11
Prospects for TB Therapeutics Targeting *Mycobacterium tuberculosis* Phosphosignaling Networks

Yossef Av-Gay and Tom Alber

11.1
Introduction

Tuberculosis (TB), an infectious disease, is one among the numerous leading causes of death worldwide, killing approximately 1.7 million people annually. In the past decade, the global incidence of TB has risen to approximately nine million cases annually, with 80% of the patients in sub-Saharan Africa or Asia [1]. Drugs to treat TB have been available for the past six decades, yet eradication of the disease is not on the horizon.

Due to the chronic nature of the infection, current therapy for TB takes at least 6 months to treat drug-sensitive infections. Monotherapies are ineffective, necessitating treatment using a cocktail of four or more drugs. A combination of isoniazid (INH), rifampin (RMP), ethambutol (EMB), and pyrazinamide (PZA) are the first-line anti-TB agents. Second-line drugs have also been developed, but they are more expensive, less effective, and more toxic in comparison to first-line drugs. These antibiotics include ethionamide (ETH), streptomycin (STR), cycloserine, *p*-aminosalicylic acid (PAS), capreomycin, amikacin, kanamycin, quinolones (such as ciprofloxacin), and clofazime. This group is used when the first-line drugs become ineffective due to developed resistance or toxicity such as hepatotoxicity resulting from INH treatment.

Current treatment practices have led to a rapidly increasing incidence of drug resistant TB (http://www.cdc.gov/mmwr/preview/mmwrhtml/00020964.htm; http://www.who.int/tb/strategy/en/). Chromosomal mutations within the *M. tuberculosis* genome [2] have engendered resistance to every anti-TB drug available. Among the new cases, approximately 20% show resistance to multiple drugs [3]. As a result, even current combinatorial therapies are becoming less effective and less capable of reducing the development of further resistance. The emergence of multidrug resistant (MDR; resistant to at least two first-line treatments) and extensively drug-resistant (XDR; additionally resistant to at least three second-line treatments) strains, which account for up to 35% of MDR strains [3], create an urgent need for novel therapies and diagnostics to speed up the treatment and to combat MDR, XDR, and latent TB.

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Indeed, after 40 years with no new TB drugs introduced to the market, in the last decade, a combined public–industry effort [4, 5] has resulted in a growing number of drug candidates that are currently in clinical development. These new compounds include Tibotec's diarylquinone TMC207, which targets the bacterial ATP synthase [6] and nitroimidazopyrans (such as PA-824 and OPC-67683 (Otsuka Pharmaceuticals)) that appear to target synthesis of polypeptides and fatty acids essential for cell wall integrity.

The prospect for continuing emergence of resistance necessitates further development of anti-TB drugs that act by novel mechanisms. Recent evidence suggests that bacterial phosphosignaling systems may provide multiple valid targets for developing new drugs.

11.2
Rationale for Ser/Thr Protein Kinases and Protein Phosphatases as Drug Targets

M. tuberculosis survives for long periods in challenging host environments, including the phagocytic compartment of macrophages. Through a complex set of strategies, *M. tuberculosis* evades the antimicrobial defenses of the host (reviewed in Ref. [7]). These pathogenic strategies include preventing the acidification of phagosome [8], inhibiting phagosome maturation [9, 10], interfering with antigen presentation [11] and cytokine signaling [12], mounting vigorous stress response [13], and expressing resistance mechanisms to counter nitrosative damage [14]. During the acute phase of infection, the bacteria multiply inside the lungs, and the host responds by turning on adaptive immunity to control the bacterial growth. There is growing evidence that the long-term survival of *M. tuberculosis* is associated with adaptation to a phenotypically resistant form in response to exposure to nitric oxide within the activated macrophages or to low-oxygen tension in the granuloma [15, 16], which represent by itself another frontier for tuberculosis therapy. Thus, *M. tuberculosis* responds to host stimuli by mediating appropriate cellular responses during the entire course of the infection.

Four main families of proteins mediate phosphorylation signal transduction pathways in *M. tuberculosis*. These systems provide means of molecular adaptation in response to external stimuli. The classic bacterial signaling machinery comprises the "two-component" systems. Eleven such systems have been identified, each consisting of a histidine kinase and a response regulator [17, 18]. The ATP binding sites of two-component kinases are considered by several experts too shallow to target with inhibitors. The second family was discovered a decade ago [19] and contains 11 "eukaryotic-like" serine/threonine protein kinases (STPKs) called PknA-PknL [20–22]. Antagonizing the protein kinases is a Ser/Thr phosphatase encoded by the *pstP* gene. This PP2C-family enzyme is homologous to the environmental-sensing phosphatase, Rv1663, which regulates transcription through alternative sigma factors. The fourth family encodes a pair of protein tyrosine phosphatases (PTPs), PtpA and PtpB, which are thought to act within host cells to interrupt signaling pathways [23–25].

