Structure-Based Drug Design for Tuberculosis: Challenges Still Ahead

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Review

Structure-Based Drug Design for Tuberculosis: Challenges Still Ahead

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Abstract: Structure-based and computer-aided drug design approaches are commonly considered to have been successful in the fields of cancer and antiviral drug discovery but not as much for antibacterial drug development. The search for novel anti-tuberculosis agents is indeed an emblematic example of this trend. Although huge efforts, by consortiums and groups worldwide, dramatically increased the structural coverage of the *Mycobacterium tuberculosis* proteome, the vast majority of candidate drugs included in clinical trials during the last decade were issued from phenotypic screenings on whole mycobacterial cells. We developed here three selected case studies, i.e., the serine/threonine (Ser/Thr) kinases—protein kinase (Pkn) B and PknG, considered as very promising targets for a long time, and the DNA gyrase of *M. tuberculosis*, a well-known, pharmacologically validated target. We illustrated some of the challenges that rational, target-based drug discovery programs in tuberculosis (TB) still have to face, and, finally, discussed the perspectives opened by the recent, methodological developments in structural biology and integrative techniques.

Keywords: tuberculosis; structure-based drug design; target-based drug design; PknB; PknG; DNA gyrase; antibiotic

1. Introduction

Although the drug discovery process has historically relied on high-throughput screening (HTS) to identify hits to be developed into drug candidates, either on a given target (in most cases, an essential enzyme) or through whole-cell screenings, it still remains an experimentally laborious, expensive, and time-consuming process. Unlike traditional drug discovery, however, drug design is not necessarily based on the screening of large libraries, which is intrinsically a trial and error process, but builds on the available knowledge for a given biological target. A particular, well-known case of drug design is called structure-based drug design (SBDD), which uses the three-dimensional structural knowledge of the target to find or optimize molecules that can bind to the target with high affinity and selectivity. The potential of using structural information for discovering candidate drugs was apparent from the early days of structural biology [1], but it took several years to achieve the first successful examples, i.e., human immunodeficiency virus (HIV) protease inhibitors [2] and carbonic anhydrase inhibitors for the treatment of glaucoma [3].

A three-dimensional model of the target is, therefore, a prerequisite for SBDD. The structure can be obtained experimentally in different ways, in most cases by X-ray crystallography, although nuclear magnetic resonance (NMR) and, more recently, cryo-electron microscopy (cryo-EM) have also attracted attention [4], especially following the spectacular increase in the resolution capabilities of single-particle cryo-EM, which is commonly referred to as the resolution revolution [5,6]. For targets whose experimental structure is elusive, in silico structure prediction is also routinely performed and

can be achieved, whenever suitable models are available, through homology modeling from a closely related homologous protein [7].

The identification of potential drug binding sites can be obvious when ligand-bound target structures are available. However, the capability to detect binding sites for substrates or modulators becomes highly relevant not only for targets for which no ligand-bound three-dimensional structure is available, but also to detect allosteric sites or protein–protein interaction surfaces that might be specifically targeted [8], and for these, dedicated libraries are now available [9]. Considering that binding site identification is not always straightforward, tools have been developed to infer "druggable" pockets from the identification of concave regions—that could accommodate drug-size molecules—by screening for appropriate binding properties, such as volume, hydrophobicity, hydrogen bonding, energy potential, solvent accessibility, and desolvation energy [10].

The search for potential ligands on a given target can then follow two different approaches, commonly referred to as ligand-based drug design (LBDD) or target-based drug design (TBDD). LBDD does not require, a priori, direct structural knowledge of the target, but relies on the identification of hits, usually congeneric compounds, with an established biological effect; hit-to-lead optimization can then proceed through the definition of appropriate chemical descriptors of the series, quantitative structure-activity relationship analysis (QSAR), and pharmacophore modeling [11], all followed by experimental validation. QSAR-based virtual screening approaches, alone or in combination with HTS screenings, can also be used to enlarge the panel of bioactive compounds against the target or pathway of interest, increasing the overall hit rate [12]. TBDD, in contrast, uses the physical-chemical constrains from the target three-dimensional structure, and possibly a well-defined binding pocket, to perform virtual screening of libraries, either of natural or synthetic compounds, usually applying appropriate filters like compliance to Lipinski's rules or QSAR models, or to design molecules de novo in a step-wise manner. A particular case of de novo molecule design is fragment-based drug design (FBDD), in which low-affinity target binding molecules are identified from appropriate libraries, using a variety of biophysical approaches, and then merged or linked together using the available three-dimensional information to achieve larger binders with improved properties [13,14].

It is common knowledge in the field that although SBDD approaches have proven to be successful for non-transmissible diseases and viral diseases, they have proven to be much less effective in the antibiotic discovery field [15,16]. Unfortunately, tuberculosis represents no exception to this trend [17], despite the considerable progress achieved during the last twenty years in the understanding of the pathogen molecular physiology, starting from the seminal publication of the *M. tuberculosis* genome sequence in 1998 [18], the extensive structural genomics campaigns during the following years [19–21], the advances in understanding host-pathogen interactions and the development of the disease [22,23], and notwithstanding the development of genetic and biochemical tools to allow the in vivo and in vitro validation of targets [24,25].

