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Therapeutic potential of promiscuous targets in *Mycobacterium tuberculosis*

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Abstract

In the field of tuberculosis drug development, the term “promiscuous” was coined to collectively describe targets that repeatedly show up in whole-cell screenings. With the current climate leaning towards the exclusion of these targets in future drug screens, this review discusses and clarifies misconceptions surrounding this classification, the prospects of developing compounds targeting promiscuous targets, and their potential impact on tuberculosis drug development. The dominance of these targets in cell-based screens reflect not only bias introduced by experimental setup, but also some of the pathogen’s greatest vulnerabilities. Coupled with favourable predictions of their *in vivo* efficacies and synergism with other TB drugs, these targets open opportunities to be explored for the development of rational drug combination for tuberculosis.

Highlights

- Target promiscuity is merely a reflection of bias in screening assays
- Promiscuous targets represent pathogen’s vulnerabilities both *in vitro* and *in vivo*
- Their inhibitors have therapeutic potential for inclusion in rational drug combinations

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Introduction

Tuberculosis is notoriously difficult to treat due to the complex physiopathology of the disease and the presence of subpopulations of non-replicating, phenotypically drug resistant bacteria that can persist that in the host for decades [1]. As a result, the current regimen recommended by WHO requires treatment for a minimum of 6 months with daily antibiotic doses for uncomplicated cases [2]. Coupled with adverse reactions associated with prolonged treatment, patients’ adherence to treatment plan is a major factor attributed to treatment failure, which then contributes to the emergence of drug-resistant TB. The recent increase in incidences of multi-drug resistant (MDR) and extensively-drug resistant (XDR) tuberculosis cases is a cause for global concern as these strains evade conventional therapy. The lack of a steady pipeline of new antitubercular drugs leaves multi-drug resistant infections with an unacceptable prognosis. However, the lacklustre drug pipeline is not entirely attributable to neglect. Following the publication of the complete genome sequence of *M. tuberculosis* in 1998 [3], screening of multiple small-molecule libraries led to the discovery novel drug/target pairs and several clinical-stage drug candidates.

In the race to discover novel antituberculars, two main screening approaches were adopted, namely target-based and whole-cell screening. Target-based screening selects for inhibitors with specific activity against gene products and was widely explored due to the availability of the pathogen’s genomic information as well as experimental methods such as gene replacement or saturation transposon mutagenesis [4]. This approach has been successful for clinically-validated drug targets [5-7], as well as for unconventional approaches to boost the potency of ethionamide [8, 9]. However, those efforts were met with little success for less characterized target spaces due to limited translatable pharmacological outcomes.

On the other hand, whole-cell screening assays, which screens for compounds based on their effect on pathogen's physiological phenotypes such as growth or survival, yielded several promising compounds acting on novel targets. Notably, the two drugs recently approved for clinical use against MDR/XDR TB – bedaquiline and delamanid – were both discovered in whole-cell screenings [10, 11]. Numerous preclinical and clinical-stage drug candidates were also discovered using this approach. Interestingly, several targets have been identified repeatedly in distinct screens [12]. These targets were marked "promiscuous" due to their apparent non-specific vulnerability to inhibition by multiple chemical scaffolds. The term "promiscuous" generally carries a negative connotation, and the therapeutic value of these frequent targets is being rightfully questioned by the TB drug development community. Nevertheless, the central question that ultimately remains is the essentiality of those targets during infection. Since these "promiscuous" targets reflect metabolic pathways that are susceptible to chemical inhibition, at least under screening conditions, they cannot be ignored. This mini review will present and clarify some misconceptions related to the use of the term promiscuous targets, the vulnerability of those targets to chemical inhibition, and how future rational drug combination may benefit from the inclusion of drugs targeting promiscuous targets.

