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# Bottlenecks and opportunities in antibiotic discovery against *Mycobacterium tuberculosis*

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Tuberculosis (TB) persists as a major global health issue and a leading cause of death by a single infectious agent. The global burden of TB is further exacerbated by the continuing emergence and dissemination of strains of *Mycobacterium tuberculosis* resistant to multiple antibiotics. The need for novel drugs that can be used to shorten the course for current TB drug regimens as well as combat the persistent threat of antibiotic resistance has never been greater. There have been significant advances in the discovery of *de novo* TB treatments, with the first TB-specific drugs in 45 years approved for use. However, there are still issues that restrict the pipeline of new antitubercular chemotherapies. The rate of failure of TB drug candidates in clinical trials remains high, while the validation of new TB drug targets and subsequent identification of novel inhibitors remains modest.

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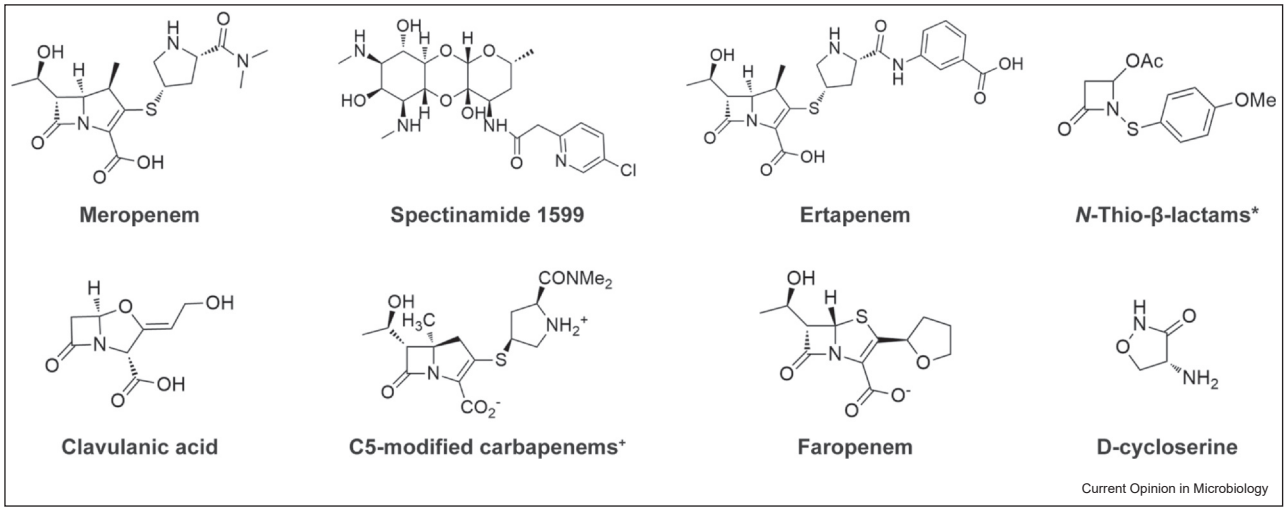
## Introduction

Infection by the slow-growing, obligate human pathogen, *Mycobacterium tuberculosis*, leads to the complex disease tuberculosis (TB). The estimated extent of *M. tuberculosis* infection is 25% of the world's population, with the vast majority of these leading to the latent form of TB, defined as persistent host immune response to stimulation by antigens of the pathogen with no clinical manifestation of the active disease [1]. In 2020, approximately 10 million people contracted active TB, while 1.5 million died as a result of the disease. Positively, the rates of new TB cases and deaths have declined by 11% and 9.2%, respectively, since 2015. Despite the positive decline in TB cases and deaths, these changes are not sufficient to satisfy the World Health Organization (WHO) End TB Strategy milestones.