The purpose of this review was not to provide an exhaustive overview of the capabilities now offered by in silico approaches for antibiotic development against *M. tuberculosis*, already reviewed elsewhere [26,27], nor to make a survey of the current state of structural knowledge of the pathogen proteome and the experimental structures relevant for drug discovery, for which we point the reader to very recent, extensive work [28]. Rather, we focused here on three emblematic case studies of *M. tuberculosis* targets that attracted most efforts for anti-tuberculosis compound development by HTS campaigns, computer-aided, and structure-driven compound identification: the serine/threonine (Ser/Thr) kinases—protein kinase (Pkn)B and PknG—two amongst the most known, supposedly promising new targets offered by the post-genomic era, and the DNA gyrase, the 'old' but the well-proven target of fluoroquinolones.

2. Protein Ser/Thr Kinases as Drug Targets

Protein phosphorylation is a well-known, widespread mechanism for signal transduction and regulation of several biological functions. Protein kinases have long been known as major drug

targets [29], especially for the treatment of cancer, where the deregulation of signaling mechanisms is a hallmark of the disease [30]. Protein kinases are not only pharmaceutical targets in cancer chemotherapy but also for the treatment of parasitic infections, ranging from the ones caused by Trypanosomatids or *Leishmania* [31] to malaria [32]. Since the sequence of *M. tuberculosis* H37Rv genome was first reported in 1998 [18], the pathogen has been known to possess eleven genes coding for Hanks-type Ser/Thr kinases [33], named from *pknA* to *pknL* (but no *pknC*), providing one of the first challenges to the paradigm of prokaryotic cell signaling as being entirely driven by two-component systems. One gene coding for a transmembrane Ser/Thr phosphatase, *pstP*, was also identified as lying on the same cluster as *pknA* and *pknB*, forming a putative operon [34], in addition to two genes (*ptpA* and *ptpB*) coding for Tyr phosphatases [35]. Nine out of eleven Ser/Thr kinases (all but PknG and PknK) were predicted to be integral membrane proteins, all sharing the same topology with an N-terminal catalytic domain in which the archetypal Hanks motifs could be identified [33], a single transmembrane segment and a very variable extracellular, C-terminal domain, hypothesized to be acting as a signal sensing domain. The role of such C-terminal domains is, in most cases, still puzzling, although, in some kinases like PknB, it has been suggested to be involved in the kinase activation process, possibly by controlling the kinase oligomerization state as a function of the external signal [36]. Work from Tom Alber's group in Berkeley indeed demonstrated the role of the 'back-to-back' dimerization to promote an active kinase conformation, not only for PknB [37,38] but also for PknD [39], notwithstanding the observation that mutations in the dimerization interface do not abolish kinase activity, as reported by separate groups [37,40]. PknB was also the first *M. tuberculosis* kinase for which the crystal structure of the catalytic domain was described in 2003 [41,42]. Indeed, this first crystal structure confirmed the overall conservation of the bi-lobed protein kinase fold and the initial assumptions about the presence of the known structural features of eukaryotic protein kinases, as predicted by the detection of the Hanks motifs [42], thus underlining the common origin of eukaryotic and prokaryotic kinases. Following these first milestones and the genetic proof of the in vitro essentiality of *pknB* coming both from transposon mutagenesis [43] and targeted studies [44,45], PknB then attracted the attention of tuberculosis (TB) research community as an ideal target for structure-based drug design. The community's interest rise when considering the increasing evidence, over the years, of its role in the control of peptidoglycan synthesis and cell division (recently reviewed in [46]). The first description of the potential use of PknB inhibitors as anti-mycobacterial agents reported the compound H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine; Table 1), well before any bacterial protein kinase structure was available [47]. Once the PknB catalytic domain structure was solved, virtual screening carried out on a library of about 40,000 compounds for hits into the PknB adenosine triphosphate-binding (ATP-binding) pocket was performed. The screening led to identifying mitoxantrone, a chemotherapeutic agent known for its DNA intercalating properties, as a sub-micromolar PknB inhibitor [48], similar to staurosporine, K-252-a, and K-252-b (Table 1), who were identified as hits after testing a few commercially available eukaryotic kinase inhibitors [44]. The crystal structure of the PknB-mitoxantrone complex was the first showing the kinase catalytic domain in complex with a non-ATP analog, kinase inhibitor. Two crucial, hydrogen bonding interactions were evidenced between the mitoxantrone hydroxyl groups and main chain atoms from the PknB hinge region that connects the two kinase lobes, i.e., the carbonyl oxygen of Glu93 and the amino group of Val95 [48], opening the way to compound optimization (Figure 1A,B). Several other PknB inhibitors were proposed in the course of the following years, starting from hits developed either from known kinase inhibitors like staurosporine analogs [37], 2-aminopurine and its derivatives, including organometallic compounds [49]), or from hits obtained from HTS on public or proprietary libraries using GarA as the substrate [50–53], or phytocompounds [54] (Table 1). Most of the published work deals with compounds inhibiting the kinase in the micro and sub-micromolar range but with limited activity on mycobacteria. The exception to this trend is IMB-YH-8 (Table 1), a compound that, despite showing an IC_{50} (half maximal inhibitory concentration) on PknB in the 20 μM range, shows good selectivity for mycobacterial PknB and PknA and a MIC (minimal inhibitory concentration) in

