Signalling Inhibitors Against *Mycobacterium tuberculosis* – Early Days of a New Therapeutic Concept in Tuberculosis

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Abstract: Tuberculosis causes nearly two million deaths per year world-wide. In addition multidrug-resistant mycobacterial strains rapidly emerge so novel therapeutic approaches are needed. Recently, several promising mycobacterial target molecules were identified, which are involved in bacterial or host cell signalling e.g. the serine/threonine protein kinases, PknB and PknG, NAD kinase and the NAD synthetase. Here we describe some early efforts in the development of novel signal transduction inhibitory anti-mycobacterial drugs using a multiple target approach, with special emphasis on the kinase inhibitory field. Initially, we are using the Nested Chemical Library™ (NCL) technology and pharmacophore modelling. A hit-finding library, consisting of ~19000 small molecules with a bias for prototypic kinase inhibitors from our NCL library and commercial sources was virtually screened against these validated target molecules. Protein structures for the virtual screening were taken from the published three dimensional crystal structures of the enzymes. The hits from the virtual screening were subsequently tested in enzymatic assay systems. Potent hits were then tested for biological activity in macrophages, infected with mycobacteria. The final goal of this exercise is not only to identify potent anti-mycobacterial substances, but also a common pharmacophore for the mycobacterial target PknG in combination with PknB, NAD kinase and/or NAD synthetase. This common pharmacophore still needs to be a unique pharmacophore for the mycobacterial target proteins over human off-targets. Such a pharmacophore might then drive the optimization of a completely new profile of an antibiotic agent with activity against latent mycobacteria and resistance mycobacterial strains.

Keywords: *Mycobacterium tuberculosis*, PknA, PknB, PknG, NAD kinase, NAD synthetase.

INTRODUCTION

Each year, 1% of the global population is infected by the most widespread bacterium, *Mycobacterium tuberculosis*. 10% of those individuals infected with mycobacteria develop active tuberculosis (TB). Although the majority carries mycobacteria without becoming sick, any weakening of the immune system, e.g. caused by physiological stress, HIV infection or malnutrition, increases the chance that the bacilli will become active [1].

Latent infections might stay silent for any period of time, from weeks to decades, before it actually develops into active disease. Therefore latent mycobacterial infections present a particular socioeconomic health risk, potentially leading to a disease outbreak without geographical or chronological boundaries [2].

Existing therapy of non-resistant, active TB consists of the treatment cocktail of four drugs: isoniazid, rifampicin, pyrazinamide and ethambutol, given over a continuous period of at least 6 months [3]. Multi-drug resistance (MDR) reflects the resistance at least against the two mainstay medications, isoniazid and rifampicin.

In 1993, the WHO declared TB as a global emergency and 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 [4]. Although a success in many nations, less than a quarter of the active cases of TB in the world receive this treatment. Due to long treatment duration compliance is a major issue and non-compliance results in a large number of treatment failures and leads to the emergence of drug resistant *M. tb*. strains.

TB gains additional importance as a serious threat due to the general globalization, which led to an increased amount of international travel, promoting the spread of TB and MDR-TB, especially in developing countries, where MDR-TB in particular is a serious concern [5].

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MDR-TB is an epidemiological problem, presenting a huge worldwide challenge. Treatment of active MDR-TB requires chemotherapy for up to 2 years, frequently surgical intervention and has high rates of treatment failure and death. Second-line drugs (such as fluoroquinolones, p-aminosalicylic acid, kanamycin, cycloserine, ethionamide, amikacin, capreomycin, thiacetazone) used to treat drug resistant TB are in general less effective, more toxic and also more expensive than the first-line drugs. These liabilities of the second line treatments underscore the need for novel and more effective anti-mycobacterial agents, which might combat not only MDR-TB, but also latent TB.

The evolution of MDR-TB as well as the fact that proliferating organisms and persistent organisms are hypothesized to be in two entirely different physiological states [6] turn the identification and development of a single agent, combining all these features, into an enormous task. Potentially successful strategies shall either aim at a) combining the traditional antibiotics with a novel drug or b) applying the multiple-target approach. Provided the more recent success of signal transduction inhibitors (see below), we decided to focus our efforts on the generation of novel anti-TB drugs on this area.

The progress in understanding the mechanisms of signal transduction has been astonishingly rapid and in the last few years important themes and principles of signal transduction have emerged. Defects in signalling have been found as the underlying cause of cancer and other human diseases [7].

Significant success has also been achieved regarding the development of small molecule kinase inhibitors resulting in at least 8 different chemical entities on the market for the treatment of various cancers. More than one hundred different small molecule kinase inhibitors are currently under clinical investigation with a significant fraction in therapeutic areas other than cancer [8]. Infectious diseases are another field of research, where small molecule kinase inhibitory compounds are directed to block and prevent disease progression [9].

Genome sequence data revealed that the *M. tuberculosis* genome contains 11 eukaryotic-like serine/threonine protein kinases (PknA, PknB, PknC, PknD, PknE, PknF, PknG, PknH, PknJ, PknK, PknL) which are regulators of metabolic processes, including transcription, cell development and interaction with host cells [10,11].

Three of these 11 protein kinases – PknA, PknB and PknG – showed to be required for mycobacterial growth [12,13]. The eukaryotic-like serine/threonine PknA and B are receptor like transmembrane proteins [14] while PknG is a soluble protein [15].

