter assay (REMA). Single dose drug susceptibility of *C. glutamicum* was determined in a 384 well plate format (20 µl assay) using clear flat bottom polystyrene microplates (Corning). Compounds were mixed with an exponential phase culture diluted to OD_{600nm} of 0.001 and incubated overnight at 30 °C. Resazurin was added (2 µl of 0.025% w/v) and plates were incubated for 4 h. Bacterial growth was determined following resazurin reduction monitored by fluorescence (excitation 570 nm, emission

590 nm). Single dose drug susceptibility of *M. tuberculosis* and MIC was determined similarly, but in a 96 well plate format (100 µl assay) in clear flat bottom polystyrene microplates (Corning). After 7 days incubation at 37 °C, resazurin was added and incubated for 20 h at 37 °C before fluorescence reading.

3.6 Macrophages Infection Assay

THP1 cell line was differentiated with 20 ng/ml PMA, aliquoted and grown overnight in RPMI containing 10 % FBS and 1 % glutamine in 5 % CO2 at 37 °C. *Mycobacterium bovis* BCG were opsonized with human serum and used to infect macrophages at a ratio of 10 bacteria per cell. Infection was carried out for 2 hours, followed by washing with fresh media. To kill non-internalized bacteria, 100 µg/ml gentamicin was added to the medium. Inhibitors were added daily at final concentration of 10 µM and were incubated with infected macrophages for 24 and 48 hours. For viability test the media was replaced after washing with media with no antibiotics, infected macrophages were collected, washed with warm PBS and plated onto 7H10 plates supplemented with 10% OADC for colony forming units measurements (CFU's).

3.7 KinaTor™ (Target Fishing)

The derivatized compound was attached to the solid matrix by the chemists based on a previous publication. The inactivated *M. tuberculosis* lysate was provided by EPFL. The column used for the affinity chromatography experiment was Superdex 75 HR 10/300 GL. The resin was equilibrated with 5 ml of 20 mM HEPES; 0,25 % Triton X-100; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 M NaCl containing solution (v=100 µl/min). 10 ml of the TB lysate was flowed through with v=50 µl/min flow rate. After washing (3 ml, 20 mM HEPES; 0.25 % Triton X-100; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 150 mM NaCl, v=100 µl/min), the proteins were eluted with 3 ml of 20 mM HEPES; 0.25 % Triton X-100; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 150 mM NaCl, 10 mM ATP, 20 mM $MgCl_2$ and 1 mM inhibitor containing solution (v=50 µl/min). The affinity chromatography was also performed using inhibitor contained and inhibitor free resins. The proteins were identified by ESI-QTOF MS at the Department of Medicinal Chemistry, University of Szeged.

4 Results

4.1 Target-based drug development

4.1.1 Compound screening against PknB

4.1.1.1 Radiometric PknB kinase assay

In the radiometric PknB kinase assay the focused compound library (EVL) was tested (~1,000 molecules) first at 50 μM, in duplicates. The hit molecules (85 molecules) were tested at 5 μM, as well, and the IC_{50} of 35 inhibitors was determined in duplicates with 8 points titration curve. The IC_{50} and MIC values of the hit compounds are shown in **Table 1**.

Table 1 IC_{50} and MIC values of the hit molecules

ID	IC_{50} (μM)	MIC (μM)
VIC-12112	0.274	49
VIC-12147	2.37	>34
VIC-16212	0.82	>55
VIC-16641	0.258	49
VIC-17103	0.584	>47

4.1.1.2 Virtual screening

4.1.1.2.1 Structure-activity relationship

One of the main steps of the drug development is the determination of structure-activity relationship which can happen based on the experience of the chemist and also by different software.

To perform this model, pIC_{50} values were approximated from 1,050 individual data points of percent inhibition and 30 different IC_{50} values. Data points with negative percent inhibition values were omitted. The final database contained 606 data points. We have screened a virtual library (~70,000 compounds) with the best model in order to come up with novel patentable scaffolds.

4.1.1.2.2 Docking

In case of the PknB kinase 3 crystal structures available in the PDB (Protein Data Bank) database were used for docking (1MRU, 2FUM, 1O6Y). The effect of the predicted best 271 compounds was investigated in biochemical assay and in some cases the IC_{50} was also determined.

4.1.1.3 Methods based on fluorescence polarization

However the sensitivity of the radiometric methods is very good, they have the disadvantage that special laboratory conditions are needed, to perform the assay takes long time and needs special preparation (storage of the isotope labelled substrate, half-life etc.). The aim of my work was to set up non-radiometric biochemical assay methods adaptable for MTS/HTS screening.