the sub-micromolar range [53]. Compounds highly active on both PknB and PknA have also been reported recently by others in the form of substituted quinazolines, and, for a compound derived from this series (a pyrimidine analog), the crystal structures of the respective complexes with both kinases have been described [55] (Figure 1, Table 1). Both structures underline the common binding mode in the ATP pocket and the crucial interaction with the hinge region (Figure 1B). Despite the inhibition constant for both kinases falls in the nanomolar range, these dual-targeting inhibitors display promising but yet limited antibacterial effect with MIC on *M. tuberculosis* in the lower micromolar range [55]. A different PknB inhibitor for which a crystal structure in complex with the target is available is GSK690693 (Figure 1C, Table 1), also identified through a virtual screening approach on known kinase inhibitors [56]. This compound, member of the imidazopyridine aminofurazans class, also displays conserved features in its binding mode to the PknB hinge region (Figure 1B), and sub-micromolar affinity to the kinase, but no significant antimycobacterial activity on *Mycobacterium smegmatis* or *Mycobacterium bovis* BCG (bacillus Calmette-Guerin). However, the MIC is significantly lowered if the compound is associated with a sub-MIC50 concentration of meropenem, suggesting a synergistic action between PknB inhibitors and β-lactams [56].

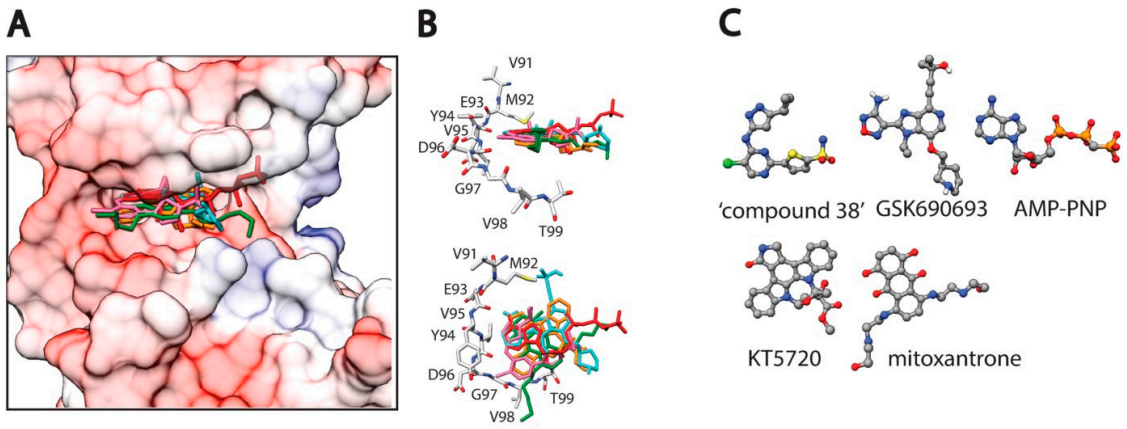


Figure 1. Experimental structures of protein kinase B (PknB)-inhibitor complexes. (**A**) Electrostatic surface from the X-ray structure of PknB in complex with AMP-PCP (β,γ-methyleneadenosine 5′-triphosphate) in red (pdb: 1O6Y), superimposed with the coordinates of four other PknB complexes with, respectively, mitoxantrone in green (pdb: 2FUM), KT5720 in orange (pdb: 3F69), GSK690693 in cyan (pdb: 5U94), and 'compound 38' in pink (pdb: 6B2P). This compound has been reported as active on both PknA and PknB [55]. (**B**) Side and top view of the superimposed ligands with the hinge region from the pdb entry 6B2P. Kinase residues are indicated. (**C**) Ball-and-stick representation of the ligands kept in the same relative binding orientation as in (**B**) and colored by element.

Table 1. *M. tuberculosis* PknB inhibitors.

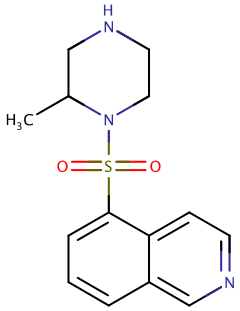
Family	Name	Structure	IC_{50} (μM)	Reference
Isoquinolines	H-7		ND	[47]

