Tuberculosis drug discovery and emerging targets

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Current tuberculosis (TB) therapies take too long and the regimens are complex and subject to adverse effects and drug–drug interactions with concomitant medications. The emergence of drug-resistant TB strains exacerbates the situation. Drug discovery for TB has resurfaced in recent years, generating compounds (hits) with varying potential for progression into developable leads. In parallel, advances have been made in understanding TB pathogenesis. It is now possible to apply the lessons learned from recent TB hit generation efforts and newly validated TB drug targets to generate the next wave of TB drug leads. Use of currently underexploited sources of chemical matter and lead-optimization strategies may also improve the efficiency of future TB drug discovery. Novel TB drug regimens with shorter treatment durations must target all subpopulations of *Mycobacterium tuberculosis* existing in an infection, including those responsible for the protracted TB treatment duration. This review proposes strategies for generating improved hits and leads that could help achieve this goal.

Keywords: tuberculosis drugs; drug regimens; nonreplicating; *Mycobacterium tuberculosis*; drug discovery

Introduction

The long treatment duration, emergence of resistant strains, adverse effects from many of the existing drugs, and challenges in treating tuberculosis (TB) patients receiving antiretroviral therapy for human immunodeficiency virus (HIV) coinfection, underlie the desperate need for new TB drug regimens. Considering the length of drug development and attrition rates throughout this process, new TB drug candidates are urgently required to ensure the generation of novel treatment regimens.

Regarding efficacy, an ideal new regimen should be rapidly bactericidal and possess potent sterilizing activity to achieve stable cure in a shorter time period than the typical 6 months required for the current standard of care for drug-sensitive TB. To achieve such a treatment, it is expected that a regimen would need to kill all subpopulations of *Mycobacterium tuberculosis* (Mtb) that exist in clinical TB and are thought to vary in replication rate. In addition, new regimens should include drugs that demonstrate novel mechanisms of action (MOA) to ensure effectiveness against strains that are resistant to existing TB drugs. The regimens should also be well tolerated and possess absorption, distribution, metabolism, and excretion (ADME) properties suitable for coadministration with anti-HIV agents and appropriate for once-a-day oral dosing, optimally within a fixed-dose combination. It is also imperative that new drugs be available at relatively low cost to remain accessible to all high-burden countries.

Recent advances in understanding the molecular biology of Mtb—the causative agent for TB—have been significant, driven largely by the whole-genome sequencing of the bacterium in 1998. Knowledge of the complete Mtb genome sequence enabled scientists to establish the number of essential genes both *in vitro* and *in vivo*, to study genome-wide DNA microarrays for patterns of gene expression under various growth conditions, and to more rapidly identify targets of new compounds via identification of the mutated genes of compound-resistant mutants. The use of elegant gene knockdown techniques has allowed *in vitro* and *in vivo* validation of potential drug targets by demonstrating the effect of the depletion of a specific target.
From a private sector investment perspective, there is limited commercial potential for new TB drugs and therefore this indication has not been an area of intense activity. In addition, the recent exodus of much of the pharmaceutical industry from antibacterial drug discovery has proven to be a loss for TB drug research and development, as the TB field no longer benefits from drug candidates being developed against other bacteria. Fortunately, some government and nongovernment organizations have taken the initiative to fill this gap, with a turning point occurring with the creation of a number of not-for-profit product-development partnerships, such as the Global Alliance for TB Drug Development (TB Alliance) that formed in 2000. Since then, several additional consortia have formed, including the TB Drug Accelerator, the Lilly Early TB Drug Discovery Initiative, and European Commission funded groups such as More Medicines for TB (MM4TB) and Orchid. Academic and government laboratories are also involved in translational medicine of basic research to TB drug discovery. The TB field has recently experienced some positive developments with the approval of bedaquiline (TMC-207, Sirturo™ from Janssen), by the U.S. Food and Drug Administration (FDA) at the end of 2012,14 (and by European Medicines Agency (EMA) in March of 2014) and delamanid (OPC-67683, Deltyba™ from Otsuka) by the EMA in December of 2013,15 both indicated for multidrug-resistant TB. However, the current drug candidate pipeline16 is woefully thin and provides little substrate for the development of drastically treatment shortening drug regimens that will truly change the TB treatment paradigm.

In this article, rather than reviewing the ongoing hit/lead generation efforts that have provided the current TB global pipeline (a subject of some excellent recent reviews, such as Refs. 22 and 23), we focus on the lessons learned from screening efforts of the recent past and how these can be applied for greater future success. We then highlight the lead-generation efforts and TB drug targets that we believe to be the most promising and worthy of more attention. Specifically, we discuss advances in screening technologies that are expected to produce the next generation of progressable hits against Mtb, highlighting the potential for natural products as a source for novel hits, and mechanism-based and structure-based drug discovery for the generation of anti-TB leads. We then focus on a selection of novel drug targets that have been recently validated such that their inhibition should eliminate persisters (a subpopulation of organisms in an infection that is phenotypically resistant to killing by most drugs and thought to be responsible for protracted treatment durations and relapse) and contribute to treatment shortening in novel regimens.