4.1.1.3.1 PknB Transcreener® ADP^2 FP technology

Around 300 molecules coming from the structure-activity relationship and the docking were tested in PknB Transcreener® ADP^2 FP assay. The IC_{50} and MIC values of the hits can be found in **Table 2**

The molecules were tested first at 10 µM and the IC_{50} values of the hits with higher inhibition than 75 % were determined in duplicates with 8 points titration curve.

The PknB Transcreener® ADP^2 FP assay was validated with the hit molecules from the radiometric kinase assay.

Table 2 The IC_{50} and MIC values of the hits from the PknB Transcreener® ADP^2 FP assay

ID	IC_{50} (µM)	MIC (µM)
VIC-12150	0.6211	>100
VIC-16135	4.98	>100
VIC-16640	0.99	>100
VIC-16719	0.27	>100
VIC-17494	0.129	>100
VIC-17499	0.688	>100

Unfortunately the molecules that inhibited PknB in the biochemical assay did not have inhibitory effect on *M. tuberculosis*.

4.1.2 Inhibitors against PknG

4.1.2.1 Virtual screening

4.1.2.1.1 Docking

To perform the docking for PknG only one crystal structure was available in the PDB (2PZI). Screening the Vichem, Tripos and WDI databases (19,033) 521 molecules were selected for further biochemical test.

4.1.2.2 Methods based on fluorescence polarization

4.1.2.2.1 PknG Transcreener® ADP² FP technology

Screening the predicted molecules in the PknG Transcreener® ADP² FP assay 12 molecules had lower IC_{50} than 1 µM (**Table 3**), but none of these inhibited the growth of the pathogen.

Table 3 The IC_{50} and MIC values of PknG inhibitors

ID	IC_{50} (µM)	MIC (µM)	ID	IC_{50} (µM)	MIC (µM)	ID	IC_{50} (µM)	MIC (µM)
VIC-15473	0.42	>100	**VIC-16027**	0.07	>100	**VIC-16113**	0.02	>100
VIC-15662	0.06	>100	**VIC-16048**	0.23	>100	**VIC-16204**	0.56	>100
VIC-15844	0.69	>100	**VIC-16077**	0.1	>100	**VIC-16284**	0.26	>100
VIC-15870	0.24	>100	**VIC-16092**	0.47	>100	**VIC-16315**	0.01	>100

Due to the important role of the PknG in the phago-lysosomal fusion some of the hit molecules were also tested on infected macrophages. Out of the 3 inhibitors shown in **Figure 1** the VIC-16315 inhibited the coloning forming in 40 %, until the VIC-20017 reference compound (AX20017, IC_{50}=0.3 µM, MIC>100 µM), used for the docking as well, showed only 26 % inhibition. The VIC-15844 rather activated the coloning forming than inhibited (116 % CFU).

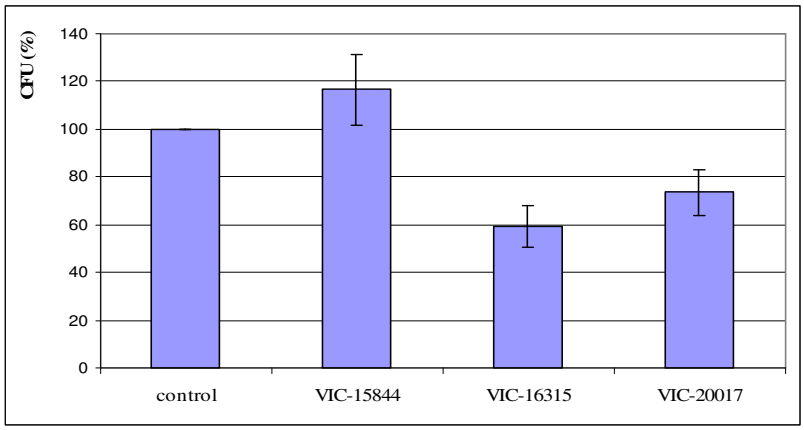

Figure 1 Infected macrophage assay with PknG inhibitors

4.1.3 Inhibitors agains PknA

4.1.3.1 Methods based on fluorescence polarization

4.1.3.1.1 *PknA HTRF® Transcreener® ADP technology*

We screened the EVL compound library at 10 μM concentration in duplicates using the HTRF method. The **Table 4** contains the IC_{50} and MIC values of the inhibitors.