Table 1. *Cont.*

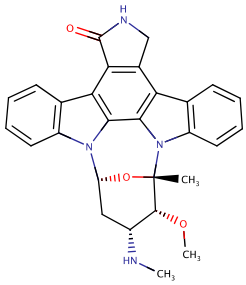
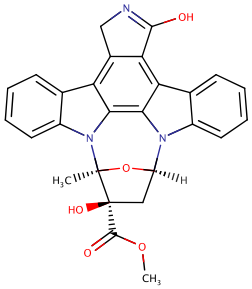
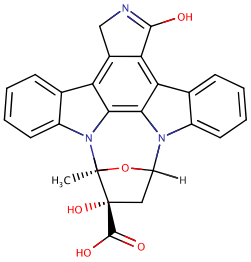
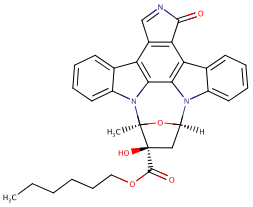
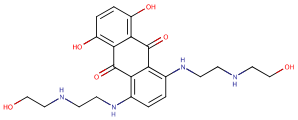
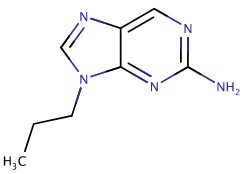
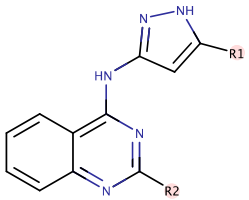
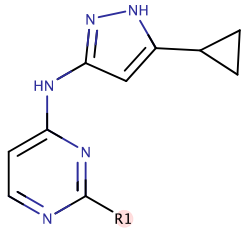
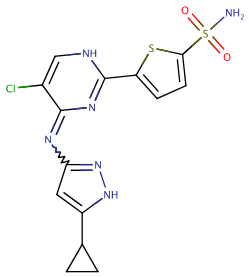
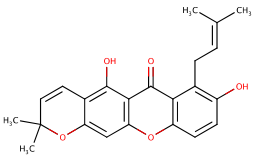
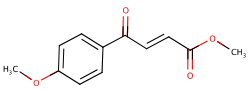
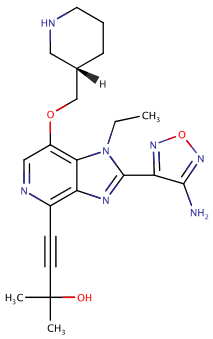
Family	Name	Structure	IC_{50} (μM)	Reference
Staurosporine analogues	Staurosporine		0.6	[44]
	K-252-a		0.096	
	K-252-b		0.106	
	KT5720		~1 [a]	[37]
Anthracenediones	Mitoxantrone		0.8	[48]
Aminopurines	2-A9P		1300	[49]

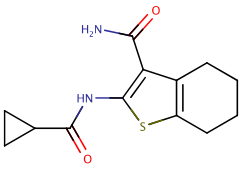
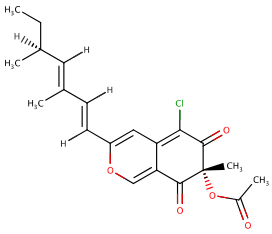
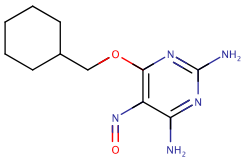
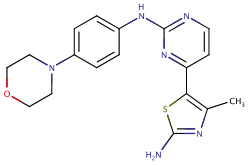
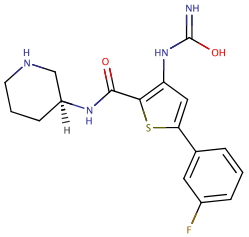
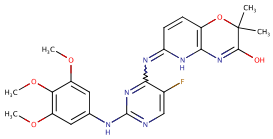
Table 1. *Cont.*

Family	Name	Structure	IC_{50} (μM)	Reference
Quinazolines	Disubstituted series		≤ 1.1	[51]
Pyrimidines	Disubstituted (-R1 also as -NHR1)		≤ 0.4	[51]
	'Compound 38'		Ki ~ 1 nM	[55]
Phytocompounds	Demethylcalabaxanthone		ND	[54]
4-oxo-crotonic acid derivatives	IMB-YH-8		20.2	[53]
Imidazopyridine aminofurazans	GSK690693		0.34	[56]

ND: not determined. [a] measured on a PknB mutant (M145L, M155V) [37].