The current status and recent history in hit/lead generation for TB

Screening efforts: the past, present, and future

As in the antibacterial field in general, despite significant effort being expended on Mtb target-based biochemical screens,17 no TB drug or drug candidate has emerged. Problems with target-based screens include their propensity to identify hits with potency against the target but that do not inhibit bacterial growth because target inhibition does not translate into bacterial killing for a variety of reasons, including poor penetration and efflux. Conversely, whole-cell screening has yielded notable successes for TB drug discovery. As a consequence, it has become the mainstay of TB hit generation. Mtb whole-cell screening is an approach in which compound libraries are screened for their ability to inhibit bacterial growth. These screens have been carried out against both replicating and nonreplicating Mtb under a variety of culture conditions in an effort to identify compounds that kill the multiple subpopulations of Mtb that exist in an infection. Whole-cell screening has the advantage that it is not target specific, thus it enables screening against the organism’s entire set of targets at once. Results of such a screen, which effectively allows the organism’s physiology to inform discovery efforts, yield accessible targets and sensitive pathways that would otherwise be unpredicted. This approach also overcomes cell-penetration and -efflux issues (i.e., getting and keeping compounds in cells) of target-based screens. However, whole-cell screening is not without its challenges. For example, target identification can be problematic and if not successful it can limit the ability to quickly improve the potency of hit compounds. A good example of whole-cell screening success is the compound TMC-207;18 other examples include a diarylcoumarin series that targets the acyl–acyl carrier protein synthetase activity of FadD3219,20 and the indolcarboxamides NITD-304.
and NITD-349, presumed to inhibit MmpL3 from the sequencing of resistant mutants. All of these compounds are efficacious in vivo against murine TB.

Perhaps the earliest publicly accessible whole-cell screening results were from the National Institute of Health’s effort to acquire and test compounds against Mtb (Tuberculosis Antimicrobial Acquisition and Coordinating Facility, TAACF). More recently, several pharmaceutical companies have carried out phenotypic screens, for example GSK recently reported a set of 177 Mtbageactive compounds. Some hits were further investigated in the hit-to-lead phase and have been made public. The Novartis Institute of Tropical Diseases (NITD) has published results of a hit-to-lead program based on their selected screens. AstraZeneca has also carried out phenotypic screening and published some results. Similar screens against Mtb are underway by members of the TB Drug Accelerator consortium and MM4TB, among others.

The whole-cell screens published thus far largely used standardized aerobic culture conditions that favor replicating Mtb. More recently, specialized whole-cell screens have been carried out or are ongoing, including those that use culture conditions considered relevant to in vivo infection or screens against Mtb inside host cells. The expectation is that these screens will identify inhibitors that will be active in vivo and against persisters. Screens against Mtb in macrophages have been successful; the late preclinical compound Q203 is one example. It is an imidazo[1,2-a]pyridine-3-carboxamide derivative that targets QcrB, a component of the CytB1 complex of the respiratory chain. Interestingly, this series was also discovered through screens under standard culture conditions and for inhibitors of ATP homeostasis under nonreplicating conditions. Other cell-based specialized screens recently reported or are ongoing include those for inhibitors of ATP homeostasis or pH homeostasis and against engineered Mtb strains with modulated expression of the target of interest. These screens are an improvement because inhibitors against targets, pathways, or processes already proven essential for TB are likely to be discovered via a whole-cell approach, thus combining the advantages of validated targets with whole-cell screening. Because most of the whole-cell screens against Mtb were undertaken in the last 5–10 years, it may be of some use to analyze the leads that emerged as well as the screens that identified them. Some interesting phenomena are emerging.

First, similar compounds have been identified as hits many times by various organizations; these compounds are listed in Table 1. Some examples include structurally closely related imidazo[1,2-a]pyridine-3-carboxamides (e.g., Q203) that were shown to kill Mtb by at least four different phenotypic screens performed by different organizations; tetrahydropyrazolo[1,5-a]pyrimidine-3-carboxamides were selected as hits in at least three different screens; and urea analogues containing an adamantyl group or a norbornylmethylene group were identified as hits in two different screens. The redundant identification of these hits may reflect the similarity of both the libraries of the various organizations and the screening methods.

Second, the same recurring mechanistic targets have been identified for different hits. Representative promiscuous targets are summarized in Table 2. For example, at least five structurally divergent chemical series were shown to have the same target, DprE1, an enzyme involved in isomerization of decaprenylphosphoryl-β-d-ribose to decaprenylphosphoryl-β-d-arabinose, an important cell wall component. Similarly, structurally divergent compounds were shown to share MmpL3 as the predominant resistant determinant; MmpL3 is a cell membrane transporter of trehalose monomycolate. The reasons for this phenomenon are not clear, but it may be that these are the most accessible and vulnerable targets under the screening conditions used, and they are mainly cell surface or membrane-bound proteins; the phenomenon of lipophilic compounds and membrane-localized targets has been pointed out in two recent review articles. The main argument Stanley and Goldman present is that lipophilic compounds may partition and concentrate in the membranes higher than in the intracellular environment. These observations are a reminder that the methodology and conditions of a screen determine the results and the hits identified. Researchers thus may have to rethink their screens and the compound libraries they use because the lipophilic and high molecular weight properties of certain hits may preclude successful subsequent downstream development. It is also the case that highly lipophilic and high molecular weight
Table 1. Compounds identified in multiple whole-cell screens