The Ser/Thr protein kinases afford promising therapeutic targets. Recent interest in drugs that inhibit human protein kinases has led to the development of large, kinase-specific chemical libraries and a wealth of knowledge about how to develop potent, selective inhibitors. The *M. tuberculosis* STPK domains are all <30% identical in sequence to the closest human homologues (CDC2HS and CK2α), making development of selectivity a relatively low hurdle. Inhibitors that target PknG or PknD in preference to the other *M. tuberculosis* STPKs have been reported [26, 27]. Two of the *M. tuberculosis* STPKs are essential for bacterial growth, and eukaryotic kinase inhibitors block mycobacterial growth *in vitro* [28, 29]. These studies provide traditional genetic and chemical validation for targeting the *M. tuberculosis* STPKs.

While pharmaceuticals that inhibit the human protein kinases have been developed successfully, the protein phosphatases have proven to be more difficult targets for medicinal chemistry. Nonetheless, the general Ser/Thr protein phosphatase, PstP, may be essential for growth, and the protein tyrosine phosphatase, PtpB, may be required to establish latent infections [25].

In this chapter, we review the recent literature about the structure, the function, and the inhibition of these promising targets. An outstanding review of the *M. tuberculosis* Ser/Thr protein kinases has been published recently by Takiff and colleagues [30].

11.3
Drug Target Validation by Genetic Inactivation

In the past decade, mycobacterial researchers developed methods to knock out genes in *M. tuberculosis*, providing powerful tools to explore the genetic determinants of pathogen physiology, infectivity, and virulence. Genetic studies have validated signaling elements as potential drug targets and provided insights into their essentiality to disease. Two of *M. tuberculosis* STPKs, PknA and PknB, were shown to be essential for *M. tuberculosis in vitro* growth [31]. Transposon mutagenesis of the whole *M. tuberculosis* genome also suggested that the deletion of *pknG* might also be lethal [32], but subsequent targeted deletions showed that the *pknG* knockout was viable but severely attenuated in animals [33]. In contrast, *pknB* could be deleted only in a merodiploid [31]. In an elegant study of *M. smegmatis* antisense knockdown strains, Husson and coworkers demonstrated that reduced levels of PknA or PknB attenuate growth rates and cause dramatic changes in cell morphology [34]. Unexpectedly, transposon insertions in the genes for the other eight *M. tuberculosis* STPKs did not attenuate growth in mouse spleen [35]. These results may indicate that the other STPKs are not essential; they serve redundant functions or influence processes (such as persistence or reactivation) that have yet to be surveyed in model organisms.

In vivo infection studies using *M. tuberculosis* mutants deleted for individual STPKs have established roles of specific kinase activities at specific stages of infection. For example, an *M. tuberculosis* mutant deleted for *pknG* displays decreased growth upon infection in immunocompetent mice and causes delayed mortality in SCID mice [33]. Using *M. bovis* BCG and *M. smegmatis* as model systems, Pieters and coworkers reported that PknG prevents phagosome-lysosome fusion [27]. The

relevance of these functions for wild-type *M. tuberculosis* infections and the biological mechanisms of PknG signaling await additional experimentation. In *M. tuberculosis*, PknG kinase acts as a sensor of nutritional stress, and plays a role in regulating glutamine/glutamate levels [33].

In contrast to PknG, Av-Gay and coworkers established that the PknH kinase plays a growth regulatory role during late stages of the infection cycle in mice [36]. Significantly, the *pknH* deletion mutant displayed increased resistance to acidified nitrite treatment and replicated to much higher numbers. Thus, the *pknH* deletion caused a hypervirulent phenotype compared to wild-type H37Rv. These studies supported the model that PknH senses nitric oxide stress produced by inducible nitric oxide synthase in the macrophage. Reduced oxygen tension and increased nitric oxide exposure are two conditions encountered by bacilli *in vivo* that may promote latency [37]. In *M. tuberculosis*, the sensor histidine kinases DosS and DosT sense oxygen tension by monitoring redox conditions and hypoxia, respectively [38]. Mutants lacking DosS and DosT are unable to activate expression of genes regulated by the latency-linked, stress response regulator, DosR. The established roles of some components of the two-component signaling systems in mediating latency [37, 39] and the hypervirulent phenotypes of mutants in a variety of pathways provides a broad scope for the biochemical mechanism of the growth regulating functions of PknH. Identification of the components of metabolic pathways regulated by PknH may help define new downstream drug targets.