On the other hand, another *M. tuberculosis* kinase that attracted as much attention for its potential druggability is PknG, a soluble Ser/Thr kinase that was described, by Jean Pieters and coworkers, as a virulence factor secreted into the human macrophage, where it would inhibit the phagosome-lysosome fusion [57]. Most notably, in the same work, the authors showed that chemical inhibition of PknG by the compound AX20017 (a tetrahydrobenzothiophene identified by HTS and found to inhibit PknG with IC_{50} in the sub-micromolar range; Table 2) led to the accumulation of *M. tuberculosis* inside lysosomes [57]. Noteworthy, the 2.4 Å resolution crystal structure of an N-terminal truncated form of PknG in complex with AX20017, published later [58], shows a great similarity between the AX20017 binding to PknG and the binding of ATP and mitoxantrone to PknB. Not only the compound occupies the adenine binding pocket but also makes similar interactions with the main chain atoms of residues Glu233 and Val235 (Figure 1). It was only a few years later that further structural work allowed to elucidate the binding mode of ATP to PknG and suggested a regulatory role of the rubredoxin-like domain by a reversible occlusion of the active site entrance [59]. These initial findings paved the way to further structure-based lead optimization and undoubtedly generated excitement for the potential chemical targeting of this kinase, which would allow preventing the arrest of phagosome maturation, directing *M. tuberculosis* to lysosomes. Attempts to develop more potent compounds starting from AX20017 yielded sub-micromolar inhibitors, some of which had no activity in macrophage assays [50], and no other structure of a PknG-inhibitor complex has so far been reported. It is, however, worth noting that, more than ten years later, the role of PknG in arresting the phagosome maturation is still elusive. Although progress has been made in identifying the SecA2 system as responsible for the export of PknG outside *M. tuberculosis* [60,61], and interference by PknG on the host Rab7l1 signaling pathway has been reported [62], it is largely accepted that several mechanisms, and not a single virulence factor, contribute to the *M. tuberculosis* capability to escape the phagocytic route and survive into macrophages [22,63]. Moreover, an increasing amount of evidence has since validated PknG as a key signaling element in the control of central metabolism, as first shown in *Corynebacterium glutamicum* [64,65], then in mycobacteria [66,67], where genetic and metabolomic evidence point to a role of PknG in regulating the 2-oxoglutarate node according to nutrient availability [68]. Alternative roles of PknG in biofilm formation, adaptation to oxidative stress [69], and hypoxia [70] have also been proposed. Therefore, the observed effects of the lack of PknG on the phagosome-lysosome fusion, and the decreased viability of a *pknG*-deprived *M. tuberculosis* mutant, both in vitro and in the mouse model [71], may not necessarily indicate a direct interference of the kinase on the host signaling pathways but could be ascribed to metabolic alterations caused by the depletion of PknG [68,72]. During the last years, further search for PknG inhibitors has allowed identifying sclerotiorin, an azaphilone derivative isolated from *Penicillium* sp. ZJ27 (Table 2), a known inhibitor of other unrelated enzymes like lipoxygenase, that, despite showing an IC_{50} on PknG around 76 μM and no inhibition on *M. tuberculosis* growth in vitro, displays capability to partially reduce the growth of *Mycobacterium bovis* BCG inside macrophages, and to enhance the effect of rifampicin [73]. On the other hand, the recently described PknG inhibitor NU-6027 (2,6-diamino-4-cyclohexylmethoxy-5-nitrosopyrimidine; Table 2) was identified from phenotypic screening as having an MIC99 value of 1.56 μM on *M. bovis* BCG and shown to partially inhibit PknG and PknD autophosphorylation in vitro [74]. Another series of four compounds, all inhibiting PknG in the micromolar range, was also recently reported after the screening of an 80-compound, commercially available kinase inhibitor library (Table 2); three of them were found to promote the transfer of mycobacteria to lysosomes, and two to inhibit *M. bovis* BCG growth in macrophages [75]. None of the reported compounds, however, issued from a structure-based screening approach.

Table 2. *M. tuberculosis* PknG inhibitors.

Family	Name	Structure	IC_{50} (μM)	Reference
Tetrahydrobenzothiophenes	AX20017		0.39 5.5	[57] [75]
Azaphilones	Sclerotiorin		76	[73]
Pyrimidines	NU-6027		ND	[74]
	CYC116		35.1	[75]
Thiophenes	AZD7762		30.3	[75]
Methoxybenzenes	R406 (benzenesulfonate) and its free base R406f (also known as Tamatinib)		8.0 (R406) 16.1 (R406f)	[75]

ND: not determined.

3. DNA Gyrase: A Validated Drug Target

Topoisomerases are enzymes that participate in the overwinding or underwinding of DNA. They are commonly split into two classes, depending on the number of DNA strands cut during a single catalysis cycle: class I topoisomerases, which include eukaryotic topoisomerase I, prokaryotic topoisomerase IIIa, or reverse gyrase among others, and class II topoisomerases, whose members include (but are not restricted to) topoisomerase IV and DNA gyrase [76]. In *M. tuberculosis*, DNA gyrase is a heterotetrameric nanomachine, consisting of two GyrB and two GyrA subunits, and is essential for DNA replication, transcription, and repair in living cells [77].

M. tuberculosis is an exception in the prokaryotic world because of the presence of only one type I and one type II topoisomerase, whereas most other eubacteria have two enzymes of each type. In the canonical situation, DNA gyrase and topoisomerase IV, both type IIA topoisomerases, have distinct specific activities: DNA gyrase removes positive supercoils that accumulate ahead of

replication forks [78], whereas topoisomerase IV decatenates replication intermediates [79]. Thus, in *M. tuberculosis*, DNA gyrase being the unique topoisomerase IIA, it must be able to carry out both activities in vivo [80]. Furthermore, with no other topoisomerase IIA in *M. tuberculosis*, the DNA gyrase is the unique target of antibiotics from the fluoroquinolones (FQ) family (quinolones with higher cellular penetration efficiency), which bind and stabilize the DNA–DNA gyrase complex. Stabilization of this ternary complex leads to the cytotoxic accumulation of double-strand cleaved DNA fragments within the cell, inducing bacterial death [81]. Crystallographic structures of the isolated domain and the cleavage core, either in its *apo* form or co-crystallized with several FQ, brought crucial information to elucidate the mode of binding of these drugs [82–87]. The ratio FQ/DNA–DNA gyrase in the ternary complex is two FQ molecules per complex, with one molecule binding in each cleavage site, wedging between the two ends of the cleaved DNA strands on both halves of the complex, in a drug-binding pocket whose walls are formed by DNA base stacking (Figure 2A) [82]. The emergence of strains with multidrug resistance (MDR) phenotype, or extensively-drug resistance (XDR) phenotype in which drug resistance includes this family of antibiotics, has made improving the activity of this family of drugs by using structural data to be of utmost importance. The main goals of these studies are to increase specific drug/enzyme interactions, but also to find new molecules that inhibit this validated target without cross-resistance with FQ [88].