It was demonstrated that PknA participates in regulating morphological changes associated with cell division [16-18], PknB is essential to sustain mycobacterial growth and the overexpression causes morphological changes and major growth in mycobacterial cells which produce defects in the cell wall synthesis and in cell division [13,17]. PknG is a secreted protein kinase that prevents phagosomal-lysosomal fusion [19]. We have chosen PknB as a target for inhibition in order to be able to block mycobacterial growth and proliferation with a PknB inhibitor. PknG has been picked, since its inhibition leads to the eradication of mycobacteria from macrophages, one of the major reservoirs of non-dividing mycobacteria [20,21]. The aim is to generate either selective inhibitors for these 2 kinases or one inhibitor specifically inhibiting both of these mycobacterial kinases without any cross-reactivity into the human kinome. This optimization strategy seems to be perfectly reasonable, since PknB and PknG are significantly different from the human protein kinase family in terms of their overall homology [11,22].

NAD(P) biosynthesis is considered an additional important source of novel targets for the development of antibacterial agents [12,23]. NAD(P) is a cofactor with a dual function that is involved in both energy and signal transduction in all organisms [24-26] and unbalancing its homeostasis results in a severe alteration of cell viability eventually leading to cell death [27]. *Mycobacterium tuberculosis* has been reported to be highly sensitive to any depletion of NAD(P) homeostasis [28,29].

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 [30]. *M. tuberculosis* NAD kinase displays an allosteric kinetics and the capability to use both ATP and inorganic-polyphosphates as phosphoryl donors [31,32] therefore showing relevant functional differences with human NAD kinase, a strictly ATP dependent enzyme with hyperbolic kinetics [33]. The enzyme is essential for the growth even in multidrug-resistant *M. tuberculosis* strains. Consequently it is an attractive target for novel antitubercular agents [12,34,35].

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 [36]. Inhibitors of *B. subtilis* NAD showing promising antibacterial activity have been published [37,38] and the *M. tuberculosis* enzyme was shown to be essential for the growth of *M. Tuberculosis* [12]. We consider NAD synthetase as an exploitable target for the identification of novel antitubercular agents [39-41].

Just as for the serine/threonine kinases our strategy is to either generate selective inhibitors against NAD kinase and NAD synthetase or to identify a common pharmacophore, which allows the generation of dual specificity inhibitor without any cross-reactivity to the human homologues.

Recently an extensive characterization of NAD biosynthesis in *M. tuberculosis* has been reported, aiming at the identification and validation of specific NAD biosynthetic enzymes as sensible targets for the development of antituberculotic drugs. The careful analysis conducted revealed that those enzymes of the pathway that are involved in both *de novo* synthesis and recycling of NAD are actually validated targets against replicating and non-replicating *M. tuberculosis*. NAD synthetase and NAD kinase are involved in both *de novo* synthesis and recycling on NAD in *M. tuberculosis*. Therefore, both enzymes are highly promising targets for the treatment of tuberculosis [42].

In this article we present a new concept and some preliminary data on a multiple target approach of signalling inhibition of *M. tuberculosis*. We focus on the above de-

scribed targets (PknB, PknG, NAD kinase, NAD synthetase) that we selected for this multiple target approach to identify novel compounds capable of killing resistant bacteria as well. The idea is to identify a common pharmacophore for at least two of these targets. This joint pharmacophore based lead molecule has to be efficient on the bacterial targets and it should not show side effects on human cells. In this approach the inhibition of additional human kinases should be acceptable (excluding the untouchables e.g. InsR). On the other hand inhibiting two (or more) survival signals of the bacteria (e.g. PknB) should result in elimination of the bacteria and decreases the chance of generation of resistance mutations. Because of network signalling in normal cells the off-target inhibition of host cell kinase pathway(s) should be compensated with alternative signalling pathways in the host cells. In this case we endeavour at least 10 times better inhibition on the bacterial kinase targets then on the human off-target(s). Our primary target is PknG. Due to its role in the phago-lysosomal fusion, PknG inhibition is considered to be essential in this multiple target approach, since it is the target with a clear role in latency and persistence. By applying such a new strategy to the generation of potentially novel treatment modality, we hope to avoid drug-resistance, to shorten the overall treatment duration for infected patients and to eradicate non-proliferating sources of mycobacteria from the patients.

The host cell kinase Akt1 was identified as a new target for MDR-TB recently and several Akt1 inhibitors were reported as inhibitors of MDR-TB infected macrophages in colony-forming units assay [43].

The above described multi-targeted approach is the extension of the multi-targeted monotherapeutic antibacterials to the signaling pathway targets [44].

MATERIALS AND METHODS

Compounds

The tested compounds were selected from the Vichem's Nested Chemical Library™ (NCL) and the Tripos database.

Vichem Chemie Research Ltd. (Budapest, Hungary) developed a unique hit finding method based on its NCL technology [45]. The NCL platform is a living data knowledge base together with physically available small molecule inhibitors, which was originally built from the knowledge gleaned from the experience obtained during the last 20 years of kinase inhibitory chemistry.

The NCL is based on 108 core structures, containing more than 10000 small molecules. Within this library, small sublibraries are built around 800 individual kinase inhibitor scaffolds [45,46].