<table>
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<th>Series</th>
<th>Structures</th>
<th>Investigating organization(s)</th>
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<td><img src="image1.png" alt="struct1" /></td>
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<tr>
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<td><img src="image2.png" alt="struct2" /></td>
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<tr>
<td>Adamantyl ureas</td>
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<td>Colorado State University and Tuberculosis Antimicrobial Acquisition and Coordination Facility</td>
</tr>
<tr>
<td>Indole-2-carboxamides</td>
<td><img src="image4.png" alt="struct4" /></td>
<td>Novartis Institute of Tropical Diseases, University of Illinois at Chicago, and GlaxoSmithKline</td>
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weight compounds that target membrane-localized targets seem to correlate with the potential for hepatotoxicity, mitochondrial dysfunction, and nonspecific interactions. They also affect pharmaceutical development owing to the lack of solubility and difficulty in formulation. The fact that bedaquiline (TMC-207), with a cLogP value of 7.3, was successfully developed amply demonstrates that such a compound can be developed, even though the hurdles are high. Various measures can be used to correct this problem. For example, hits with preferred physicochemical properties should be prioritized over higher potency hits with poor properties; and methods should be established to accurately determine the drug concentrations in various cellular compartments. To that point, a recently reported method for measuring the intracellular compound levels in *Mycobacterium smegmatis* may be useful in linking intracellular compound levels to bacterial killing.

Third, instead of the completely nontarget-specific whole-cell screens, target-specific, whole-cell screens using specialized strains that focus on a specific *in vivo* validated target, pathway, or process should be performed. Such screens should be designed to use the current knowledge of the most sensitive targets—including for persisters—the transport functions, and the porin systems of *Mtb* that can be exploited to transport drugs and avoid efflux.

Fourth, smaller libraries with preferred physicochemical properties should be utilized. For example, NITD scientists recently screened a library of small and polar compounds, with promising results. Fifth CPZN-45, spectinamides, and macrolides are successful chemical series in lead optimization derived from natural products. This suggests that use of natural products as a potential source for novel leads against *Mtb* should be revisited. New potential natural product leads are discussed below.

**Underexploited methods of lead generation**

**Natural products.** The utility of natural products and natural product–derived compounds in
Table 2. Promiscuous targets identified in multiple whole-cell screens

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<tr>
<td>NITD-304</td>
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</tr>
<tr>
<td>QcrB</td>
<td>Imidazo[1,2-a]pyridine</td>
<td><img src="image" alt="QcrB Structure" /></td>
</tr>
<tr>
<td>Q203</td>
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[Correction added on July 21, 2014, after first online publication: the bottom-most structure in Table 2, labeled Q203, was corrected.]

antibacterial research is well documented. There are excellent reviews on antitubercular compounds derived from natural products. Here we highlight some recent notable examples that may prove to be useful leads for TB drug discovery (Fig. 1).

Thuggacin A, isolated from the fermentation broth of the myxobacterium *Sorangium cellulosum*, is active against Mtb with an minimum inhibitory concentration (MIC) of 8.0 μg/mL and a MOA believed to be inhibition of the Mtb respiratory chain—a target of great interest at present. Ergosterol peroxide, isolated from the leaves of *Radermachera boniana*, has an MIC of 1.5 μg/mL; although it has an unusual peroxide moiety, it appears to have a reasonable selectivity, since its cytotoxicity against Vero cells is greater than 86 μg/mL.
Trichodermin A, isolated from a fungus from marine sponges, has an Mtb MIC of 0.12 μg/mL under both aerobic and hypoxic conditions; it has been shown to be active against nonreplicating mycobacteria and its MOA to be inhibition of ATP synthesis. Another peptide natural product, lariatin A, shows a potent inhibitory activity against Mtb, with an MIC of 0.39 μg/mL. Isolated from...
the soil bacterium *Rhodococcus jostii*, lariatin A forms an unusual “lasso” structure in which its tail passes through a ring structure; its MOA is speculated to be inhibition of cell wall biosynthesis.61 A thiostrepton-class compound isolated from *Nocardia pseudobrasiliensis*, nocardiathiocin is highly potent against Mtb, with an MIC ranging from 0.025 to 1.56 μg/mL. A known natural product, pyridomycin was recently demonstrated to have the same MOA as isoniazid (i.e., inhibition of Mtb InhA, the enoyl-ACP reductase involved in mycolic acid biosynthesis);62 structural studies indicate that this compound blocks both the cofactor- and substrate-binding pockets.63 These examples indicate that natural products, although mostly neglected by current efforts due to cost and other factors, remain a relevant source of leads for TB drug development. Importantly, natural products occupy a chemical space that is very different from that covered by the various small molecules currently being screened within most pharmaceutical company libraries. Although medicinal chemistry to improve ADME and toxicity properties of an initial natural product hit may be challenging, such compounds can provide novel templates and indicate vulnerable targets for simplified, smaller, more developable analogues. Natural products discovered for non-Mtb bacteria may also be a source of future anti-TB leads.53,54

**Mechanism- and structure-based drug design**

There are numerous examples of mechanism- or structure-based drug designs64 in TB research, only some of which will be mentioned here. An interesting case of structure-guided design is the recently reported malate synthase (GlcB) inhibitors that are analogues of phenyl-dikitoacids, designed based on the enzyme X-ray crystal structure. One analog demonstrated efficacy against murine TB, as discussed in the section on targets below.65 FASII enoyl-ACP reductase (InhA) is the well-validated target of the TB drug isoniazid and has been a target of rational drug design.66 Based on the methylthiazole compounds discovered at GSK,67,68 scientists at AstraZeneca have published their investigation of the binding mode of these agents to InhA.69 This is part of an effort to discover direct InhA inhibitors that, unlike isoniazid, do not require activation by KatG.70 Defective KatG activation is the dominant resistance determinant in isoniazid-resistant Mtb clinical isolates.