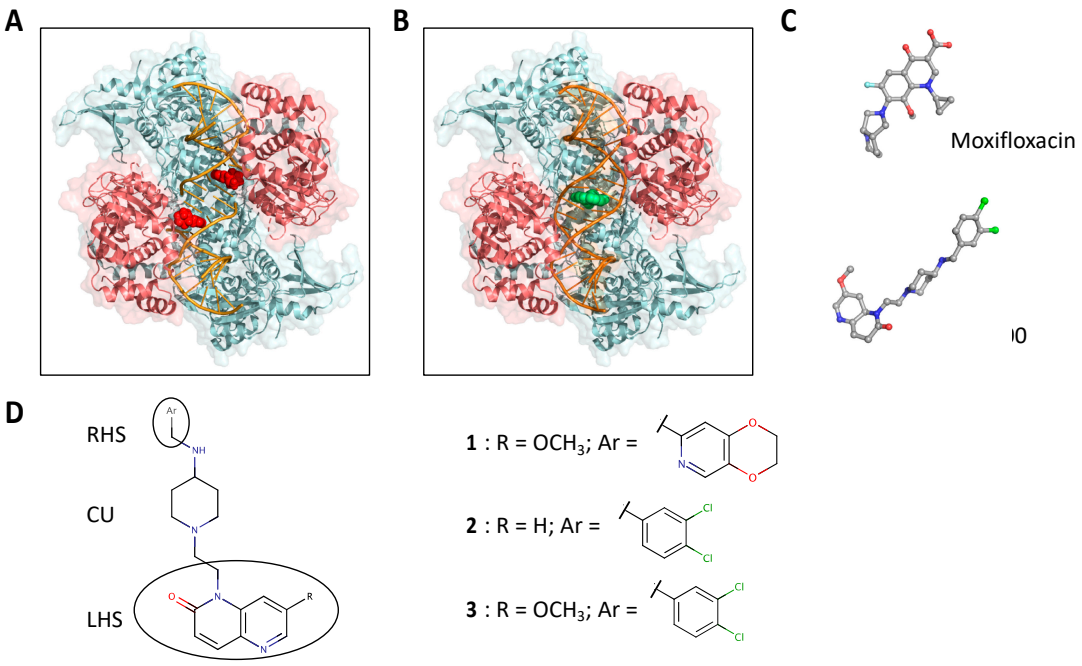


Figure 2. Inhibitors of DNA gyrase. (**A**) Top view of the X-ray structure of *M. tuberculosis* gyrase cleavage core in complex with DNA and moxifloxacin. Protein is shown in cartoon representation with transparent surfaces, with GyrB in blue and GyrA in pink. DNA is shown in orange, with moxifloxacin as red spheres (pdb: 5BS8). (**B**) Top view of the model of a complex of GSK000 (green spheres) with *M. tuberculosis* gyrase cleavage core and uncleaved DNA (in orange), based on the crystal structure of GSK299423 with *Staphylococcus aureus* gyrase (pdb: 2XCR). The same representation, as in panel A, was used for the protein. (**C**) Ball-and-stick representation of the ligands colored by element. (**D**) Generic chemical structure of the novel bacterial topoisomerase inhibitor (NBTI) series of compounds. Analog 1 is a classical NBTI, whereas analogs 2 and 3 combine the three favorable features for *M. tuberculosis* DNA gyrase inhibitor (MGI).

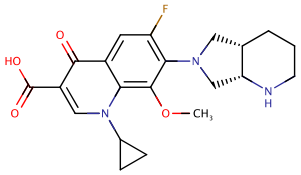
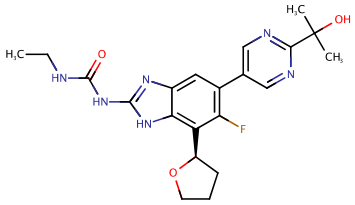
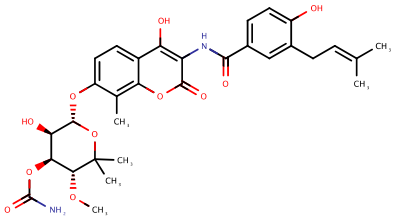
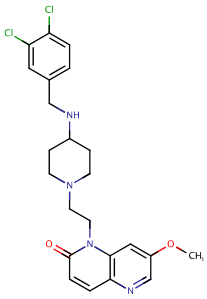
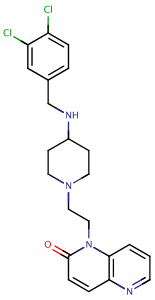
A significant amount of effort has thus been invested in discovering new *M. tuberculosis* gyrase inhibitors, mostly centered on the ATP- and DNA-binding sites, and particularly using in silico methods based on the emerging gyrase structural information, as extensively reviewed by Nagaraja