Compounds from the Tripos database are commercially available.

The logP and logS values were calculated with ACD Labs v.6.02 and v.6.04 respectively.

Proteins

PknB and GarA were expressed in *E. coli* and purified as described [47]. *Mycobacterium tuberculosis* PknG was cloned and purified from *E. coli*. Details will be described elsewhere [48]. Recombinant *B. subtilis* and *M. tuberculosis* NAD synthetase were prepared following published procedures [27,41]. Recombinant *M. tuberculosis* NAD kinase was expressed and purified by applying a published protocol [32]. Akt1 was purchased from ProQinase.

Virtual Screening

Crystal structures were prepared for docking by removing waters and adding hydrogen atoms in Sybyl 6.9.1 (Tripos Associates: St. Louis, MO). PDB IDs of the crystal structures used in this study: 1Y3I, 1NSY, 1MRU, 2FUM, 1O6Y, 2PZI. *M. tuberculosis* NAD synthetase consists of 679 residues that appear organized into an N-terminal domain endowed with glutaminase activity that confers to the mycobacterial enzyme the capability of using glutamine as a nitrogen source and a C-terminal synthetase domain, where NAD synthesis takes place. On the other hand the *B. subtilis* NAD synthetase is a strictly ammonia dependent enzyme that contains only a synthetase domain consisting of 272 residues. 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.

Since the crystal structure of *M. tb.* NAD synthetase was not available at the start of this project, we used the structure of the *B. subtilis* enzyme in complexed with substrates [39,49]. Docking calculations were performed by means of the docking program FlexX 1.20.1 [50]. FlexX was run with default settings. Thirty docking poses were saved per ligand. Docking poses were scored by the own scoring function of FlexX as well as by the ones available in the CScore package in Sybyl. Random decoys were collected from the World Drug Index (WDI). These compounds as well as ATP and known inhibitors (actives) were docked to the binding site to select the most efficient scoring function combination for ranking the docked poses for a ligand, and for ranking the best poses for all ligands. In some cases, multiple scoring function combinations were found to select known actives efficiently from the random decoys. Here, we used a consensus scoring method, i.e. compounds highly ranked by all the chosen scoring function combinations were selected as virtual hits. Besides, the top ranked compounds from all individual scoring function combinations were also selected. Virtual screening was performed on three of the target proteins (PknB, NAD kinase, NAD synthetase) with our in-house compound library, pre-filtered by kinase-like features. A pre-filtered library of the Leadquest database (Tripos) was also virtually screened against the three crystal structures. Virtual hits were carefully analyzed by their docking poses at the binding site. False positives, e.g. ligands partially positioned outside the binding site, were excluded from further investigations. Compounds that formed significant interactions with binding site residues were chosen for subsequent *in vitro* testing. Finally we proposed some hundred compounds for biological testing. The visualisations of docking results were prepared by MOE 2005.06 (Molecular Operating Environment, Chemical Computing Group, Inc.: Montreal, Canada, 2005).

Biochemical Assay Methods

PknB Kinase Assay

The radioactive PknB kinase assay was performed as described [51], using [γ-^{33}P]ATP. The reaction was carried out in the presence of GarA, the physiological substrate of PknB, on 96-well plates at room temperature for 30 minutes. The spots on the P81 paper were visualized and quantified with PhosphorImager system (Storm, Molecular Dynamics).

All compounds were first tested at 10μM concentration and IC_{50} values were calculated using KaleidaGraph software.

PknG Kinase Assay

Radioactive Kinase Assay

The PknG kinase activity was determined as described [51] in 96-well plate using [γ-^{33}P]ATP. The reaction was conducted using myelin basic protein (MBP) at room temperature for one hour.

The radioactive signal was determined with a μbeta microplate counter.

Non-Radioactive Kinase Assay

The non-radioactive PknG assay was performed with Transcreener™ Kinase assay method (BellBrook Labs) in 384 microtiter plates in total volume of 10 μl. In case of the determination of % of inhibition the final concentration of the compounds was 10 μM. The final PknG concentration was 30 nM and 0.17 mg/ml GarA was used as substrate. The ATP (Sigma) concentration was used around the Km_{app} (10 μM). The buffer contained 20 mM MOPS (Sigma) pH:7.5, 1 mM DTT (Bio-Rad), 10 mM $MnCl_2$ (Sigma), 0.01% Brij 35 P (Fluka), 5% glycerol (Sigma) and 2 mM EGTA (Sigma). The kinase solution was incubated for 60 min at room temperature.

The IC_{50} values were calculated with XLFit (IDBS) software.

NAD Kinase Assay

NAD kinase activity was determined in a coupled assay by monitoring the production of NADP in the reaction, through its conversion to NADPH in the presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase (GPDH) from bakers' yeast. Excess of GPDH was used to ensure rapid conversion of NADP to NADPH so that rate-limiting step in the system was the NAD kinase reaction. While excess of GPDH decreased the possibility that inhibition of GPDH gives a false result, this assumption was verified by confirming that the NAD kinase inhibitors did not inhibit GPDH.