Another approach to modern drug design, fragment-based drug discovery, has recently succeeded in generating inhibitors of Mtb pantothenate synthetase (Pts).71 In this study, 1250 fragments were initially screened by a thermal-shift method and NMR experiments. The initial 39 hits were further characterized by isothermal titration calorimetry and X-ray crystallography to further categorize them into fragments binding at the adenine pocket, those binding at the pantoate pocket, and a fragment binding deep in the pantoate pocket. One fragment has been elaborated to provide potent inhibitors of Pts.72

**Emerging targets**

**Targeting MTB iron acquisition and storage**

Iron is an essential nutrient for all living organisms including pathogenic bacteria in an infection, but the mammalian immune system employs various methods to restrict a pathogen’s access to iron.73 Various mammalian proteins are involved in iron homeostasis and their effect on the immune system is discussed in an excellent review;74 they include hepcidin, lactoferrin, siderocalin, haptoglobin, hemopexin, Nramp1, ferroportin, and the transferrin receptor, which underscores the importance of this nutrient in the host–pathogen relationship. Iron is sequestered in the human body by intracellular ferritin and extracellular transferrin.75 During infection, Mtb localizes inside host macrophages,76 where it has access to transferrin-bound iron. Mtb secretes two classes of siderophores, mycobactins77 and carboxymycobactins,78 to bind iron, obstructing it from the mammalian iron-binding proteins, and then internalizes the iron-laden siderophores through its receptors.

**Iron acquisition.** It was recently demonstrated that mbtE deletion mutants are unable to synthesize mycobactins and are attenuated for growth in vitro, macrophages, and guinea pigs, highlighting the importance of mycobactin biosynthesis for the growth and virulence of Mtb and establishing this pathway as a potential target for the TB drug development.79 Mycobactin analogs that inhibited MbtA, another mycobactin biosynthesis enzyme, have shown in vitro Mtb activity superior to first-line TB drugs.80
Deletion mutants of Mtb lacking *irtA* and *irtB* are attenuated in human macrophages and mouse lungs.\(^8^1\) *IrtAB* genes encode the ABC transporter IrtAB involved in the efficient transport and utilization of iron from Fe-carboxymycobactin in Mtb. The *irtAB* genes are located in a chromosomal region previously shown to contain genes regulated by iron and the major iron regulator IdeR.

Mutants of Esx-3, a secretion system that works in concert with the ItrAB ABC transport system to take up iron-laden siderophores, have been shown to be severely impaired for growth in macrophages, indicating Esx-3 as another potential drug target in iron acquisition.\(^8^2\) A novel siderophore-export system consisting of MmpS4/MmpL4 and MmpS5/MmpL5 was recently identified in Mtb by Wells et al., and an *mmpS4/S5* deletion mutant was shown to be attenuated in mouse infection models, indicating mycobactin efflux as a potential drug target.\(^8^3\)

An Mtb heme-uptake system has been defined\(^8^4\) that consists of the secreted protein Rv0203 and the transmembrane proteins MmpL3 and MmpL11; recent experiments showed that Rv0203 transfers heme to both MmpL3 and MmpL11 during Mtb heme uptake,\(^8^5\) making these proteins potential targets for TB drugs.

**Iron storage.** Although iron acquisition is required for the growth and virulence of Mtb, it seems that proper iron storage within the pathogen is just as crucial. Excess free iron becomes toxic, catalyzing the production of reactive oxygen radicals that could lead to oxidative damage. Mtb has two iron storage proteins, bacterioferritin (BfrA)\(^8^6\) and a ferritin-like protein (BfrB),\(^8^7\) which have been shown to be essential for Mtb protection against oxidative stress, growth in macrophages, and virulence in guinea pigs.\(^8^8\) Mtb lacking ferritin suffers from iron-mediated toxicity, is unable to persist in mice, and is highly susceptible to killing by antibiotics,\(^8^9\) demonstrating that endogenous oxidative stress can enhance antibiotic killing.

Various investigators have targeted siderophores for the development of novel TB drugs. One approach has been to develop agents that directly inhibit enzymes involved in siderophore synthesis.\(^9^0\) Another approach targets the iron-dependent regulator protein (IdeR) that represses siderophore synthesis genes and virulence factors when sustainable iron levels have been achieved;\(^9^1\) dysregulation of IdeR would lead to excess iron and oxidative damage or reduce virulence and enhance bacterial killing. The structural basis for iron activation and IdeR binding to DNA has been recently reported, and these insights have enabled the structure-based design of agents that target IdeR function. Small peptides that either enhance IdeR repression or inhibit IdeR dimerization demonstrate that IdeR activity can be rationally modulated.\(^9^2\)

The totality of the data reported here indicates that the biosynthesis, transport, and utilization of siderophores are potential targets for Mtb drug discovery, as Mtb survival and virulence seem to be directly related to iron availability. Indeed, the host already uses iron metabolism against pathogens: siderocalins are host proteins that sequester iron-laden siderophores\(^9^3\) as a defense mechanism.