and coworkers [89]. Although the ATP binding sites have been less successfully exploited as antibacterial targets, with the exception of the natural products, such as coumarins [90,91], it is worth noting that a candidate drug emerged from these studies. SPR720 (Table 3) is an orally bioavailable prodrug of SPR719, an aminobenzimidazole inhibitor of both gyrase and topoisomerase IV. Indeed, this novel class of antimicrobials targets the ATPase subunits of gyrase and topoisomerase IV, and it was optimized using SSBD and structure-activity relationship (SAR) studies of potency against both Gram-positive and some Gram-negative bacterial species [92,93]. Crystal structures of novobiocin (Table 3) bound to *Escherichia coli* GyrB subunit served as a starting point for ligand optimization [94], while further optimization of the metabolic profile led to the identification of SPR720 (formerly VXc-486) [95], whose suitability to be a drug candidate for the treatment of tuberculosis and non-tuberculosis mycobacterial infections was thoroughly evaluated [96,97]. The compound completed Phase I clinical trials in 2019 (study ID NCT03796910, sponsored by Spero Therapeutics, LLC), aimed at evaluating the safety, tolerability, and pharmacokinetics (PK) profile in healthy volunteers. Moreover, an in vivo combination of SPR720 with rifampicin and pyrazinamide (two first-line drugs used in TB treatment) showed comparable efficacy to the combination of three drugs, including moxifloxacin, rifampicin, and pyrazinamide [98].

Work on the DNA-binding site has also been productive, as GlaxoSmithKline (GSK) created a new class of type IIA topoisomerase inhibitors, called novel bacterial topoisomerase inhibitors (NBTIs) (Table 3) [99]. The crystallographic structures of the DNA-bound *Staphylococcus aureus* DNA gyrase with either NBTI or FQ, all obtained at high resolution, revealed that the binding sites for each drug are different [100]. Indeed, the NBTI 'bridges' the DNA and a transient non-catalytic pocket on the two-fold axis at the GyrA dimer interface, remaining close to the active sites and FQs binding sites (Figure 2B). As NBTIs display relatively poor activity against the *M. tuberculosis* DNA gyrase, the antitubercular profiles of 3000 compounds, representative of the chemical diversity of this family, were evaluated by high-throughput phenotypic screenings in vitro and in vivo [101]. MIC determination on *M. tuberculosis* H37Rv showed a high hit rate (68% of compounds with MIC values lower than 10 μM), with the most potent derivatives matching or even improving the MIC values for currently used TB drugs, including last-generation FQs. Overall, 29% of the compounds had MICs of < 1 μM, and 18% had MICs of < 0.1 μM. By using structural data provided by the work of Bax et al., the general structures of these TB-active compounds were divided into three different regions, each of them interacting with one of the three topologically important target-gyrase interacting regions [100]. While a left-hand side (LHS) is responsible for key contacts with the gyrase DNA substrate, a right-hand side (RHS) is embedded into the enzyme, potentially contributing to the protein target selectivity (Figure 2D). Last, a central linker unit (CU) establishes key interactions with the gyrase and offers the opportunity to modulate key physicochemical properties (Figure 2D). These SAR observations helped to schematize synthesis and to rationalize how to balance antimycobacterial potency with oral exposure, safety, and synthetic complexity, leading to the identification of the 7-substituted-1,5-naphthyridin-2-one core as a privileged LHS binder, the N-ethyl-4-aminopiperidines as a linker, and monocyclic aromatic rings with different substitution patterns as the best RHS binding option, in contrast to NBTIs bearing bicyclic rings as the RHS (Figure 2D, analogs 2 and 3) [101]. This work led to the identification of a subclass of naphthytidone/aminopiperidine-containing compounds that displayed activity against *M. tuberculosis*, both in vitro and in the mouse model, known as '*M. tuberculosis* DNA gyrase inhibitors' (MGIs) due to structural and activity differences with respect to NBTIs [101]. More recently, the mechanism of action of two such compounds, i.e., GSK000 and GSK325 (Figure 2C; Table 3), was assessed on the *M. tuberculosis* gyrase, showing that MGIs greatly enhanced DNA cleavage mediated by the bacterial enzyme, but they induced only single-stranded DNA breaks [102]. Their mechanism of action involves stabilizing covalent gyrase-cleaved DNA complexes and appears to suppress the ability of the enzyme to induce double-stranded breaks. Furthermore, these compounds maintained activity against mutant versions of DNA gyrase, bearing the three most commonly observed FQs resistance mutations, but displayed no activity against human topoisomerase IIα [102], suggesting good potential

as candidate drugs, especially in the presence of FQ resistance. Modeling studies were carried out by the authors using the crystal structure of the *M. tuberculosis* gyrase cleavage core and the NBTI crystal structure complex of *S. aureus* gyrase [100] (Figure 2B). Most importantly, these studies allowed to confirm that FQs and MGIs do not share the same binding site and bind in a mutually exclusive manner (Figure 2), considerably reducing the risk of developing cross-resistance phenotypes.

Table 3. *M. tuberculosis* DNA gyrase inhibitors.

Family	Name	Structure	IC_{50} (μM)	Reference
Fluoroquinolones	Moxifloxacin		1 2.5	[102] [101]
SPR	SPR720 (VXc-100, VXc-486)		ND	[92,93]
Coumarins	Novobiocin		0.5	[80]
NBTI	GSK000		0.5 5.4	[102] [101]
	GSK325		10.98	[101]

ND: not determined.