TB is a treatable disease, but its chemotherapy requires an intensive first-line regimen, consisting of a combination of four drugs: rifampicin, isoniazid, ethambutol, and pyrazinamide over an initial two-month period, followed by a further six-month continuation of rifampicin and isoniazid treatment [2]. Chemotherapy for the treatment of drug-resistant TB is not quite as standardized, primarily due to its multiple types (mono-, multi-, or extensively drug-resistant) each of which requiring a distinct regimen. These pharmacologically diverse diseases are divided into five different categories by the WHO. However, most of the recommended regimens to treat a drug-resistant *M. tuberculosis* infection involve a combination of the second-line TB drugs, which include bedaquiline, linezolid, fluoroquinolones, D-cycloserine, delamanid, meropenem, *para*-aminosalicyclic acid, pretomanid, and ethionamide [3]. Recent successes with bedaquiline-containing drug combinations, most notably the 2019 US Food and Drug Administration approval or the BPaL regimen (bedaquiline in combination with pretomanid and linezolid), highlight how dynamic antitubercular treatment, particularly for drug-resistant TB, has become [4]. This dynamism is predominantly enabled by the availability of new TB drugs, which promises to continue with more new and repositioned antibiotics in late-stage clinical development. Despite these successes, second-line TB drug regimens can take anywhere from six months to over two years to treat drug-resistant *M. tuberculosis* infections [5].

TB treatment is complex due to a number of different factors, including host health and immune status, pharmacological interactions with other drugs, toxicity due to chronic use, as well as the diversity of physiological states adopted by *M. tuberculosis* during TB pathogenesis [6]. The treatment of a latent *M. tuberculosis* infection is a particular challenge as the physiological states adopted by the bacilli limit the efficacy of most TB drugs. To achieve the WHO End TB Strategy, new TB treatments must be effective against both actively replicating *M. tuberculosis*, as well as the latent form of the bacillus. Host toxicity of the current TB drugs has remained a major concern, with severe side effects such as blindness, deafness, and organ failure of the liver and kidneys.

**Figure 1**




Old molecules, new antitubercular drugs. Chemical structures of the compounds discussed in the drug-repurposing section. *The *N*-thio-β-lactam example is molecule 1 g, disclosed in the report by Martelli and colleagues [10]. +The C5-modified carbapenem example presented is molecule 10a from the study carried out by Gupta and coworkers [8].

The safety profile of the newly approved antitubercular chemotherapies, including bedaquiline and linezolid, remains an issue with several clinically relevant adverse events reported, including QT prolongation and peripheral neuropathy [7]. These toxicities, as well as emerging reports of bedaquiline and linezolid-resistant strains of *M. tuberculosis*, highlight the continuing need to develop new TB drugs and regimens [8,9].

*M. tuberculosis* drug-resistance mechanisms include cell-envelope penetration, efflux, enzymatic modification, and decreased activation [10]. These resistance processes collectively represent a significant bottleneck for the development of new antitubercular chemotherapies. The issues presented by drug-efflux mechanisms in *M. tuberculosis* have recently been thoroughly reviewed by Remm and coauthors [11]. The activation/inactivation and metabolism mechanisms employed by *M. tuberculosis* for xenobiotics are complex, dynamic, and are yet to be fully characterized. While these mechanisms will not be explored in this article, the bottlenecks presented could also be exploited as opportunities for new drug discovery.

This minireview aims to summarize and critically evaluate a few important bottlenecks, as well as highlight opportunities for modern antitubercular discovery.

## Old molecules and classic targets with great potential

Drug repurposing represents a "risk-free" and economically streamlined platform to identify new applications to existing molecules. The TB drug-discovery community has fully embraced this concept, yet very few compounds have reached clinical trials [12,13]. Drug repurposing would perhaps better deliver results if it was viewed as a route to novel lead compounds, instead of "ready-to-go" drugs. A few important examples are discussed below to illustrate this point.

The two most successful drug-repurposing efforts for *M. tuberculosis* are clavulanic acid and penems ( Figure 1). Enzymological experiments demonstrated that clavulanic acid and certain penems are irreversible inhibitors of *M. tuberculosis* β-lactamase BlaC [14,15]. This indicated for the first time the potential to inhibit the chromosomally encoded BlaC and sensitize *M. tuberculosis* to β-lactam antibiotics. Indeed, analysis of the combination of meropenem (MEM) and clavulanate revealed potent and quick sterilization of cultures both at normoxic and hypoxic conditions [16]. These studies opened the TB and nontuberculous mycobacteria drug-discovery field to the use of clavulanate/penems as antimycobacterial agents [17–19].