**Targeting MmpL**

The Mtb genome contains 13 genes that encode 12 RND (resistance, nodulation, and cell division) proteins designated *mycobacterial membrane protein large* (*MmpL*). RND proteins transport a variety of cationic, anionic, or neutral compounds, including various drugs, heavy metals, aliphatic and aromatic solvents, bile salts, fatty acids, detergents, and dyes, across the cytoplasmic membrane.\(^9^4\) In a heroic attempt to decipher the role of MmpL proteins in Mtb, mutant strains inactivated in 11 *mmpL* genes were generated.\(^9^6\) Susceptibilities to a variety of drugs were unaffected in all the MmpL-deficient strains. One gene, *MmpL3*, was shown to be essential for growth, and mutants in four genes, *MmpL4, MmpL7, MmpL8*, or *MmpL11*, were attenuated in mice. The role of MmpL7 in virulence is likely explained by the findings that this polypeptide is required for the transport of phthiocerol dимyocеросате, a known virulence factor, to the cell surface.\(^9^7,9^8\) MmpL8 was found to be involved in sulfatide biogenesis and virulence,\(^9^9\) and an Mtb mutant with disruptions in *mmpL4, mmpL5, mmpL7, mmpL8, mmpL10*, and *mmpL11* showed significant attenuation in mice.\(^1^0^0\) As discussed above, Wells et al.\(^8^3\) recently demonstrated the direct involvement of MmpL4 and MmpL5 in siderophore export; the involvement of MmpL3 and MmpL11 in heme uptake has also been demonstrated, thus establishing the importance of these proteins in iron homeostasis and as potential...
targets for TB drug development. MmpL3, the only MmpL protein that has been shown to be essential, has also been shown to be the resistance determinant for a variety of unrelated drug candidates currently being evaluated,\textsuperscript{101,102} as well as to be involved in the transport of mycolic acids across the cell membrane onto the cell surface.\textsuperscript{38,103} Efflux of azole antibiotics in Mtb has been associated with mutations in \textit{Rv0678}, which transcriptionally regulates the efflux system encoded by the \textit{mmpS5–mmpL5} efflux system.\textsuperscript{104}

The above studies clearly indicate the importance of this protein family for growth, transport of vital cell wall components with some involvement in virulence, and perhaps the efflux of small molecules that are toxic to the bacterial cell. It is therefore interesting to imagine a single molecule that might inhibit a variety of MmpL proteins and potentially affect growth, virulence, and sensitivity to other drugs. This would be reminiscent of β-lactam antibiotics that target penicillin-binding proteins and affect peptidoglycan biosynthesis and sensitivity to other drugs. A concerted search for such an inhibitor might uncover a natural product capable of such if MmpLs are as important in soil-dwelling mycobacteria. MmpLs are membrane proteins and may thus be more accessible for targeting than cytosolic enzymes, a potential explanation for the various compounds with reported associations with MmpL3. More studies are required to determine if different MmpLs are important under different \textit{in vivo} conditions and their potential involvement in pathogenesis and drug efflux in the various environments of the diseased lung.

**Targeting cholesterol metabolism**

Mounting evidence suggests that Mtb uses the host's cholesterol as a source of carbon and energy during infection. Strains defective in cholesterol transport or degradation are attenuated in activated macrophages and Mtb requires several genes involved in cholesterol catabolism for full virulence in animal models.\textsuperscript{105–108} It is unclear what carbon source for Mtb is most important during an infection, but host cholesterol use by Mtb has been demonstrated\textsuperscript{109,110} and appears to be particularly important during the chronic phase of infection.\textsuperscript{107}

Host cholesterol has been shown to be involved in a human Mtb infection,\textsuperscript{111} and high levels of cholesterol in the diet have been shown to significantly enhance the bacterial burden in the lung.\textsuperscript{112} Specifically, cholesterol is required for phagocytosis of mycobacteria by macrophages.\textsuperscript{113,114}

The intracellular growth operon, the \textit{igr} locus, encodes enzymes of cholesterol catabolism in the Mtb genome\textsuperscript{115} the primary function of which is the degradation of the 2′-propanoate side chain. Inactivation of the \textit{igr} operon results in reduced Mtb growth on cholesterol alone or in combination with glycerol, indicating that cholesterol or its metabolites are toxic to the \textit{igr} mutants. The \textit{igr} locus encodes a cytochrome P450 (\textit{cyd}125), two acyl-CoA dehydrogenases (\textit{fadE28} and \textit{fadE29}), two conserved hypothetical proteins (\textit{Rv3541–2c}), and a putative lipid-carrier protein; the locus is essential for growth in macrophages and critical in the chronic phase of infection.\textsuperscript{116} An enzyme encoded by \textit{fadA5} has been described that catalyzes the thiolysis of acetoacetyl-CoA and is required for growth on cholesterol and virulence in the late stages of an infection.\textsuperscript{105}