Genetic knockout and antisense knockdown studies have been also carried out for PknD [40], PknE, [36, 41] PknF [42], PknI, and PknJ (Av-Gay and coworkers, unpublished results). These kinases seem to be nonessential for growth in culture media *in vitro*.

Whole-genome transposon mutagenesis suggested that the *M. tuberculosis* phosphatases are not essential for growth *in vitro* or *in vivo* [32, 35]. Nonetheless, attempts to make precise deletions of the Ser/Thr phosphatase PstP (Rv0018c) in both *M. smegmatis* and *M. tuberculosis* have failed in multiple laboratories. Although such negative results should be interpreted with caution, it appears that PstP is essential for *M. tuberculosis* growth. The knockout of *ptpB* produced growth attenuation in activated but not resting macrophages [25]. While the *ptpB* deletion mutant survived as well as isogenic wild-type strains in the early stages of infection in guinea pigs, bacterial growth was attenuated after 6 weeks. This phenotype suggested that PtpB antagonizes the adaptive immune response, perhaps by antagonizing interferon-γ signaling [25]. Although these studies hint at roles for the *M. tuberculosis* PTPs in long-term bacterial survival, the phosphatases require further validation to be considered among the most promising pharmaceutical targets.

11.4
STPK Mechanisms, Substrates, and Functions

Genetic and biochemical studies suggest that PknB is the best candidate STPK target for development of a sterilizing inhibitor. PknB was the first bacterial STPK to be

studied in detail, and orthologues are the most widespread of any STPK in other bacterial genera [43]. Thus, inhibitors of *M. tuberculosis* PknB may have applications to treat infections of other species, including *Staphylococcus aureus*. This pathogen is predicted to produce a PknB orthologue as well as more than 30 additional STPKs. In *M. tuberculosis*, PknB was shown to be a functional, autophosphorylated kinase expressed *in vitro* and *in vivo* in alveolar macrophages from a patient with tuberculosis [44]. PknB and the PstP phosphatase encoded in the same operon work as a functional pair [45], and they are thought to control mycobacterial cell growth [20]. The PknB extracellular sensor domain comprises four PASTA repeats that are thought to bind intermediates in cell wall biosynthesis [46]. Nonetheless, the signals that activate PknB or any bacterial STPK are not yet defined.

Dimerization [26] and autophosphorylation of a conserved motif called the activation loop [43, 45] activate PknB, PknD, and other *M. tuberculosis* receptor STPKs. The dimerization interface, on the opposite side of the N-lobe relative to the ATP binding site, comprises a conserved allosteric surface [26] that holds the kinase active sites away from each other in the dimer. A similar activating dimer interface was found in the human double-stranded-RNA-dependent protein kinase, PKR [47, 48]. Consistent with the back-to-back orientation of kinase domains (KDs) in the activated dimers, *M. tuberculosis* STPK autophosphorylation is an intermolecular reaction that is inhibited by mutations in the dimer interface [26].

The crystal structures of the PknB KD complexed with nonhydrolyzable nucleotides provided the first views of bacterial STPKs [43, 49]. The structures showed dramatic similarities between the folds, nucleotide binding sites, nucleotide conformations, and regulatory features of bacterial and eukaryotic STPKs. These similarities support a universal activation mechanism of Ser/Thr protein kinases in prokaryotes and eukaryotes [43]. The back-to-back KD dimer now observed in three different crystal forms of PknB [29] was also observed in the crystal structure of the phosphorylated KD of the *M. tuberculosis* receptor STPK, PknE [50]. The ATP binding site of the activated, nucleotide-free PknE KD adopted a conformation incapable of nucleotide accommodation and provided evidence that nucleotide exchange involves large conformational rearrangements of the ATP binding site. These results suggested that each STPK might adopt multiple conformations that could be targeted by distinct inhibitor classes. The active, back-to-back dimer formed by the PknB and PknE KDs was not seen in the structure of PknG, the first structure of a soluble bacterial STPK [51]. Instead, PknG contained folded N- and C-terminal extensions that may regulate the KD.

The substrates of the *M. tuberculosis* STPKs and phosphatases determine the functions of these proteins and also highlight possible targets for orthogonal inhibitors. Based on the number of phosphoproteins detected in *Corynebacterium glutamicum*, a related actinomycete [52], the upper bound for the number of STPK substrates in *M. tuberculosis* was estimated to approach 800 proteins [21]. Alternatively, the *C. glutamicum* phosphoproteins may reflect the most abundant substrates, or substrates modified with stable phosphates that do not play regulatory functions.