Another elegant example of repurposing is spectinomycin. Spectinomycin, a ribosomal inhibitor, is not sufficiently potent to be used as an antimycobacterial agent [20]. Instead of pursuing a molecule that does not have the desired properties, Lee and colleagues used spectinomycin as a starting point to generate and screen for improved compounds against *M. tuberculosis*. Building on important lessons learned from Gram-negative antibiotic discovery and medicinal chemistry, the team created improved analogs (spectinamides) (Figure 1), which are poor substrates for mycobacterial efflux pumps, such as

Rv1258c [21]. Spectinamides, in a molecule- and target-centered drug-discovery program rooted in excellent medicinal chemistry, are currently in development as promising antitubercular agents [22–24].

D-cycloserine (DCS) is the only resistance-proof antitubercular drug in clinical use (Figure 1) [25]. Yet, it causes central nervous system (CNS)-related toxicity in a subset of patients and is hence a second-line drug. Many academic and pharmaceutical groups have tried, unsuccessfully, to identify and optimize DCS analogs or similar compounds. We have recently demonstrated that DCS inhibition in *M. tuberculosis* differs from the accepted mechanism of action, which can partially explain the lack of success in identifying improved analogs of DCS. DCS engagement with D-Ala:D-Ala ligase appears to be the most lethal interaction, instead of inhibition of its other target, alanine racemase [25–27]. These findings were further confirmed by the demonstration that DCS is slowly hydrolyzed by alanine racemase [28]. Armed with this knowledge, new drug-discovery programs might succeed in identifying hydrolysis-proof, improved DCS analogs that interact with both targets, but do not cause CNS toxicity. Interestingly, Zandi and Townsend also observed that MEM undergoes both reversible reaction and nonhydrolytic off-loading reactions with *M. tuberculosis* L,D-transpeptidases [29]. Together, these two studies suggest that target-catalyzed antibiotic degradation is perhaps a more general phenomenon and therefore an important parameter for improved generations of antibiotics.

Collectively, these examples illustrate the critical importance of understanding kinetic and mechanistic aspects of antibiotic-enzyme engagement and how poor drugs can be redesigned into excellent compounds through elegant medicinal chemistry.

## Single-nutrient utilization, high risk, and low gain

*M. tuberculosis* metabolism is complex and dynamic, and therefore it represents both a unique opportunity and a significant bottleneck in drug-discovery campaigns [30–33].

For example, we now understand that *M. tuberculosis* simultaneously cometabolises multiple carbon and nitrogen sources ( Figure 2) [34,35]. Furthermore, in many cases, the transporters and enzymes responsible for nutrient uptake and catabolism have been identified [34–43]. It is therefore not surprising that inhibitors aimed at blocking metabolism of a single nutrient are not efficacious. For example, work from Wilburn and colleagues demonstrated that even well- validated cyclic adenosine monophosphate (cAMP) small- molecule activators, which lead to complete interruption of cholesterol utilization by *M. tuberculosis* in vitro, are only weakly active during infection [44]. Lack of efficacy is likely due to the presence of other carbon sources, such as fatty acids, pyruvate/lactate, and amino acids. Although apparent, until we understand exactly the relative importance of different nutrients utilized by *M. tuberculosis* during infection, discovery of antibiotics targeting single-nutrient utilization is a high-risk, low-gain area.

Another example of this challenge deals with glycerol catabolism. Although glycerol is the best carbon source for *M. tuberculosis* in vitro, its abundance in humans is very low. It is therefore not surprising that glycerol utilization and its inhibition by compounds such as the pyrimidine–imidazole class of glycerol kinase inhibitors are devoid of antitubercular activity during experimental infection [45].