The other phase of cholesterol catabolism in Mtb is the degradation of the A–D rings. The 3-ketosteroid 9α-hydroxylase (\textit{KshAB}) has been identified as a virulence factor involved in cholesterol ring degradation.\textsuperscript{117} In a seminal study utilizing bioinformatics, it was shown that 51 rhodococal genes specifically expressed during growth on cholesterol were conserved within an 82-gene cluster in Mtb, and that mycobacteria grown on cholesterol upregulate \textit{hsaC} and \textit{kshA}.\textsuperscript{110} Transposon mutagenesis studies suggested that the genes encoding HsaA and HsaD are essential for intracellular survival of Mtb in human macrophages.\textsuperscript{118} HsaC, an iron-dependent extradiol dioxygenase, has been shown to be a virulence determinant in guinea pigs that influences dissemination, persistence, and the extent of disease pathology.\textsuperscript{106} Interestingly, cholesterol metabolism was demonstrated to be lethal to an \textit{hscA} Mtb mutant, consistent with catechol toxicity. HsaD, the cholesterol meta-cleavage product hydrolase,\textsuperscript{119} has been associated with the survival of Mtb in macrophages.\textsuperscript{118} HsaAB, an enzyme that transforms 3-HSA to 3,4-DHSA in cholesterol catabolism, has also been identified.\textsuperscript{120} Also, FadD3 has been shown to be a 3α-H-4α (3′-propanoate)-7αβ-methylhexahydro-1,5-indanedione (HIP)-CoA synthetase that initiates catabolism of steroid rings C and D after side-chain degradation is complete.\textsuperscript{121}
The rhodococcal Mce4 system is required for steroid uptake and is upregulated upon growth on cholesterol. On the basis of this, it was predicted that all Mce4 systems are steroid transporters, and that in Mtb they transport cholesterol during an infection. Indeed, the involvement of the Mtb mce4-encoded transport system in cholesterol import has been demonstrated, and mce4 deleted mutants have been shown to be severely attenuated in both macrophages and mouse infections models, establishing cholesterol import and metabolism as potential therapeutic targets.

From the above experimental data, it seems that while cholesterol catabolism by Mtb is important during infection, if ineffectively metabolized, cholesterol may be toxic to the pathogen. Importantly, both phenomena can be exploited for TB drug discovery, for drugs that inhibit cholesterol metabolism or those that inhibit enzymes that remove the toxic intermediates of cholesterol metabolism. In contrast to these observations, it was recently reported that cholesterol is not required as an nutritional source during infection.

**Targeting the Mtb ClpP protease**

The ClpP proteases (cutinase-like proteins, chaperon-linked proteases, or caseinolytic proteases) have been of interest as drug targets since the antibacterial activity of an acyldepsipeptide (ADEP) that binds the Clp protease of *Bacillus subtilis*, was demonstrated. Clp proteases are conserved proteases involved in the degradation of damaged, poorly formed, and unfolded proteins.

*Mtb* encodes two ClpP homologs, ClpP1 and ClpP2, which form a self-compartmentalized protease consisting of two heptameric rings stacked on top of each other, enclosing a catalytic chamber. Within the chamber, which can be reached through two axial pores, each of the 14 identical monomers possesses a serine protease active site. To gain activity, the ClpP protease multimer associates with hexameric rings of Clp ATPases forming the proteolytic complex. The ATPase subunits (ClpC1 and ClpC2) are responsible for recognition, unfolding, and translocation of peptides into the ClpP degradation chamber. The resulting structure is the chamber in which the active sites are sequestered from the cytoplasm to exclude native proteins and control access to the proteolytic chamber.

A recent *Mtb* global transcriptional analysis identified approximately 100 genes involved in resumption of replication upon reaeration following hypoxia, which included a transcription factor, the Clp protease regulator orthologue ClgR. *Mtb* ClgR was shown to activate the transcription of at least 10 genes, including four that encode protease systems (ClpP1/C, ClpP2/C, PtpA, and HtrA-like protease Rv1043c) and three that encode chaperones (Acr2, ClpB, and the chaperonin Rv3269). clgR deletion mutants were attenuated for growth in macrophages, and for controlling the phagosomal pH, compared to wild-type organisms.

Recent studies have demonstrated that ClpP1 and ClpP2 are essential for growth and form a mixed complex that degrades missense and prematurely terminated peptides, as depletion of the protease specifically led to growth reduction in the presence of antibiotics that increase errors in translation. A recent study confirmed that ClpP1 is essential for *Mtb* growth in vitro and that the previously described ClpP activators (ADEPs, discussed below) are active against *Mtb*. *Mtb* ClpP has thus been validated as a drug target that could be exploited because of its novel mechanism—in the presence of ADEPs—of bacterial killing. It has been shown that ADEP binding leads to a change of ClpP structure, allowing access of unfolded proteins to the proteolytic chamber in the absence of the regulatory Clp ATPases, thus effectively dysregulating this protease system and leading to unregulated proteolysis and bacterial death. It was recently demonstrated that ADEP4-activated ClpP becomes nonspecific in its proteolytic activity and kills *Staphylococcus aureus* persisters by degrading over 400 proteins, causing cells to self-digest. A similarly efficacious dysregulator of *Mtb* ClpP would represent a truly novel mechanism of sterilizing chronically infected lungs, contribute to shortening of treatment durations in novel TB drug regimens, and be effective against resistant disease.

Alternatively, a structurally diverse series of β-lactone inhibitors has been shown to form a covalent adduct at the ClpP2 catalytic site and inhibit *Mtb* growth. This finding provides yet another pharmacological validation of the ClpP protease as a potential drug target, but in this case the mechanism is by inhibition of ClpP instead of by dysregulating ClpP activity as occurs with ADEPs.
**Targeting central carbon metabolism**

Mtb adapts its metabolism to the environmental conditions to which it is exposed. Several metabolic enzymes have been validated as drug targets; the multifunctionality of some of these enzymes makes them of particular interest.

