An overview on the potential antimycobacterial agents targeting serine/threonine protein kinases from *Mycobacterium tuberculosis*

Matteo Mori[a], José Camilla Sammartino[b], Luca Costantino[c], Arianna Gelain[a], Fiorella Meneghetti[a], Stefania Villa[a], Laurent Roberto Chiarelli[*b]

[a]*Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, via L. Mangiagalli 25, 20133 Milano; Italy;* [b]*Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", Università degli Studi di Pavia, via Ferrata 9, 27100 Pavia; Italy;* [c]*Dipartimento Scienze della Vita, Università degli Studi di Modena e Reggio Emilia, via Campi 103, 41121 Modena; Italy.*

Abstract: *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), still remains an urgent global health issue, mainly due to the emergence of multi-drug resistant strains. Therefore, there is a pressing need to develop novel and more efficient drugs to control the disease. In this context, targeting the pathogen virulence factors, and particularly its signal mechanisms, seems to be a promising approach. An important transmembrane signaling system in *Mtb* is represented by receptor-type serine/threonine protein kinases (STPKs). *Mtb* has 11 different STPKs, two them, PknA and PknB, have been reported as essential, while PknG and PknH are important for virulence and adaptation, since they have been shown to be fundamental for the growth of the pathogen in infection models. Therefore, STPKs represent a very interesting group of pharmacological targets in *M. tuberculosis*. In this work, the principal inhibitors of these mycobacterial STPKs will be presented and discussed. In particular, medicinal chemistry efforts have been focused on discovering new antimycobacterial compounds, targeting three of these kinases, namely PknA, PknB and PknG. Generally, the inhibitory effects on the enzymes did not correlate with a significant antimycobacterial action in whole-cell assays. However, compounds with activity in the low micromolar range have been obtained, demonstrating that targeting *Mtb* STPKs could be a new promising strategy for the development of drugs to treat TB infections.

Please provide corresponding author(s) photograph

Keywords: Tuberculosis, antitubercular drugs, virulence inhibition, transmembrane signal transduction, Serine/Threonine Protein Kinases, kinase inhibition.

1. INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is an intracellular pathogen and the causative agent of tuberculosis (TB). Current anti-TB therapies tend to be rather effective against the treatment of this pathology, but their use is correlated with significant disadvantages, including several forms of toxicities, drug resistance and severe side effects [1]. One of the biggest problems is the development of multi-drug resistant *Mtb* strains [2, 3]. Moreover, the available treatments are sometimes inefficient at cleansing the body of all the bacteria, so some organisms can endure the pharmacological therapy [4]. Therefore, there is a great need to exploit new druggable targets [5, 6] and to develop more efficient anti-TB agents to control the disease.

*Address correspondence to this author at the Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", Università degli Studi di Pavia, via Ferrata 9, 27100 Pavia; Italy; Tel: ++39-382-987241, E-mail: laurent.chiarelli@unipv.it

In this context, targeting essential virulence factors, like *Mtb* protein kinases, seems to be a very promising approach. Signal transduction in *Mtb* involves two-component systems driven by histidine kinases [7, 8], and 11 eukaryotic-like serine/threonine kinases [9]. In addition, *Mtb* produces and secretes one Tyr kinase, PtkA [10], which is required for *Mtb* growth in macrophages [11]. While this kinase has not been considered for drug discovery yet, it is interesting to note that the substrate of PtkA appears to be the phosphatase PtpA, an essential protein for the inhibition of the acidification and maturation of host macrophages [12]. PtkA is able to transfer phosphate groups to the tyrosine phosphatase PtpA, although the role of PtpA phosphorylation in *Mtb* physiology remains to be fully elucidated [10].

While the two-component sensor system, composed of a His kinase and a response regulator, has not been studied in-depth for the development of drugs [13, 14], there is a growing interest in the discovery of new compounds able to inhibit *Mtb* serine/threonine protein kinases.

2. SERINE/THREONINE PROTEIN KINASES

XXX-XXX/14 $58.00+.00

Signal transduction is a fundamental mechanism that connects extracellular signals to intracellular regulatory mechanisms, allowing the adaptation of the organisms to changes in environmental conditions. Thanks to this system, an extracellular signal is sensed by a protein (sensor), transduced across the membrane, and then converted in an intracellular response. Reversible protein phosphorylation is one of the major mechanisms by which signal transduction can be achieved.

In bacteria, the most widely used mechanism of signal transduction is generally the two-component system driven by histidine kinases [15]. This system consists of two separate proteins, in which the sensor is a transmembrane protein that, upon the binding of a ligand, can regulate through phosphorylation the transducer, usually a transcription factor, which controls the expression of specific genes.

Another important transmembrane signaling system is regulated by the receptor-type serine/threonine protein kinases (STPKs). This class of proteins is also called "eukaryotic-like" for its structural similarity to the well-known eukaryotic serine/threonine kinase domains, despite their low sequence homology (< 30%) [16]. STPKs generally consist of an extra-cytosolic receptor domain and an intracellular kinase domain. Once the extracellular signal is sensed by the receptor domain, the cytosolic kinase domain phosphorylates substrate proteins, such as transcription factors, enzymes, structural proteins etc. This process then promotes a cascade of downstream modifications, leading to an alteration of the cellular metabolism.

Differently from the two-component system, STPKs are usually poorly distributed among bacteria, with the exception of *Acidobacteria*, some *Cyanobacteria* and *Actinobacteria*, including *Mycobacteria* [17]. Indeed, *Mtb* has 11 different STPKs [18], indicating the importance of this signal transduction mechanism. Among them, PknA, PknB, PknG and PknL were found to be highly conserved among mycobacteria, suggesting their involvement in key physiological processes in *Mtb*. Nevertheless, only two STPKs (PknA and PknB) have been reported as essential for *Mtb* growth *in vitro* [19], while the non-essential kinases PknG and PknH are required for growth in animal models or in macrophages *in vitro*, thus playing an important role in virulence and adaptation [20, 21].

Based on their sequence, STPKs have been grouped in five homology clusters, clades I-V, encompassing PknA-PknB-PknL, PknF-PknI-PknJ, PknD-PknE-PknH, PknG, and PknK, respectively [22]. Two STPKs (PknG and PknK) are soluble proteins, while the others have been proposed to be transmembrane proteins. In bacterial STPKs, and differently from the eukaryotic ones, the catalytic domains are at the N-terminus, and linked to the sensor C-terminal domain through a single transmembrane helix [23]. N-terminal domains are bilobed, with the N-lobe consisting of a curled β-sheet and a long α-helix, and the C-lobe of α-helices [24-28]; all of them are characterized by the presence of the 11 conserved subdomains, which are common to eukaryotic protein kinases, despite the low sequence identity [16, 22]. These subdomains are characterized by conserved motifs, endowed with specific functions. In particular, subdomains 1-4 and part of 5 comprise the N-terminal lobe of the kinase domain, responsible for ATP binding, whereas subdomains 5-11 are responsible for the binding of the protein substrate and the transfer of the phosphate group [15].

The bacterial STPKs are characterized by the presence of an arginine which precedes a conserved aspartic acid in the active site [29]. Similarly to many eukaryotic STPKs, they are activated by the auto-phosphorylation of specific amino acids located in the activation loop, a conserved region bordered by the Asp-Phe-Gly and Ala-Pro-Glu amino acid sequences [28, 30, 31]. The activation mechanism involves the phosphorylation of Ser/Thr residues in the activation loop upon binding of ATP at the active site [32, 33], leading to a conformational change from the catalytically inactive dephosphorylated state to the active phosphorylated form [29]. Biochemical and structural studies of the catalytic domains of PknA, PknB, PknE and PknI suggest a regulation of mycobacterial STPKs through a "back-to-back" dimerization of the N-lobes of the kinase domains [34, 35]. The dimerization, promoted by the binding of the sensor domain to a ligand, stabilizes the active conformation of the kinase domain, facilitating intermolecular autophosphorylation, thus producing a fully active kinase domain, able to phosphorylate the substrates. Finally, the dephosphorylation by PstP restores the inactive unphosphorylated monomeric form [35].

Depending on the specificity of their substrates, *Mtb* STPKs play different biological roles, being involved in a large variety of processes such as transcriptional regulation, cell wall biosynthesis, cell division and cell metabolism [23]. Therefore, the understanding of the biological role of STPKs is strictly related to the characterization of their physiological substrates. However, with the exception of PknA, PknB and PknG, which are relatively well-studied, STPK substrates are still poorly characterized. In this context, recent advances in proteomics and phospho-proteomics approaches started shedding light on the STPK-protein phosphorylation network, that regulates the mycobacterial cell cycle [36, 37].

2.1. PknA and PknB

PknA and PknB, the only two essential STPKs [19], are respectively encoded by the *Rv0015c* and *Rv0014c* genes, which are present in a conserved operon. This operon includes *pstP*, encoding the only known *Mtb* Ser/Thr phosphatase [30], *rodA* encoding a peptidoglycan synthase involved in the control of cell shape and elongation [38], and *pbpA* coding for a class B penicillin-binding protein, involved in cell wall biosynthesis. Due to their location in this operon, which is in the proximity of the origin of replication, PknA, PknB and PstP were supposed to regulate cell division and growth [23, 39]. PknB indeed plays a fundamental role in cell shape regulation and hypoxia-induced cellular replication [40, 41], and it is essential for the growth and survival of *Mtb* in the host [42]. Moreover, it was demonstrated that the transcription of *pknA* and *pknB* is increased in the exponential growth phase [43] or during

macrophage infection [44, 45], while *pknB* was found to be down-expressed in a nutrient starvation model of *Mtb* persistence [46].

The two kinases are reported to be involved in several pathways, for instance in peptidoglycan synthesis, and in morphological changes associated with cell division [47, 48]. The extra-cytoplasmic domain of PknB comprises four penicillin-binding proteins and STPK-associated (PASTA) domains, predicted to bind peptides of peptidoglycan [49], whose major function seems to reside in proper PknB localization, at mid-cell and cell poles [50].

In particular, it was recently found that PknB phosphorylates the pseudo-kinase domain of MviN, which then recruits the FhaA protein through its forkhead-associated (FHA) domain, essential for peptidoglycan synthesis [47]. Moreover, they also have a role in other pathways, such as central carbon metabolism, through the phosphorylation of the regulatory protein GarA [51, 52], or in mycolic acid synthesis, by regulating the activity of MabA, KasB, and InhA [53-55]. Finally, PknA was shown to inhibit cell division protein FtsZ, which is involved in septum formation [56].

Despite the available information about the substrates of PknA and PknB, a deep comprehension of the roles of the two kinases in *Mtb* physiology is still lacking. However, different approaches employing integrated proteomics, phosphoproteomics, transcriptomics, lipidomics and metabolomics analyses have recently been used, providing new insights into the different signaling pathways involving PknA and PknB [37, 57]. These studies revealed that the STPK-protein interaction map includes a number of different binding proteins, involved in different functions. For instance, the role of PknA and PknB in several pathways related to cellular growth, including ATP synthesis and electron transport has been evidenced, highlighting a previously undescribed role in the regulation of translation. Moreover, the kinases also showed a role in the regulation of the genes encoding VapC and MazF toxins, which block the translation in response to different stresses [37, 57]. These results further emphasize the value of PknA and PknB as targets for the development of anti-TB drugs.