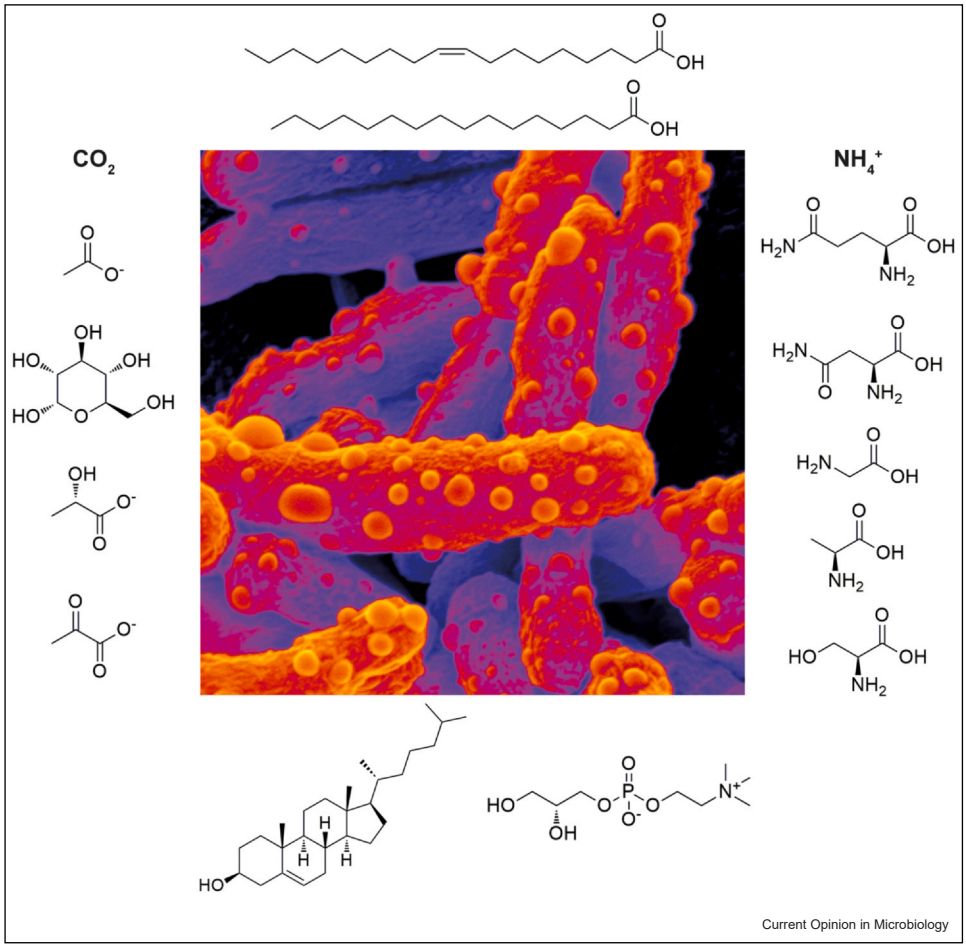
Studies such as these have demonstrated beyond doubt that *M. tuberculosis* is not a "picky eater". A quantitative characterization of nutrient preferences should be a primary focus of the TB research community, as a more thorough understanding of *M. tuberculosis* metabolism will surely yield opportunities for methodical, *de novo* antitubercular drug discovery.

## *M. tuberculosis* cell-envelope permeability, a key challenge in antitubercular discovery

The difficulties of translating inhibitors identified and optimized using target-based drug- discovery approaches to bacterial cells were seminally highlighted by Payne and colleagues [46]. These challenges remain, with one of the primary causes believed to be the permeability of the bacterial cell envelopes. The complex *M. tuberculosis* cell envelope is atypical among bacteria, reflecting the extreme environments that the bacilli encountered originally in environmental niches. Not only is the dense and elaborate cell envelope highly dynamic, it is also tightly regulated, with only a fraction of the transporters of related species [47,48]. The challenges of gaining access to *M. tuberculosis* by crossing the cell envelope and some strategies for addressing these issues are discussed below, and are shared with Gram-negative bacteria.