Promiscuous targets side-lined for the discovery of novel TB drugs

In multiple whole-cell screenings carried out in recent years, MmpL3, QcrB, and DprE1 appeared to be highly vulnerable to chemical inhibition by several unrelated chemical series [13]. *MmpL3* codes for a flippase required for the transport of trehalose monomycolate (TMM) across the plasma membrane in *M. tuberculosis* [14]. 1,5-diphenyl pyrrole derivative BM212 was the first compound identified which binds to MmpL3 [15, 16]. Shortly following the discovery, the transporter was also found to be susceptible to inhibition by adamantyl urea compound AU1235 [17], ethylenediamine SQ109 [18], indolcarboxamides [19], Spirocycles [20] and various other chemical classes. While a direct interaction between MmpL3 and inhibitors has been demonstrated [16], it remains to be determined if all MmpL3 inhibitors act through a direct inhibition of MmpL3, or indirectly by dissipating charge partition across the plasma membrane. Nevertheless, MmpL3 remains an attractive drug target for tuberculosis and other pathogenic mycobacteria [21, 22]. *QcrB* encodes for the cytochrome b subunit of the cytochrome bc_1, which is part of a supercomplex terminal oxidase in the electron transport chain inhibited by the clinical-stage drug candidate Q203 [23]. QcrB is also the binding target of a series of imidazopyridine amide compounds [24, 25-27], 2-(quinolin-4-yloxy)acetamides [28], and phenoxy alkyl benzimidazoles [29]. Additionally, drugs such as Zolpidem and Lansoprazole were also found to target the mycobacterial QcrB [30, 31]. DprE1 is part of the DprE1-DprE2 complex, which catalyses the epimerisation of decaprenyl-phospho-ribose (DPR) to decaprenyl-phospho-arabinose (DPA) [32], a key precursor involved in arabinan biosynthesis. Nitrobenzothiazinones belong to a class of compounds which were found to inhibit DprE1 [33]. They were first identified in 2009, subsequently a variety of classes of compounds such as pyrazolopyridones [34] and carboxyquinoxalines [35] quickly emerged. Due to their susceptibility to inhibition by diverse chemical classes, these targets were collectively described as "promiscuous".

The striking commonality amongst these promiscuous targets is their location within the mycobacterial cell envelope. This elevated frequency of identifying targets localised at the membrane showcases the inherent biases introduced by the experimental setup. In screening assays, biases can stem from the culturing and testing conditions as well as the phenotypic characteristics used to differentiate potential hits from the pool. In this instance, assuming positive binding to their targets, compounds with easily accessible targets or good cell membrane permeability would possibly be enriched, while compounds with inaccessible

targets may be underrepresented due to the intrinsic impermeability of the mycobacterial cell envelope. Another possible bias that may explain the enrichment for compounds targeting oxidative phosphorylation and certain targets involved in cell-wall synthesis is the incubation time. For practical reasons, the incubation time of small-molecules with the mycobacteria rarely exceeds 3 days in a cell-based campaign [36, 37], which is short relative to the generation time of slow-growing mycobacteria. Coupled with the frequent use of intracellular ATP quantification as a surrogate marker for growth, small-molecules triggering a rapid depletion in ATP level are involuntarily enriched during those screening campaigns. This is the case for QcrB inhibitors as they trigger a rapid ATP depletion under normoxic growth conditions [23, 38]. It is interesting to note that most MmpL3 inhibitors can collapse the transmembrane proton concentration gradient (ΔpH), and/or the membrane potential (Δψ), leading to a reduction in bacterial intracellular ATP levels [39]. Interestingly, DprE1 inhibitors were not frequently identified during large scale phenotypic screens using ATP as read-out, but other unidentified biases may be responsible for the apparent high vulnerability to chemical inhibition of this target. Because these targets often dominate the list of hit compounds, some attempts to systematically exclude them from hit lists were initiated to identify the otherwise buried hits targeting novel drug targets. Examples include the use of bioreporters to exclude agents targeting cell-wall synthesis [40] and strains hyper-susceptible to QcrB inhibitors [26].