2.2 PknG

PknG is probably the most studied actinobacterial STPK, because of its pivotal role in virulence [20, 21] and its involvement in the industrial production of glutamate by *Corynebacterium glutamicum* [58]. In particular, PknG is important for its roles in *Mtb* pathogenicity, being involved in the inhibition of the phagosome-lysosome fusion in infected macrophages [21, 59]. In addition, PknG also plays a role in cellular metabolism, by phosphorylating the FHA protein GarA [60, 61], involved in the regulation of the tricarboxylic acid cycle and the glutamate biosynthesis [20, 52]. Furthermore, PknG was recently demonstrated to phosphorylate *in vivo* the 50S ribosomal protein L13, important for the formation of the biofilm and in the maintenance of the redox homeostasis [62, 63]. However, these two substrates alone cannot account for the several roles proposed for PknG, and for the phenotype of the knock-out *Mtb* strain [20, 36]. Indeed, PknG also seems to be involved in resistance to antimicrobial agents [64], glycogen metabolism [65] and biosynthesis of rhamnose [66].

The most recent studies, performed by integrated proteomics and phosphoproteomics approaches, identified a number of previously undescribed potential PknG binding proteins, including the S50 ribosomal protein L2 [67] or MurC [37], highlighting the role of this STPK also in amino acid, lipid and cell wall biosynthesis. Moreover, PknG was also found to interact with the RelB-like and VapB antitoxin proteins, suggesting a role in the adaptation to changes in environmental conditions, including dormancy [67, 68].

From a structural point of view, PknG is a multidomain protein, containing an unstructured N-terminal extension, a rubredoxin (Rdx)-like domain, adjacent to the catalytic site, and a C-terminal dimerization domain containing tetratricopeptide repeats [27]. Differently from most *Mtb* STPKs, PknG is not an RD kinase [29]; as such, it is not regulated by phosphorylation of the activation loop. Conversely, four autophosphorylation sites are present in the N-terminal extension, which represent the interaction sites with the FHA domain of GarA [69]. Moreover, the Rdx domain, containing two CxxCG motifs which act as ligands of a divalent metal ion, and are associated with the kinase domain, forms a deep substrate-binding cleft [27]. Thus, the ability to bind metals as well as the absence of phosphorylation in the activation, suggest a possible redox-dependent regulation [27, 63]. Recently, biochemical and structural studies have proposed a regulation of the PknG kinase activity based on its substrate GarA, in which the N-terminal extension and the tetratricopeptide repeats domain of PknG optimize the affinity for the substrate, and Rdx regulates the kinase activity by restricting the accessibility to the active site [70]. Even if the precise mechanism of regulation still needs to be completely elucidated, this particular regulation of the kinase activity, relying on substrate selectivity, paves the way for the development of new STPK inhibitors, endowed with novel modes of action [70].

2.3 Others *Mtb* STPKs

Currently, relatively little is known about the substrates and functions of the remaining *Mtb* STPKs, especially because they are not essential for *Mtb* growth [9]. Nevertheless, all of these transmembrane receptors have important roles, particularly in virulence and/or adaptation [7].

For instance, PnkD, although not essential, was demonstrated to contribute to the survival of the bacteria in phosphate-poor environments [71, 72]. Indeed, *pknD* gene is located in an operon which also contains *pstS2*, encoding a phosphate-binding protein, in a locus comprising several genes involved in phosphate transport [73]. Moreover, PknD was shown to phosphorylate *Rv0516c*, a putative regulator of the sigma factor SigF [74], thus probably having a role in osmosensory signaling pathway [75]. The crystal structure of the extracellular sensor domain of PknD has been solved,

showing a six-bladed β-propeller, built on a remarkably rigid and symmetric framework [76]. Each blade contains four antiparallel β-strands, with the first strand in the center of the structure and the last strand at the outer edge. The most variable residues in the blade subdomains are in a cluster forming a "cup" motif, which is analogous to the ligand-binding surface of several other β-propeller proteins; this suggests that PknD could bind different ligands, and then transmit signals through quaternary structure conformational changes of the intracellular kinase domain [76]. Interestingly, PknD proved to be essential in animal models for central nervous system TB, the most serious form of the disease, primarily affecting young children [77]. In particular, PknD was found to be necessary for invasion of brain endothelia, probably assisting bacterial adherence by the association with host extracellular matrix components. These findings demonstrate that PknD is an important element in the mechanism of bacterial invasion and virulence, though it can be contrasted by a specific antiserum, thus representing a promising therapeutic potential [77, 78]. Indeed, the vaccination with recombinant *Mtb* PknD was demonstrated to prevent TB meningitis in a guinea pig model [78].

PknE, PknH, PknI and PknK, toghether with PknG, have important roles in *Mtb* intracellular survival [21, 79-82]. For instance, PknE has been demonstrated to promote the suppression of apoptosis, as shown by the use of a *pknE*-deleted *Mtb* strain [79, 83], and to be involved in adaptive responses [84] and HIV co-infection [85]; PknH is implicated in *Mtb* dormancy [86] and in the regulation of cell wall biosynthesis [87], while PknI plays a role in the redox homeostasis during oxidative stress [88]. Moreover, advances in integrated proteomic approaches allowed to assign novel biological functions to the different STPKs. For instance, the involvement of PknE in the regulation of cell division and in transport, the role of PknI in carbohydrate biosynthesis, and the implication of PknJ in nucleotide biosynthesis were elucidated [37, 57].

These new results further highlight the great potential of targeting STPKs, for the identification of novel antitubercular drugs.

3. STPKs AS TARGETS FOR THE DEVELOPMENT OF ANTITUBERCULAR AGENTS

Since the identification of these kinases in the genomic sequence of *Mtb* in 1998 [18], significant developments have been made in the definition of their role in the physiologic regulation of *Mtb* growth and survival. Therefore, STPKs represent a very interesting group of pharmacological targets in *M. tuberculosis*. Among these kinases, PknA, PknB and PknG have been targeted by medicinal chemistry efforts, with the aim of disclosing new antimycobacterial compounds.

In the following paragraphs, the principal inhibitors of these mycobacterial STPKs will be presented and discussed.

3.1 PknA inhibitors

PknA and PknB are crucial for cell wall formation and resuscitation from dormancy; in addition, they perform multiple regulatory functions in metabolism and adaptation to environmental stress [41, 43, 89, 90]. Both PknA and PknB are attractive drug targets since these kinases are essential for bacterial growth [19, 39, 91]. Few compounds have been identified as PknA inhibitors so far. A screening of the Nested Chemical Library disclosed 4 compounds belonging to different chemical classes, able to inhibit PknA, provided with a low *in vitro* activity (% inhibition at 100 μM: between 37 and 86%) [92]. These data were used for subsequent molecular modeling studies [93], but no biological data have been hitherto reported.

3.2 PknB inhibitors

The role of PknB in *Mtb* survival was investigated through experiments of gene editing: the expression of wild-type *pknB* from pRBexint-*pknB* was sufficient to rescue knock-out *pknB* mutants, showing the importance of this enzyme [37].

A study to identify potential PknB inhibitors was carried out, and the effects of the selected compounds on *Mtb* growth were evaluated. Considering that the physiologic substrates of the enzyme were still to be clearly identified, the preliminary assays (performed on a panel of 18 commercially available inhibitors of eukaryotic STPKs) were carried out using myelin basic protein (MBP) as surrogate substrate [30, 44]. Even though MBP can be phosphorylated at five different sites by PknB [31], it is considered to be a poor substrate. The putative physiologic substrates of PknB were then discovered through a proteomic approach in a soluble protein extract of *Mtb*, allowing the identification of GarA, a protein containing the FHA domain, as the best candidate [60]. The screening identified **staurosporine (1)** and two derivatives, **K-252-a (2)** and **K-252-b (3)** (Figure 1), characterized by an indole-carbazole chromophore moiety, thought to be responsible for the interaction with the ATP binding site, as the most potent inhibitors: the IC_{50} values were 0.6 ± 0.05 μM for **(1)**, 96 ± 7 nM for **(2)**, and 106 ± 6 nM for **(3)** [39].

The antimycobacterial activity of these compounds was assayed against *Mtb* H37Rv, *M. smegmatis* mc^2155 and *M. aurum* A+ (which is usually extremely susceptible to antitubercular drugs). The results showed that **(2)** is capable of inhibiting the growth of both *Mtb* H37Rv and *M. smegmatis* mc^2155 at a concentration of 20 μM, while its MIC against *M. aurum* A+ was found to be 5 μM. **(1)** showed inhibitory effects on *Mtb* H37Rv, with MIC values in the range 25 μM - 50 μM. **(3)** could not inhibit the growth of the mycobacterial strains at the maximum tested concentration (40 μM), probably due to its poor permeability through the cell wall [39]. In order to evaluate the relationship between the inactivation of PknB and the inhibition of *Mtb* growth, the *pknB* gene and the nearby regions were cloned in the mycobacterial replicating vector pOMK. The resulting plasmid, pOMK-*pknB*, was introduced in *M. smegmatis* mc^2155 and the overexpression of PknB was evaluated through Western blotting. As a control, *M. smegmatis* mc^2155 was transformed with the empty vector, and the resistance of the modified

mycobacteria against **(2)** was evaluated. In two independent experiments, the MIC calculated for the strains of *M. smegmatis* mc^{2}155 transformed with pOMK-*pknB* were two-fold higher than those obtained with the control strain, confirming that PknB is the actual target of **(2)** *in vivo* [39].

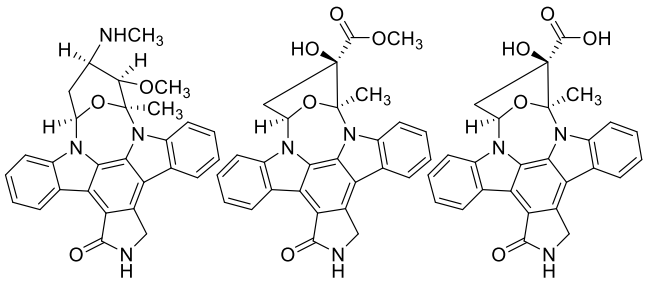


Fig. (1). Structures of compounds (1), (2) and (3).

Considering the structural similarity between STPKs and eukaryotic kinases [94], as well as the high degree of conservation of the active site of these enzymes, the activity of known eukaryotic kinase inhibitors was also evaluated on STPKs. A significant example is represented by **2-Aminopurine** (**4**) in Figure 2. It is an adenine analogue which is known to inhibit multiple cellular targets and several eukaryotic kinases [95, 96]. Since **(4)** inhibits eukaryotic kinases at concentrations in the millimolar range, this compound was assayed on mycobacterial STPKs. Kinase inhibition *in vitro* assays showed an IC_{50} value of 2.2 mM [97], which is four-fold higher than the value obtained on the eukaryotic kinase PKR (10 mM) [98].

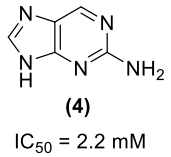


Fig. (2). Structure of compound (4).