The enzyme inhibition assay was carried in a standard cuvette/standard spectrophotometer with a total reaction volume of 200 μl. The reaction buffer contained 100 mM TRIS-HCl buffer (pH 8.0), 100 mM KCl, 100 mM NaCl, 20 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, 1 mM glucose-6-phosphate, 0.2 U of yeast GPDH, 0.25 mg/ml bovine serum albumin and 15 μg of *M. tuberculosis* NAD kinase enzyme. The GPDH activity was assayed in a total reaction volume of 200 μl containing 100 mM TRIS-HCl buffer (pH 8.0), 100 mM NaCl, 100 mM KCl, 4 mM NADP, 1 mM glucose-6-phosphate and 0.2 U of yeast GPDH. All reagents were purchased from Sigma.

UV absorbance at 340 nm was used as detection method. All compounds were first tested at 100μM concentration and IC_{50} values were calculated using KaleidaGraph software.

NAD Synthetase Assay

NAD synthetase activity was determined in a coupled enzyme assay by monitoring the production of NAD in the reaction through its conversion to NADH in the presence of ethanol and alcohol dehydrogenase (ADH) from bakers' yeast. Excess ADH was used to ensure rapid conversion of NAD to NADH so that rate-limiting step in the system was the NAD synthetase reaction. While excess ADH decreased the possibility that inhibition of ADH gives a false result, this assumption was verified by confirming that the NAD inhibitors did not inhibit ADH.

The enzymes inhibition assay was carried in a standard cuvette/standard spectrophotometer with a total reaction volume of 200 μl. The assay system contained 60 mM HEPES buffer (pH 8.5), 10 mM $MgCl_2$, 22 mM KCl, 10 mM NH_4Cl, 1.1 mM NaAD, 1.5 mM ATP, 80 μl ethanol 1% v/v, yeast alcohol dehydrogenase at 0.025 mg/ml, 0.5 mg/ml bovin serum albumin and 15 μg of *M. tuberculosis* NAD synthetase. The ADH activity was assayed in a total reaction volume of 200 μl containing 60 mM HEPES buffer (pH 8.5), 0.5 mM NAD, 80 μl ethanol 1% v/v, 5 μg of ADH and 100 μg of bovine serum albumin. All reagents were purchased from Sigma.

UV absorbance at 340 nm was used as detection method. All compounds were first tested at 100 μM concentration and IC_{50} values were calculated using KaleidaGraph software.

Akt1 Assay

The assay was performed with IMAP FP technology in 384 microtiter plates (Corning 3676, Sigma). Akt1 kinase activity was assayed at 10 μM compound concentrations in duplicates, in total volume of 8 μl. The final Akt1 concentration was 5 nM. 5FAM-GRTGRRNSI-NH_2 (purchased from G. Mező, Peptidechemistry Research Group of Hungarian Academy of Sciences, Eötvös Loránd University, Budapest) was used as substrate at final concentration of 100 nM. The final ATP concentration was used around the Km_{app} (7.42 μM).

The reaction buffer contained 20 mM MOPS (Sigma) pH: 7, 1 mM DTT (Bio-Rad), 2 mM $MgCl_2$ (Sigma), 0.4 mM $MnCl_2$ (Sigma), 0.01 Titon X-100 (Sigma). The kinase reaction was incubated for 60 min at room temperature and stopped by addition of 15 μl IMAP binding solution (100% Progressive binding buffer A, 0% Progressive binding buffer B, 1:400 IMAP binding reagent dilution).

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% CO_2 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

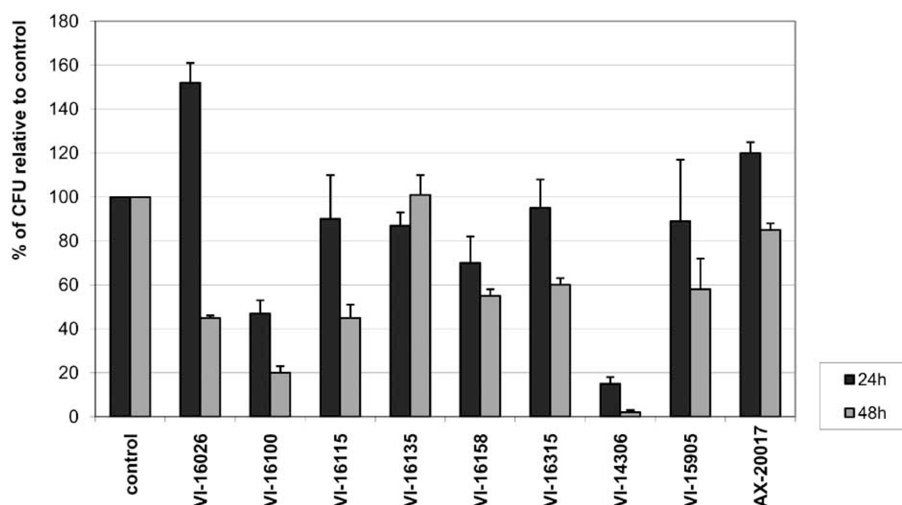

Fig. (1). Infected macrophage assay with PknG inhibitors after 24 and 48 hours.

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).

Toxicity Assay

The toxicity of the compounds was determined as described [52].