Initially, in response to the perceived failure of target-based antibacterial drug discovery, a number of whole-cell phenotypic screens were carried out most notably to identify inhibitors of *M. tuberculosis* [49]. These screens, against either chemical diversity compound collections or focussed sets of molecules for specific targets, have been relatively successful, as demonstrated by the increasing numbers of inhibitor series for targets such as DprE1 and MmpL3 [50]. However, these proto-chemogenomic approaches have, to date, failed to identify a broad cross section of targets vulnerable to small-

**Figure 2**




An up-to-date view of *M. tuberculosis* nutrition. *M. tuberculosis* takes up a diverse selection of nutrients that are subsequently used to fuel metabolism. The chemical structures presented in this image are a selection of the molecules acquired from the extracellular environment by *M. tuberculosis*. The molecules are (moving clockwise from $CO_2$), oleic acid (top), palmitic acid (bottom), ammonium, L-glutamine, L-asparagine, glycine, L-alanine, L-serine, α-glycerylphosphorylcholine, cholesterol, pyruvate, lactate, dextrose, and acetate. The central panel is a scanning electron micrograph image of *M. tuberculosis,* depicting bacilli covered in microvesicles. The production of microvesicles by *M. tuberculosis* has previously been characterized as a means to export molecules that can modulate the immune response as well as a mechanism to enhance iron acquisition (J Bacteriol. 2014 Mar;196(6):1250–6. doi: 10.1128/JB.01090-13.). The central panel image is courtesy of Dr. Maximiliano G. Gutierrez (The Francis Crick Institute).

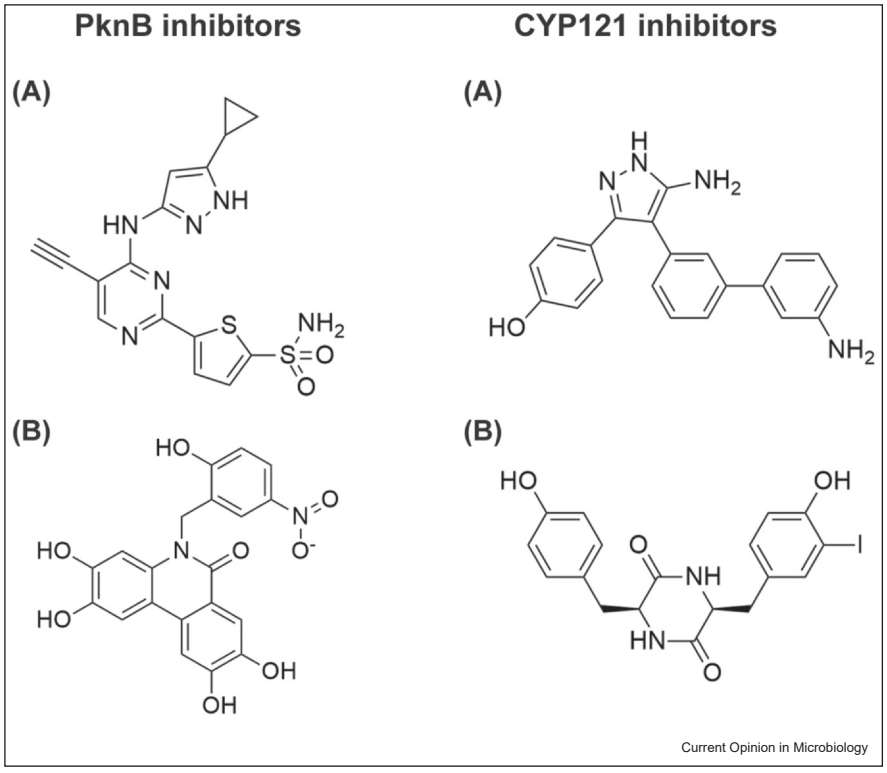
molecule inhibition, with identification of compounds for the aforementioned apparently promiscuous targets predominating [51–53].