This result demonstrated that **(4)** can also act as a competitive inhibitor of mycobacterial kinases and in particular as a PknB inhibitor; thus, this compound was selected to develop more potent inhibitors [97]. The first compounds were designed, based on the fact that metal ions have been used for centuries as anti-infectives. Indeed, *in vivo* literature data indicate that metallic ions can inhibit the bacterial growth, by interfering with the enzymatic machinery. Therefore, the activity of several divalent transition metal ions (dissolved in DMSO), namely Zn^{2+}, Ag^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} e Hg^{2+}, on PknB autophosphorylation was evaluated. Using a luminescence kinase assay, the amount of unused ATP was quantified to provide an inversely proportional correlation with the autophosphorylation activity. In the presence of Cd^{2+}, Cu^{2+} e Ag^{2+}, PknB autophosphorylation was almost completely abolished, even at 50 μM concentration; Zn^{2+} and Hg^{2+} exerted a partial inhibitory activity, while the presence of Ni^{2+}, Fe^{2+} and Co^{2+} did not produce any significant effect.

The activity of metallic salts in aqueous solution was also investigated to determine the influence of their ionization in polar solvents on PknB inhibition. With the only exception of Cd^{2+}, all other metallic ions showed a comparable inhibition, confirming that their action is not significantly influenced by the solvent [97].

In order to determine if the inhibitory activity of these ions was selective for PknB, their effect was also evaluated on other STPKs, like PknG and PknL. The experiments showed that, among the six tested metallic ions, Ag^{2+} was capable of inhibiting both PknG and PknL, while Hg^{2+} was active only on PknL. These results proved that Ag^{2+} and Hg^{2+} act as broad-spectrum STPK inhibitors. Circular dichroism was employed to determine if PknB maintained its structural integrity when exposed to polar metallic ions at a concentration of 50 μM. This experiment showed that metallic ions inhibit the kinases by binding their active site, without inducing any structural deformation [97]. Then, new coordination complexes were developed as inhibitors of mycobacterial STPKs, to exploit their advantages over the classical organic inhibitors, i.e. the variability of oxidation states and the different spatial dispositions. Previously, Feng *et al.* developed highly specific kinase inhibitors against GSK3R, PAK1 and PIM1 by introducing ruthenium Ru(II) in the structure of **(1),** which was otherwise a non-specific kinase inhibitor [99]. Inspired by this observation, a similar metal-complex derivatization approach was employed for **(5)** (Figure 3), an equipotent analog of **(4)**. Moreover, other transition metals were considered, to develop organometallic inhibitors provided with an increased specific inhibition and a better ability to cross membranes. Differently from the parent compound, **(5)** bears a propyl group at the N-9 position, which increases its solubility in organic solvents, thus allowing the reaction of complex formation. The ligand *per se* did not lead to a significant improvement of the inhibitory activity: **(5)** exhibited an IC_{50} value of 1.3 mM against PknB, much like the parent compound **(4)** (2.2 mM).

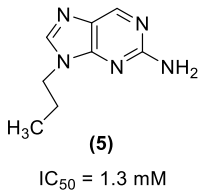


Fig. (3). Structure of compound (5).

Six metal complexes **(6) - (11)** were synthesized. The addition of the metal to the ligand **(5)** significantly increased the potency of the compound, shifting the IC_{50} values from the millimolar to the micromolar range. All compounds inhibited PknB activity with an IC_{50} value in the micromolar range (5-20 μM), at a concentration of 50 μM. Additionally,

the complex with Hg^{2+} **(9)** proved to be able to inhibit PknG and PknL, while the complex with Ag^{2+} **(11)** was active only on PknL. The remaining complexes did not exhibit any significant effect on PknG or PknL. Overall, these data indicate that the inhibition pattern of the metal complexes is very similar to that observed for the uncoordinated ions. Specifically, **(9)** and **(11)** proved to be particularly effective against *Mtb* in whole-cell assays.

In order to determine whether the inhibition of PknB was due to a competitive binding to the ATP-binding pocket or to the effect of metal ions on the kinase active site, the inhibitory activity of **(6)** - **(11)** was examined against glucose-1-phosphate adenylyltransferase from *Thermotoga maritima*, an enzyme harboring a similar ATP binding pocket as *Mtb* kinases. **(6)** - **(11)** did not show any inhibition of activity against this enzyme up to 50 μM concentration. This observation supports the hypothesis that the inhibitory activity of **(6)** - **(11)** is due to an effect of the metal ions on the kinase active site. The organometallic complexes developed in this study exhibited both *in vitro* and *in vivo* inhibitory effects. To assess the antibacterial activity of these complexes on other strains, MIC values against *M. smegmatis*, *B. subtilis* and *E. coli* were determined. The MIC values of **(9)** and **(11)** increased in all these three strains, suggesting that the target is specific to *Mtb*; notably, *M. smegmatis* has only 4 STPKs, in *E. coli* these enzymes are absent and *B. subtilis* has only one homolog of PknA. Therefore, it is plausible to theorize that the strong inhibitory effect observed in *M. tuberculosis* may be due to the inhibition of *Mtb*-specific STPKs [96].

Table 1. IC_{50} (PknB) and MIC (*Mtb*) values of the metal complexes of (5), (6) - (11).

Complex	IC_{50} (μM)	MIC (μM)
(6)	6.8 ± 0.18	> 50
(7)	10.5 ± 0.28	> 50
(8)	10.2 ± 0.47	> 50
(9)	5.0 ± 0.19	6.25
(10)	16.6 ± 0.16	> 50
(11)	12.9 ± 0.11	12.5

An HTS screening allowed the investigation of a new class of compounds, leading to PknB inhibitors. PknB was cloned and purified, and then used in an *in vitro* kinase assay to evaluate the activity of 970 compounds from the NCL library. The IC_{50} values of the 20 most potent inhibitors were determined. Five representative terms **(12)-(16)** are shown in Figure 4, and the obtained results were all comparable [100].

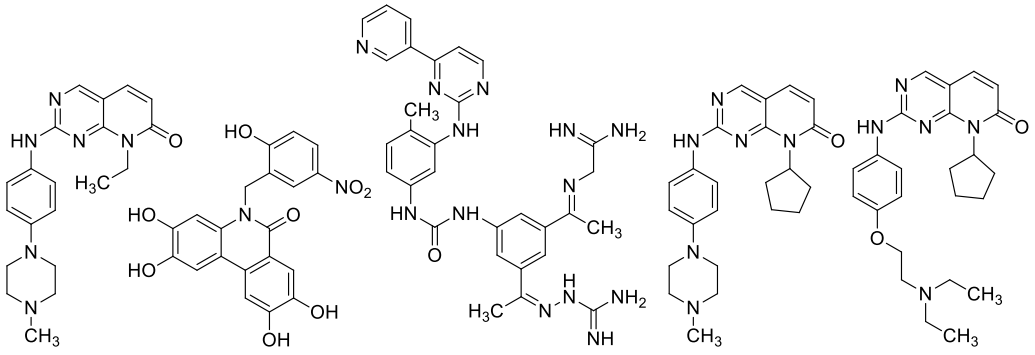
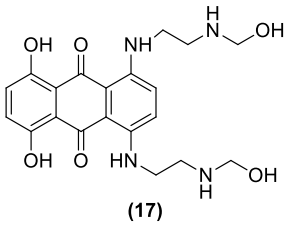


Fig. (4). Structures of compounds (12)-(16).

Wehenkel and co-workers observed that **mitoxantrone (17)** (Figure 5), an anthraquinone derivative commonly used in cancer treatment, is a PknB inhibitor capable of preventing mycobacterial cell growth [101]. The crystal structure of the complex demonstrates that mitoxantrone is an ATP-competitive inhibitor of PknB, suggesting a strategy for drug design based on ATP-competitive lead compounds, such as mitoxantrone derivatives.

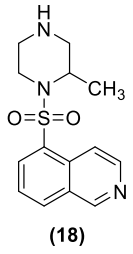


(17)

IC_{50} = 0.8 ± 0.05 µM
MIC = 400 µM

Fig. (5). Structure of compound (17).

The kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine **H7 (18)** in Figure 6 was found to be able to inhibit the growth of two strains of mycobacteria, the slower *M. bovis* Bacille Calmette-Guerin (BCG) and the faster *M. smegmatis* mc^2155, in a dose-dependent manner [102]. Millimolar concentrations of **(18)** can induce a 40% decrease in the growth of *M. bovis* BCG. Moreover, **H7** determines a 2log decrease in the viable count of *M. smegmatis* within 48 hours, and a 50% reduction in the viable count of *M. bovis* within 10 days. At micromolar concentrations, the compound proved to be a potent inhibitor of PknB. **(18)** also showed an interesting inhibitory activity on RNA polymerase II, a protein involved in the growth and development of the mycobacterium [103].



(18)

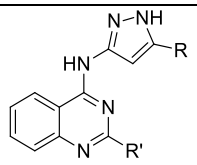
81% inhibition at 6 µM

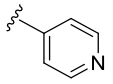
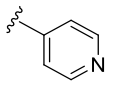
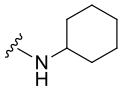
Fig. (6). Structure of compound (18).

As **(18)** exhibits a variable effect on eukaryotic kinases, *in vitro* tests were performed to verify that mycobacterial STPKs were the actual targets of the compound; PknB was used as the model enzyme, and MBP was employed as the artificial substrate. These experiments showed that a 6 µM dose of **(18)** is capable of inducing an 81% inhibition of PknB-mediated MBP phosphorylation, compared to the control [102]. This result corroborates the hypothesis of a correlation between mycobacterial STPKs and the regulation of cellular growth [23]. Additionally, **(18)** could also be active on other members of the STPK family [102].

Substituted aminopyrimidine-based compounds have recently emerged as new inhibitors of PknB. The inhibitory activity of this series was evaluated by determining their effect on the phosphorylation of the substrate GarA *in vitro* [60]; an interesting compound **(19)**, showing an IC_{50} value of 1.35 µM, was identified and employed as a lead for further studies [104]. A SAR exploration of this compound was performed, starting from 2,4-dichloroquinazoline. The most potent compounds of the series were **(20)** and **(21)** (Table 2) [104]. The antimycobacterial activity of this series was evaluated through an Alamar blue cell viability assay [105]: all compounds proved to be weakly active or completely inactive. This discrepancy was attributed to the physico-chemical characteristics of these molecules, which prevent the crossing of the mycobacterial cell wall.

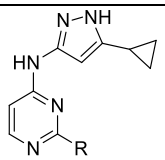
Table 2. Structure and IC_{50} values of compound (19) and its derivatives (20) and (21).



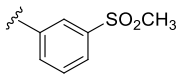
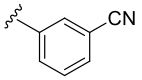
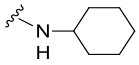
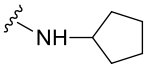
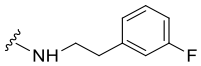
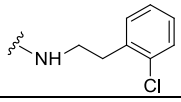
	R	R'	IC_{50} (µM)
(**19**)	CH_3		1.35
(**20**)			0.322
(**21**)			0.107

Moreover, the significant binding of these compounds to plasma protein (> 99%) drastically lowers the amount of free compound. In order to improve the activity of these inhibitors *in vivo*, many efforts were made to lower their logD values and explore the effect of this variation on the activity. The quinazoline ring was substituted with a pyrimidine, and many aryl and amino groups were introduced at position 2, maintaining the cyclopropylaminopyrazole ring at position 4. This strategy afforded the most potent compounds **(22) - (27)**, reported in Table 3 [104].