Table 4 Activity of the PknA inhibitors in biochemical and whole cell based assays

ID	IC_{50} (μM)	MIC (μM)
VIC-6557	42	>200
VIC-15739	19	>200
VIC-16317	5.2	>200
VIC-16819	10	>200

4.1.4 Inhibitors against NADK

4.1.4.1 Virtual screening

4.1.4.1.1 Docking

Due to the lack of published data about *M. tuberculosis* NADK inhibitors we considered the docking a good starting point for NADK inhibitor development.

We performed the docking based on the crystal structure of the *M. tuberculosis* NADK in complex with NAD (1Y3I).

After the screening of the 19,033 compounds, 49 molecules were selected for *in vitro* NADK screening. Unfortunately none of these molecules had inhibitory effect measured by spectrophotometer.

The spectrophotometric assay is considered as a low throughput method that is why I preferred to set up an assay suitable for MTS/HTS screening.

4.1.4.2 Methods based on fluorescence polarization

4.1.4.2.1 NADK Transcreener® ADP² FP technology

Screening the EVL library on NAD kinase the compounds were tested at 98 μM concentration in duplicates. Surprisingly none of the 1,000 molecules showed inhibitory effect which is contradictory to the experience taken in other kinase assays.

This result can be explained by a paper published in 2009 where the authors describe that the perfect NAD kinase inhibitors are the NAD analogues instead of the ATP ones. The di-(8-bromo-adenosine)-disulfide NAD analogue published in this paper was synthesize and tested in the NADK Transcreener® ADP² FP assay as a reference compound (VIC-23779).

Table 5 The published and measured IC_{50} and MIC values of the reference NAD analogue

ID	Molecular weight	IC_{50} (μM) published	IC_{50} (μM) measured	MIC (μM)
VIC-23779	722.40	19	97.4	>100

The **Table 5** shows that the published IC_{50} of the reference molecule is difference from the measured value in the NADK Transcreener® ADP² FP assay which can be explained with the fact that the assay method used in the paper was different and the assay conditions are missing from the description (NAD, NADK concentration, etc.). Looking at the MIC value it is evident that however the reference compound has some inhibitory effect in biochemical system; it does not inhibit *M. tuberculosis*.

4.1.5 Inhibitors against NADS

4.1.5.1 Virtual screening

4.1.5.1.1 Docking

At the beginning of my work the crystal structure of the *M. tuberculosis* NAD synthetase was not available, only the one of *B. subtilis* NADS but as the sequence alignment of *M. tuberculosis* and *B. subtilis* synthetase domains revealed a sequence similarity of 40% with strict conservation of all residues demonstrated to play a role in catalysis, we decided to use the *B. subtilis* NADS structure for docking.

After the virtual screening of 19,033 compounds 47 were selected as potential NADS inhibitors for *in vitro* biochemical test.

4.1.5.2 Spectrophotometric assay

After testing the NADS inhibitor candidates predicted by docking the lowest IC_{50} value was around 50 µM, which is not very prominent, but optimizing the compounds several molecules disposed of low micromolar IC_{50}. The IC_{50} and MIC values of the best NADS inhibitors can be found in **Table 6**.

Table 6 NADS inhibitorok IC_{50} és MIC értéke

ID	IC_{50} (µM)	MIC (µM)	ID	IC_{50} (µM)	MIC (µM)	ID	IC_{50} (µM)	MIC (µM)
VIC-4450	18	99	**VIC-12592**	8	51	**VIC-12979**	5.1	99
VIC-9982	18	64	**VIC-12595**	5	56	**VIC-12990**	4	26
VIC-11647	17	65	**VIC-12965**	10	35	**VIC-13017**	12.5	69
VIC-11649	14	64	**VIC-12976**	6.2	127	**VIC-13268**	3.5	33
VIC-12553	13	72	**VIC-12977**	4.5	67	**VIC-15108**	45	36

4.1.5.3 Methods based on fluorescence polarization

4.1.5.3.1 NADS Transcreener™ AMP/GMP FP technology

Independently from the results of the spectrofotometric method, the ~ 1,000 molecules of the focused library of Vichem (EVL) and additional 700 compounds were tested at

98 µM using the MTS/HTS method. Some of the potent inhibitors were also tested on *M. tuberculosis* (**Table 7**).