RESULTS AND DISCUSSION

PknG has been selected as the primary target for the search of a common pharmacophore because of its important role in preventing the phago-lysosomal fusion and the maintenance of the phagosomes as a reservoir for non-proliferating mycobacteria [19]. Previously we tested more than 1000 compounds of Vichem's NCL, a chemically diverse kinase inhibitor library. IC_{50} values of the most potent compounds and hits were determined in a radioactive kinase assay [51]. Based on the structures and on the macrophage results we continued the optimization around the initial hits focused on improving their drug-likeliness and the efficacy. Optimized compounds from those medicinal chemistry cycles achieved in IC_{50} values for PknG in the 10-300 nM range on the isolated target.

Subsequently, selected members of the optimization efforts were incubated for 24 and 48 hours at a concentration of 10 µM with human macrophages, infected with mycobacteria. Many of them proved to be effective, resulting in a low percentage of colony forming units relative to the control (the inhibitory properties of selected compounds are shown in Fig. **1**). VI-14306 shows 80% inhibition after 24 hours and 99% inhibition after 48 hours comparing to the control CFU value, which makes it a very interesting and potent lead compound. Another promising inhibitor seems to be the VI-16100, which inhibits 80% of the colonies after 48 hours of incubation. The inhibition on the Akt1 kinase by these inhibitors was also measured to clarify the relevance of PknG target (Table **1**). Our PknG inhibitors had no effect on Akt1 and they also had no effect on bacteria containing mutant

Table 1. PknB, PknG and Akt1 Inhibitory Values and Toxicity of the Compounds Measured in Macrophage Assay

ID	PknG % of inhibition at 10 µM	PknG IC_{50} (µM)	PknB % of inhibition at 10 µM	PknB IC_{50} (µM)	Toxicity % of PI staining	Akt1 % of inhibition at 10 µM
VI-16026	98	0.03	6.5	ND	66.4	12.1
VI-16100	98	0.02	53.5	ND	97.3	7.9
VI-16115	96	0.02	21.6	ND	23.0	5.8
VI-16158	99	0.04	8.2	ND	15.5	<1
VI-16135	98	0.05	80.3	3.85	8.3	22.1
VI-16315	99	0.01	21.5	ND	4.2	4.6
VI-14306	99	0.16	37.8	ND	98.7	5.5
VI-15905	98	0.05	17.7	ND	95.8	<1
AX-20017	90	0.3	9.8	ND	7.9	2.3

ND: The IC_{50} value of the compound with a % of inhibition lower than 75% was not determined.

PknG (data not shown). In summary, starting from the original 4,5,6,7-Tetrahydrobenzo[b]thiophene series [19], we have generated PknG inhibitors with good potency in the macrophage infection model but most of the compounds had some cellular toxicity except the VI-16315 which is one of our potent multiple target inhibitors.

The VI-16315 is not the most potent inhibitor with 60% of a remaining CFU value after 48 hours, but is a very interesting compound from the perspective of the common pharmacophore strategy. It shows IC_{50} values of 12 nM in the PknG assay and 55 μM in the NAD synthetase assay and has no significant cellular toxicity in human cells (Table **1** and Table **2**). This inhibitor is a promising starting point to come up with effective multiple target inhibitory compounds, yet further optimization cycles are clearly needed to improve potency at least by 4 orders of magnitude. The selectivity of this compound for the mycobacterial NAD synthetase compared to the human homolog is significant (Table **2**).

To further characterise the ATP binding site of PknG from the inhibitors point of view we performed a docking study. We used the available crystal structure of PknG (2PZI) [21]. The previously identified selective PknG hit compounds and the dual target hits were docked into the 2PZI structure to understand the binding mode of these hit compounds.

Fig. (**2**) shows the binding mode of the compound VI-16315 at the ATP binding site of PknG and the NaAD (Nicotinic acid adenine dinacleotide) binding site of NAD synthetase.

Another dual target inhibitor (VI-16135) has been identified for PknB and PknG. This compound has low toxicity but it does not have an effect on infected macrophages, so further optimization is needed.

PknG inhibitors, like AX-20017, have been demonstrated to promote the phagosomal-lysosomal fusion, but they might not necessarily induce the elimination of the bacteria located in the blood stream or in the granules. In contrast, PknB inhibitors have the potential to act as bactericidal compounds [53].

To identify potent inhibitors against PknB from a wide range of kinase inhibitor molecules, we built a docking model and performed structure-based virtual screening. We used three available crystal structures of the PknB (1MRU, 2FUM, 1O6Y) [53-55] and performed the docking of the ATP, staurosporine and the mitoxantrone to each of the crystal structures. We applied 30 actives and 3000 random decoys derived from WDI and docked all of them to the ATP binding site of PknB to determine the enrichment factors and select the best scoring function combinations. Enrichment was best for the 1MRU structure, which was 11.6 at the top