4. General Remarks

Several challenges need to be overcome for therapeutic molecules to be active against *M. tuberculosis.* Due to its metabolic adaptability and its known capabilities to occupy different niches inside the human host, from free aerobic bacteria to granulomas, an ideal molecule should be able to act against bacteria in very different, environmentally adapted states. The unique structure and composition of the *M. tuberculosis* cell wall are also well known to act as a barrier for potentially active compounds. To be active against latent and active infections, an ideal drug candidate should thus be able to reach bacteria in all the tubercular lesions and niches inside the human host [103], permeate the mycobacterial cell wall or be actively internalized, and deliver the desired bioactivity in bacteria under different metabolic states. To overcome these limitations and reduce the risk of further resistance development, TB drugs are delivered in combinations, starting from the standard DOTS (directly observed therapy short-course). Drug combinations, however, have to take in due account the bioavailability and pharmacokinetic properties of the single components and their associations, as extensively treated in this same journal issue [104].

In the course of the last ten years, it has become increasingly clear that some of the criteria commonly used to classify a given *M. tuberculosis* biological process, in many cases, an enzymatic step in a pathway, as a 'good' target for drug development, needed to be revised. This is particularly true for in vitro genetic essentiality. Even targets confirmed to be essential by the construction of specific conditional mutants do not necessarily show a good 'druggability', even when highly potent hits become available. The case of PknB is an emblematic example: despite the gene was early shown to be essential for *M. tuberculosis* growth, first by transposon mutagenesis [43], then through attempts to generate a knock-out mutant [44], yet several research groups, including ours, had to face the problem of the lack of correlation between hit potency in vitro and efficacy in vivo [50–52]. Similar issues were also reported for other *M. tuberculosis* kinase inhibitors, in addition to the cross-reactivity sometimes reported towards eukaryotic kinases, and the cytotoxicity shown by some compounds on human cell lines [105]. In addition, although the availability of crystal structures of both PknB and PknG in complex with hits issued from medium-throughput screens (mitoxantrone and AX20017, respectively) has looked as a promising 'proof-of-concept' [48,58], further structure-based work on these kinases has, so far, failed to produce suitable drug candidates. Many reasons might be evoked to explain the lack of *M. tuberculosis* kinase inhibitors that display significant antibacterial activity, ranging from the poor capacity to penetrate the mycobacterial cell and reach their target to the kinase redundancy in *M. tuberculosis* and their, relatively poor, substrate specificity [46], which prompted groups to seek for multiple kinase inhibition [105]. Yet, the main causes should perhaps be looked for in our, still limited, knowledge of mycobacterial molecular physiology and the regulatory networks in *M. tuberculosis*, in which Ser/Thr kinases are actors of a complex interplay [46,106]. Indeed, the activation mechanisms of both PknB and PknG are still a matter of speculation, and changes to *pknB* expression, either as depletion or overexpression, have been reported to alter the bacterial growth significantly [34]. Given the known *M. tuberculosis* metabolic plasticity, whose complexity has only recently started to be elucidated, thanks to the development of genetic and 'omics' tools [107], and the adaptability of the pathogen in the course of infection, it is now largely accepted that genetic essentiality of a putative target in laboratory conditions is not necessarily an indication of chemical vulnerability [15], and even more in clinical conditions. For these reasons, phenotypic screens have increasingly been employed in TB drug development to the detriment of target and structure-based methods, including for lead optimization [15], and the vast majority of candidate TB drugs that have been able to enter clinical trials in the last years were issued from this kind of screens [17,108].

Nevertheless, structure-based and computer-aided drug design maintain a clear potential for the future development of new anti-tubercular drug candidates. For instance, a recent, very promising success of structure-based and fragment-based approaches in TB drug discovery is the development of the so-called ethionamide boosters directed against the EthR repressor, one of which has been shown to be active both in vitro and in vivo [109]. In addition, the recent developments in X-ray

crystallography [110], cryo-EM [4], and integrative structural biology methods [111] will all contribute to increasing the number of tools available to tackle the challenges that lay ahead. These opportunities are well exemplified by the case of mycobacterial DNA gyrase that, considering our capacity to obtain high-resolution structures of an almost full-length form of the *M. tuberculosis* enzyme [112], and the recent, high-resolution cryo-EM structure of *E. coli* gyrase in complex with NBTI [113], let us believe that SBDD will deliver a key contribution to developing new compounds against this 'old' but validated target. High-resolution snapshots of the complete mycobacterial gyrase machinery, especially if in complex with representative members of each known family of inhibitors, might revolutionize our knowledge of this key target and substantially increase chances of improving our therapeutic bullets. For instance, combining crystallographic and cryo-EM data could allow to perform structure-guided drug design to target these flexible complexes and identify new conformations of mycobacterial gyrase that could not, otherwise, be obtained by conventional structural methods. More generally, the integration of biophysical and structural biology data, with the notable contribution of high-resolution EM, will allow the drug discovery pipelines to work on a higher complexity level that was previously not achievable, now looking at targets in their larger biological context (e.g., complexes or cellular compartments). It is, therefore, foreseeable that, despite the technical challenges, target-based and structure-based approaches will have increasing relevance in future drug discovery and will give significant contributions in the search for new tuberculosis drugs.

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