Moreover, if the 11 *M. tuberculosis* STPKs are more specific than the 4 homologous *C. glutamicum* enzymes, the number of authentic substrates in *M. tuberculosis* may be significantly smaller. A computational search of the *M. tuberculosis* genome using the phosphorylation-site sequence in the PknH activation loop revealed 40 potential substrates for this kinase [53]. Some of these candidates were verified to be phosphorylated by the PknH KD *in vitro*.

For the other STPKs, a growing number of candidate substrates have been identified in studies using *in vitro* and *in vivo* assays. These substrates include transcriptional regulators, membrane channels, enzymes, and regulatory proteins (reviewed by Greenstein *et al.*, 2006). Some of these studies, however, have nominated candidates that fail to fulfill characteristics expected for authentic targets of phosphoregulation. The *in vitro* phosphorylation sites identified in PbpA, DacB1, and MmpL7 [40, 53, 54], for example, are predicted to be outside the bacterial cell. In such an extracellular location, it is hard to imagine how the STPK and the PstP catalytic domains, which are intracellular, could perform continuous regulatory functions. It remains to be seen if phosphorylation of extracellular sites occurs prior to protein transport or is a prerequisite for protein secretion. In addition, candidate substrates that are phosphorylated by numerous kinases or are phosphorylated inefficiently *in vitro* may be the targets of nonspecific modifications [55, 56]. Limited selectivity or modest catalytic efficiency could result from the absence of the complete STPK or substrate protein in the native context [57]. Weak phosphorylation, however, may be suppressed in the presence of competing authentic substrates. Moreover, substoichiometric phosphorylation would be incapable of dramatically changing the total metabolic activity of the candidate substrate protein. Efficient phosphorylation of a large excess of substrate, on the other hand, provides more confidence that the target is functionally significant [56, 58, 59].

In our experience, many proteins are weakly phosphorylated by the purified KDs, and incomplete phosphorylation *in vitro* falls short of providing a strong case that the modification plays a regulatory role *in vivo*. Similarly, because PstP rapidly dephosphorylates cognate and noncognate substrates *in vitro*, dephosphorylation by PstP provides only part of the case for functional relevance. Stronger evidence for the function of a particular phosphorylation reaction might include a change in activity upon phosphorylation [58, 60], occurrence of the phosphorylated substrate protein *in vivo* [60] or detection of a phenotype resulting from mutations of the phosphorylation site [34].

To date, a small number of substrates have been shown to be phosphorylated efficiently *in vitro* and to respond to perturbations of STPKs *in vivo*. Importantly, autophosphorylation of the KDs appears to be an authentic regulatory reaction. Supporting this conclusion, mutations of phosphorylation sites in the PknB activation loop inactivated the KD *in vitro*, multiple receptor STPKs are expressed with activation loop phosphates, dephosphorylation with PstP inactivates the KDs, several STPKs are phosphorylated *in vivo*, and expression of catalytically dead and active PknD produced distinct patterns of auto- and transphosphorylation *in vivo* [26, 44, 45, 61]. Consistent with a regulatory network, the PknA and PknB KDs

phosphorylate each other *in vitro* [34]. The sites of these cross-phosphorylations and any regulatory effects on kinase functions remain to be defined.

In vivo studies have implicated several substrates for the *M. tuberculosis* STPKs. Pioneering work by Kang *et al.* identified Wag31, Rv1422 and PknA as substrates for PknB [34]. Effects of expressing (or depleting) *pknA*, *pknB*, and *wag31* in *M. smegmatis* provided evidence that PknA and PknB regulate cell shape in mycobacteria. The transcriptional regulator, EmbR, is an *in vivo* substrate for PknH in *M. tuberculosis* [62, 63], similar to the phosphorylation of the AfsR regulator by the AfsK kinase in *Streptomyces* [64]. Although the apparent effects on activity are modest, all purified *M. tuberculosis* STPK KDs except those of PknG, PknI, and PknJ phosphorylate the FAS II components KasA and KasB *in vitro*, and these fatty-acid biosynthetic enzymes are phosphorylated *in vivo* in *M. bovis* BCG [60]. The PknD kinase efficiently phosphorylates the anti-anti-sigma factor homologue Rv0516c on Thr2 *in vitro* and *in vivo*, and this phosphorylation alters *in vitro* binding to the homologous regulator, Rv2638 [58]. Activation by overexpression of PknD in *M. tuberculosis* altered the expression of more than 100 genes, including genes regulated by sigma F, providing evidence that PknD converts environmental signals into a transcriptional response. Combined with a growing number of studies of STPK functions in other bacteria [65, 66], these studies support the idea that the *M. tuberculosis* receptor kinases regulate diverse physiological processes.