Table 2. NAD Kinase and NAD Synthetase Inhibitors

ID	*M. tb.* NAD synthetase % inhibition at 100 μM	*M. tb.* NAD synthetase IC_{50} (μM)	*M. tb.* NAD kinase % inhibition at 100μM	*M. tb.* NAD kinase IC_{50} (μM)	Human NAD kinase % inhibiton at 100 μM
VI-3242	75	67	<1	ND	46
VI-6026	100	50	<1	ND	<1
VI-6569	100	50	<1	ND	76
VI-8995	50	80	<1	ND	100
VI-9311	65	72	<1	ND	40
VI-11085	59	69	<1	ND	32
VI-12507	98	55	<1	ND	100
VI-12524	77	67	<1	ND	90
VI-12538	82	64	<1	ND	100
VI-12539	90	60	<1	ND	90
VI-13047	67	71	<1	ND	100
VI-13632	56	58	100	53	<1
VI-15108	63	73	<1	ND	10
VI-15666	76	62	<1	ND	90
VI-15765	52	94	<1	ND	100
VI-15901	64	66	<1	ND	50
VI-15929	40	102	<1	ND	46
VI-16047	65	76	<1	ND	20
VI-16315	85	55	<1	ND	32
VI-16581	71	72	<1	ND	10
VI-16816	87	63	<1	ND	65
VI-17731	65	82	<1	ND	<1

ND: The IC_{50} value of the compound with a % of inhibition lower than 75% was not determined.

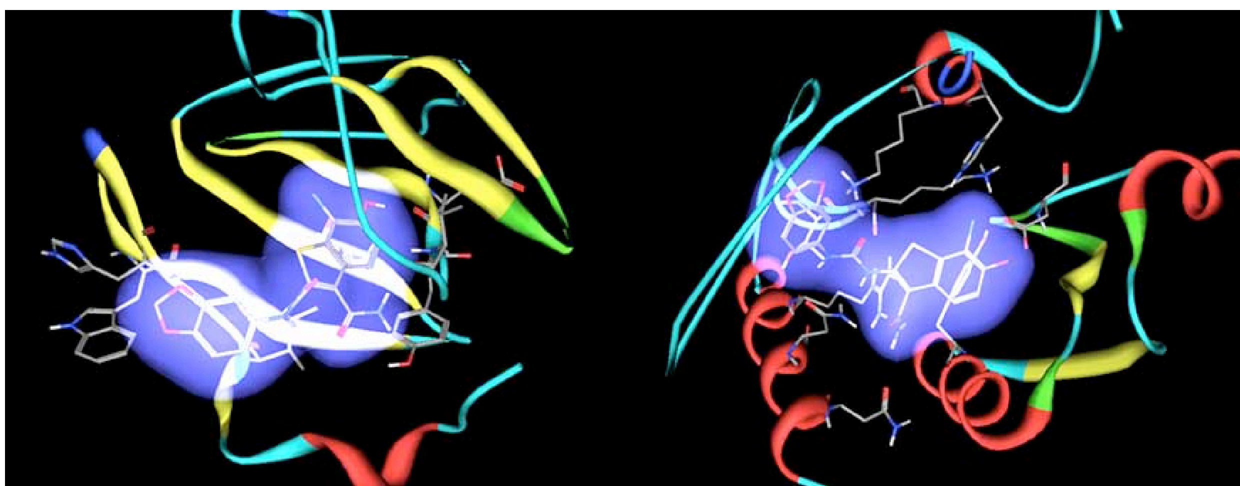

Fig. (2). Compound VI-16315 in the ATP binding site of PknG (left) and in the NaAD binding site of NAD synthetase (right).
Important residues of PknG, NAD synthetase and VI-16315 are shown as stick representations. The molecular surface of the inhibitor is shown in blue. The protein backbone is shown as tube representation.

2% value. 19033 compounds were virtually tested on the 1MRU structure. These molecules were derived from the Vichem and Tripos chemical libraries. After virtual screening 209 Vichem and 51 Tripos compounds were selected as candidate inhibitors for subsequent *in vitro* confirmation.

The candidate compounds were tested in radioactive PknB kinase assay using [γ-^{33}P]ATP and GarA as the putative physiological substrate of PknB [56].

These compounds were also tested against PknG, using the non-radioactive kinase assay, and against Akt1 kinase as well (Table **3**). Each of the presented compounds has an IC_{50} value in the nanomolar range on PknB. VI-15662 and VI-17494 are very interesting compounds, showing good inhibitory effect on PknB and PknG according to the multiple target concept.

To increase the chance to identify a potent and novel antitubercular drug that can limit the insurgence of resistance, we decided to include in our drug-discovery process, other targets responsible for cell viability in *M. tuberculosis*. NAD kinase and NAD synthetase are two coupled enzymes in the NAD(P) biosynthesis that were both shown to be essential for *M. tuberculosis* growth [12].

There are no effective published mycobacterial NAD kinase or NAD synthetase inhibitors known, but the crystal structures of the enzymes have been published [34,35,39]. In analogy to the hit finding strategy for PknB inhibitors, we performed structure-based virtual screening on these enzymes.