The failures of many target-based drug-discovery projects for *M. tuberculosis* macromolecules have not been because of poor compounds. Invariably, the studies carried out to identify and optimize these inhibitors have used well-established drug-discovery approaches or tried to capitalize on the accumulated knowledge of how to drug a specific target class.

The use of an established drug-discovery approach, fragment-based inhibitor identification, was utilized to identify molecules for the *M. tuberculosis* essential cytochrome P450s (P450s or CYPs) enzyme, CYP121. A number of chemically diverse inhibitor classes, optimized using elegant structural biology-aided medicinal chemistry, have subsequently been identified, many with low-nanomolar affinities (Figure 3). Unfortunately, this approach has not been able to translate these affinities into potent antibacterial inhibition [54,55].

Another well-established *M. tuberculosis* drug target pursued using target-based discovery is the eukaryotic-like serine–threonine kinase PknB. A primary attraction of this target is that it may be possible to capitalize on the wealth of chemotherapeutic knowledge accumulated

**Figure 3**



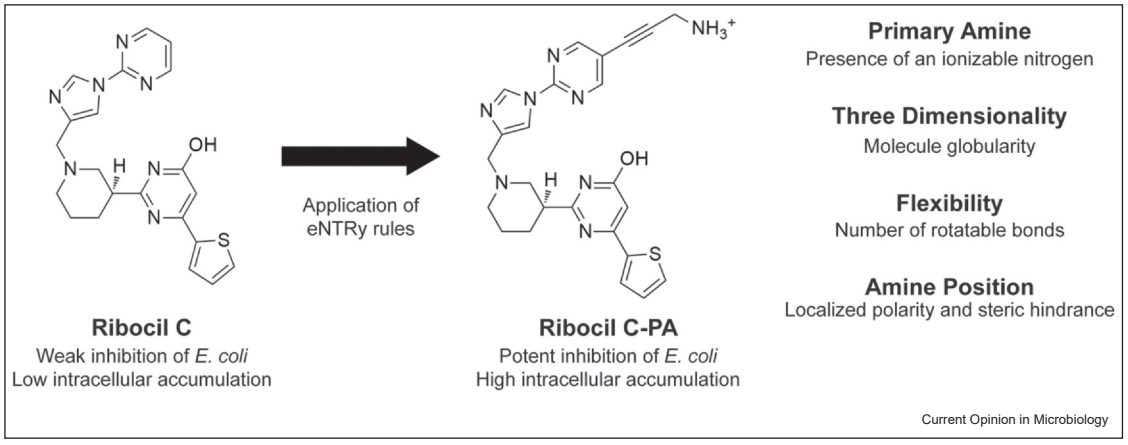

Potent inhibitors of *M. tuberculosis* enzymes with low or no antitubercular activity. Chemical structures of the compounds discussed in the cell-envelope permeability section. PknB inhibitor **(a)** is molecule 49 from [44], **(b)** is compound 11 from [46]. CYP121 **(a)** is molecule 25a from [43], **(b)** is compound 9 from [42].

from the many studies of the mammalian members of this enzyme class. Unfortunately, the development of PknB inhibitors has so far failed to deliver cell-active compounds (Figure 3). The primary source of this failure is believed to be *M. tuberculosis* cell-envelope permeability, which has rendered several chemically diverse inhibitor series, mostly with low-nanomolar affinities for this genetically validated target, nonefficacious against the bacterium [56,57].

The challenge of translating inhibitor potency against recombinant bacterial targets into whole-cell antibacterial activity is well-established. However, studies are starting to emerge that employ novel strategies to circumvent these issues. A set of physicochemical traits have been defined that when satisfied predispose compounds for accumulation in *Escherichia coli* (*E. coli*) [58]. These "eNTRy rules" have been used to convert deoxynybomycin, from a Gram-positive-only inhibitor, to a broad-spectrum antibiotic that is active against a diverse panel of multidrug-resistant Gram-negative pathogens. Hergenrother and colleagues then went on to use these physicochemical rules to modify the antibiotic ribocil C (Figure 4), which is inactive against Gram-negative bacteria, to facilitate intracellular accumulation in *E. coli*, while maintaining target potency, ultimately leading to whole-cell activity [59].