The main motivation behind exclusion of “promiscuous targets” in new screens is to focus on otherwise neglected target spaces overcrowded by the presence of inhibitors of promiscuous targets [40]. Identifying novel target spaces offers a few key advantages: it ensures that the new hits do not fall under a rapidly expanding/saturating category of “me-too” drugs with same binding targets, and they greatly reduce the risk of cross-resistance to drugs of similar MOAs. However, as mentioned above, the hits yielded from any screening assay are pre-filtered by the inherent biasness of the assay design. In a phenotypic cell-based screening it is apparent that oxidative phosphorylation and cell wall biosynthesis are the most essential for *in vitro* growth. Hence if the testing conditions of drug screens remain unchanged, the likelihood of discovery of novel target space by merely excluding promiscuous targets is questionable.

Adapting screening assays to simulate real infections could be useful to find novel targets. For instance, phenotypically drug resistant subpopulation of persisters is the Achilles’ heel of current treatment and remains a gap to be filled. The study of mycobacterium biology is still ongoing, but factors such as low oxygen tension, nutrient starvation, and acidic conditions have been shown to drive mycobacteria into dormancy [41]. By simulating the persisters’ *in vivo* microenvironment, targets essential for the persister phenotype can then be significantly upregulated, thus increasing the representation of compounds targeting this particular subgroup. For instance, the development of multi-stress model for high throughput screening aims to mimic multiple physiological conditions in the microenvironment surrounding non-replicating mycobacteria such that the compounds’ *in vivo* efficacy can be better predicted in the screening process [42]. Various teams have successfully discovered compounds with novel targets by making various modifications to the screening assay: screening in non-replicating mycobacteria [43]; using sensitized strains by method of gene knockdown [44]; and alternative pathway screening in whole-cells [45, 46]. Targeted screens using mycobacterial inverted membrane vesicles led to the discovery of novel inhibitors targeting oxidative phosphorylation [47, 48]. Despite these studies showcasing success in this approach, modifying the screening assay should be approached with careful consideration because of the implicit risk of introducing unpredicted biases. Validation of the screening protocol is hence always necessary prior to launching of actual screening to maximise the probability of success in translating *in vitro* potency into *in vivo* efficacy.

A place for promiscuous targets in the current drug discovery landscape

What then, would the role of these promiscuous targets be in the current drug development landscape? The dominating presence of aforementioned promiscuous targets reflects the essential role of oxidative phosphorylation, especially cytochrome bc_1:aa_3, and cell wall biosynthesis in mycobacterial growth *in vitro*. The apparent promiscuity of DprE1, QcrB, and MmpL3 informs us of the pathogen's greatest metabolic vulnerabilities. Furthermore, the potency of their inhibitors in infection models reflect the essentiality of these targets during infection [18, 23, 24, 31, 33]. Several inhibitors of these promiscuous targets have shown promise for further development. For instance, Q203 (QcrB inhibitor) and PBTZ169 (DprE1 inhibitor) are currently in clinical development [49, 50], whereas promising preclinical drug candidates targeting MmpL3 are under development [51]. Promiscuous targets have therefore the capacity to be developed clinically. Given the high attrition rate in drug development, having an expendable pool of small-molecules targeting promiscuous targets would be helpful in the development of the most ideal medicines. With the end goal of treating and eventually eradicating TB in mind, to disregard these promiscuous targets in future drug screens would be highly counter-intuitive.

While concerns have been raised about further developing drugs targeting cell-wall biosynthesis, eliminating drugs targeting this target space is not necessarily desirable since attractive druggable targets remain to be explored. Even though they have limited effect on the non-replicating subpopulation, approved cell-wall biosynthesis inhibitors such as isoniazid are bactericidal and rapidly reduce bacterial load in open lesions. This fast-acting characteristic of cell-wall synthesis inhibitors is crucial in improving patient's condition rapidly and reducing transmission. PA-824 and delamanid inhibit both aerobic respiration and cell wall biosynthesis and they are currently either in clinical development or approved for clinical use [52, 53]. Additionally, out of the 17 compounds listed as anti-TB medicines in the WHO model list of essential medicines [54], five of these drugs target cell wall biosynthesis. The dominating presence of this antibiotic class in current and future TB drugs highlights the vulnerability of replicating mycobacteria, and further exploration of inhibitors targeting this weak point of mycobacteria is still very much relevant.