Aiming to find effective and selective NAD kinase inhibitors, we performed docking of the ATP and the above mentioned WDI subset of 3000 compounds into the binding site of the kinase (1Y3I) by FlexX. After the selection of the best scoring functions combinations based on ranking ATP compared to random decoys, we virtually screened the same compound libraries (Vichem, Tripos) as for PknB. The 50 best predicted compounds were then tested in the biochemical NAD kinase assay (Table **2**). For all the inhibitors we tested on NAD kinase or NAD synthetase and for which the IC_{50} is reported (in the form of inhibitor concentration versus

Table 3. Inhibitory Values of the Potent Compounds Against PknB, PknG and Akt1

ID	PknB % of inhibition at 10 µM	PknB IC_{50} (µM)	PknG % of inhibition at 10 µM	PknG IC_{50} (µM)	Akt1 % of inhibition at 10 µM
VI-437	91.5	0.337	39.3	ND[b]	52.3
VI-9501	99.1	0.210	ND[a]	ND[b]	58.9
VI-11963	98.4	0.205	ND[a]	ND[b]	68.9
VI-12150	99.6	0.621	ND[a]	ND[b]	63.5
VI-12177	97.6	0.088	ND[a]	ND[b]	80.6
VI-15662	82.4	0.870	88.8	0.6	46.8
VI-16743	94.2	0.445	49.2	ND[b]	10.4
VI-17494	97.6	0.129	75.3	3.0	16.7
VI-17499	92.4	0.688	43.3	ND[b]	8.1

ND[a]: We got extremely high values of % of inhibition, so it is possible that the compound interferes with the non-radioactive assay system.
ND[b]: The IC_{50} value of the compound with a % of inhibition lower than 75% was not determined.

Table 4. Molecular Weight and Predicted logP and logS Values of the Compounds

ID	Molecular weight g/mol	logP	logS g/l at 25 °C
VI-437	397.35	1.05 (±0.74)	0.29
VI-3242	328.37	3.07 (±0.59)	0.026
VI-6026	452.38	3.11 (±0.49)	9.4×10^{-3}
VI-6569	275.26	1.74 (±0.36)	47.10
VI-8995	321.34	3.09 (±1.58)	0.082
VI-9311	470.58	3.52 (±0.75)	2.1×10^{-4}
VI-9501	660.75	0.85 (±0.86)	0.019
VI-11085	284.30	-2.99 (±0.82)	18.76
VI-11963	714.82	1.42 (±0.79)	3.9×10^{-3}
VI-12150	503.57	1.88 (±0.86)	0.015
VI-12177	1032.76	9.10 (±1.12)	6.2×10^{-9}
VI-12507	482.51	5.58 (±0.64)	6.7×10^{-5}
VI-12524	382.42	3.45 (±0.57)	7.2×10^{-3}
VI-12538	489.54	4.82 (±0.63)	3.4×10^{-4}
VI-12539	459.51	4.74 (±0.61)	4.5×10^{-4}
VI-13047	488.59	4.37 (±0.45)	52.87
VI-13632	492.38	3.17 (±0.56)	1.9×10^{-3}
VI-14306	355.83	2.64 (±0.73)	0.020
VI-15108	438.53	4.52 (±1.00)	0.077
VI-15662	396.45	2.31 (±0.69)	3.70
VI-15666	355.83	3.04 (±0.74)	0.012
VI-15765	471.53	3.49 (±0.72)	0.023
VI-15901	439.54	4.53 (±0.65)	1.0×10^{-3}
VI-15905	290.34	3.28 (±1.42)	6.7×10^{-3}
VI-15929	354.47	2.45 (±0.61)	48.27
VI-16026	293.35	3.73 (±1.42)	9.7×10^{-3}
VI-16047	383.39	3.96 (±1.49)	4.3×10^{-4}
VI-16100	355.21	3.78 (±1.43)	8.4×10^{-3}
VI-16115	324.79	3.84 (±1.42)	5.5×10^{-3}
VI-16135	295.32	2.37 (±1.43)	0.031
VI-16158	359.23	4.54 (±1.44)	2.7×10^{-4}
VI-16315	405.82	5.36 (±1.44)	3.6×10^{-4}
VI-16581	470.55	1.59 (±0.78)	0.024
VI-16743	365.44	1.45 (±0.93)	2.59
VI-16816	470.58	2.15 (±0.92)	0.13
VI-17494	497.44	3.30 (±1.01)	0.021
VI-17499	460.58	2.49 (±1.01)	0.038
VI-17731	432.44	2.45 (±0.94)	2.66
AX-20017	264.35	2.48 (±0.56)	0.048

% of inhibition) we observed a linear behaviour that suggests linear kinetics with a Hill coefficient of 1 with no sign of co-operation.

Since NAD synthetase has both, a NaAD and an ATP binding site, we docked the structures of the published inhibitors [37] and ATP into both of them. After docking the

