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Review

Drug Target Validation in Polyamine Metabolism and Drug Discovery Advancements to Combat Tuberculosis

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Abstract

Bacterial natural ecological niches are characterized by variations in the availability of nutrients, resulting in a complex metabolism. Their impressive ability to adapt to changeable nutrient conditions is possible through the utilization of large amounts of substrates. Recent discoveries in bacterial metabolism have suggested the importance of polyamine metabolism in bacteria, particularly in those of the order *Actinomycetales*, in enabling them to survive in their natural habitats. This makes such enzymes promising targets to inhibit their growth. Since the polyamine metabolisms of soil bacteria of the genus *Streptomyces* and the human pathogenic *Mycobacteria* are surprisingly similar, target-based drug development in *Streptomyces* and *Mycobacterium* spp. is an alternative approach to the classical search for antibiotics. The recent development of drugs to treat epidemic diseases like tuberculosis (TB) has gained attention due to the occurrence of multidrug-resistant strains. In addition, drug repurposing plays a crucial role in the treatment of various complex diseases, such as malaria. With that notion, the treatment of TB could also benefit from this approach. For example, molecular chaperones, proteins that help other proteins to fold properly, are found in almost all living organisms, including the causative agents of TB. Therefore, targeting these molecules could help in the treatment of TB. We aim to summarize our knowledge of the nitrogen and carbon metabolism of the two closely related *actinobacterial* genera, *Streptomyces* and *Mycobacterium*, and of the identification of new potential drug targets.

Keywords: nitrogen assimilation; drug targets metabolism; *Streptomyces*; carbon metabolism; *Mycobacterium*

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1. Tuberculosis as a Global Treat

1.1. Common Features of Pathogenic and Non-Pathogenic Actinobacteria

The *Actinobacteria* (*Actinomycetota* or *Actinomycetes*) are a phylum that contains a group of Gram-positive aquatic and terrestrial bacteria with high GC content. This phylum includes bacteria that are of great importance for the environment, industry, and medicine. Representative genera include *Streptomyces*, *Mycobacterium*, *Actinomyces*, *Frankia*, *Arthrobacter*, *Corynebacterium*, *Micrococcus*, *Micromonospora*, *Nocardia*, and *Propionibacterium*. Certain soil *Actinobacteria* are endosymbionts, e.g., *Frankia* spp., that are able to fix nitrogen and obtain in exchange access to the plant's nutrients. Members of the genera *Corynebacterium*, *Mycobacterium*, *Streptomyces*, and *Rhodococcus* are human pathogens [1]. Some examples of

actinobacterial pathogens include *Mycobacterium tuberculosis* (causes tuberculosis), *Mycobacterium leprae* (causes leprosy), *Corynebacterium diphtheria* (causes diphtheria), *Tropheryma whipplei* (causes Whipple's disease), *Gardnerella* spp. (cause bacterial vaginosis), *Streptomyces scabiei* (causes potato scab), and *Rhodococcus fascians* (causes leafy gall syndrome) [1–3].

Actinomycetota contains *Streptomyces*, which is one of the largest of the bacterial genera, containing contributors to the biological buffering of soils and bacteria producers of many antibiotics and other secondary metabolites. *Actinomycetes*-derived antibiotics crucial for medicine include anthracyclines, aminoglycosides, tetracyclines, macrolides, glycopeptides, etc. [3,4]. *Streptomyces* spp. share with pathogens from the genus *Mycobacterium* an almost identical core metabolism, which makes organisms like *S. coelicolor* a model system for mycobacterial research and translational medicine [2–5].

Actinobacteria reside in ecological niches under the conditions of long-lasting nutrient limitation and constant competition with other organisms [6]. As non-motile bacteria, *actinomycetes* are not able to move towards more nutrient-rich habitats; thus, they have multiple mechanisms to adapt to the environment. On the one hand, they can use diverse nutrient sources, including mono- and polyamines. On the other hand, they possess different adaptation mechanisms to be able to adapt to rapid changes in nutrient availability [4–6].

1.2. Distribution of Tuberculosis and Its Pathogenesis

Tuberculosis (TB) remains the most frequent, infectious, long-persisting, and difficult-to-treat disease with a very high mortality rate. In 2023, about 10.0 million people (range, 9.0–11.1 million) developed TB disease, with an estimated 1.25 million deaths [7]. Treatments of multidrug-resistant tuberculosis (MDR-TB) infections are particularly concerning because of their poor safety and efficacy, extended duration, and high cost. There is no effective anti-TB vaccine available that can prevent TB in adults. Developed in the 1930s, the bacilli Calmette–Guérin (BCG) vaccine was developed to prevent severe forms of TB in children, and it is still widely used today. While some antibiotics are effective in tuberculosis treatment, these drugs target only a limited number of functions in the cell [8].

The recommended treatment for tuberculosis is based on a combination of four antibiotics: ethambutol (EMB), rifampicin (RIF), isoniazid (INH), and pyrazinamide (PZA). All these were introduced nearly 60 years ago. This cocktail of four drugs should be administered for at least 6 months, but the therapy can last up to 24 months. The treatment consists of two phases: the initial phase, comprising administering the four drugs for two months, and then the continuation phase treatment with RIF and INH for a further four months to eliminate the dormant bacteria [9]. Multidrug-resistant tuberculosis (MDR-TB) has been reported on all continents, e.g., about 500,000 people developed rifampicin-resistant TB (RR-TB) in 2023 [7]. The identification of pathways that are required for the in vivo growth of mycobacteria would provide novel targets for the design of effective anti-TB agents active against MDR-TB [10].