Table 3. Structures, IC_{50} and MIC (Mtb) values of compounds (22)-(27)



R	IC_{50} (µM)	MIC (µM)

(22)		0.086	63
(23)		0.087	125
(24)		0.084	63
(25)		0.115	63
(26)		0.084	63
(27)		0.064	125

Recently, phytomolecules have gained popularity as potential scaffolds for the development of novel anti-TB drugs, deriving from natural sources [106]. Copp and Pearce described 333 natural products for the treatment of TB, including lipids and fatty acids, simple aromatic compounds, phenols, quinones, peptides, alcaloids, terpenes, steroids etc. [107]. Three of the most effective compounds included **cryptolepine hydrochloride (28)**, **ermanine (29)** and **demethylcalabaxanthone (30)**, shown in Figure 7. **(28)** is an alkaloid, extracted from a Western African plant, called *Cryptolepis sanguinolenta* [108]. **(29)** (5,7-dihydroxy-3',4'-dimethoxyflavone) derives from *Tanacetum microphyllum*; it has anti-inflammatory properties and can reduce ear edema in many murine models [109]. **(30)** is a xantonic derivative obtained by powders, fresh arils and seeds of *Garcinia mangostana*, as a result of repeated methanol extractions [110]. QSAR analyses on these molecules showed that they could be optimal candidates for the development of new drugs [111]. Protein-ligand interactions were investigated; the binding experiments showed that all these molecules could be potential inhibitor of the target protein PknB [111]. However, the available data about these phytomolecules as novel antimycobacterial agents are quite limited [112].

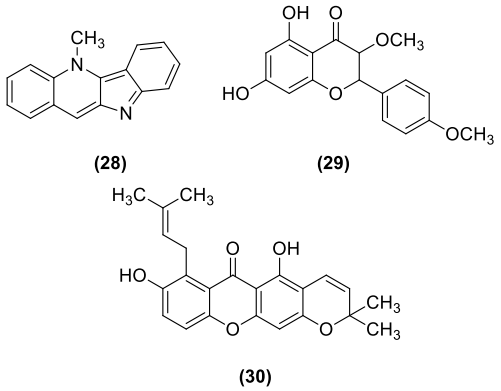


Fig. (7). Structures of phytomolecules (28)-(30).

Recently, compound **IMB-YH-8 (31)** (Figure 8) was identified as a PknB inhibitor, with the potentiality to become a lead for the development of new antitubercular agents. Indeed, it is capable of inhibiting both PknB autophosphorylation and dose-dependent PknB-induced GarA phosphorylation. Conversely, it does not inhibit human Akt1 or other Ser/Thr kinases from *Mtb*, with the only exception of the homologous PknA. **(31)** binds PknB with moderate affinity: molecular docking experiments revealed that it interacts with the catalytic domain of the enzyme. **(31)** also proved to be able to influence the transcriptional activity of *Mtb*, inducing changes in PknB-modulated SigH regulatory pathways. Additionally, the compound was found to be effective on both resistant and multi-drug resistant *Mtb* isolates. To confirm the inhibitory activity of **(31)** on PknB, its effect on PknB autophosphorylation and on PknB-induced GarA phosphorylation was measured through a non-radioactive assay [113]. PknB autophosphorylation occurs at its Ser and Thr residues [31, 44, 114], while the reaction on its substrate, the protein GarA, occurs at a single N-terminal threonine (Thr22) [60]. **(31)** inhibited PknB autophosphorylation with an IC_{50} value of 20.2 μM. Phosphorylated and unphosphorylated GarA proteins were separated through Mn^{2+}–Phos-tag SDS-PAGE: in this technique, performed in the presence of $MnCl_2$, phosphorylated proteins migrate more slowly as a consequence of their phosphate group being bound by the Phos-tag (20 μM). The treatment with **(31)** resulted in an increased intensity of the band corresponding to unphosphorylated GarA, proving that the compound is indeed capable of inhibiting PknB-induced GarA phosphorylation in a dose-dependent fashion.

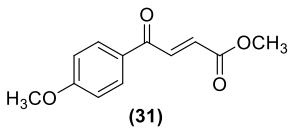


Fig. (8). Structure of compound (31).

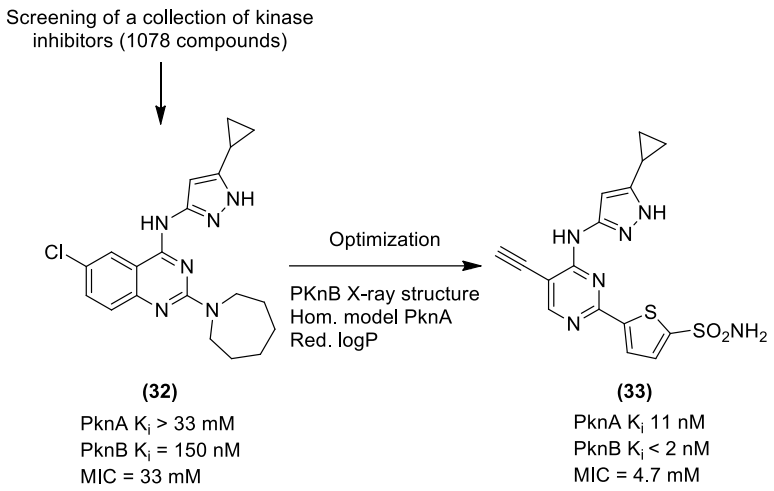


Fig. (9). Discovery and structure of the PknA/B *Mtb* dual inhibitor (33).

As PknB is involved in cell wall synthesis and cell shape regulation [43, 47, 115], the effect of **(31)** on PknB inhibition *in vivo*, and its impact on cellular morphology were evaluated [113]. *Mtb* H37Rv cells were treated with the inhibitor, and then analyzed through scansion electron microscopy. While untreated cells were rod-shaped with a smooth surface, the administration of 1 µg/mL of **(31)** for 5 days resulted in a weakening of the structural integrity of the cells. Indeed, a significant amount (61.5%) of lysed cells was observed in the treated *Mtb* H37Rv strains. As PknB is known to be essential to guarantee the integrity of the cell, these findings support the hypothesis that **(31)** acts by inhibiting PknB action *in vivo* [113].

3.3 PknA and PKnB inhibitors (dual inhibitors)

A screening performed on a library of kinase inhibitors led to the discovery of a compound provided with inhibitory activity towards PknB on *Mtb*. The optimization of this lead **(32)**, performed on the basis of the crystal structure of PknB and on a model of PknA, led to the discovery of the first PknA - PknB dual inhibitor (Figure 9) [116]. The optimized compound achieved potent inhibition of both kinases. Interestingly, a binding pocket unique to mycobacterial kinases was identified. Structural modifications designed to fill this pocket resulted in a 100-fold differential against a broad selection of mammalian kinases. Reducing the lipophilicity further improved the antimycobacterial activity, with the most potent compound **(33)** achieving a MIC of 4.7 µM against the H37Ra *Mtb* strain.

3.4 PknG inhibitors

The ability of *Mtb* to survive in macrophages and establish a long-term infection in the host is one the main determinant of its virulence [117, 118]. In this context, PknG plays a key role in maintaining the integrity of the phagosome within macrophages. Thus, PknG inhibition leads to lysosome-phagosome fusion, which, in turn, determines the degradation of the pathogen [21].

Considering the importance of this target, the identification of PknG inhibitors has been the subject of several studies [119, 120]. The homologous enzyme from *E. coli* was cloned and used as a model, while MBP was chosen as its substrate; then, 1000 compounds from the kinase inhibitor library (NCL) were screened by a radioactive biochemical PknG kinase assay. Among these compounds, **AX20017 (34)** emerged as a potent PknG inhibitor (Figure 10) [100].

The inhibitory activity of **(34)** against the 11 known mycobacterial kinases revealed that the compound is highly selective for PknG, and it does not affect human kinases.

The 2.4 Å x-ray crystal structure of PknG in complex with **(34)** was reported [27], revealing that the inhibitor is buried deep within the adenosine-binding site (residues Pro-85-Met-94), targeting an active conformation of the kinase domain. Remarkably, although the topology of the kinase domain is reminiscent of eukaryotic kinases, the binding pocket of **(34)** is surrounded by a set of amino acid side chains that are not found in any human kinase.

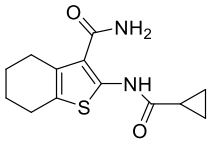
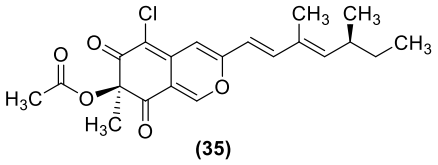


Fig. (10). Structure of compound (34).

This lead was optimized to improve its ADME parameters, affording a series of derivatives which were tested by the same assay: the best compounds exhibited IC_{50} values in the range 90-500 nM. The selected inhibitors also underwent a lysosome transfer assay, to verify their ability to induce lysosome-phagosome fusion within macrophages. Some derivatives showing IC_{50} values in the sub-nanomolar range against PknG proved to be inactive; the best

compounds, which proved to be active both against PknG and in the macrophage assay, were AX14585 (IC_{50} = 0.4 μM) and AX35510 (IC_{50} = 0.2 μM) [100].

As marine microorganisms have always provided a significant number of secondary metabolites, endowed with antitumoral and antimicrobial properties [121-123], **Sclerotiorin (35)** (Figure 11) was tested against PknG, showing good IC_{50} and k_i values (76.5 ± 3.9 μM and 27.2 ± 7.8 μM, respectively) [119]. In order to determine the binding affinity of **(35)**, an efficient *in vitro* approach, based on microscale thermophoresis (MST), was employed [124]. The dissociation constants (K_D) of PknG-**(34)** and PknG-**(35)** were 8.5 ± 0.2 mM and 11.4 ± 0.7 mM respectively, revealing that both the inhibitors are moderate PknG inhibitors [119]. Then, the cytotoxicity of **(34)** and **(35)** was evaluated through an MTT assay, against HeLa and MCF-7 cells, and against J774A macrophages. The viability of the cells treated with sclerotiorin was above 90% even at 100 mM, revealing that the inhibitor does not influence cell growth. On the contrary, **(34)** proved to be toxic on HeLa and MCF-7 cells, with IC_{50} values of 200 mM and 91.3 mM, respectively; conversely, it did not influence the growth of J774A macrophages, showing an 89% cell viability at 100 mM. However, **(35)** proved to be ineffective at inhibiting the growth of *Mtb*, showing that PknG is not essential for the survival of the pathogen *in vitro* [119]. In brief, **(35)** proved to be able to inhibit PknG autophosphorylation and reduce the growth of intracellular mycobacteria in a dose-dependent manner, without provoking significant side effects on mammal cells. The fact that the compound is effective only on intracellular microorganisms [21] might prevent the development of resistant strains. Moreover, it could also be extremely efficient *in vivo*, since it would seem to be able to inhibit the kinases in the macrophage, without crossing the mycobacterial membrane. The association of **(35)** with rifampicin resulted in a reduction of the mycobacterial growth, directly proportional to the concentration of **(35)** [119]. Therefore, the moderate PknG inhibitory activity, the ability to reduce mycobacterial growth inside macrophages and the low cytotoxicity of **(35)** strongly support the use of this compound in antitubercular therapy.