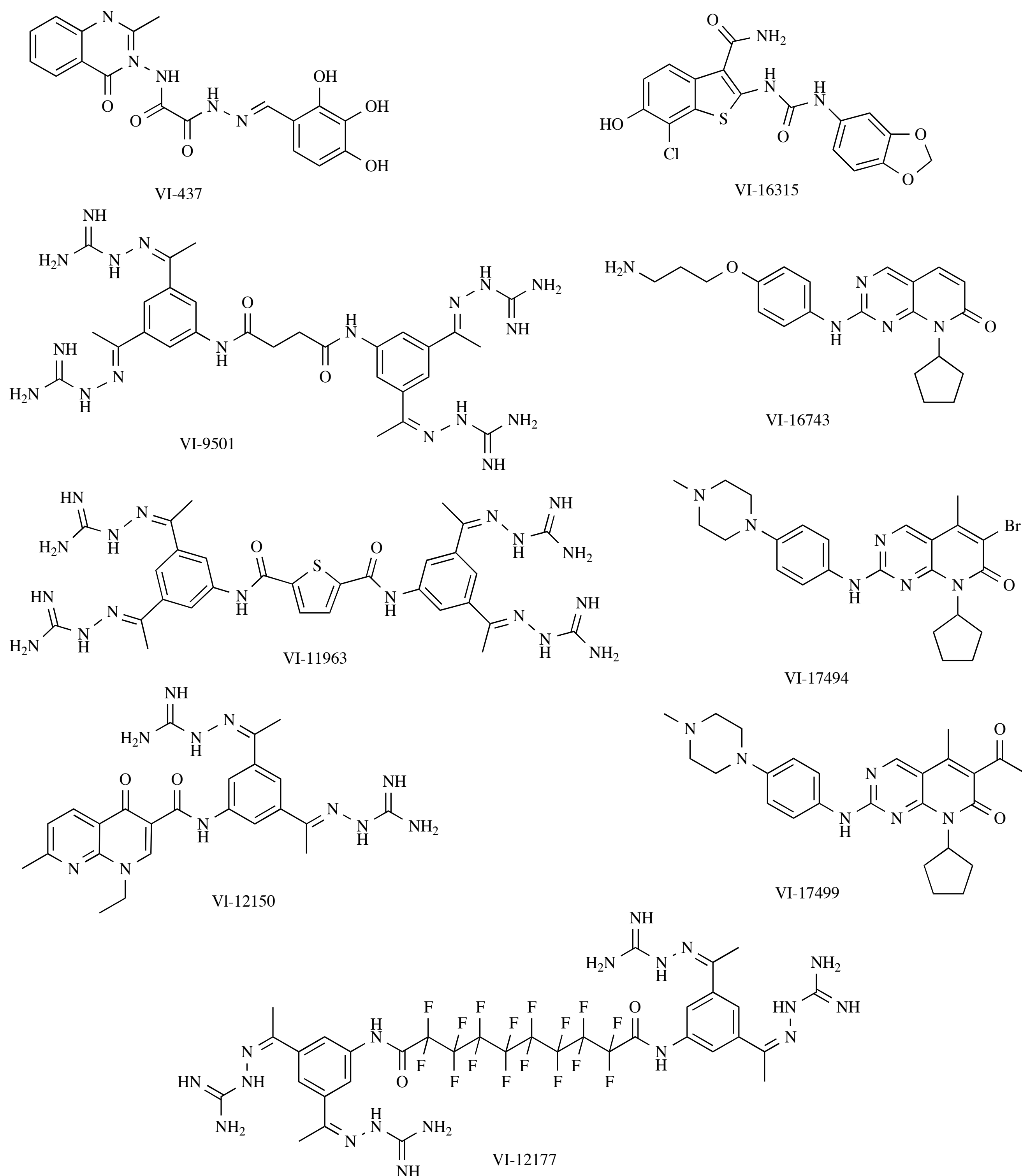

Fig. (3). The chemical structures of the most important hit compounds.

inactive compound set as well, we selected the best scoring function combinations based on ranking the inhibitors and ATP compared to the random decoys and we again screened the 19033 compounds. 50 candidate compounds were selected for NAD synthetase assay.

Although the compounds selected through the structure-based virtual screenings, were divided in two subgroups, i.e. the putative NAD synthetase and putative NAD kinase inhibitors, we decided to test all of them, at a concentration of 100 μM, against both enzymes and against the human NAD kinase as well, to check the selectivity of the compounds. A remarkable difference was observed in case of several compounds in the inhibition of NAD kinase or NAD synthetase (Table **2**). Out of the 100 tested compounds, 22 showed inhibitory activity against NAD synthetase but only one

inhibited significantly NAD kinase. The average IC_{50} of the confirmed inhibitors is in the range of 50-80 µM and therefore further chemical optimization, based on the currently being determined 3D structure of the complex with the respective target, will be required. Moreover, chemical optimization will be carried out for those hits showing to affect *M. tuberculosis* survival judged by the determination of the MIC that inhibits 99% of the growth of *M. tuberculosis*. It is worthwhile to note that the NAD kinase inhibitor, VI-13632, also inhibits NAD synthetase at a comparable rate. Based on these structures effective multiple target inhibitors should be developed by further optimization.

The molecular weight and the calculated logP, logS values are shown in Table **4**.

The chemical structures of the most important compounds effective on one or two of the selected targets are depicted in Fig. (**3**).

SUMMARY

Antibiotic resistant tuberculosis has become a major health threat worldwide. A promising strategy for treating resistant tuberculosis might be to aim for novel targets, including signalling related targets and at the same time trying to inhibit multiple targets with the same compound. We have selected and optimized potent PknG inhibitors, which *via* inducing phago-lysosomal fusion might destroy latent and even drug-resistant bacteria, residing in the macrophages. As second-tier targets, we have selected PknB, NAD kinase and NAD synthetase. For these three targets, we performed 3D structure based virtual screening, selected some promising hit compounds and indeed identified some compounds, which inhibit PknG and another target from this group. In the next series of optimization cycles, we will try to develop these multiple target pharmacophores further in terms of balanced potencies or near equipotent activities on their respective targets, while making sure that they are not inhibiting any human off-targets. This clearly is a major challenge for structure-based compound optimization, but in case of success, this strategy might lead to a completely novel treatment modality for one of the most devastating infectious disease.

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