The main agent causing tuberculosis, *Mycobacterium tuberculosis* (*tb*), which belongs to the phylum *Actinobacteria*, is an example of an intracellular pathogen, which is well adapted to survive and persist within human macrophages [9,11]. Bacteria that are able to colonize, survive, or manipulate macrophages aside from *M. tuberculosis* include *Klebsiella pneumonia*, *Salmonella typhimurium*, *Brucella abortus*, and *Acinetobacter baumannii* [9–11]. Recent studies have suggested that intracellular pathogens are able to employ strategies to manipulate human macrophage differentiation, particularly their basic cell metabolism. They can induce a metabolic shift in macrophages from the M1 (a kill/inhibit) to the M2 (a repair/heal) state during infection. This causes a time-dependent up-regulation of the metabolic regulator (PPARγ) in infected macrophages, resulting in increased expression of M2 markers and the down-modulation of the M1 response [12–14]. PPARγ induces the arginine metabolism,

leading to the synthesis of spermine from putrescine via spermidine [12]. Of all bacteria that colonize macrophages, *M. tuberculosis* is the most dangerous infectious agent. It has been shown that virulent *M. tuberculosis* strains are able to diverge away from the production of nitric oxide while enhancing the synthesis of polyamines, which are necessary for the reparative functions of the host macrophages [9–13]. Even though this elevated polyamine production has been considered beneficial for the host, it may be even more advantageous for the invader, serving as a supply of nutrients and energy to the pathogen [12–14].

2. Polyamine Metabolism in *M. tuberculosis* as a Part of Nitrogen Metabolism for Survival and Pathogenicity

2.1. Nitrogen Assimilation and Control in Mycobacterium tuberculosis

Nitrogen metabolism was extensively investigated in pathogenic *Actinobacteria* belonging to *Mycobacterium*. These include soil bacteria like *M. smegmatis*, as well as human pathogens like *M. leprae* (caused leprosy) and *M. tuberculosis* (causative agent of tuberculosis). In *M. tuberculosis*, the nitrogen assimilatory enzyme, the glutamine synthetase GlnA1, has been associated with virulence and pathogenicity [15–17]. Understanding nitrogen assimilation in this bacterium allows us to understand infection mechanisms as well as aid in the development of new therapeutic strategies to control *M. tuberculosis* and multidrug-resistant strains. In contrast to *S. coelicolor*, *M. tuberculosis* does not have an active glutamate dehydrogenase (GDH) enzyme in the central nitrogen assimilation pathway. Therefore, the glytamine synthetase (GS)/glutamate synthase (GOGAT) route is the only way to assimilate nitrogen [15]. The *M. tuberculosis* genome contains one glutamine synthetase-encoding gene *glnA1* (also referred to as *glnA*). Furthermore, it resembles three GS-like enzymes encoded by *glnA2*, *glnA3*, and *glnA4*. GlnA1 belongs to the GSI type of glutamine synthetases [15–17].

Despite the fact that all GS-like enzymes were demonstrated to be active in cells, only GSI encoded by *glnA1* was reported to be essential for the growth of *M. tuberculosis*. The bifunctional adenylyl transferase GlnE can down-regulate the activity of the GlnA1 enzyme under nitrogen excess. GlnE deadenylylates GlnA1 and restores GS activity under nitrogen starvation. In contrast to *E. coli*, GlnE activity in *M. tuberculosis* is not regulated by GlnK and GlnD. At the transcriptional level, GlnR controls nitrogen assimilation, being a homolog of the global transcriptional regulator of nitrogen metabolism (GlnR) from *S. coelicolor*. AmtR [18,19], a putative TetR-like transcriptional regulator, was also found in *M. tuberculosis*, featuring only 27.9% amino acid sequence identity to AmtR from *C. glutamicum* [5]. In *M. tuberculosis*, the global regulator GlnR regulated the transcription of *amtB-glnK-glnD*, *gltBD*, and *nirBD* operons as well as the transcription of *glnA* [20].

2.2. GS-like Enzymes GlnA2, GlnA3, and GlnA4 in M. tuberculosis

The function of GS-like enzymes GlnA2, GlnA3, and GlnA4 in *M. tuberculosis* have remained uncharacterized, although they were reported to be non-essential for cellular growth [15–17]. The in silico analysis of *glnA*-like genes across the *actinobacterial* genomes revealed that *glnA3* and *glnA4* genes, encoding proteins that might be involved in colonization, persistence, and survival in diverse habitats, might have a common *glnA* ancestor [21,22]. In *M. tuberculosis*, in silico homology protein modeling revealed that the structure of GlnA2Mt, GlnA3Mt, and GlnA4Mt is very similar to the structure of GlnA1Mt but also respective homologs in *S. coelicolor*—GlnA2Sc, GlnA3Sc, GlnA4Sc—indicating high confidence for their homology [23–31].

It was shown that the growth of *M. tuberculosis* similarly to *S. coelicolor* was impaired by the polyamine spermine [6,23,32,33]. In enzymatic in vitro assays, it was determined that GlnA3Mt (Rv1878) possesses gamma-glutamylspermine synthetase catalytic

activity [23,34]. It was further shown that purified GlnA3Mt preferred spermine as a substrate over monoamines, polyamines (cadaverine, putrescine, spermidine), and amino acids, suggesting that GlnA3Mt plays a specific role in the detoxification of the polyamine spermine [23].

2.3. Polyamine Metabolism in Actinobacteria

Polyamines are positively charged molecules with a hydrocarbon chain and multiple amino groups. Polyamines are molecules derived mainly from the amino acids arginine, ornithine, lysine. These are small aliphatic polyvalent cations [35]. Polyamines are present in virtually all organisms. The most common cellular polyamines are cadaverine, putrescine, spermidine, and spermine. These compounds are able to interfere with negatively charged molecules, such as DNA, RNA, polyphosphate, and phospholipids. Intracellular polyamine levels have to be regulated since polyamine imbalance can dramatically change cell homeostasis [35,36]. Polyamine excess was reported to be toxic for both eukaryotic and prokaryotic organisms, causing apoptosis [36]. However, polyamine metabolism has remained almost not investigated in *actinobacteria*, although the de novo biosynthesis of spermine has been reported in *Mycobacterium* [37–41].