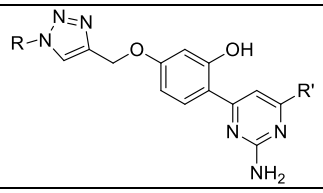
(35)

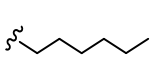
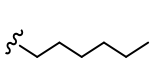
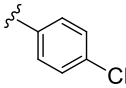
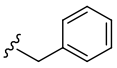
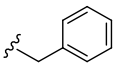
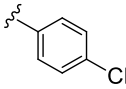
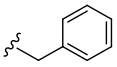
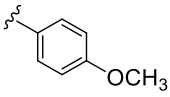
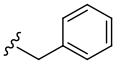
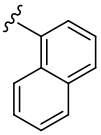
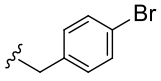
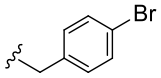
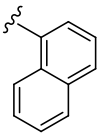
IC_{50} = 76.5 ± 3.9 μM

Fig. (11). Structure of (35).

A set of triazolylmethoxy aminopyrimidines were investigated as potential PknG inhibitor; their activity against the purified enzyme was evaluated (Table 4) using MBP as a substrate and (**34**) as reference inhibitor. The enzymatic activity was assessed by measuring the amount of ADP produced by PknG, using the ADP-Glo luciferase reporter kit. All tested compounds had different substituents at N1 of the 1,2,3-triazole and at position 6 of the pyrimidine ring; the best results were obtained when the triazole bore a *n*-hexyl, benzyl or 4-Br-benzyl group, and when a phenyl, 4-Cl-phenyl, 4- methoxyphenyl or naphthyl moiety was attached to position 6 of the pyrimidine. The positive control (**34**) showed a 41% (41 ± 0.02) inhibition at 100 μM, in the selected biological assay; the activities of the candidate inhibitors are reported in Table 4. Despite most of them displayed a comparable or better potency with respect to the reference compound, only (**36**) proved to possess antimicrobial properties, showing a MIC value of 50 μg/mL against *Mtb* H37Rv [120].

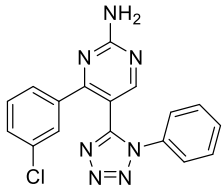
Table 4. Structures and% Inhibition at 100 μM of compounds (36)-(43).



	R	R'	% Inhibition at 100 μM
(**36**)			23 ± 1.86
(**37**)			43 ± 2.94
(**38**)			29 ± 3.03
(**39**)			53 ± 0.61
(**40**)			43 ± 0.61
(**41**)			35 ± 5.97
(**42**)			41 ± 0.78
(**43**)			34 ± 6.10

A recent virtual screening study led to the identification of compound (**44**) (Figure 12), which showed a 43% inhibition against PknG at 25 μM. Moreover, it was

able to inhibit the growth of *M. bovis BCG* and it was found to be non-toxic against the host macrophages. These findings suggest that its scaffold could be further explored for the development of novel PknG inhibitors [125].



(44)

43% inhibition at 25 μM

Fig. (12). Structure of compound (44).

4. CONCLUSION

A number of researches is now available, concerning the importance of *Mtb* Ser/Thr kinases as drug targets for the development of novel anti-TB agents. These enzymes appear to be implicated in bacterial growth, virulence and adaptation, supporting the role of these enzymes in bacterial growth and providing the proof-of-concept about their druggability. Until now, the crystal structures of PknA, PknB, PknE, PknG and PknH have been solved [24, 25, 27, 76, 126, 127].

In vitro inhibitory activities show that these enzymes possess a druggable active site. In several cases, the inhibitory activity on the isolated enzyme did not correlate with the antimycobacterial effect in whole-cell assays. This discrepancy is probably due to the highly lipophilic nature of the mycobacterial cell wall (high content of mycolic acids), which lowers the permeability of the pathogen to xenobiotics. Despite this problem, MIC in the low micromolar range have been obtained for several PknA, PknB and PknG inhibitors, showing that it is possible to find compounds that target *Mtb* Ser/Thr kinases able to inhibit mycobacterial growth.

In conclusion, even if the available data concerning the activity of these compounds on infected macrophages and *in vivo* in infected animals, as well as information regarding their selectivity with respect to mammalian enzymes are still limited, it would seem that targeting *Mtb* Ser/Thr kinases could be a promising new strategy for the development of novel drugs for the treatment of *Mtb* infections.

LIST OF ABBREVIATIONS

FHA: forkhead-associated domain; MBP: myelin basic protein; MIC: Minimal Inhibitory Concentration; *Mtb*: *Mycobacterium tuberculosis*; STPKs: serine/threonine protein kinases; TB: tuberculosis.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

This research was supported by the Italian Ministry of Education, University and Research (MIUR): Dipartimenti di Eccellenza Program (2018–2022) - Dept. of Biology and Biotechnology "L. Spallanzani", University of Pavia and by the University of Milan (Linea B).

REFERENCES

[1] Yee, D.; Valiquette, C.; Pelletier, M.; Parisien, I.; Rocher, I.; Menzies, D. Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis. *Am. J. Respir. Crit. Care. Med.*, **2003**, *167*(11), 1472-1477.

[2] Faustini, A.; Hall, A.J.; Perucci, C.A. Risk factors for multidrug resistant tuberculosis in Europe: a systematic review. *Thorax*, **2006**, *61*(2), 158-163.

[3] Mulu, W.; Mekonnen, D.; Yimer, M.; Admassu, A.; Abera, B. Risk factors for multidrug resistant tuberculosis patients in Amhara National Regional State. *Afr. Health. Sci.*, **2015**, *15*, 368-377.

[4] Zhang, Y. The magic bullets and tuberculosis drug targets. *Annu. Rev. Pharmacol. Toxicol.*, **2005**, *45*, 529-564.

[5] Chiarelli, L.R.; Mori, G.; Esposito, M.; Orena, B.S.; Pasca, M.R. New and old hot drug targets in tuberculosis. *Curr. Med. Chem.*, **2016**, *23*(33), 3813-3846.

[6] Meneghetti, F.; Villa, S.; Gelain, A.; Barlocco, D.; Chiarelli, L.R.; Pasca, M.R.; Costantino, L. Iron acquisition pathways as targets for antitubercular drugs. *Curr. Med. Chem.*, **2016**, *23*(35), 4009-4026.

[7] Johnson, B.K.; Abramovitch, R.B. Small molecules that sabotage bacterial virulence. *Trends Pharmacol. Sci.*, **2017**, *38*(4), 339-362.

[8] Bem, A.E.; Velikova, N.; Pellicer, M.T.; Baarlen, P.; Marina, A.; Wells, J.M. Bacterial histidine kinases as novel antibacterial drug targets. *ACS. Chem. Biol.*, **2015**, *10*(1), 213-224.

[9] Prisic, S.; Husson, R.N. *Mycobacterium tuberculosis* Serine/Threonine Protein Kinases. *Microbiol. Spectr.*, **2014**, *2*(5), MGM2-0006-2013.

[10] Bach, H.; Wong, D.; Av-Gay, Y. *Mycobacterium tuberculosis* PtkA is a novel protein tyrosine kinase whose substrate is PtpA. *Biochem. J.*, **2009**, *420*(2), 155-160.

[11] Wong, D.; Li, W.; Chao, J.D.; Zhou, P.; Narula, G.; Tsui, C.; Ko, M.; Xie, J.; Martinez-Frailes, C.; Av-Gay, Y. Protein tyrosine kinase, PtkA, is required for *Mycobacterium tuberculosis* growth in macrophages. *Sci. Rep.*, **2018**, *8*(1), 155.

[12] Fanzani, L.; Porta, F.; Meneghetti, F.; Villa, S.; Gelain, A.; Lucarelli, A.P.; Parisini, E. *Mycobacterium tuberculosis* low molecular weight phosphatases (MPtpA and MPtpB): from biological insight to inhibitors. *Curr. Med. Chem.*, **2015**, *22*(27), 3110-3132.

[13] Zheng, H.; Colvin, C.J.; Johnson, B.K.; Kirchhoff, P.D.; Wilson, M.; Jorgensen-Muga, K.; Larsen, S.D.; Abramovitch, R.B. Inhibitors of *Mycobacterium tuberculosis* DosRST signaling and persistence. *Nat. Chem. Biol.*, **2017**, *13*(2), 218-225.

[14] Gupta, R.K.; Thakur, T.S.; Desiraju, G.R.; Tyagi, J.S. Structure-based design of DevR inhibitor active against nonreplicating *Mycobacterium tuberculosis*. *J. Med. Chem.*, **2009**, *52*(20), 6324-6334.

[15] Wehenkel, A.; Bellinzoni, M.; Graña, M.; Duran, R.; Villarino, A.; Fernandez, P.; Andre-Leroux, G.; England, P.; Takiff, H.; Cerveñansky, C.; Cole, S.T.; Alzari, P.M. Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. *Biochim. Biophys. Acta.*, **2008**, *1784*(1), 193-202.

[16] Hanks, S.K.; Hunter, T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.*, **1995**, *9*(8), 576-596.

[17] Pérez, J.; Castañeda-García, A.; Jenke-Kodama, H.; Müller, R.; Muñoz-Dorado, J. Eukaryotic-like protein kinases in the prokaryotes and the myxobacterial kinome. *Proc. Natl. Acad. Sci. U. S. A.*, **2008**, *105*(41), 15950-15955.

[18] Cole, S.T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S.V.; Eiglmeier, K.; Gas, S.; Barry, C.E.; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M.A.; Rajandream, M.A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J.E.; Taylor, K.; Whitehead, S.; Barrell, B.G. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, **1998**, *393*(6685), 537-544.

[19] Sassetti, C.M.; Rubin, E.J. Genetic requirements for mycobacterial survival during infection. *Proc. Natl. Acad. Sci. U. S. A.*, **2003**, *100*(22), 12989-12994.

[20] Cowley, S.; Ko, M.; Pick, N.; Chow, R.; Downing, K.J.; Gordhan, B.G.; Betts, J.C.; Mizrahi, V.; Smith, D.A.; Stokes, R.W.; Av-Gay, Y. The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth in vivo. *Mol. Microbiol.*, **2004**, *52*(6), 1691-1702.

[21] Walburger, A.; Koul, A.; Ferrari, G.; Nguyen, L.; Prescianotto-Baschong, C.; Huygen, K.; Klebl, B.; Thompson, C.; Bacher, G.; Pieters, J. Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science*, **2004**, *304*(5678), 1800-1804.

[22] Narayan, A.; Sachdeva, P.; Sharma, K.; Saini, A.K.; Tyagi, A.K.; Singh, Y. Serine threonine protein kinases of mycobacterial genus: phylogeny to function. *Physiol. Genomics*, **2007**, *29*(1), 66-75.

[23] Av-Gay, Y.; Everett, M. The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends Microbiol.*, **2000**, *8*(5), 238-244.

[24] Gay, L.M.; Ng, H.L.; Alber, T. A conserved dimer and global conformational changes in the structure of apo-PknE Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *J. Mol. Biol.*, **2006**, *360*(2), 409-420.

[25] Ortiz-Lombardía, M.; Pompeo, F.; Boitel, B.; Alzari, P.M. Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *J. Biol. Chem.*, **2003**, *278*(15), 13094-13100.