Effective rational drug combinations: the future of tuberculosis medicine

Following the approval of bedaquiline and delamanid for clinical use in the recent years, the benefit of introducing new drugs to the current regimen for MDR and XDR TB is becoming clearer. Despite the novelty of their mechanisms of action, incidences of resistance to bedaquiline and delamanid rapidly surfaced only 3 years after approval for clinical use for MDR and XDR TB [55, 56]. It is thus clear that while developing novel drugs is crucial in controlling the spread of MDR and XDR tuberculosis, it is inadequate as a standalone approach to fully address the problem. It is indeed a drug combination – ideally comprising of several rationally-designed agents – that will contribute to solve the problem of drug resistance. Not only can a well-designed drug combination suppress the emergence of resistant strains by combining several drugs targeting specific populations of the pathogen, a rational combination therapy has potential in shortening treatment time. Identification of favourable drug combinations from the small pool of advanced drug candidates has some benefit, but reliance on serendipity is simply too unpredictable to be depended on as a long-term solution. By conscientiously planning drug combination as part of the development process in the early stages of drug discovery, future combinations are more likely to work synergistically, therefore improving the overall sustainability of these novel treatment regimens. Therefore, when developing a new drug against tuberculosis, novelty of its MOA is ideal, but the most crucial consideration should be how the compound acts when administered with other drugs. In dealing with a metabolically plastic pathogen like *M. tuberculosis*, a magic bullet of a cure is unlikely to come by, thus emphasis should be placed on developing drugs that can act in

combination with other drugs. In this context, promiscuous targets have shown promise to be used as part of a combination therapy. BTZ043, an agent targeting DprE1, interact positively with various antitubercular drugs such as rifampicin, isoniazid, moxifloxacin, bedaquiline and pretomanid [57]. MmpL3 inhibitors from the chemotypes of indolcarboxamides and adamantyl ureas demonstrated synergistic action with rifampin, bedaquiline, clofazimine, and beta-lactams [58]. These studies reinforce the notion that inhibitors of these assumed promiscuous targets are not only relevant, they also hold potential to be incorporated in a rational drug combination to treat MDR and XDR tuberculosis. Arguments have been raised against further development of agents targeting QcrB due to the target's undesirable biological properties. Indeed, QcrB inhibitors have been demonstrated to be inactive against some clinical strains [26], to be bacteriostatic, as well as ineffective against non-replicating mycobacteria [59]. However, the recent understanding of the reason behind Q203's lack of bactericidal potency led to the discovery of a synthetic lethal interaction between cytochrome bc_1:aa_3 and the cytochrome *bd* oxidase [59]. Furthermore, the combination of QcrB inhibitors with other agents targeting the mycobacterial electron transport chain enhanced the killing efficacy in *M. tuberculosis* [60], therefore opening avenues for the development of a rational drug combination targeting oxidative phosphorylation.

These promiscuous targets have consistently promised favourable or even synergistic activities with traditional anti-TB drugs as well as potential drug candidates, thus it is only logical to continue the search for the most ideal inhibitors of these targets with desirable safety and pharmacokinetic profiles compatible with human use.

Conclusion

The word "promiscuous" is often used in a pejorative sense and casts a negative light on these identified targets, making them appear redundant and unoriginal. However, the fact remains that these targets' promiscuity reflects the biggest vulnerabilities in *Mycobacterium tuberculosis* growth and survival. Coupled with the fact that inhibitors of these targets are efficacious *in vivo*, act synergistically with multiple anti-TB drugs, and are coupled with a favourable toxicological profile, they present opportunities waiting to be harnessed for the development of innovative, rational drug combinations for tuberculosis.

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Combination of several drugs targeting different components of the oxidative phosphorylation pathway is synergistic.