Polyamines have been implicated in a wide range of biological processes, and their intracellular level is elevated predominantly during exposure to several stress conditions [35,42]. Thus, intracellular polyamine concentrations are tightly regulated by cell metabolic pathways [35,36], as polyamine excess is toxic for all organisms and may lead to cellular death [37,38]. Polyamines can interact with negatively charged molecules (e.g., RNA, DNA, polyphosphate, proteins, phospholipids) [43–47]. An imbalance in polyamine metabolism can drastically affect cellular homeostasis.

Polyamine assimilation is required to utilize them as a C/N source under deficiency conditions and for detoxification under excess. Polyamine catabolism has been studied in some bacterial species, revealing that the polyamine utilization pathway is not universal for all bacteria and is usually interconnected with prior polyamine detoxification. This process has been studied extensively in Gram-negative bacteria like *E. coli* and *P. aeruginosa* POA1 [36,48–50], which can catabolize polyamines via the aminotransferase pathway [51] or the γ-glutamylation pathway [48–51] In *P. aeruginosa*, the direct oxidation pathway for putrescine and the spermine/spermidine dehydrogenase pathway have been described: in *E. coli* and *B. subtilis*—the acetylation pathway for spermidine [50,52–54]. In the *actinobacterial* model organism *Streptomyces coelicolor*, the γ-glutamylation pathway has also been described [55].

Hardly anything is known about polyamine utilization in *M. tuberculosis*, but it has been hypothesized that it may occur via the γ-glutamylation pathway described in the model actinobacterium *S. coelicolor* [21], which is a close relative of *M. tuberculosis*. Recently, it has been reported that *M. tuberculosis* growth was inhibited by spermine [23,56]. *M. tuberculosis* is very well adapted to survive within macrophages [57–59]. The long-term persistence of *M. tuberculosis* within the macrophage is in part a consequence of an equilibrium between the nutritive needs of the host and the pathogen [11]. The presence of high amounts of the intracellular polyamine (specifically spermine) in macrophages may lead to the growth arrest and cell death of *M. tuberculosis* [60]. However, polyamines may provide a nutrient source (nitrogen and carbon) that can be exploited by *M. tuberculosis*. GlnA3Mt was demonstrated to play a specific role in the detoxification of spermine, as well as additional factors (spermine transporters) that are essential for the survival of *M. tuberculosis* during spermine stress [23].

3. Current Tuberculosis Drug Targets and Validated Drug Candidates in *M. tuberculosis*

Common drug targets in *Actinobacteria* and *M. tuberculosis* include metabolic enzymes (e.g., protein kinases, proteases, esterases, and phosphatases), membrane transport proteins, ion channels, and nucleic acids [61–63]. Conventional antibiotics function by killing the bacteria or by inhibiting growth. Modern antibiotics mostly affect the same cellular targets as peptidoglycan synthesis, the cytoplasmic membrane, translation, nucleic acid replication, and transcription [64]. The development and widespread use of antibacterial compounds have resulted in the rapid occurrence of resistance mechanisms in pathogens to existing drugs. Bacterial antibiotic resistance is based on some principal mechanisms, including the production of proteins to inactivate the antibiotic; mutation in the target-site protein receptor or the ribosomal subunit leading to ineffective drug binding; and changes in transport proteins preventing antibiotic entry as well as active compound efflux from cells [63–69].

There is thus an urgent need to discover new strategies for developing novel antibacterial drugs. *Actinobacteria* offer numerous secondary metabolites of enormous importance for the healthcare and food industries [70]. An effective strategy is the identification of bacterial proteins that may be drug targets for antibiotics. In this way, essential bacterial proteins or pathways (e.g., key proteins for the viability, growth, replication, or survival of the pathogen) that do not have human homologs can be identified [41,71–78].

In *M. tuberculosis* drug discovery, there are currently several popular drug targets in the primary carbon, nitrogen, phosphate, and sulfur metabolism, in the DNA replication machinery, in protein synthesis, and in other mechanisms, including targets such as GyrA/B, QcrB, ATP synthase, DprE1, FadD32, Pks13, MmpL3 [9], and GlnA3Mt [23] (Figure 1).

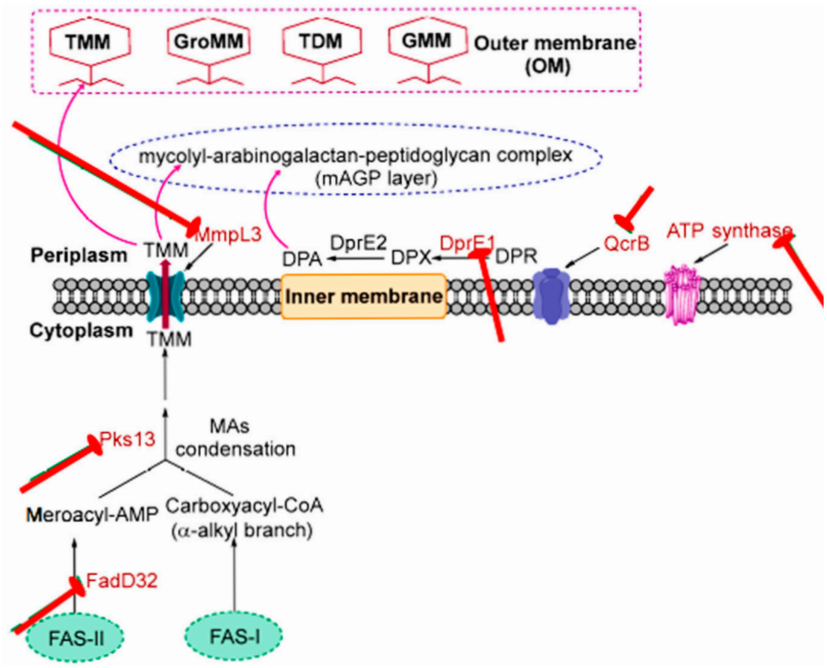


Figure 1. Drug targets in *M. tuberculosis* sorted according to their mechanism of action (adapted from [9], MDPI, 2023).

There are molecular targets that are involved in the pathways of macromolecular synthesis. From antibiotics in the main classes used in systemic monotherapy, only a few targets belong to essential enzymes. Most systemic monotherapeutic agents have nonprotein targets and interfere with the ribosomal RNA, membranes, or cell wall synthesis. Up-to-date intracellular enzymes can be targeted by β-lactams and fluoroquinolones. Many

antibiotics do target single essential enzymes. Clinical resistance to antibacterial agents is usually caused by horizontal gene transfer, with a contribution from the in vitro type of resistance that has been observed in a few cases. These investigations have led to the multitarget hypothesis. Successful systemic monotherapeutic agents should have low levels of endogenous single-step resistance. This is due to their targeting of the products of multiple genes or pathways [7–84].

3.1. Targeting DNA Replication and Protein Synthesis (Transcription and Translation)

A well-tested therapeutic approach targets DNA replication and protein synthesis. Many compounds and chemical inhibitors from this category have been used in the first and second lines of therapy, e.g., to combat mycobacterial infections. These compounds include streptomycin, amikacin, kanamycin, and capreomycin, which target translation; fluoroquinolones, which target DNA unwinding and replication; and rifampin, which targets transcription [62]. Only a few antibacterials are used in systemic monotherapy. Rifampicin, trimethoprim, and sulfamethoxazole are used in combination [62]. Among single-enzyme inhibitors, only fosfomycin is currently used as a monotherapy, but mainly for urinary tract infections (UTIs). Some inhibitors targeting DNA replication, transcription, and translation have been studied in *M. tuberculosis*: SPR719/SPR720, GSK3036656 (GSK 656, GSK 070), oxazolidinones linezolid, sutezolid, delpazolid, and TBI-223 [62].

3.2. Targeting Cell Wall/Peptidoglycan Biosynthesis

The cell wall of pathogenic *Actinobacteria*, e.g., *M. tuberculosis*, consists of the mycelial–arabinogalactan–peptidoglycan (mAGP) complex and has been observed as a potential drug target too. The disruption of peptidoglican biosynthesis has been demonstrated in *M. tuberculosis* MDR-Mtb and XDR-Mtb by cycloserine, a known broad-spectrum antibiotic, which binds to d-alanine–d-alanine ligase (Ddl). Further drugs targeting cell wall biosynthesis include enduracidin, ramoplanin (a lipoglycodepsipeptide), and teixobactin. Combination therapy with clavulanate, carbapenem, and meropenem showed potent activity against *M. tuberculosis* [85–88].

DprE1—decaprenyl phosphoryl-β-D-ribose oxidase—is a flavoprotein and a key enzyme in *mycobacterial* cell wall biosynthesis. DprE1 has been identified as the target of a novel class of inhibitors, 1,3-benzothiazin-4-ones (BTZs). DprE1 functions in concert with decaprenylphosphoryl-D-2-keto erythropentose reductase (DprE2), generating an arabinose precursor, which has an important role in the synthesis of the mycobacterial cell wall polysaccharides lipoarabinomannan and arabinogalactan. DPA biosynthesis was demonstrated to take place in the periplasmic space of the mycobacterial cell wall. The extracytoplasmic localization of DprE1 makes it more accessible to drugs. It was shown that the formation of DPA is inhibited by DprE1. Furthermore, DrpE1 provokes cell lysis and mycobacterial cell death [9].

3.3. Targeting Arabinogalactan

Another potential target is arabinogalactan, which is composed of a polysaccharide backbone (galactose and arabinose sugar moieties) and is contently linked to the peptidoglycan layer and the mycolic acid layer. Targeting arabinogalactan biosynthesis by ethambutol disrupts the arabinogalactan assembly. The fatty acyl-AMP ligase 32 (FAAL32 or FadD32) and polyketide synthase 13 (Pks13) are crucial enzymes in the biosynthetic machinery of MAs, which are the major integral lipid components of the exceptionally fortified cell wall of *M. tuberculosis*. After FadD32/Pks13 crosstalk takes place, resulting in the formation of TMM, these MA precursors are then flipped into periplasm with the help of the inner membrane protein MmpL3. The mycolyl portion gets anchored to arabinogalactan, the major cell wall polysaccharide that is linked to peptidoglycan [9,88].

Mycobacterial membrane proteins have been recognized as potential drug targets in *M. tuberculosis*, the inhibition of which can be achieved using the drugs SQ109, NITD-304, NITD-349, and BM212/BM635 [89]. The compounds isoniazid (INH) and ethionamide (ETH) have been found to inactivate InhA (enoyl-ACP reductase), which is involved in mycolic acid biosynthesis. Further antitubercular drugs targeting mycolic acid synthesis include the indazole sulfonamide GSK 724 (DG167 or GSK3011724A), inhibiting β-ketoacyl-ACP synthase (KasA), and delamanid and pretomanid [62,90].

3.4. Targeting Cytochrome B Subunit QcrB

QcrB—the cytochrome b subunit of the cytochrome bc1 complex—was reported as a target in *M. tuberculosis* [9,62]. The cytochrome bc1 complex is a crucial component of the respiratory electron transport chain, which is required for ATP synthesis. Thus, the inhibition of this complex changes the ability of *M. tuberculosis* to generate energy. A phenotypic screening of about 100,000 potential antimycobacterial compounds resulted in the identification of inhibitor candidates in the form of imidazopyridine amides (IPAs). An optimized IPA derivative Q203 demonstrated growth inhibition against the DS *M. tuberculosis* H37Rv strain (MIC50 = 2.7 nM) and MDR and XDR *M. tuberculosis* clinical isolates in vitro (MIC90 < 0.43 nM for most DR strains) [9,62].

3.5. Targeting Clp Proteases

One chemotherapeutic strategy is targeting proteostasis and proteolysis enzymes, e.g., in *M. tuberculosis*, the proteasome, the two ATP-dependent proteases, and the caseinolytic protease (Clp) (Table 1). ClpP forms a complex with AAA + ATPase chaperone proteins, such as ClpC1 or ClpX. The ClpP proteolytic complex consists of a functional central channel, which is a stacked assembly formed by two ClpP heptamers—ClpP1 and ClpP2. The opening of the central channel is capped by AAA + ATPases, such as ClpX or ClpC. These transfer chaperone substrate proteins into the ClpP central channel and align the catalytic triad for proteolysis [91–93].

The disruption of proteolytic complexes (ClpP/ClpX or ClpP/ClpC) can result in the inhibition of proteolysis, its activation, uncontrolled proteolysis, and the decoupling of ClpC's or ClpX's chaperone activity from ClpP1P2 proteolysis. Since *clpp1p2* genes have been demonstrated to be essential for the *M. tuberculosis* pathogen and, unlike bacterial proteases, ClpP is not cellular in humans but mitochondrial, these proteins have become promising drug targets. Currently proposed compounds targeting the ClpP1P2 proteases include peptidyl boronate (bortezomib analogs, which also inhibit Mtb20S proteasome) and β-lactones. Compounds that target ClpX/ClpC include cyclic acyldepsipeptide (ADEP). ClpC can be targeted by *Actinomycetes*-derived cyclic peptides, such as ecumicin, cyclomarin A, rufomycin, and lassomycin [62].

A summary of validated drug targets is depicted in Table 1.

Table 1. Proteins in *M. tuberculosis* that are validated drug targets.

Target	Function	References
Gyrase B, rRNA, Leucyl tRNA synthetase, RNA Polymerase	DNA replication and protein synthesis	[62]
MurX, l,d-traspeptidases, l,d-transpeptidases + β lactamase, Lipid II	peptidoglycan biosynthesis	[88]
WecA, DprE1 (Covalent inhibitors), DprE1 (Noncovalent inhibitors)	arabinogalactan biosynthesis	[88]

Table 1. *Cont.*

Target	Function	References
InhA, MmpL3, β-ketoacyl-ACP synthase (kasA), Inhibition of methoxy and keto mycolic acid (exact target unknown)	mycolic acid biosynthesis	[9]
ATP synthase (AtpE), Cytochrome bc1/aa3 super, NDH-2, MenA, MenG, Isocitrate lyase (ICL)	energy metabolism	[92]
ClpC	proteolysis	[91]
Glutamine synthetase GlnA1	Primary metabolism, glutamine synthesis	[94–97]
Gamma-glutamylpolyamine synthetase GlnA3	Polyamine metabolism	[23]

3.6. Targeting Primary Metabolism

3.6.1. Targeting Carbon Metabolism

Central carbon metabolism (CCM) includes the enzyme-mediated transformation of carbon through glycolysis, the tricarboxylic acid (TCA) cycle, and gluconeogenesis to incorporate carbon into the bacterial metabolism; the genes encoding these reactions have been found to be conserved [5,98]. Recent studies have revealed distinctive elements of *actinobacterial* CCM that have helped these organisms survive in specific ecological niches. Especially important is knowledge of CCM in *Actinobacterial* pathogens, such as *M. tuberculosis*, in which carbon metabolism has evolved to serve interdependent physiologic and pathogenic roles [98].

Carbon metabolism in *M. tuberculosis* has been extensively investigated in association with mutations of essential CCM enzymes and the opportunity to design inhibitors based on transition state analogs. Recent advances in inhibitor design have demonstrated that the active sites of enzymes involved in carbon metabolism are often sub-saturated and therefore more susceptible to inhibitors. Broad-spectrum antibiotics in clinical use nowadays include fluoroquinolones, inhibiting bacterial DNA gyrases, and trimethoprim (TMP), targeting dihydrofolate reductases (DHFRs). Newer drugs include diarylquinoline TMC207, which inhibits the C subunit of its ATP synthase [58]; rhodamine, inhibiting DlaT; triazospirodimethoxybenzoyls, targeting Lpd; and oxadiazole-2-ones that inhibit the proteasome [99]. The DNA gyrase GyrA/B is considered to be a hot drug target as a conserved type II topoisomerase enzyme, essential for DNA transcription, recombination, and replication in *M. tuberculosis*. The inhibition of the DNA gyrase, consisting of two subunits, A and B, results in impaired DNA replication and bacterial death. A promising drug candidate for GyrA/B inhibition is SPR720 [9].

The challenge in the validation of CCMs as potential drug targets is that metabolic enzymes can exist at levels higher than those needed to support cell viability. Most enzymes of carbon metabolism serve diverse, interconnected pathways that are subject to strict regulation. The validation of *actinobacterial* enzymes of carbon metabolism as potential drug targets requires biochemical knowledge of pathways that define the functionality of enzymes of interest combined with the quantitative level of inhibition needed to achieve the death of a pathogen [5,98].

3.6.2. Targeting Sulfur Metabolism

Extracellular sulfated metabolites play a regulatory role in cell–cell and host–pathogen communication [100]. The acquisition and metabolism of sulfur is essential for diverse

Actinobacterial species; e.g., it is a key determinant in the virulence and survival of *Mycobacterium* spp. [101].

Microbial sulfur metabolic pathways are largely absent in humans. Thus, sulfur metabolism, including amino acid biosynthetic pathways and downstream metabolites, represents a potential and unique target for drug development [100]. Sulfur metabolic pathways are required for virulence in different pathogenic *actinobacteria*, e.g., *M. tuberculosis* [102]. Mutants in genes of mycobacterial sulfur metabolism have been demonstrated to have an impaired ability to persist and cause disease [103]. Recent investigations provided molecules that inhibit the PAPS- and substrate-binding domains of Sts, as well as synthetic bisubstrate analogs that have been employed [104]. Such compounds incorporate elements from the cofactor, PAPS, and the substrate, resulting in specificity via critical interactions within the binding pocket of the enzyme. Inhibitor potency is achieved by linking structures that mimic substrates. For example, bisubstrate-based compounds were identified [104] as inhibitors of EST. Other approach includes the kinase inhibitors. It was proposed that ATP derivatives might also function as ST inhibitors [105]. Recent studies identified potent inhibitors of β–arylsulfotransferases (β-AST-IV) [106]. Furthermore, isoquinoline sulfonamides have also been tested for inhibitory activity against STs, consisting of EST, NodH, and GST-2, as well as two inhibitors of Golgi-resident tyrosyl protein ST-2 (TPST-2) [105]. Recent advances in structure-based drug design and high-throughput screening will greatly facilitate the discovery of new inhibitors for STs and other sulfonucleotide-binding enzymes.

3.6.3. Targeting Phosphate/ATP Metabolism

Pathogenic *Actinobacteria* like *M. tuberculosis* generate ATP via two inter-linked metabolic pathways: oxidative phosphorylation and substrate-level phosphorylation. Since pathogens like *M. tuberculosis* need higher basal energy, oxidative phosphorylation has proved essential for *M. tuberculosis* [107]. The current tuberculosis pipeline has numerous drug candidates with targets in these pathways [108]. For targeting oxidative phosphorylation, compounds such as bedaquiline (BDQ) and diarylquinoline, which block ATP synthesis, have been reported. A combination therapy of BDQ, linezolid (BpaL), and pretomanid was evaluated in clinical trials and approved for treating XDR-Mtb or MDR-Mtb. Furthermore, BDQ with pyrazinamide (PZA), pretomanid, and MOX has been studied for its safety, efficacy, and shortened treatment duration in drug-sensitive (DS) and drug-resistant (DR) tuberculosis [62,88,107].

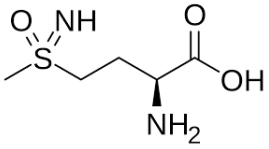
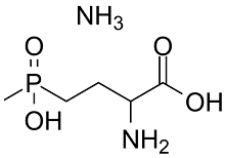
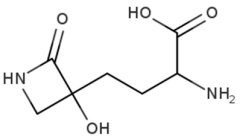
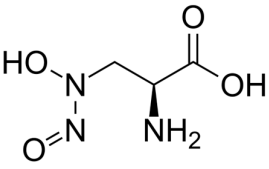
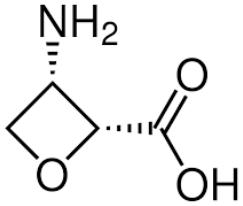
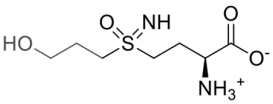
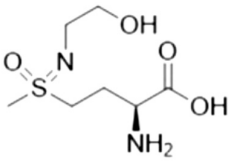
The diarylquinoline BDQ was recently approved as an anti-TB drug, and it was reported to elicit its activity through the inhibition of the c subunit of the mycobacterial ATP synthase enzyme. It disrupts energy metabolism, decreasing intracellular ATP levels in *M. tuberculosis*. Two diarylquinolines, TBAJ-587 and TBAJ-876, were identified as compounds with anti-TB activity against the H37Rv strain in vitro [9].

3.6.4. Targeting Nitrogen and the Metabolism of Polyamines

An important drug target evaluated in *Actinobacteria* is the glutamine synthetase (GS) GlnA. The in silico analysis of *glnA*-like genes across the mycobacterial genomes revealed that *glnA1* might have evolved to specialized *glnA*-like genes, such as the GS-like enzyme-encoding genes *glnA2*, *glnA3*, and *glnA4*, which encode proteins that may be involved in colonization and survival in many diverse habitats [4,5]. GS-like genes have been found in *actinobacterial* pathogens like *M. tuberculosis Rhodococcus jostii*, as well as plant symbiotic bacteria like *Frankia alni* or plant pathogens like *S. scabies*. Validated inhibitors targeting GSs include methionine sulfoximine (MSO), phoshpinothricin (PPT), and some synthetic inhibitors. Recently, the first inhibitor of GS-like proteins was introduced [109] (Table 2).

Another group is the larger hydrophobic heterocycles that compete for the ATP targeting of the nucleotide-binding site, such as purine analogs. Besides GSs, also GOGAT and GDH in *M. tuberculosis* have been identified as imported drug targets, with the azaserine inhibitor being able to disrupt GOGAT [110,111].

Table 2. Characterized GS and GS-like inhibitors targeting *M. tuberculosis*. MSO and PPT are some examples of GS inhibitors available on the market. All compound figures (PubChem) adapted from [112], Nucleic Acids Res., 2025.

Name	Mode of Action	Structure	References
Methionine sulfoximine (MetSox/MSO)	Potent, ATP-dependent inactivator of GS		[113]
Phosphinothricin (PPT)	Potent, ATP-dependent inactivator of GS that is produced as part of a tripeptide antibiotic by *Streptomyces viridochromogenes*.		[114]
Tabtoxinine β-lactam	Potent, ATP-dependent inactivator of GS produced by *Pseudomonas* pv. *tabaci*		[115]
Alanosine	Antibiotic produced by *Streptomyces alanosinicus*		[94]
Oxetin	Antibiotic produced by *Streptomyces* sp., inhibitor of GS		[94]
7b (PPU301)	Synthetic inactivator of the GS-like enzyme GlnA4 from *Streptomyces coelicolor*		[109]
PPU268	Synthetic inactivator of the GS-like enzyme GlnA2 from *Streptomyces coelicolor*		[110]

Some alternative metabolic pathways were shown to be effectively targeted. These include, for instance, mono- and polyamine biosynthesis and utilization. A group of inhibitors that target polyamine biosynthesis has been introduced, including D,L-α-difluoromethylornithine (DFMO), the putrescine analogs 3-aminooxy-1-aminopropane (APA) and 1,4-diamino-2-butanone (DAB), the spermine analog MDL 27,695 (N,N′-bis(3-((phenylmethyl)amino)propyl)-1,7-diaminoheptane), the agmatine analog 1-guanidinooxy-3-aminopropane (GAPA), and others. However, the development of drugs targeting monoamine ethanolamine and polyamine utilization has been far less investigated. The

only drug that is able to target these pathways in *Actinobacteria* so far was proposed recently [109].

4. Drug Repurposing Targeting Polyamines in *M. tuberculosis*

Drug repurposing that targets polyamines in *Actinobacteria* is a promising area of research in the fight against tuberculosis (TB). As mentioned, polyamines such as spermidine and spermine are essential for various cellular functions, including growth, motility, and stress response [4]. One notable role of polyamines is their regulation of reactive oxygen species (ROS) during stressful conditions, which helps protect cells from oxidative damage. Generally, stress conditions can increase the expression of genes involved in polyamine biosynthesis. This, in turn, elevates polyamine levels, thereby enhancing stress tolerance. Polyamines play a crucial role in complex signaling pathways that activate various stress responses. This includes the interaction of signaling molecules like abscisic acid (ABA) and nitric oxide (NO), which together help organisms respond effectively to stressful conditions. Both genetic engineering and the exogenous application of the polyamine approach in plants have shown that they can mitigate the effects of environmental stresses [116,117]. Taken together, there have been strategies to exploit the molecules that are produced in response to stressful conditions, such as molecular chaperones, which are known for their involvement in protein folding and regulation. Some drugs can be used or tested against these molecules to inhibit their activities, effectively shutting down the growth and differentiation of *Actinobacteria* as a strategy to develop a treatment for tuberculosis [110].

4.1. Reactive Oxygen Species (ROS) as Drug Targets for TB

One of the key factors in the pathogenesis and survival of *Mycobacterium tuberculosis* is the presence of highly reactive molecules known as reactive oxygen species (ROS) [118]. These molecules serve two primary roles in biological systems: they act as signaling molecules and can also cause cellular damage. Different types of ROS include superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen. When present in high concentrations, ROS can induce oxidative stress, resulting in damage to DNA, proteins, and lipids [119]. Complex diseases, such as cancer, cardiovascular diseases, and neurodegenerative disorders, are associated with elevated levels of ROS. Additionally, the malaria-causing parasite *Plasmodium falciparum* also utilizes these molecules to its advantage. ROS are byproducts of the metabolism of this obligate parasite and can lead to oxidative damage to cellular components. To survive, *Plasmodium falciparum* has developed strategies to regulate ROS levels effectively. Two important enzymes that are employed by the malarial parasite as antioxidants include superoxide dismutase (SOD) and glutathione peroxidase to detoxify ROS [120]. Therefore, blocking these enzymes could lead to high levels of ROS that can lead to the death of the parasite. Antimalarial drugs such as artemisinin and its derivatives could contribute to the generation of ROS within the cellular system of the parasite, which could cause cellular damage and lead to parasite death [121]. Therefore, the drug repurposing approach is very important, and this approach may be used to combat TB too because the *Mycobacterium* depends on ROS for its growth. Thus, testing drugs that target ROS could lead to the death of these bacteria.

4.2. The Synergy of Abscisic Acid and Nitric Oxide as a Therapeutic Target

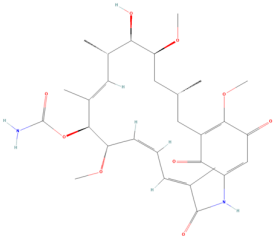
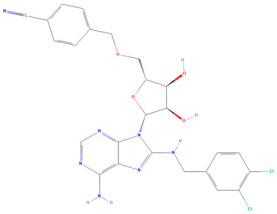
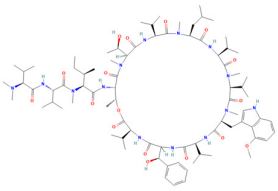
Abscisic acid (ABA) is a plant molecule known for its role in plant stress responses and development. However, these molecules are also produced in different microorganisms, such as *actinobacteria*, and their role is to influence stress responses and secondary metabolite production [120]. On the other hand, nitric oxide (NO) is a versatile molecule that plays a crucial role in the physiological processes of both prokaryotic and eukaryotic organisms. Its

role in *actinobacteria* is regulating secondary metabolism and morphological differentiation. The synergistic interaction between ABA and NO in *actinobacteria* is an emerging area of research [107]. However, few studies conducted thus far show that both molecules are involved in stress responses. For example, in plants, ABA can induce NO production and, therefore, mediates different stress responses. It is believed that the same mode of action could exist in *actinobacteria*, where ABA and NO could cooperate to enhance stress tolerance [122]. This could be a very good starting point for developing a treatment for TB.

4.3. Molecular Chaperones as Drug Targets

Molecular chaperones or heat shock proteins are found in almost all living organisms, from plants to bacteria. Their role is to help other proteins to fold properly and prevent aggregation [123]. These molecules are divided into different subclasses, namely, small heat shock proteins and middle and major heat shock proteins with various roles in cellular homeostasis (Table 3).

Table 3. Summary of heat shock proteins in *M. tuberculosis* and therapeutic strategies. All compound figures (PubChem) adapted from [112], Nucleic Acids Res., 2025.

Name	Roles	Action	Inhibitor	Structure	References
Hsp90 (Grp94)	Holdase	These compounds bind to the N-terminal ATP-binding domain of Hsp90, inhibiting its chaperone activity	Geldanamycin and its derivatives		[124]
Hsp70 (Dnak)	Foldase	A small molecule inhibitor that binds to the ATPase domain of Hsp70, inhibiting its activity	VER-155008		[125]
ClpB	Holdase	A cyclic peptide that targets the ClpC1 ATPase component of the Clp protease complex, enhancing its ATPase activity but preventing proteolysis	Ecumicin		[126]

4.4. Targeting Chaperone Networks

Molecular chaperones function as cooperative partners in the folding of newly synthesized polypeptides. For some, their role is to identify client proteins and activate the ATPase domain of the folding chaperones, thus opening the binding site for the client protein to bind for folding purposes (Figure 2). Almost all living organisms require this system to maintain balanced cellular function. From plants to bacteria, molecular chaperones are crucial for functional activities in cell signaling and proteostasis [123]. Therefore, these molecules are ideal drug targets for various diseases, like malaria, cancer, and TB. The idea is that, if the molecular chaperone network is targeted, it can lead to cell death, thus eliminating TB.