11.5
M. tuberculosis STPK Inhibitors

The sequence motifs and structural similarities shared among the eukaryotic and *M. tuberculosis* STPKs suggested that similar compound classes might inhibit the eukaryotic and prokaryotic family members. This concept, however, received experimental confirmation well before the structure of a bacterial STPK was determined. The initial chemical validation of *M. tuberculosis* STPKs as potential drug targets was provided by analysis of the effects of nonspecific inhibitors of eukaryotic STPKs on mycobacterial growth *in vitro*. The kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), a H-series compound developed to bind eukaryotic kinases, not only inhibited the purified PknB in a biochemical assay but also attenuated *M. bovis* BCG and *M. smegmatis* growth *in vitro* [28]. Although H7 concentrations in excess of 100 μM were needed, which most likely induce off-targets effects and the direct physiological target in the bacteria was not identified experimentally, these studies began to establish the case that STPK inhibitors can sterilize mycobacteria in culture.

A similar approach of surveying available inhibitors of eukaryotic kinases led to the identification of PknB as a target of mitoxantrone and PknD as a target of SP006125 [29, 58]. SP600125, a c-Jun N-terminal kinase inhibitor, shows an IC_{50} for the purified PknD KD of 30 nM (C. Miecskowski and Tom Alber unpublished results), binds more weakly to other *M. tuberculosis* KDs and inhibits PknD activity *in vivo* [58]. Mitoxantrone, an anthraquinone derivative developed for cancer therapy,

inhibits the PknB kinase domain with an IC_{50} of 0.8 +/− 0.05 μM and attenuates mycobacterial growth *in vitro* [29]. Mitoxantrone (which also reacts with DNA) showed MIC values of 100 μM and 400 μM for *M. smegmatis* and *M. tuberculosis*, respectively. Ectopic expression of PknB in *M. smegmatis* increased the MIC twofold. Because this effect was small, caution is warranted in concluding that PknB is the cellular target of the inhibitor. Overall, these results support the view that a more potent, selective PknB inhibitor may block growth *in vivo* [29].

The first commercial program to develop unique compounds specifically targeting a mycobacterial kinase was carried out by Axxima Pharmaceuticals AG, a German company focusing on developing novel kinase inhibitors for therapeutic treatment of infectious diseases. Using combined screening and medicinal chemistry strategies, Axxima developed AX20017, a tetrahydrobenzothiophene compound that specifically inhibits PknG kinase activity. By inhibiting PknG in *M. bovis* BCG, AX20017 promoted phagolysosome fusion in macrophages, leading to bacterial killing in host cells without affecting the viability of the macrophages [27]. Conceptually, the development of an inhibitor that limits disease processes but does not directly kill *M. tuberculosis in vitro* was unique and challenging to the drug development community. The company produced a series of related compounds targeting PknG, AX14585, and others that were more stable, but development of these kinase inhibitors ceased when the company was sold to GPC Biotech. These tetrahydrobenzothiophene hit compounds are still not potent enough to be active in animal model of infections and require subsequent medicinal chemistry optimization.

The modes of inhibition of mitoxantrone and AX20017 were revealed by the cocrystal structures of these compounds bound, respectively, to the kinase domains of PknB and PknG [29, 51]. Both compounds lodged in the ATP binding sites of the target KDs (Figure 11.1). The two compounds, however, exploited distinct regions of the ATP binding sites that overlapped the position of the adenine ring (Figure 11.2). In particular, AX20017 made contact with the hinge region of PknG [51], while mitoxantrone made contact with residues that recognize the adenine and ribose moieties of the nucleotide and also the "front" of the ATP binding site [29]. Mitaxantrone binding stabilized a conformation of the PknB KD distinct from the nucleotide complex. In the inhibitor complex, the P loop, which recognizes the ATP phosphates, dropped into the active site to make inhibitor contacts (Figure 11.2c). This conformation, which resembles the P loop conformation in the structure of nucleotide-free PknE [50], illustrates the flexibility of the PknB ATP binding site (even in the phosphorylated KD) that can allow recognition of different ligands. Overall, these cocrystal structures illustrate that potency and selectivity can be enhanced by exploiting distinct regions of the ATP binding sites of different KDs.