Table 7 The IC_{50} and MIC values of the inhibitors coming from the NADS Transcreener™ AMP/GMP FP screening

ID	IC_{50} (µM)	MIC (µM)	ID	IC_{50} (µM)	MIC (µM)	ID	IC_{50} (µM)	MIC (µM)
VIC-12730	4.91	ND	**VIC-13597**	15.71	ND	**VIC-13734**	13.78	>100
VIC-12734	5.41	ND	**VIC-13708**	6.77	ND	**VIC-14185**	10.30	>100
VIC-12735	10.18	ND	**VIC-13716**	8.59	ND	**VIC-14201**	9.72	>100
VIC-13592	5.64	ND	**VIC-13720**	7.81	ND	**VIC-15740**	15.22	>100
VIC-13594	12.45	ND	**VIC-13722**	9.45	ND	**VIC-16319**	19.42	20
VIC-13595	10.37	ND	**VIC-13731**	10.94	ND	**VIC-16889**	18.53	ND

4.2 Whole cell-based drug development

During the last ten years, pharmaceutical companies have based their strategy on target-based approaches that have met with very poor outcomes as all TB drug candidates that are currently in clinical trials were first selected on the basis of their antibacterial activity. In consequence, whole cell-based screening approaches are being reconsidered as a means of identifying new active scaffolds as well as finding new targets.

In the whole cell-based screening the resazurin reduction microtiter assay was applied, where ~12,000 compounds were tested on *C. glutamicum* and ~1,000 on *M. tuberculosis.* The rationale for using the fast-grower *C. glutamicum* in this study stems from the fact that it has a similar cell wall structure, a much smaller genome than *M. tuberculosis*, and as opposed to *M. smegmatis*, contains only four STPK (PknA, PknB, PknG, PknL).

Based on these screenings 17 molecules inhibited the growth of *M. tuberculosis*, from which 3 compounds were selected for further drug development.

Table 8 Inhibitors of *M. tuberculosis* (M = mutagen)

ID	Screening score		Genotoxicity 10 mM	Citotoxicity MCC (µM)	MIC (µM)
	***M. tuberculosis* H37Rv**	***C. glutamicum* ATCC 01332**			
VIC-7777	0.9	-0.1	-	>100	3.1
VIC-9376	1	0.5	-	>100	3.1
VIC-18469	0.5	0.2	-	100	6.25

In this Ph.D. thesis I only show the work on VIC-9376 and VIC-18469.

4.2.1 VIC-9376 and derivatives

Based on the structure similarities between our nitroquinoxalines and the antituberculotic BTZ043 in preclinical phase, we wanted to determine whether the VIC-9376 also targets the same protein that BTZ043. Both the VIC-9376 and the BTZ043 were tested on different wild type and BTZ resistant strains. However BTZ043 is more potent than VIC-9376, the inhibitory effect of both molecules is abolished on BTZ resistant strains. This result strongly suggests that VIC-9376 and its derivatives also target DprE1.

4.2.2 VIC-18469 and derivatives

Benzoquinoxalines have some structural similarity with the clofazimine, drug used in combination with rifampicin and dapsone for the treatment of leprosy, and potent inhibitor against *M. tuberculosis* NDH-2 in the biochemical system of collaborative partners. Based on this fact the inhibitory effect of some of our benzoquinoxaline hits were tested on NDH-2 *in vitro*. However the VIC-18469 did not inhibit NDH-2, two of its derivatives (VIC-12955 and VIC-13202) had prominent activity ($IC_{50(12955)}$=0.54 μM; $IC_{50(13202)}$=4.14 μM).

4.3 Target identification

The major disadvantage of the whole cell-based screening is that the mode of action and the target protein of the selected inhibitor are unknown. To answer these questions is often a very difficult issue and means a big challenge for the researcher.

4.3.1 KinaTor™

The KinaTor™ technology was already applied successfully in our research group in close collaboration with the Max Planck Institute for the identification of different human kinases. Based on this I thought to use this technology for the identification of *M. tuberculosis* target proteins.

However the MIC value of our KinaTor™ compound (VIC-14385) is not the best, it is close to the MIC of the most effective benzoquinoxaline, thus I applied it for the identification of target proteins.

I performed the affinity chromatography using resin conjugated with VIC-14385 and blank resin as well, to eliminate the non-specific binding proteins. 8 proteins were identified by ESI-QTOF MS (**Table 9**).