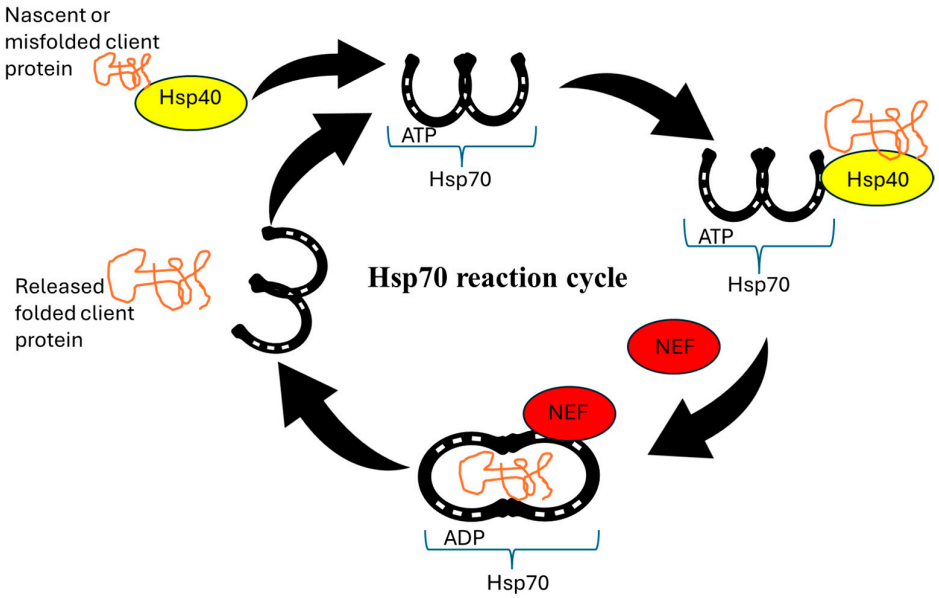


Figure 2. Summary of Hsp70 reaction. Hsp40, as a cochaperone, binds to the client protein or unfolded protein and hands it over to Hsp70 (ATP form) for folding purposes. The nucleotide exchange factor binds to Hsp70, thus catalyzing the dissociation of ADP. Finally, ATP binds to Hsp70, inducing the opening of the lid, thereby enabling substrate release (adapted from [127], Frontiers, 2018).

5. Combination Therapies for the Treatment of Tuberculosis

Combination therapy has received significant attention in the pharmaceutical industry as a strategy to combat complex diseases. In combating malaria, this approach has been proposed to address the problem of drug resistance, particularly in Sub-Saharan Africa and other regions where malaria is prevalent. Various studies have indicated that combination therapy could serve as a crucial solution not just for malaria, but also for other diseases like tuberculosis (TB) [128]. In the case of tuberculosis, combination therapy typically involves using multiple antibiotics to effectively treat the infection and prevent the development of drug resistance. For instance, one effective regimen combines rifapentine and moxifloxacin, which is typically administered for four months [129]. Recently, it has been demonstrated that the polyamine spermine can enhance the activity of currently available and WHO-approved tuberculosis drugs, such as isoniazid, rifampicin, aminosalicylic acid, and bedaquiline [130]. Table 4 gives a brief overview of some combination treatments for infections caused by *Actinobacteria*.

Table 4. Summary of some common combination therapies for *M. tuberculosis* treatment.

Use Case	Mechanism	Combination	References
Multidrug-resistant strains	Synergistic effect	Colistin + Rifampicin	[131]
Severe infections	Enhanced efficacy	Tigecycline + Sulbactam	[132]
Carbapenem-resistant strains	Broad-spectrum activity	Meropenem + Polymyxin	[133]
General use in resistant infection	Synergistic effect	Beta-lactam + Aminoglycoside	[134]
Reducing resistance development	Targeting different pathways	Fluoroquinolone + Beta-lactam	[135]
General use in resistant infection	Enhancing effect of spermine on conventional drugs	Spermine + isoniazid, rifampicin, aminosalicylic acid, bedaquiline	[130]

6. Conclusions: Perspectives on Drug Target Validation in Polyamine Metabolism and Tuberculosis Drug Discovery

Antibacterial agents must meet a variety of criteria to be successful: selectivity, safety, no cross-resistance with existing therapeutics, pharmacological properties, and low propensity for rapid resistance selection. The search for molecular targets/target proteins for new inhibitors and antibiotics requires careful analysis of the target's role in metabolism and proper inhibitor design. Essentially, a lack of close human homologs, a lack of target-based cross-resistance, and the presence of important pathogens can be predicted based on the target. However, the choice of a single protein or enzyme target may also increase the likelihood of resistance selection. Thus, specificity is of importance to develop a potent drug. These potential problems must be resolved for the success of novel target-based discovery.

Increasing human movement and migration worldwide promote the spread of MDR-TB to a global extend, making the disease epidemic. Nowadays, several drugs and vaccines are undergoing trials, but the development pipelines are progressing slowly. The investigation of the cellular metabolism of mycobacteria, leading to the identification of new drug targets, can provide a subsequent rationale and effective design for new anti-TB and anti-MDR-TB agents [9,11,136]. The antibiotics normally applied in tuberculosis therapy combat classical bacterial targets like cell envelope synthesis or transcription and translation. Proteins from polyamine metabolism would contribute completely novel targets, and their inhibition would offer new perspectives for innovative combination therapy. Furthermore, known problematic resistance mechanisms should not protect *M. tuberculosis* from the inhibition of polyamine metabolism-related proteins. The inhibition of mycobacterial proteins from polyamine metabolism would constitute a novel strategy to control tuberculosis and potentially other mycobacterial infections by overcoming the natural polyamine resistance of *M. tuberculosis* and simultaneously supporting the natural immune response of the host during the infection.

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