While PknB currently affords the most promising phosphosignaling target in *M. tuberculosis*, several programs have developed selective inhibitors of the *M. tuberculosis* PTPs, PtpA, and PtpB. Waldmann and coworkers used a new approach inspired by natural-product pharmacophores to discover PTP inhibitors [67–69]. Using this "biology-oriented synthesis" approach, PtpB inhibitors with IC_{50} values as low as 360 +/− 12 nM were developed [69]. In contrast, Serono Pharmaceuticals screened libraries of compounds directed against eukaryotic phosphatases for

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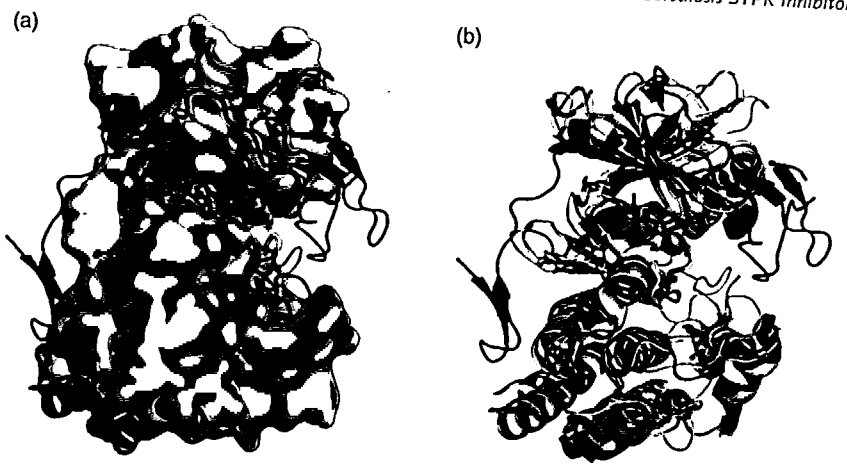
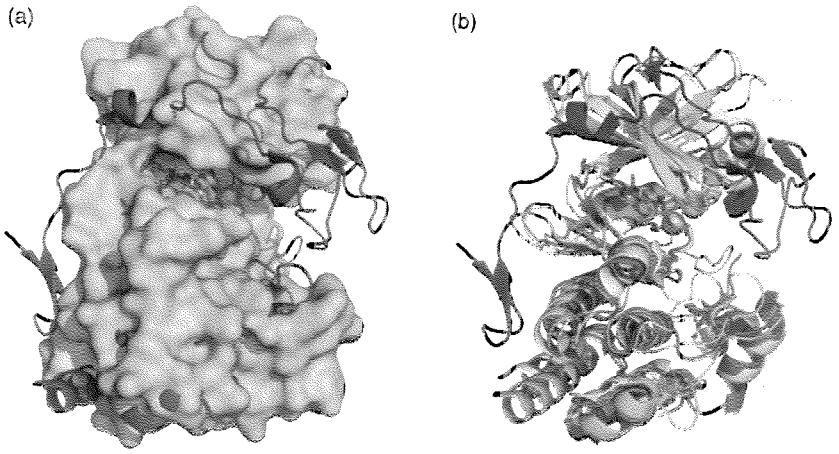


Figure 11.1 Inhibitors occupy the ATP binding site of PknB and PknG. (a) AMPPCP, mitoxantrone and AX20017 (sticks) superimposed on the surface of PknB KD and the ribbon representation of the PknG KD and N-terminal extension. To focus on the shared elements, the C-terminal domains of PknG were omitted. The N-terminal extension of PknG starts on the back of the C-lobe (bottom left) and drapes over the N-lobe, filling the putative binding site for protein substrates. (b) Superimposed ribbon diagrams of the kinase domains in the AMPPCP (light gray) and inhibitor (medium gray) complexes of PknB and PknG (dark gray), with the N-terminal extension of PknG shown in darker gray. Although the overall folds of the PknB and PknG kinase domains in the complexes are similar, the backbone root mean square deviation (rmsd) is 2.6 Å. Thus, the inhibitors target distinct conformations and surfaces.

inhibitors of the *M. tuberculosis* homologues [70]. Improving potency using medicinal chemistry led to the development of oxalylamino-methylene-thiophene sulfonamide (OMTS), a PtpB inhibitor with and IC_{50} of 440 nM. Remarkably, this compound showed more than 65-fold selectivity for PtpB compared to all human PTPs tested. The cocrystal structure of OMTS bound to PtpB revealed a large change in conformation of the enzyme compared to the product complex and showed two molecules of the inhibitor bound in the active site [70]. The structure supported the idea that the selectivity of OMTS may arise from inhibitor contacts with every residue in the phosphate binding loop, which is conserved in many human phosphatases but differs in three positions in PtpB.

Ellman and coworkers have developed a novel screening method, called substrate activity screening (SAS), and applied it to the *M. tuberculosis* phosphatases to produce the most potent PtpB inhibitor reported to date [70]. Rather than the traditional approach of assaying a compound library for inhibitors, the SAS approach identifies hits in a chemical library of substrates. Because the library compounds must be turned over by the enzyme, false positives due to nonspecific inhibition or denaturation are eliminated and compounds that target the active site are identified. For the PTPs, the substrate library comprises a collection of *O*-aryl phosphates, which are relatively straightforward to synthesize from commercially available building blocks.



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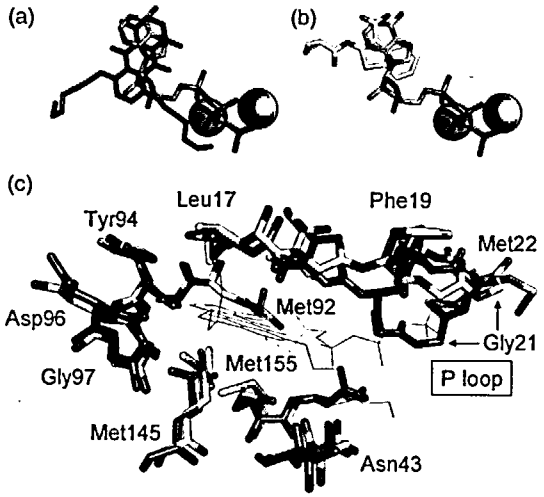

Figure 11.2 Recognition of *M. tuberculosis* STPK inhibitors. (a) Ligands from the superimposed PknB complexes of mitoxantrone (blue, 2FUM) and Mg:AMPPCP (spheres: green, 1O6Y). The inhibitor samples the volume occupied by the adenine and ribose rings of the nucleotide [29, 49]. (b) Nucleotide from the PknB:Mg:AMPPCP complex (spheres: green, 1O6Y) superimposed on the inhibitor from the PknG:AX20017 complex (white and gray atoms, 2PZI). The PknG inhibitor [51] samples a distinct region of the ATP binding site. (c) ATP binding site of PknB (sticks) adjusts to the binding of AMPPCP (light gray) and mitoxantrone (dark gray). Ligands are shown as lines. The P loop (*top right*) slumps into the active site in the inhibitor complex, filling the region occupied by the terminal phosphates of the bound nucleotide. Met92, Met155, and Asp156 adopt distinct rotamers in the nucleotide and inhibitor complexes, while the other residues in the binding site adopt similar conformations. The backbone rmsd of the PknB KDs in the superimposed complexes is 0.83 Å.

In the second step of SAS, the best substrates are converted to inhibitors by replacing the labile phosphoryl group with an inhibitory "warhead." For the PTPs, focusing on the best substrate scaffolds limits the more challenging synthesis of inhibitors to a small set, speeding the overall development process. Using SAS, a PtpB inhibitor with a molecular weight of 433 Da and a K_i value of 220 +/− 30 nM was found. This isoxazole inhibitor showed from 35 to >225 selectivity against *M. tuberculosis* PtpA and a panel of four eukaryotic PTPs. The small size of this compound leaves scope for additional modifications to engineer improved properties [70].

A major hurdle for the development of *M. tuberculosis* PTP inhibitors is to demonstrate efficacy *in vivo*. Neither *ptpA* nor *ptpB* are essential for growth in culture, as judged by whole-genome transposon mutagenesis [35] and the growth rate of the *ptpB* deletion mutant [25]. As a result, the PTP inhibitors are expected to have no effects on *M. tuberculosis* growth or viability *in vitro*. The attenuation of the *ptpB* deletion strain in activated macrophages and guinea pigs [25], suggests that cell based or animal assays will be required to establish the validity of these targets and drive further development.