[26] Rakette, S.; Donat, S.; Ohlsen, K.; Stehle, T. Structural analysis of *Staphylococcus aureus* serine/threonine kinase PknB. *PLoS One*, **2012**, *7*(6), e39136.

[27] Scherr, N.; Honnappa, S.; Kunz, G.; Mueller, P.; Jayachandran, R.; Winkler, F.; Pieters, J.; Steinmetz, M.O. Structural basis for the specific inhibition of protein kinase G, a virulence factor of *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.*, **2007**, *104*(29), 12151-12156.

[28] Young, T.A.; Delagoutte, B.; Endrizzi, J.A.; Falick, A.M.; Alber, T. Structure of *Mycobacterium tuberculosis* PknB supports a universal activation mechanism for Ser/Thr protein kinases. *Nat. Struct. Biol.*, **2003**, *10*(3), 168-174.

[29] Johnson, L.N.; Noble, M.E.; Owen, D.J. Active and inactive protein kinases: structural basis for regulation. *Cell*, **1996**, *85*(2), 149-158.

[30] Boitel, B.; Ortiz-Lombardía, M.; Durán, R.; Pompeo, F.; Cole, S.T.; Cerveñansky, C.; Alzari, P.M. PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Mol. Microbiol.*, **2003**, *49*(6), 1493-1508.

[31] Durán, R.; Villarino, A.; Bellinzoni, M.; Wehenkel, A.; Fernandez, P.; Boitel, B.; Cole, S.T.; Alzari, P.M.; Cerveñansky, C. Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of *Mycobacterium tuberculosis* Ser/Thr protein kinases. *Biochem. Biophys. Res. Commun.*, **2005**, *333*(3), 858-867.

[32] Pereira, S.F.; Goss, L.; Dworkin, J. Eukaryote-like serine/threonine kinases and phosphatases in bacteria. *Microbiol. Mol. Biol. Rev.*, **2011**, *75*(1), 192-212.

[33] Nolen, B.; Taylor, S.; Ghosh, G. Regulation of protein kinases; controlling activity through activation segment conformation. *Mol. Cell.*, **2004**, *15*(5), 661-675.

[34] Greenstein, A.E.; Echols, N.; Lombana, T.N.; King, D.S.; Alber, T. Allosteric activation by dimerization of the PknD receptor Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *J. Biol. Chem.*, **2007**, *282*(15), 11427-11435.

[35] Lombana, T.N.; Echols, N.; Good, M.C.; Thomsen, N.D.; Ng, H.L.; Greenstein, A.E.; Falick, A.M.; King, D.S.; Alber, T. Allosteric activation mechanism of the *Mycobacterium tuberculosis* receptor Ser/Thr protein kinase, PknB. *Structure*, **2010**, *18*(12), 1667-1677.

[36] Nakedi, K.C.; Nel, A.J.; Garnett, S.; Blackburn, J.M.; Soares, N.C. Comparative Ser/Thr/Tyr phosphoproteomics between two mycobacterial species: the fast growing *Mycobacterium smegmatis* and the slow

growing *Mycobacterium bovis* BCG. *Front. Microbiol.*, **2015**, *6*, 237.
[37] Wu, F.L.; Liu, Y.; Jiang, H.W.; Luan, Y.Z.; Zhang, H.N.; He, X.; Xu, Z.W.; Hou, J.L.; Ji, L.Y.; Xie, Z.; Czajkowsky, D.M.; Yan, W.; Deng, J.Y.; Bi, L.J.; Zhang, X.E.; Tao, S.C. The Ser/Thr protein kinase protein-protein interaction map of *M. tuberculosis*. *Mol. Cell. Proteomics.*, **2017**, *16*(8), 1491-1506.
[38] Meeske, A.J.; Riley, E.P.; Robins, W.P.; Uehara, T.; Mekalanos, J.J.; Kahne, D.; Walker, S.; Kruse, A.C.; Bernhardt, T.G.; Rudner, D.Z. SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature*, **2016**, *537*(7622), 634-638.
[39] Fernandez, P.; Saint-Joanis, B.; Barilone, N.; Jackson, M.; Gicquel, B.; Cole, S.T.; Alzari, P.M. The Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth. *J. Bacteriol.*, **2006**, *188*(22), 7778-7784.
[40] Molle, V.; Kremer, L. Division and cell envelope regulation by Ser/Thr phosphorylation: *Mycobacterium* shows the way. *Mol. Microbiol.*, **2010**, *75*(5), 1064-1077.
[41] Ortega, C.; Liao, R.; Anderson, L.N.; Rustad, T.; Ollodart, A.R.; Wright, A.T.; Sherman, D.R.; Grundner, C. *Mycobacterium tuberculosis* Ser/Thr protein kinase B mediates an oxygen-dependent replication switch. *PLoS Biol.*, **2014**, *12*(1), e1001746.
[42] Chawla, Y.; Upadhyay, S.; Khan, S.; Nagarajan, S.N.; Forti, F.; Nandicoori, V.K. Protein kinase B (PknB) of *Mycobacterium tuberculosis* is essential for growth of the pathogen in vitro as well as for survival within the host. *J Biol Chem*, **2014**, *289*(20), 13858-13875.
[43] Kang, C.M.; Abbott, D.W.; Park, S.T.; Dascher, C.C.; Cantley, L.C.; Husson, R.N. The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. *Genes Dev.*, **2005**, *19*(14), 1692-1704.
[44] Av-Gay, Y.; Jamil, S.; Drews, S.J. Expression and characterization of the *Mycobacterium tuberculosis* serine/threonine protein kinase PknB. *Infect. Immun.*, **1999**, *67*(11), 5676-5682.
[45] Singh, A.; Singh, Y.; Pine, R.; Shi, L.; Chandra, R.; Drlica, K. Protein kinase I of *Mycobacterium tuberculosis*: cellular localization and expression during infection of macrophage-like cells. *Tuberculosis*, **2006**, *86*(1), 28-33.
[46] Betts, J.C.; Lukey, P.T.; Robb, L.C.; McAdam, R.A.; Duncan, K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.*, **2002**, *43*(3), 717-731.
[47] Gee, C.L.; Papavinasasundaram, K.G.; Blair, S.R.; Baer, C.E.; Falick, A.M.; King, D.S.; Griffin, J.E.; Venghatakrishnan, H.; Zukauskas, A.; Wei, J.R.; Dhiman, R.K.; Crick, D.C.; Rubin, E.J.; Sassetti, C.M.; Alber, T. A phosphorylated pseudokinase complex controls cell wall synthesis in mycobacteria. *Sci. Signal.*, **2012**, *5*(208), ra7.
[48] Kang, C.M.; Nyayapathy, S.; Lee, J.Y.; Suh, J.W.; Husson, R.N. Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria. *Microbiology*, **2008**, *154*(Pt 3), 725-735.
[49] Shah, I.M.; Laaberki, M.H.; Popham, D.L.; Dworkin, J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell*, **2008**, *135*(3), 486-496.
[50] Mir, M.; Asong, J.; Li, X.; Cardot, J.; Boons, G.J.; Husson, R.N. The extracytoplasmic domain of the *Mycobacterium tuberculosis* Ser/Thr kinase PknB binds specific muropeptides and is required for PknB localization. *PLoS Pathog.*, **2011**, *7*(7), e1002182.
[51] Nott, T.J.; Kelly, G.; Stach, L.; Li, J.; Westcott, S.; Patel, D.; Hunt, D.M.; Howell, S.; Buxton, R.S.; O'Hare, H.M.; Smerdon, S.J. An intramolecular switch regulates phosphoindependent FHA domain interactions in *Mycobacterium tuberculosis*. *Sci. Signal.*, **2009**, *2*(63), ra12.
[52] Ventura, M.; Rieck, B.; Boldrin, F.; Degiacomi, G.; Bellinzoni, M.; Barilone, N.; Alzaidi, F.; Alzari, P.M.; Manganelli, R.; O'Hare, H.M. GarA is an essential regulator of metabolism in *Mycobacterium tuberculosis*. *Mol. Microbiol.*, **2013**, *90*(2), 356-366.
[53] Molle, V.; Gulten, G.; Vilchèze, C.; Veyron-Churlet, R.; Zanella-Cléon, I.; Sacchettini, J.C.; Jacobs, W.R.; Kremer, L. Phosphorylation of InhA inhibits mycolic acid biosynthesis and growth of *Mycobacterium tuberculosis*. *Mol. Microbiol.*, **2010**, *78*(6), 1591-1605.
[54] Veyron-Churlet, R.; Zanella-Cléon, I.; Cohen-Gonsaud, M.; Molle, V.; Kremer, L. Phosphorylation of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein reductase MabA regulates mycolic acid biosynthesis. *J. Biol. Chem.*, **2010**, *285*(17), 12714-12725.
[55] Vilchèze, C.; Molle, V.; Carrère-Kremer, S.; Leiba, J.; Mourey, L.; Shenai, S.; Baronian, G.; Tufariello, J.; Hartman, T.; Veyron-Churlet, R.; Trivelli, X.; Tiwari, S.; Weinrick, B.; Alland, D.; Guérardel, Y.; Jacobs, W.R.; Kremer, L. Phosphorylation of KasB regulates virulence and acid-fastness in *Mycobacterium tuberculosis*. *PLoS Pathog.*, **2014**, *10*(5), e1004115.
[56] Thakur, M.; Chakraborti, P.K. GTPase activity of mycobacterial FtsZ is impaired due to its transphosphorylation by the eukaryotic-type Ser/Thr kinase, PknA. *J. Biol. Chem.*, **2006**, *281*(52), 40107-40113.
[57] Carette, X.; Platig, J.; Young, D.C.; Helmel, M.; Young, A.T.; Wang, Z.; Potluri, L.P.; Moody, C.S.; Zeng, J.; Prisic, S.; Paulson, J.N.; Muntel, J.; Madduri, A.V.R.; Velarde, J.; Mayfield, J.A.; Locher, C.; Wang, T.; Quackenbush, J.; Rhee, K.Y.; Moody, D.B.; Steen, H.; Husson, R.N. Multisystem analysis of *Mycobacterium tuberculosis* reveals kinase-dependent remodeling of the pathogen-environment interface. *MBio*, **2018**, *9*(2), e02333-17.
[58] Niebisch, A.; Kabus, A.; Schultz, C.; Weil, B.; Bott, M. Corynebacterial protein kinase G controls 2-oxoglutarate dehydrogenase activity via the phosphorylation status of the OdhI protein. *J. Biol. Chem.*, **2006**, *281*(18), 12300-12307.

[59] Zulauf, K.E.; Sullivan, J.T.; Braunstein, M. The SecA2 pathway of *Mycobacterium tuberculosis* exports effectors that work in concert to arrest phagosome and autophagosome maturation. *PLoS Pathog.,* **2018**, *14*(4), e1007011.

[60] Villarino, A.; Duran, R.; Wehenkel, A.; Fernandez, P.; England, P.; Brodin, P.; Cole, S.T.; Zimny-Arndt, U.; Jungblut, P.R.; Cerveñansky, C.; Alzari, P.M. Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. *J. Mol. Biol.,* **2005**, *350*(5), 953-963.