The enzymes of the glyoxylate shunt, isocitrate lyase (ICL) and malate synthase (GlcB), have long been and remain of interest to TB drug discovery. The glyoxylate shunt serves to bypass the two CO$_2^-$ generating steps of the tricarboxylic acid (TCA) cycle when carbon is limiting, including during growth on fatty acids. ICL-deficient Mtb cannot establish an infection in mice, and _icl_ is up-regulated during infection. Although this suggests reliance on this pathway and subsistence on fatty acids _in vivo_, the importance of ICL may result from its several roles: ICL in the glyoxylate shunt, methyl-ICL in the methylcitrate cycle of propionyl-CoA metabolism, ICL in ATP homeostasis during adaptation to slow growth, and ICL in succinate generation for proton motive force (PMF) maintenance under hypoxia. Perhaps owing to the small polar active site of ICL only weakly efficacious ICL inhibitors have been reported to date. Efforts continue to target ICL, including via target-based whole-cell screening. Conversely, novel, efficaciousphenyl-diketo acid inhibitors of Mtb GlcB were recently discovered. As for ICL, GlcB appears to play unexpected metabolic roles in carbohydrate and cholesterol metabolism—another example of the metabolic flexibility of Mtb.

Phosphoenolpyruvate carboxykinase (PEPCK), like ICL, is required by Mtb for growth on fatty acids and survival in rodents, and it is induced during infection. PEPCK catalyzes the first committed step of gluconeogenesis, by which sugars are synthesized from TCA intermediates during growth on fatty acids. As with ICL, the importance of PEPCK _in vivo_ suggests subsistence on fatty acids. However, the glycolytic enzyme glucokinase is also required for survival of Mtb in mice, suggesting a requirement for metabolism of carbohydrates during late infection. No drug discovery efforts have been reported yet against these interesting targets.

Two better explored targets are lipoamide dehydrogenase (Lpd) and dihydrolipoamide acyltransferase (DlaT). DlaT and Lpd function as components of pyruvate dehydrogenase, which supplies glycolysis-derived acetyl-CoA to the TCA cycle. Both are also components of the Mtb peroxynitrite reductase/peroxidase, which functions to resist host-reactive nitrogen intermediates. Lpd also functions within branched-chain amino acid metabolism, perhaps explaining the more profound attenuation for growth in mice upon disruption of _lpdC_ compared to _dlaT_. Efforts to target both Lpd and DlaT are ongoing, the latter motivated by the demonstration that DlaT-deficient Mtb fails to establish an infection in guinea pigs. Discovery efforts focused on DlaT have identified inhibitors that are selectively active against nonreplicating Mtb.

Finally, the maltosyltransferase GlgE was recently identified as a target of interest. This enzyme participates in a pathway from trehalose to α-glucan, and GlgE-deficient Mtb dies _in vitro_ and in mice due to accumulation of its toxic substrate, maltose-1-phosphate. This pathway also exhibits a synthetic lethal interaction with the glucosyltransferase Rv3032, suggesting that synergistic TB drugs may be discovered by targeting components of these pathways.

**Targeting energy generation: inhibitors of the respiratory chain and ATP synthesis**

ATP synthesis and PMF generation are among the best-validated Mtb drug targets owing to the clinical successes of the ATP synthase inhibitor TMC207 (bedaquiline, Sirturo) and the cornerstone TB drug pyrazinamide, which disrupts the PMF. PMF and ATP homeostasis are required by replicating and nonreplicating Mtb, under a variety of conditions and efforts to target these processes include pathway screens using membrane particles, cell-based screens for ATP homeostasis disruptors, target-based screens, and repurposing of existing drugs known to inhibit this pathway.

Mtb operates a branched respiratory chain, the components of which operate variably, depending on the environment. A menaquinone pool is reduced by NADH dehydrogenase (Ndh, provided by a 14-subunit NdhI complex and single subunit NdhII) and succinate:menaquinone oxidoreductase (Sdh). Electrons flow from the menaquinone pool to a quinol oxidase (cytochrome _bd_ oxidase) or to a cytochrome _bcl_–_aa3_ oxidoreductase supercomplex wherein a _bcl_ c-type cytochrome reductase transfers electrons to the terminal _aa3_-type oxidase. Both oxidases use molecular oxygen as the terminal electron acceptor. In the absence of respiration, Mtb...
maintains the PMF and ATP synthesis using secreted succinate generated by fumarate reductase and/or produced by ICL.

TB drug discovery efforts have focused on NdhII, encoded in Mtb by ndh and ndhA. The phenothiazines (a class of CNS drugs) have anti-Mtb activity in vitro and in vivo and their Mtb target is NdhII, pharmacologically validating this enzyme. In addition, the leprosy drug clofazimine, which is efficacious against murine TB, targets NdhII as part of its mode of action. The existence of transposon mutants of ndhA, but not ndh, suggests the relative importance of the ndh-encoded component of NdhII. However, a novel compound identified through a screen for ATP synthesis inhibitors appears to target NdhA. The importance of NdhI and Sdh for Mtb energy metabolism is not fully understood, although recent evidence suggests a crucial role for Sdh under hypoxia.

The biosynthesis of menaquinone is expected to be critical under aerobic and hypoxic conditions. Menaquinone is synthesized from chorismate by a series of at least eight enzymes. Of these, MenC, MenD, and MenE appear to be essential in vitro. A recent report of anti-Mtb MenB inhibitors cites a personal communication (from C.M. Sassetti) that MenB is essential. A report on MenA inhibitors active against replicating and non-replicating Mtb mentioned an unpublished demonstration (by D. Schnappinger) that MenA is essential for growth of Mtb in mice, providing genetic validation for MenA and menaquinone biosynthesis in general as a drug target. Target-based efforts are ongoing against Mtb MenA, MenB, and MenE.

The cytochrome b subunit (QcrB) of the cytochrome bc1-aa3 complex has recently emerged as a target of interest. Previous studies indicated that QcrB is essential and that the aa3-type cytochrome c oxidase is important for anaerobic growth, while the less bioenergetically favorable cytochrome bd oxidase is more important for microaerobic growth. However, disruption of Mtb cytochrome c maturation results in only impaired growth in vitro and in mice, which is partially compensated for by induction of cytochrome bd oxidase. This suggests a role for cytochrome bd oxidase during aerobic growth and in an infection, as well as limited target vulnerability of the cytochrome bc1 complex. Despite this, the QcrB inhibitor, Q203, demonstrates potent efficacy against murine TB, Q203 and other compounds confirmed or presumed to inhibit QcrB are described in more detail above.

Although Mtb QcrB is pharmacologically validated, further efficacy profiling of QcrB inhibitors is ongoing.

Following generation of the PMF, ATP synthesis occurs via F0F1 ATP synthase. Its c subunit is the target of the diarylquinoline TMC207. Efforts to target ATP synthase are limited by its complex nature. However, cell-based or membrane particle–based screens for ATP synthesis inhibitors may identify new series targeting ATP synthase.

**ROS and NOS generation**

Although somewhat controversial, one hypothesis states that all bactericidal antibiotics kill bacteria by generating reactive oxygen or nitrogen species (ROS/RNS). Recently it was shown that a relatively small change (20%) in dissolved oxygen can affect killing of bacterial persisters, a subpopulation of bacteria in an infection that is phenotypically resistant to killing by most antibiotics but still sensitive, however, to high quantities of radicals. This observation can be critical for killing Mtb in granulomas, which have hypoxic cores.

Drugs such as clofazimine and PA-824 and delamanid have been shown to generate ROS and/or RNS, which is presumably the mechanism for their activity against Mtb. Recently, the function of the Mtb deazaflavin-dependent nitroreductase (Ddn), an enzyme that activates PA-824, has been hypothesized to be what provides Mtb protection from oxidative stress and bactericidal agents; the authors of the report noted that Ddn mutants defective in the formation of deazaflavin were hypersensitive to isoniazid, moxifloxacin, and clofazimine. This suggests that any agent that can inhibit Ddn or deazaflavin biosynthesis could synergize with existing anti-TB drugs. Thus, novel mechanisms to generate ROS/NOS at sufficiently high concentrations should be investigated as potential novel therapy for TB, as long as they do not cause oxidative damage to the host cells.

**Concluding remarks**

The current status of lead generation for TB drug development is much improved compared to the situation 10–15 years ago. However, it is still slow and drastically lacking in success; significant changes
are needed to produce novel regimens with efficacy against drug-resistant TB, improved safety and shorter treatment durations. A review of the processes that have led to the current global pipeline suggests a few solutions that could be employed to improve the situation and produce better, more easily developable leads: smaller, targeted compound libraries with favorable physicochemical properties should be used for hit generation; hits with preferred physicochemical properties should be prioritized over higher potency hits with poor properties; hits with improved cell penetration and with activity against multiple subpopulations of Mtb should be prioritized. When possible, leads that can penetrate caseum and have efficacy within hypoxic lesions should be prioritized; and natural products should be revisited to allow the identification of currently unexplored chemical matter.

In addition, structure- and fragment-based approaches should be used wherever practicable. Instead of either target-based biochemical screening, or whole-cell-based screening, whole-cell-based, target-specific screens using specialized strains that focus on in vivo validated targets, pathways, or processes should be performed. Such screens should be designed using what is currently known about the various bacterial populations in the infected lung; the nature and metabolic properties of the organisms in an infection, including persisters; their transport systems and porins that could be exploited for drug uptake; and the most sensitive targets at the different stages of the disease. Such vulnerable pathways and processes might include the in vivo validated iron acquisition and storage pathways; central carbon, cholesterol, and energy metabolism pathways; the MmpL transport systems; and the Clp protease system. Nevertheless, because both knowledge of the biology of Mtb remains incomplete and the nature of clinical TB so complex, any current screening approach necessarily remains limited. Multiple subpopulations of bacteria, differing in their replication rate and metabolic state, exist in an infection in the multiple and changing environments of the tuberculous lung. Any one set of screening conditions is thus unlikely to include key features of all of these environments, and few individual targets and pathways are likely crucial to all physiological states of the organism and disease. It is unlikely that any in vitro or even mouse-based screen will select and identify compound series with sufficient exposure for efficacy in necrotic, hypoxic, and cavitary lesions, or eliminate viable but nonculturable organisms.

Yet, we believe that combining the lessons learned from recent and ongoing TB drug discovery efforts with emerging technologies and an evolving understanding of Mtb biology should provide a path toward safer, novel regimens with greater treatment-shortening potential.

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Conflicts of interest

The authors declare no conflicts of interest.

References


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