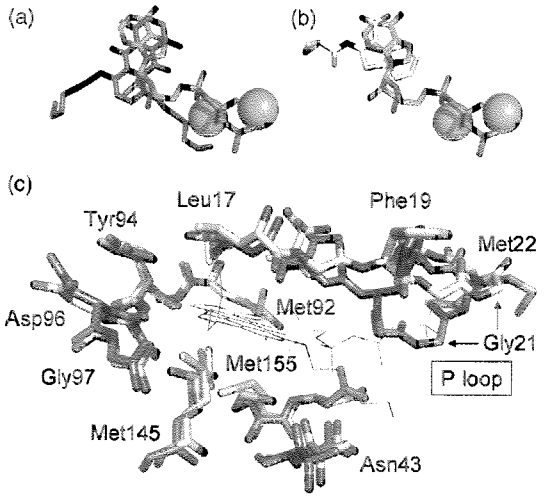

Figure 11.2 Recognition of *M. tuberculosis* STPK inhibitors. (a) Ligands from the superimposed PknB complexes of mitoxantrone (blue, 2FUM) and Mg:AMPPCP (spheres: green, 1O6Y). The inhibitor samples the volume occupied by the adenine and ribose rings of the nucleotide [29, 49]. (b) Nucleotide from the PknB:Mg:AMPPCP complex (spheres: green, 1O6Y) superimposed on the inhibitor from the PknG:AX20017 complex (white and gray atoms, 2PZI). The PknG inhibitor [51] samples a distinct region of the ATP binding site. (c) ATP binding site of PknB (sticks) adjusts to the binding of AMPPCP (light gray) and mitoxantrone (dark gray). Ligands are shown as lines. The P loop (*top right*) slumps into the active site in the inhibitor complex, filling the region occupied by the terminal phosphates of the bound nucleotide. Met92, Met155, and Asp156 adopt distinct rotamers in the nucleotide and inhibitor complexes, while the other residues in the binding site adopt similar conformations. The backbone rmsd of the PknB KDs in the superimposed complexes is 0.83 Å.

In the second step of SAS, the best substrates are converted to inhibitors by replacing the labile phosphoryl group with an inhibitory "warhead." For the PTPs, focusing on the best substrate scaffolds limits the more challenging synthesis of inhibitors to a small set, speeding the overall development process. Using SAS, a PtpB inhibitor with a molecular weight of 433 Da and a K_i value of 220 +/− 30 nM was found. This isoxazole inhibitor showed from 35 to >225 selectivity against *M. tuberculosis* PtpA and a panel of four eukaryotic PTPs. The small size of this compound leaves scope for additional modifications to engineer improved properties [70].

A major hurdle for the development of *M. tuberculosis* PTP inhibitors is to demonstrate efficacy *in vivo*. Neither *ptpA* nor *ptpB* are essential for growth in culture, as judged by whole-genome transposon mutagenesis [35] and the growth rate of the *ptpB* deletion mutant [25]. As a result, the PTP inhibitors are expected to have no effects on *M. tuberculosis* growth or viability *in vitro*. The attenuation of the *ptpB* deletion strain in activated macrophages and guinea pigs [25], suggests that cell based or animal assays will be required to establish the validity of these targets and drive further development.

11.6
Conclusions and Prospects

PknB provides the most promising bacterial phosphosignaling target for pharmaceutical development. Promiscuous inhibitors of human kinases are active against PknB, suggesting that the large chemical libraries and deep knowledge about targeting eukaryotic STPKs can be used to develop potent, selective inhibitors. The low sequence identity to the most related human kinase suggests that selectivity will be a low hurdle. High-throughput and secondary assays are well developed, and crystallographic studies to speed inhibitor development are feasible. Genetic studies indicate that *pknB* and *pknA* are essential genes, while the other *M. tuberculosis* STPKs may play interesting adaptive roles with pleiotropic effects on physiology. The scope for inhibitors that target multiple *M. tuberculosis* STPKs to shorten treatment of active TB or enable treatment of latent disease has yet to be explored. PknB orthologues are the most widely distributed bacterial STPKs, suggesting that inhibitors may be active against diverse bacterial pathogens.

Inhibitors of *M. tuberculosis* PtpB have been developed by traditional and novel approaches. These compounds show remarkable selectivity against the human phosphatases. The structures of PtpA and PtpB, as well as a cocrystal structure of the OMTS inhibitor bound to PtpB, indicate that structure-based strategies are accessible to increase potency. Improving potency and demonstrating that the inhibitors limit infection *in vivo* are key steps needed to promote development.

Overall, efforts to discover pharmaceuticals targeting bacterial phosphosignaling are just beginning. Fundamental studies of the functions and structures of these proteins have established the groundwork to make rapid progress in this field.

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Index

a
A-420983 179
A-641593 179
A-770041 179
AAL-993 177
Abelson (Abl) kin
– inhibitor 186
absorption
– gastrointestinal
absorption, distri
excretion (AD
– protein kinase i
discovery 26
ACE (angiotensin
inhibitor 90
activator protein-
active analogue a
acute myelogeno
adenine binding
adenovirus 327
AEB071 126
AEE788 120
AEW541/NPV-A
affinity chromato
– immobilized k
– kinase inhibito
AG013736 116
AG024322 127
Akt (Akt1)/ PKB
Akt/PDK-/Flt3 m
allosteric kinase
allosteric site 1
AlphaScreen-bas
AMG-548 292
AMG706 121
2-aminobenzimi
2-aminobenzoxa
2-aminoquinazo

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