[61] Rieck, B.; Degiacomi, G.; Zimmermann, M.; Cascioferro, A.; Boldrin, F.; Lazar-Adler, N.R.; Bottrill, A.R.; le Chevalier, F.; Frigui, W.; Bellinzoni, M.; Lisa, M.N.; Alzari, P.M.; Nguyen, L.; Brosch, R.; Sauer, U.; Manganelli, R.; O'Hare, H.M. PknG senses amino acid availability to control metabolism and virulence of *Mycobacterium tuberculosis*. *PLoS Pathog.,* **2017**, *13*(5), e1006399.

[62] Wolff, K.A.; de la Peña, A.H.; Nguyen, H.T.; Pham, T.H.; Amzel, L.M.; Gabelli, S.B.; Nguyen, L. A redox regulatory system critical for mycobacterial survival in macrophages and biofilm development. *PLoS Pathog.,* **2015**, *11*(4), e1004839.

[63] Tiwari, D.; Singh, R.K.; Goswami, K.; Verma, S.K.; Prakash, B.; Nandicoori, V.K. Key residues in *Mycobacterium tuberculosis* protein kinase G play a role in regulating kinase activity and survival in the host. *J. Biol. Chem.,* **2009**, *284*(40), 27467-27479.

[64] Wolff, K.A.; Nguyen, H.T.; Cartabuke, R.H.; Singh, A.; Ogwang, S.; Nguyen, L. Protein kinase G is required for intrinsic antibiotic resistance in mycobacteria. *Antimicrob. Agents. Chemother.,* **2009**, *53*(8), 3515-3519.

[65] Belanger, A.E.; Hatfull, G.F. Exponential-phase glycogen recycling is essential for growth of *Mycobacterium smegmatis*. *J. Bacteriol.,* **1999**, *181*(21), 6670-6678.

[66] Deng, J.; Bi, L.; Zhou, L.; Guo, S.J.; Fleming, J.; Jiang, H.W.; Zhou, Y.; Gu, J.; Zhong, Q.; Wang, Z.X.; Liu, Z.; Deng, R.P.; Gao, J.; Chen, T.; Li, W.; Wang, J.F.; Wang, X.; Li, H.; Ge, F.; Zhu, G.; Zhang, H.N.; Wu, F.L.; Zhang, Z.; Wang, D.; Hang, H.; Li, Y.; Cheng, L.; He, X.; Tao, S.C.; Zhang, X.E. *Mycobacterium tuberculosis* proteome microarray for global studies of protein function and immunogenicity. *Cell. Rep.,* **2014**, *9*(6), 2317-2329.

[67] Nakedi, K.C.; Calder, B.; Barnejee, M.; Giddey, A.; Nel, A.J.; Garnett, S.; Blackburn, J.M.; Da Cruz Soares, N.A. Identification of novel physiological substrates of *Mycobacterium Bovis* BCG Protein Kinase G (PknG) by label-free quantitative phosphoproteomics. *Mol. Cell Proteomics*, **2018**. [Epub ahead of print] doi: 10.1074/mcp.RA118.000705

[68] Khan, M.Z.; Bhaskar, A.; Upadhyay, S.; Kumari, P.; Rajmani, R.S.; Jain, P.; Singh, A.; Kumar, D.; Bhavesh, N.S.; Nandicoori, V.K. Protein kinase G confers survival advantage to *Mycobacterium tuberculosis* during latency-like conditions. *J. Biol. Chem.,* **2017**, *292*(39), 16093-16108.

[69] O'Hare, H.M.; Durán, R.; Cerveñansky, C.; Bellinzoni, M.; Wehenkel, A.M.; Pritsch, O.; Obal, G.; Baumgartner, J.; Vialaret, J.; Johnsson, K.; Alzari, P.M. Regulation of glutamate metabolism by protein kinases in mycobacteria. *Mol. Microbiol.,* **2008**, *70*(6), 1408-1423.

[70] Lisa, M.N.; Gil, M.; André-Leroux, G.; Barilone, N.; Durán, R.; Biondi, R.M.; Alzari, P.M. Molecular basis of the activity and the regulation of the eukaryotic-like S/T Protein Kinase PknG from *Mycobacterium tuberculosis*. *Structure,* **2015**, *23*(6), 1039-1048.

[71] Vanzembergh, F.; Peirs, P.; Lefevre, P.; Celio, N.; Mathys, V.; Content, J.; Kalai, M. Effect of PstS sub-units or PknD deficiency on the survival of *Mycobacterium tuberculosis*. *Tuberculosis,* **2010**, *90*(6), 338-345.

[72] Peirs, P.; Lefèvre, P.; Boarbi, S.; Wang, X.M.; Denis, O.; Braibant, M.; Pethe, K.; Locht, C.; Huygen, K.; Content, J. *Mycobacterium tuberculosis* with disruption in genes encoding the phosphate binding proteins PstS1 and PstS2 is deficient in phosphate uptake and demonstrates reduced in vivo virulence. *Infect. Immun.,* **2005**, *73*(3), 1898-1902.

[73] Peirs, P.; De Wit, L.; Braibant, M.; Huygen, K.; Content, J. A serine/threonine protein kinase from *Mycobacterium tuberculosis*. *Eur. J. Biochem.,* **1997**, *244*(2), 604-612.

[74] Greenstein, A.E.; MacGurn, J.A.; Baer, C.E.; Falick, A.M.; Cox, J.S.; Alber, T. *M. tuberculosis* Ser/Thr protein kinase D phosphorylates an anti-anti-sigma factor homolog. *PLoS Pathog.,* **2007**, *3*(4), e49.

[75] Hatzios, S.K.; Baer, C.E.; Rustad, T.R.; Siegrist, M.S.; Pang, J.M.; Ortega, C.; Alber, T.; Grundner, C.; Sherman, D.R.; Bertozzi, C.R. Osmosensory signaling in *Mycobacterium tuberculosis* mediated by a eukaryotic-like Ser/Thr protein kinase. *Proc. Natl. Acad. Sci. U. S. A.,* **2013**, *110*(52), E5069-5077.

[76] Good, M.C.; Greenstein, A.E.; Young, T.A.; Ng, H.L.; Alber, T. Sensor domain of the *Mycobacterium tuberculosis* receptor Ser/Thr protein kinase, PknD, forms a highly symmetric beta propeller. *J. Mol. Biol.,* **2004**, *339*(2), 459-469.

[77] Be, N.A.; Bishai, W.R.; Jain, S.K. Role of *Mycobacterium tuberculosis* pknD in the pathogenesis of central nervous system tuberculosis. *BMC Microbiol.,* **2012**, *12*, 7.

[78] Skerry, C.; Pokkali, S.; Pinn, M.; Be, N.A.; Harper, J.; Karakousis, P.C.; Jain, S.K. Vaccination with recombinant *Mycobacterium tuberculosis* PknD attenuates bacterial dissemination to the brain in guinea pigs. *PLoS One,* **2013**, *8*(6), e66310.

[79] Jayakumar, D.; Jacobs, W.R.; Narayanan, S. Protein kinase E of *Mycobacterium tuberculosis* has a role in the nitric oxide stress response and apoptosis in a human macrophage model of infection. *Cell. Microbiol.,* **2008**, *10*(2), 365-374.

[80] Kumari, R.; Singh, S.K.; Singh, D.K.; Singh, P.K.; Chaurasiya, S.K.; Srivastava, K.K. Functional characterization delineates that a *Mycobacterium tuberculosis* specific protein kinase (Rv3080c) is responsible for the growth, phagocytosis and intracellular survival of avirulent mycobacteria. *Mol. Cell. Biochem.*, **2012**, *369*(1-2), 67-74.

[81] Malhotra, V.; Arteaga-Cortés, L.T.; Clay, G.; Clark-Curtiss, J.E. *Mycobacterium tuberculosis* protein kinase K confers survival advantage during early infection in mice and regulates growth in culture and during persistent infection: implications for immune modulation. *Microbiology*, **2010**, *156*(Pt 9), 2829-2841.

[82] Papavinasasundaram, K.G.; Chan, B.; Chung, J.H.; Colston, M.J.; Davis, E.O.; Av-Gay, Y. Deletion of the *Mycobacterium tuberculosis* pknH gene confers a higher bacillary load during the chronic phase of infection in BALB/c mice. *J. Bacteriol.*, **2005**, *187*(16), 5751-5760.

[83] Kumar, D.; Narayanan, S. pknE, a serine/threonine kinase of *Mycobacterium tuberculosis* modulates multiple apoptotic paradigms. *Infect. Genet. Evol.*, **2012**, *12*(4), 737-747.

[84] Kumar, D.; Palaniyandi, K.; Challu, V.K.; Kumar, P.; Narayanan, S. PknE, a serine/threonine protein kinase from *Mycobacterium tuberculosis* has a role in adaptive responses. *Arch. Microbiol.*, **2013**, *195*(1), 75-80.

[85] Parandhaman, D.K.; Hanna, L.E.; Narayanan, S. PknE, a serine/threonine protein kinase of *Mycobacterium tuberculosis* initiates survival crosstalk that also impacts HIV coinfection. *PLoS One*, **2014**, *9*(1), e83541.

[86] Chao, J.D.; Papavinasasundaram, K.G.; Zheng, X.; Chávez-Steenbock, A.; Wang, X.; Lee, G.Q.; Av-Gay, Y. Convergence of Ser/Thr and two-component signaling to coordinate expression of the dormancy regulon in *Mycobacterium tuberculosis*. *J. Biol. Chem.*, **2010**, *285*(38), 29239-29246.

[87] Gómez-Velasco, A.; Bach, H.; Rana, A.K.; Cox, L.R.; Bhatt, A.; Besra, G.S.; Av-Gay, Y. Disruption of the serine/threonine protein kinase H affects phthiocerol dimycocerosates synthesis in *Mycobacterium tuberculosis*. *Microbiology*, **2013**, *159*(Pt 4), 726-736.

[88] Venkatesan, A.; Palaniyandi, K.; Sharma, D.; Bisht, D.; Narayanan, S. Functional characterization of PknI-Rv2159c interaction in redox homeostasis of *Mycobacterium tuberculosis*.. *Front. Microbiol.*, **2016**, *7*, 1654.

[89] Prisic, S.; Dankwa, S.; Schwartz, D.; Chou, M.F.; Locasale, J.W.; Kang, C.M.; Bemis, G.; Church, G.M.; Steen, H.; Husson, R.N. Extensive phosphorylation with overlapping specificity by *Mycobacterium tuberculosis* serine/threonine protein kinases. *Proc. Natl. Acad. Sci. U. S. A.*, **2010**, *107*(16), 7521-7526.

[90] Chou, M.F.; Prisic, S.; Lubner, J.M.; Church, G.M.; Husson, R.N.; Schwartz, D. Using bacteria to determine protein kinase specificity and predict target substrates. *PLoS One*, **2012**, *7*(12), e52747.

[91] Sassetti, C.M.; Boyd, D.H.; Rubin, E.J. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.*, **2003**, *48*(1), 77-84.

[92] Sipos, A.; Pató, J.; Székely, R.; Hartkoorn, R.C.; Kékesi, L.; Őrfi, L.; Szántai-Kis, C.; Mikušová, K.; Svetlíková, Z.; Korduláková, J.; Nagaraja, V.; Godbole, A.A.; Bush, N.; Collin, F.; Maxwell, A.; Cole, S.T.; Kéri, G. Lead selection and characterization of antitubercular compounds using the Nested Chemical Library. *Tuberculosis*, **2015**, *95 Suppl 1*, S200-206.