Table 9 Proteins identified by ESI-QTOF MS

Taxonomy	Gene	Protein	Molecular weight (Da)
Mycobacterium tuberculosis	*cfp30B*	bimodular glyoxalase	27324.5
Mycobacterium tuberculosis	*rv1261c*	nitroreductase	16738.2
Mycobacterium tuberculosis	*lprA*	lipoprotein	24885.8
Mycobacterium bovis (BCG / Pasteur 1173P2)	*atpD*	ATP synthase subunit β	53076.7
Mycobacterium bovis (BCG / Pasteur 1173P2)	*groLE1*	chaperon protein	56709.3
Mycobacterium tuberculosis	*hsaC*	iron-dependent extradiol dioxygenase	33564.4
Mycobacterium tuberculosis	*acpM*	acyl carrier protein	12506
Mycobacterium tuberculosis	*pstS1*	phosphate-binding protein	38226

5 Discussion and conclusion

During my Ph.D. work I mainly focused on the development and optimization of biochemical assays used for target-based drug development. I worked on 5 proteins (PknA, PknB, PknG, NADK, NADS) important in *M. tuberculosis* signalling. The compounds were selected for *in vitro* screening from the 12,000 molecules containing library of Vichem Chemie Ltd. The molecules are mostly ATP-like and allosteric compounds. I also performed the expression and purification of the proteins used in biochemical assays.

Acquiring and applying the PknB radiometric kinase assay on ~ 1,000 molecules I found several compounds with nanomolar IC_{50}, but unfortunately none of them proved to be inhibitor of *M. tuberculosis.* We performed QSAR analysis and *in silico* docking to find more potent inhibitors. The predicted hit molecules and the ones suggested by the chemists were tested by Transcreener® ADP^2 kinase technology. The inhibitors effective *in vitro* did not inhibited *M. tuberculosis* in the whole cell-based screen.

The docking of the 19,033 compounds was also performed in case of the PknG kinase and the predicted inhibitors were tested in Transcreener® ADP^2 kinase assay. However the biochemically active PknG inhibitors did not effect the growth of *M. tuberculosis*

some of them were tested on infected macrophages as PknG is proved to play important role in the phago-lysosomal fusion. The most potent inhibitor seemed to be the VIC-16315 inhibiting the coloning formation in 40 % after 48 hours of incubation.

In case of the PknA kinase docking was not performed due to the lack of the crystal structure, but I planed to obtain inhibitors testing the 1,000 diverse molecules containing EVL library of Vichem Ltd. I used the HTRF Transcreener ADP technology for screening, but unfortunately none of the hit molecules inhibited the pathogen in the whole cell-based assay.

The predicted molecules coming from the docking of NADS and NADK targets were tested by spectrophotometer at the University of Piemonte. Until in case of the NADK none of the molecules come out as inhibitors, the best NADS inhibitor was the VIC-15108 (IC_{50}=73 µM, MIC=50 µM) from several candidates. Based on the structures of the hit compounds other 130 were selected for further screening, from which the VIC-13268 (IC_{50}=3.5 µM, MIC=33 µM) and VIC-12990 (IC_{50}=4 µM, MIC=26 µM) molecules proved to be the most potent ones. Simultaneously with this I optimized the NADK and NADS Transcreener methods and tested the EVL molecular library. None of these molecules inhibited NADK even at a very high concentration (98 µM). This can be explained with a publication which demonstrates that the ATP-like inhibitors do not have effect on NADK in contrast with the NAD analogues.

In case of the NADS target several inhibitors were found but only few of them were tested on *M. tuberculosis*.

As a conclusion of the target-based screening it can be summarized that however several hit molecules come out from the biochemical test, none of them had significant inhibition on *M. tuberculosis*. As a solution we decided to screen the molecules directly on the whole cells and obtain those molecules which are able to penetrate the thick cell wall of the pathogen.

We screened the 12,000 molecules containing NCL library of Vichem Ltd. on *C. glutamicum* where the MIC of the hit molecules were determined on *M. tuberculosis.* In parallel with this the small, diverse library (EVL) was directly tested on *M. tuberculosis*.

After the mutagenicity and cytotoxicity tests of the 17 inhibitors of the pathogen 3 compounds were selected for further development (VIC-9376, VIC-7777, VIC-18469), from which I only focus on the VIC-9376 (nitoquinoxaline) and VIC-18469 (benzoquinoxaline) molecules in my Ph.D. thesis. Several derivatives of both

compounds were tested. The VIC-9376 has structural similarity with the BTZ043, so we were interested to know whether both compounds target the same protein. The tests on different wild type and mutant strains demonstrated that the VIC-9376 also targets the DprE1 protein.

The structure of VIC-18469 resembles that of the clofazimine, inhibitor of NDH-2 in biochemical experiments of project partners. Thus testing VIC-18469 and some derivatives on NDH-2, VIC 12955 and VIC-13202 proved to be potent inhibitors.

For the identification of the *M. tuberculosis* targets of the selected inhibitors I used the KinaTor™ method which was already successfully applied in case of human proteins. 8 proteins were identified by this technique until now, but none of them play crucial role in the surviving and growth of *M. tuberculosis*.

6 Publication list

Publications:

- Székely R, Wáczek F, Szabadkai I, Németh G, Hegymegi-Barakonyi B, Erős D, Szokol B, Pató J, Hafenbradl D, Satchell J, Saint-Joanis B, Cole ST, Őrfi L, Klebl BM, Kéri G. A Novel Drug Discovery Concept for Tuberculosis: Inhibition of Bacterial and Host cell Signalling. Immunology Letters 116:225–231, (2008)
- B. Hegymegi-Barakonyi, R. Székely, Z. Varga, R. Kiss, G. Borbély, G. Németh, P. Bánhegyi, J. Pató, Z. Greff, Z. Horváth, G. Mészáros, J. Marosfalvi, D. Erős, C. Szántai-Kis, S. Garavaglia, S. Perozzi, M. Rizzi, D. Hafenbradl, M. Ko, Y. Av-Gay, B.M. Klebl, L. Őrfi, G. Kéri. Signalling inhibitors against *Mycobacterium tuberculosis* – early days of a new therapeutic concept in tuberculosis. Curr. Med. Chem., 15:2760-2770, (2008)
- Magnet S*, Hartkoorn RC*, Székely R*, Pató J, Triccas JA, Schneider P, Szántai-Kis C, Őrfi L, Chambon M, Banfi D, Bueno M, Turcatti G, Kéri G, Cole ST. Leads for antitubercular compounds from kinase inhibitor library screens. Tuberculosis., 90:354-60. (2010) (*first authors)

Patent:

- S. Cole, R. Hartkoorn, S. Magnet, J. Pató, Gy. Kéri, L. Őrfi, P. Bánhegyi, R. Székely. Quinoxaline derivatives and their use for the treatment of mycobacterial infections (2010) P1000356

7 Acknowledgements

I would like to thank my supervisor, Prof. György Kéri the possibility to do my Ph.D. at Vichem Chemie Ltd., the strong support and the useful advices. I also thank Dr. László Őrfi the help and advice in the field of medicinal chemistry and Dr. Csaba Szántai-Kis to reveal the secrets of drug screening during these years.

I would like to acknowledge the help of all colleagues of Vichem Chemie Ltd. specially the successful work of Dr. János Pató and Dr. Dániel Erös in chemistry and the results of the QSAR analysis made by Bálint Hegymegi-Barakonyi and Dr. Zsolt Szegedi.

Within the confines of the NM4TB project I thank the group of Prof. Pedro Alzari the help in the radiometric PknB kinase assay and screening, the group of Prof. Kai Johnson for teaching me the expression and purification of the PknB, PknG and GarA proteins and thanks to Prof. Stewart Cole for the invitation to perform the PknA screening and the whole cell-based screening on *C. glutamicum.* I also would like to acknowledge the work of his colleagues in the whole cell-based screening, cytotoxicity and genotoxicity determinations. Thanks to AstraZeneca India for the results of the NDH-2 tests.

Within the confines of the TB-DRUG project I thank the group of Prof. Menico Rizzi the results of the spectrophotometric assay and also the NADK and NADS enzymes, and Prof. Peter Sander the tests on *M. tuberculosis*.

I thank Dr. József Lengyel the possibility to perform some radiometric measurements at the Central Isotope Laboratory, SOTE NET.

Thanks to Prof. Beáta Vértessy for giving me place at the Enzymology Institute to perform the expression and purification of the proteins.

I acknowledge the work of Dr. Róbert Kiss in the *in silico* screens.

Tanks to Prof. Yossef Av-Gay (University of British Columbia) for the results of the infected macrophage assay, to Proteros biostructures GmbH for the selectivity profiling, to CFRI the help in the 2D electrophoresis and to the Department of Medicinal Chemistry at the University of Szeged for the analysis of the proteins by ESI-QTOF.

Thanks to the Peptide Chemistry Research Group of ELTE-MTA for the synthesis of the peptide substrates.

I would like to thank my family and friends the priceless patience, love and support that was indispensable to achieve this Ph.D. thesis.