The prevailing drug-discovery strategies to identify new *M. tuberculosis* inhibitors, such as whole-cell phenotypic screening, have gone some way to address the lack of permeability of the cell envelope. However, a limiting factor of these approaches is that invariably the compounds screened were originally selected using rules specific for mammalian cells (Lipinski's rule of 5) [60]. While these criteria are ultimately important to treat human diseases, they may not be the most appropriate compounds to cross the cell envelope. The approaches taken by Hergenrother and coworkers demonstrate that it is possible to define a tailored set of physicochemical rules that can be used to modify compounds that were previously impermeable for Gram-negative bacteria, so that they accumulate within the bacteria and become potent inhibitors [58]. Given the wealth of compound screening data available for *M. tuberculosis*, as well as the availability of established methodologies, such as

**Figure 4**




Gram-negative "eNTRy rules" applied to modify Ribocil C. The application of Gram-negative "eNTRy rules" to develop the potent antibiotic Ribocil C-PA from Ribocil C [48]. More information, including a prediction tool to identify molecules likely to accumulate in Gram-negative bacteria, can be found at http://www.entry-way.org/.

metabolomics, to monitor the intracellular concentrations of small molecules, perhaps, the definition of a specific set of "eNTRy rules" for *M. tuberculosis* is not unattainable.

## Opportunities for future drug discovery

Despite the bottlenecks highlighted, there remains grounds for cautious optimism that the progress made in the identification of new antitubercular chemotherapies can be maintained. The current clinical development pipeline for new TB drugs is comprised of 10 ongoing trials, including new molecules and new multidrug regimens (clinicaltrials.gov). The past decade has been the most successful period for the TB drug discovery in 50 years with the approval of three new antitubercular drugs: pretomanid, bedaquiline, and delamanid [61]. However, the failure rates encountered in clinical trials before a drug candidate is approved for human use remain very high, in excess of 85% [62]. Furthermore, *M. tuberculosis* mutations conferring resistance to all the new and repurposed antitubercular chemotherapies have already been reported [63]. The need to not only maintain the current forward momentum in TB drug discovery but also to embrace new technologies to address some of the antitubercular bottlenecks highlighted in this minireview is clearly evident. The need to do more with less is highlighted by the continuing shortfall in research funding, including new drug discovery, which has been at least 50% below WHO targets for the past decade. Embracing advances in complex and data-rich approaches such as chemoinformatics, machine learning, genomics, chemoproteomics, and metabolomics, in tandem with a continually growing understanding of *M. tuberculosis* physiology, is one route to lessening the impact of this funding shortfall.

Capitalizing on the ever-increasing understanding of *M. tuberculosis* essential protein vulnerabilities [51,64], that is, not all essential gene products can be inhibited to the necessary level, and combining these findings with cutting-edge medicinal chemistry is one means by which new TB drugs can be identified. The development of spectinamides and oxazolidinones (linezolid and novel congeners) as antitubercular agents is an excellent example of successful repurposing and highlights why drug repurposing should be continually re-evaluated [65].

Further characterization of the nutrient requirements of *M. tuberculosis* is another potential opportunity for new TB drug discovery. This can and should be considered in tandem with the challenges presented by the *M. tuberculosis* cell envelope. A greater understanding of *M. tuberculosis* nutrient requirements and what small molecules are transported across the cell envelope will begin to provide a set of rules for access into the bacilli. The "eNTRy rules" for Gram-negative pathogens defined by Hergenrother and colleagues highlight how this can be achieved in a systematic manner.

Methodically combining these approaches could enable researchers to better the last decades' productivity and continually expand the arsenal of much-needed anti-TB drugs.

## Conflict of interest statement

Peter D. Craggs is a GSK employee and a visiting scientist at the Francis Crick Institute. Luiz Pedro S. de Carvalho is an employee of the Francis Crick Institute.

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