[93] Morales-Bayuelo, A. Molecular quantum similarity, chemical reactivity and database screening of 3D pharmacophores of the protein kinases A, B and G from *Mycobacterium tuberculosis*. *Molecules*, **2017**, *22*(6), E1027.

[94] Grangeasse, C.; Nessler, S.; Mijakovic, I. Bacterial tyrosine kinases: evolution, biological function and structural insights. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **2012**, *367*(1602), 2640-2655.

[95] Hu, Y.; Conway, T.W. 2-Aminopurine inhibits the double-stranded RNA-dependent protein kinase both in vitro and in vivo. *J. Interferon Res.*, **1993**, *13*(5), 323-328.

[96] Huang, J.T.; Schneider, R.J. Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine. *Proc. Natl. Acad. Sci. U. S. A.*, **1990**, *87*(18), 7115-7119.

[97] Bais, V.S.; Mohapatra, B.; Ahamad, N.; Boggaram, S.; Verma, S.; Prakash, B. Investigating the inhibitory potential of 2-Aminopurine metal complexes against serine/threonine protein kinases from *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)*, **2018**, *108*, 47-55.

[98] Huang, S.; Qu, L.K.; Cuddihy, A.R.; Ragheb, R.; Taya, Y.; Koromilas, A.E. Protein kinase inhibitor 2-aminopurine overrides multiple genotoxic stress-induced cellular pathways to promote cell survival. *Oncogene*, **2003**, *22*(24), 3721-3733.

[99] Feng, L.; Geisselbrecht, Y.; Blanck, S.; Wilbuer, A.; Atilla-Gokcumen, G.E.; Filippakopoulos, P.; Kräling, K.; Celik, M.A.; Harms, K.; Maksimoska, J.; Marmorstein, R.; Frenking, G.; Knapp, S.; Essen, L.O.; Meggers, E. Structurally sophisticated octahedral metal complexes as highly selective protein kinase inhibitors. *J. Am. Chem. Soc.*, **2011**, *133*(15), 5976-5986.

[100] Székely, R.; Wáczek, F.; Szabadkai, I.; Németh, G.; Hegymegi-Barakonyi, B.; Eros, D.; Szokol, B.; Pató, J.; Hafenbradl, D.; Satchell, J.; Saint-Joanis, B.; Cole, S.T.; Orfi, L.; Klebl, B.M.; Kéri, G. A novel drug discovery concept for tuberculosis: inhibition of bacterial and host cell signalling. *Immunol. Lett.*, **2008**, *116*(2), 225-231.

[101] Wehenkel, A.; Fernandez, P.; Bellinzoni, M.; Catherinot, V.; Barilone, N.; Labesse, G.; Jackson, M.; Alzari, P.M. The structure of PknB in complex with mitoxantrone, an ATP-competitive inhibitor, suggests a mode of protein kinase regulation in mycobacteria. *FEBS Lett.*, **2006**, *580*(13), 3018-3022.

[102] Drews, S.J.; Hung, F.; Av-Gay, Y. A protein kinase inhibitor as an antimycobacterial agent. *FEMS Microbiol. Lett.*, **2001**, *205*(2), 369-374.
[103] Kumahara, E.; Ebihara, T.; Saffen, D. Protein kinase inhibitor H7 blocks the induction of immediate-early genes zif268 and c-fos by a mechanism unrelated to inhibition of protein kinase C but possibly related to inhibition of phosphorylation of RNA polymerase II. *J. Biol. Chem.*, **1999**, *274*(15), 10430-10438.
[104] Chapman, T.M.; Bouloc, N.; Buxton, R.S.; Chugh, J.; Lougheed, K.E.; Osborne, S.A.; Saxty, B.; Smerdon, S.J.; Taylor, D.L.; Whalley, D. Substituted aminopyrimidine protein kinase B (PknB) inhibitors show activity against *Mycobacterium tuberculosis*. *Bioorg. Med. Chem. Lett.*, **2012**, *22*(9), 3349-3353.
[105] Lougheed, K.E.; Osborne, S.A.; Saxty, B.; Whalley, D.; Chapman, T.; Bouloc, N.; Chugh, J.; Nott, T.J.; Patel, D.; Spivey, V.L.; Kettleborough, C.A.; Bryans, J.S.; Taylor, D.L.; Smerdon, S.J.; Buxton, R.S. Effective inhibitors of the essential kinase PknB and their potential as anti-mycobacterial agents. *Tuberculosis*, **2011**, *91*(4), 277-286.
[106] Palomino, J.C.; Ramos, D.F.; da Silva, P.A. New anti-tuberculosis drugs: strategies, sources and new molecules. *Curr. Med. Chem.*, **2009**, *16*(15), 1898-1904.
[107] Copp, B.R.; Pearce, A.N. Natural product growth inhibitors of *Mycobacterium tuberculosis*. *Nat. Prod. Rep.*, **2007**, *24*(2), 278-297.
[108] Gibbons, S.; Fallah, F.; Wright, C.W. Cryptolepine hydrochloride: a potent antimycobacterial alkaloid derived from *Cryptolepis sanguinolenta*. *Phytother. Res.*, **2003**, *17*(4), 434-436.
[109] Martinez, J.; Silván, A.M.; Abad, M.J.; Bermejo, P.; Villar, A.; Söllhuber, M. Isolation of two flavonoids from *Tanacetum microphyllum* as PMA-induced ear edema inhibitors. *J. Nat. Prod.*, **1997**, *60*(2), 142-144.
[110] Suksamrarn, S.; Suwannapoch, N.; Phakhodee, W.; Thanuhiranlert, J.; Ratananukul, P.; Chimnoi, N.; Suksamrarn, A. Antimycobacterial activity of prenylated xanthones from the fruits of *Garcinia mangostana*. *Chem. Pharm. Bull. (Tokyo)*, **2003**, *51*(7), 857-859.
[111] Appunni, S.; Rajisha, P.M.; Rubens, M.; Chandana, S.; Singh, H.N.; Swarup, V. Targeting PknB, an eukaryotic-like serine/threonine protein kinase of *Mycobacterium tuberculosis* with phytomolecules. *Comput. Biol. Chem.*, **2017**, *67*, 200-204.
[112] Sharma, A.; Dutta, P.; Sharma, M.; Rajput, N.K.; Dodiya, B.; Georrge, J.J.; Kholia, T.; Bhardwaj, A.; Consortium, O. BioPhytMol: a drug discovery community resource on anti-mycobacterial phytomolecules and plant extracts. *J. Cheminform.*, **2014**, *6*(1), 46.
[113] Xu, J.; Wang, J.X.; Zhou, J.M.; Xu, C.L.; Huang, B.; Xing, Y.; Wang, B.; Luo, R.; Wang, Y.C.; You, X.F.; Lu, Y.; Yu, L.Y. A novel protein kinase inhibitor IMB-YH-8 with anti-tuberculosis activity. *Sci. Rep.*, **2017**, *7*(1), 5093.
[114] Mieczkowski, C.; Iavarone, A.T.; Alber, T. Auto-activation mechanism of the *Mycobacterium tuberculosis* PknB receptor Ser/Thr kinase. *EMBO J.*, **2008**, *27*(23), 3186-3197.
[115] Dasgupta, A.; Datta, P.; Kundu, M.; Basu, J. The serine/threonine kinase PknB of *Mycobacterium tuberculosis* phosphorylates PBPA, a penicillin-binding protein required for cell division. *Microbiology*, **2006**, *152*(Pt 2), 493-504.
[116] Wang, T.; Bemis, G.; Hanzelka, B.; Zuccola, H.; Wynn, M.; Moody, C.S.; Green, J.; Locher, C.; Liu, A.; Gao, H.; Xu, Y.; Wang, S.; Wang, J.; Bennani, Y.L.; Thomson, J.A.; Müh, U. Mtb PKNA/PKNB dual inhibition provides selectivity advantages for inhibitor design to minimize host kinase interactions. *ACS Med. Chem. Lett.*, **2017**, *8*(12), 1224-1229.
[117] Zahrt, T.C.; Deretic, V. *Mycobacterium tuberculosis* signal transduction system required for persistent infections. *Proc. Natl. Acad. Sci. U. S. A.*, **2001**, *98*(22), 12706-12711.
[118] Pieters, J. Evasion of host cell defense mechanisms by pathogenic bacteria. *Curr. Opin. Immunol.*, **2001**, *13*(1), 37-44.
[119] Chen, D.; Ma, S.; He, L.; Yuan, P.; She, Z.; Lu, Y. Sclerotiorin inhibits protein kinase G from *Mycobacterium tuberculosis* and impairs mycobacterial growth in macrophages. *Tuberculosis*, **2017**, *103*, 37-43.
[120] Anand, N.; Singh, P.; Sharma, A.; Tiwari, S.; Singh, V.; Singh, D.K.; Srivastava, K.K.; Singh, B.N.; Tripathi, R.P. Synthesis and evaluation of small libraries of triazolylmethoxy chalcones, flavanones and 2-aminopyrimidines as inhibitors of mycobacterial FAS-II and PknG. *Bioorg. Med. Chem.*, **2012**, *20*(17), 5150-5163.
[121] Chidananda, C.; Rao, L.J.; Sattur, A.P. Sclerotiorin, from *Penicillium frequentans*, a potent inhibitor of aldose reductase. *Biotechnol. Lett.*, **2006**, *28*, (20), 1633-1636.
[122] Chidananda, C.; Sattur, A.P. Sclerotiorin, a novel inhibitor of lipoxygenase from *Penicillium frequentans*. *J. Agric. Food. Chem.*, **2007**, *55*(8), 2879-2883.
[123] Somoza, A.D.; Lee, K.H.; Chiang, Y.M.; Oakley, B.R.; Wang, C.C. Reengineering an azaphilone biosynthesis pathway in *Aspergillus nidulans* to create lipoxygenase inhibitors. *Org. Lett.*, **2012**, *14*(4), 972-975.
[124] Wienken, C.J.; Baaske, P.; Rothbauer, U.; Braun, D.; Duhr, S. Protein-binding assays in biological liquids using microscale thermophoresis. *Nat. Commun.*, **2010**, *1*, 100.
[125] Singh, N.; Tiwari, S.; Srivastava, K.K.; Siddiqi, M.I. Identification of novel inhibitors of *Mycobacterium tuberculosis* PknG using pharmacophore based virtual screening, docking, molecular dynamics simulation, and their biological evaluation. *J. Chem. Inf. Model.*, **2015**, *55*(6), 1120-1129.
[126] Ravala, S.K.; Singh, S.; Yadav, G.S.; Kumar, S.; Karthikeyan, S.; Chakraborti, P.K. Evidence that phosphorylation of threonine in the GT motif triggers activation of PknA, a eukaryotic-type serine/threonine

kinase from *Mycobacterium tuberculosis*. *FEBS J.*, **2015**, *282*, (8), 1419-1431.
[127] Cavazos, A.; Prigozhin, D.M.; Alber, T. Structure of the sensor domain of *Mycobacterium tuberculosis* PknH receptor kinase reveals a conserved binding cleft. *J. Mol. Biol.*, **2012**, *422*(4), 488-494.

Received: Revised: Accepted: