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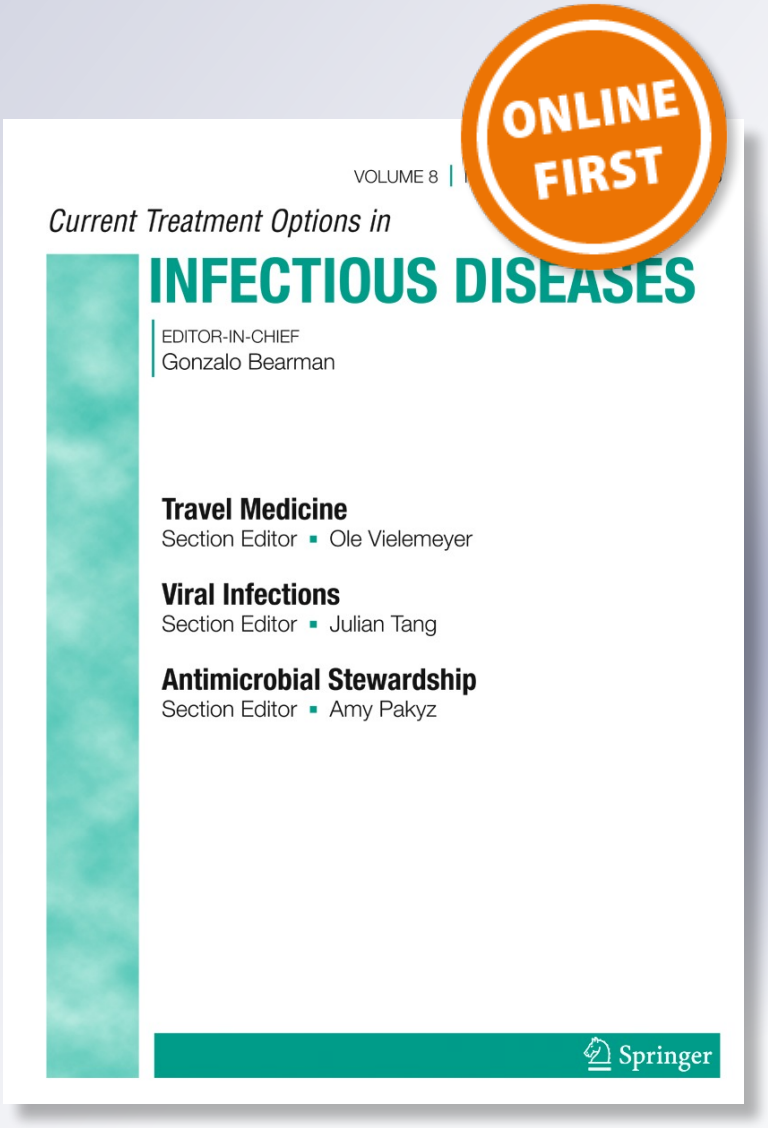

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Mycobacterial Infections (H Bach, Section Editor)

New Era of TB Drug Discovery and Its Impact on Disease Management

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Opinion statement

Tuberculosis (TB) is a devastating infectious disease that continues to plague the world, despite improved hygiene, massive vaccination efforts and an arsenal of chemotherapeutic agents. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, is a slow-growing bacterium that naturally resists most currently known antibiotics. Emergences of ever-increasing drug-resistant Mtb strains threaten our ability to control the disease. Unfortunately, lethargic drug development efforts led to the approval of only one new TB drug in the last 50 years by the US Food and Drug Administration. This dismal progress warrants a re-evaluation of approaches and methods for new TB drug discovery. Although successful in the past, the continuous use of in vitro drug discovery methods eroded recent attempts towards TB drug discovery, caused by a pathogen that inhabits human cells. Advances in recent years include the development of new intracellular screening protocols using relevant disease models. Pilot studies have yielded new lead compounds filling the pipeline for further development. Furthermore, these studies have revealed new insights to forecast changes in diagnostics and chemotherapies against this notorious infectious agent.

Introduction

The burden of TB

Tuberculosis is the world's deadliest infectious disease, on par with acquired immune deficiency syndrome (AIDS). World Health Organization (WHO) estimated that there were 9.6 million new cases of tuberculosis (TB) worldwide in

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2014, 480,000 of which suffered from multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) [1]. Despite successful efforts in reducing TB mortality rates (reduction of 47 % from 1990 to 2014 [1]), TB still claimed 1.2 million lives in 2014, 0.4 million of which were also human immunodeficiency virus (HIV) positive [1]. Contrary to common beliefs, MDR-TB and XDR-TB mortality rate has been stabilized at 5 % of all TB cases [1].

Pathogenesis of Mtb

Mycobacterium tuberculosis (Mtb) is an obligatory human pathogen which manifests primarily as lung disease, although it can spread to other organs such as the spine and joints in the case of milliary TB. Patient-to-patient transmission occurs when aerosols containing live Mtb reach the alveoli of the lungs. Resident alveolar macrophages, which act as the first defence against invading organisms, are able to engulf Mtb but fail to completely eradicate it. Instead, Mtb takes advantage of these macrophages and uses them to gain entry to the lungs. In order to do so, Mtb secretes various effectors into the macrophage to interfere with the killing mechanisms, enabling it to survive and replicate inside the phagosomes of these cells [2].

Fortunately, only 5 % of Mtb-infected individuals develop active disease, typically characterized by coughing, bloody sputum, fever, night sweats and weight loss [3]. Roughly 90 % of infected individuals develop latent TB infections (LTBI), which exhibits no clinical symptoms and are not infectious [3]. LTBI patients are usually tuberculin skin test positive, which is indicative of initial exposure, while some exhibit visible granuloma on x-rays. The granuloma is a spherical structure formed by infected and uninfected macrophages, foamy macrophages, neutrophils, dendritic cells, natural killer cells, B and T cells and fibroblasts. It is developed in an attempt to control the spread of Mtb [4]. The infection may be contained this way for decades and never actually cause TB, yet it serves as the focal point of infection once the immune system loses its control over it.

Current treatments

Currently, there are 14 available drugs for the treatment for TB patients, divided into first- and second-line drugs [5]. First-line drugs consist of isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA) and the less favourable streptomycin (SM) [5]. Second-line drugs are given in cases of resistant strains or because of toxicity and tolerability issues. These include the injectables (amikacin, capreomycin and kanamycin), fluoroquinolones (moxifloxacin, levofloxacin and ofloxacin), carbothionamides (ethionamide and prothionamide) and D-alanine analogs (cycloserine and para-aminosalicylic acid) [5].

The preferred treatment for drug-sensitive TB is a combination of RIF, INH, PZA and EMB daily for 8 weeks followed by RIF and INH daily for 18 weeks [6]. Regimen for drug-resistant TB is determined for individual patients based on the susceptibility of the Mtb strain causing the disease. The treatment length for MDR-TB is typically about 2 years and for XDR-TB is almost 3 years [7]. In the case of LTBI, a single-drug regimen of INH daily for 9 months is typically used [6].

Current challenges of TB therapeutics

TB treatments are lengthy and complicated compared to most other infections. The mandatory use of combination therapies relying on multiple antibiotics associated with prolonged treatment courses leads to excessive side effects and lack of adherence to completion of therapy. This issue is greatly amplified in patients with MDR- and XDR-TB, since the most powerful drugs with relatively lower toxicity, such as INH and RIF, are not effective against drug-resistant TB. Instead, these patients rely on more toxic, second-line TB drugs. The total cost to society per patient is 15 times higher for MDR-TB and as much as 30 times higher for XDR-TB [7]. The one and only new drug approved by FDA in the past 40 years is bedaquiline [8]. However, the toxic side effects are so severe that its use is limited as a last resort anti-TB treatment for XDR-TB or for some high-risk cases [8]. Advancement in TB therapy has been rather disappointing. It is clear that the world can benefit from novel TB therapies that are more potent and less toxic. To do so, we must develop more effective methods to identify novel drugs.

The need for disease-relevant high-throughput drug discovery

The 1940s to 1960s is considered the prime time (see Fig. 1) for TB drug discovery, as illustrated by the fact that most of the current TB drugs were discovered in this period [9]. All of these compounds were likely identified by examining their ability to inhibit Mtb growth in artificial culturing media; this method is known as phenotypic screen. Their mode of actions (MOAs) were largely mysteries until sophisticated molecular biology techniques became available. RIF, the last TB drug discovered in that period, marked the start of a 50-year drought in new TB drug discovery [11].

Since completion of the Mtb genome sequencing effort in 1998 [12], TB drug discovery methods have been divided into two main groups: (1) the bioinformatics/target-based approach and (2) the "old school" phenotypic screening approach. Enthusiasm towards the information contained in the Mtb genome database supported more rational target-based approaches to drug discovery. This approach was widely adopted in academic laboratories, where rational design appeals to both researchers and funding agencies. As such, target-based drug discovery is a natural extension of scientists' curiosity to understand biology and to develop compounds that target essential processes. Phenotypic-based approaches rely on screening processes where millions of compounds may be tested for their ability to kill Mtb. In this approach, the MOAs, i.e., the target of each hit compound, are determined afterward, if at all. Phenotypic-based approaches are typically performed by pharmaceutical companies that have large chemical libraries and automation at their disposal. A study by Payne et al. evaluated GlaxoSmithKline's experience on target-based drug discovery. Based on a collection of 300 targeted gene studies, the authors found the target-based approach to be ineffective and thus unsustainable [13]. Their experience suggested that it is easier to find a cellular target of a compound than it is to chemically engineer an antibiotic from an enzyme inhibitor; thus, phenotypic-based methods are more effective for antibacterial discovery.

In vitro screening methods have proven to be very effective for most infectious diseases because the target organisms cause topical infections or bacteraemia where the infection is spread to organs through circulating fluids.

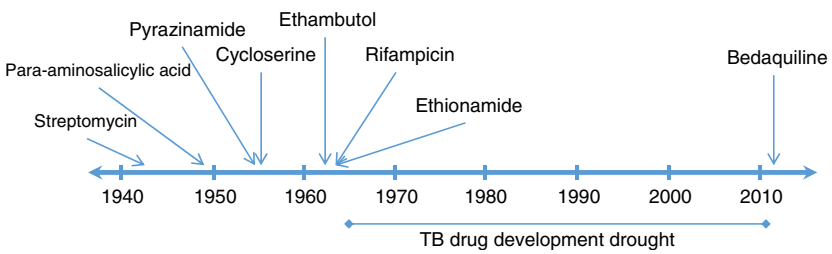

Fig. 1. Timeline for new TB drug development. Majority of TB-specific drugs were developed between 1940 and 1963 [9, 10], with an exception of bedaquiline, which was approved at the end of 2012 [8]. From 1963 to 2012 represents a near five-decade-long drought in novel TB drug discovery.

For this reason, bioavailability is less of an issue. Meanwhile, Mtb is an intracellular pathogen that resides inside the phagosomes of macrophages or in calcified granulomas. As such, TB drugs may not effectively penetrate the barriers to reach Mtb or may be inactivated by the host cell intracellular enzymes. In vitro drug discovery methods ignore the degree of complexity in pathogenesis of TB. Indeed, in recent years, the focus of TB drug development has shifted into more disease-relevant, intracellular TB drug screening methods.

Mtb residing inside macrophage phagosomes may present some challenges to traditional drug discovery processes, yet, at the same time, it provides us with alternative and exciting ways to target TB. Mtb extensively exploits the host cells by secreting various factors to interfere with host cell processes [2, 14]. Processes targeted by Mtb include antigen recognition; phagosome maturation arrest; and inhibition of apoptosis, autophagy, inflammatory response and antigen presentation [2, 14]. All of these processes are important for the macrophage and the rest of the immune system to mount effective defences against invading organisms. Compounds that target these host cell processes and Mtb-host interactions, exemplified in Fig. 2, may be desirable therapeutic agents and could advance the field towards host-directed therapy for TB [15–18].

The host may also positively modulate the efficacy of compounds that target Mtb. This is accomplished by either concentrating the active compound or providing the necessary condition for prodrug activation. Drug-concentrating effect is a rare phenomenon; the best characterized occurrence is the accumulation of macrolides, a class of antibiotics, especially in phagocytes [19–21]. Drugs that benefit from host cell concentrating effects may exhibit reduced side

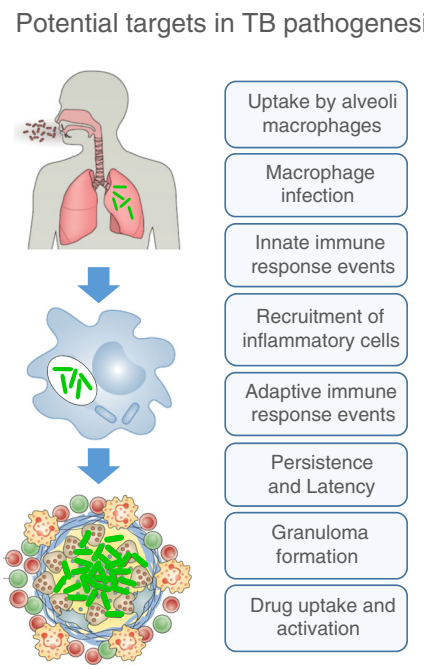

Fig. 2. Potential targets in TB pathogenesis for future drug development. TB pathogenesis is a complicated process that involves various cell types and processes. In addition to targeting Mtb biological processes, new therapies can be developed to better take advantage of Mtb-host interactions, host processes and drug activation/uptake.

effects due to the lower dosage needed to achieve bactericidal concentration [19].

Prodrug activation on the other hand is more common in TB therapy, involving both INH and PZA. These drugs are given to patients as precursors of the active compound and are activated by enzymes that catalyse reactions necessary for normal physiological function in the bacterium. Mutations in the activating enzymes, usually by altering substrate specificity, cause drug resistance. For example, INH is activated by the Mtb catalase-peroxidase KatG [22–24]. Activated INH inhibits the enzyme enoyl-[acyl-carrier-protein] reductase (InhA), which is essential for Mtb cell wall formation [25], and resistance is mainly caused by mutations in *katG* [22–24]. Therefore, prodrug activation by host cell processes would provide ways to reduce chances of resistance mutation in the activating enzyme due to the lack of selective pressure.

Recent Mtb physiology studies have identified key proteins and metabolic pathways that are essential for intracellular survival of Mtb and yet completely dispensable for in vitro growth in typical culturing media. Proteins such as PtpA [26•, 27•, 28•] and SapM [29•, 30•, 31] are secreted by Mtb to interfere with host cell signalling to create a less hostile environment. Metabolic pathways such as cholesterol degradation [32] and glutamine-glutamate metabolism controlled by PknG and GarA [33, 34] ensure proper nutrition to maintain homeostasis. These are some prime examples of Mtb processes that are suitable as drug targets and yet difficult to screen for using in vitro methods.

To maximize our effort in TB drug discovery, we should target Mtb pathogenesis in disease-relevant models of infection. As animal studies are cost-ineffective and impractical for large screening efforts, the development of intracellular high-throughput screening methods to evaluate compound efficacy is more practical and promising. Following the above, we believe that a phenotypic screening, rather than a target-based screening, in a more disease-relevant model holds more promising antimycobacterial discovery potential.

Development of intracellular high-throughput assays

General approach to intracellular high-throughput screening

Current high-throughput intracellular screening methods can be categorized into two groups: (1) high-content screening and (2) raw intensity-based screening, both following a similar theme. Monocytic cell lines are differentiated into macrophage-like cells (hereafter referred to as macrophages for simplicity), followed by infection with Mtb. The infected macrophages are then subjected to treatment with compounds of interest for chosen duration. Finally, the endpoint assay determines Mtb viability at the end of the treatment period. Some variations on the theme include choice of host cells, Mtb reporter systems and detection methods.

Typical host cell choices include human monocytic leukaemia cell line THP-1, peripheral blood monocytic cells (PBMCs) and an assortment of murine macrophages. PBMCs can vary from donor to donor and are also difficult to obtain in large quantities, thus unsuitable for high-throughput screens. Murine macrophages are not best suited for studying human-specific disease, as illustrated by evidence such as the secreted phosphatase PtpA being essential for survival in human macrophages [26•] but not in mice [35]. Therefore, the human-derived THP-1 is currently the best model that is uniform, disease

relevant and suitable for high-throughput screens.

Mtb is one of the three slow-growing pathogenic species of the genus *Mycobacterium* known so far. The minimum doubling time of Mtb inside the host cell is about 24 h [36], and it is significantly longer under suboptimal in vitro conditions. Rapid detection of live Mtb was a hurdle for high-throughput method development. Prior to development of efficient reporter systems, Mtb detection required plating of the bacteria, long incubation time and exhaustive colony counting. Current high-throughput methods mainly utilize reporting systems based on either fluorescence [37••, 38, 39, 40••, 41, 42•] or luminescence [37••] monitoring of intracellular infection. Both reporter systems allow full automation in 96- and 384-well formats [37••, 41, 42•], driving down the time required to take the final measurement to be as low as 0.1 s per well [37••]. With implementation of these improvements, it is now feasible to screen large libraries containing millions of compounds.

Imaging-based high-content screening methods

Intracellular high-throughput screening methods can benefit significantly from the development of fully automated fluorescent microscopes. Currently, there are over a dozen offerings from various companies [43]. Each system is a self-contained integrative unit combining both data acquisition and analysis. Using multiple fluorophores, the system is capable of monitoring Mtb viability and localization, macrophage cytotoxicity and phagosome-lysosome fusion from every well [43, 44]. The amount of data generated from each experiment is unmatched by other screening approaches.

High-content screening devices and associated computer hardware are expensive, and in addition, they require dedicated space outside of the BCL-3 laboratory used for Mtb research. The financial and space requirements make high-content screening far from affordable for most research facilities.

Raw intensity-based high-throughput screening methods

Raw intensity-based screening method is an interesting and lower-cost alternative to the image-based high-content screening method. This method can utilize either fluorescence or luminescence. Luminescence is a chemical energy-based light emission reaction. A strain of Mtb that overexpresses firefly luciferase is used to generate light, and the assay is commonly referred to as a luciferase assay [37••]. Signal detection is performed with a luminometer, a simpler and therefore inexpensive machine. The lower initial equipment investment makes luminescence-based methods readily accessible to most research facilities. The greatest benefit of the luciferase assay is its speed and simplicity. No additional sample manipulation and incubation are required prior to performing the final assay. Therefore, data can be collected in as little as 5 min following the compound treatment period. This extra efficiency over imaging-based methods is highly beneficial in screening campaigns involving large compound libraries.

A simpler assay also means that the acquired data would be one dimensional. In this case, the assay simply allows determination of live Mtb in each well. However, it is insufficient to accurately assess the quality of compounds since no data regarding macrophage cytotoxicity

can be obtained. Therefore, raw intensity-based assay must be supplemented with a cytotoxicity assay.

Recent high-throughput screens

Dr. Priscille Brodin's lab at the Institut Pasteur (France) was the earliest adopter of high-content screening for TB drug discovery. One hundred thirty-five active compounds were identified in a screen of a library containing 57,000 small molecules [40••]. Several compounds containing benzamide scaffold were able to inhibit intracellular Mtb at sub-micromolar concentrations. The intracellular and extracellular dose-response of this class of compounds suggested that they target a cellular process in Mtb, and compounds that were identified as positive hits in the screen were able to effectively penetrate all membrane barriers. A MOA study revealed that these benzamides inhibit synthesis of certain Mtb cell wall components. The identification of this particular class compound does not showcase the strengths of intracellular screening as these compounds would also be identified by in vitro methods. Nevertheless, this study was the first to demonstrate that high-throughput intracellular screening is possible for TB drug discovery. A follow-up screen by Pethe et al., using the same approach, identified a compound (Q203) which has an unusually low half-maximal inhibitory concentration (IC_{50}) of 0.3 nM against intracellular Mtb [45••]. In contrast, the in vitro IC_{50} was found to be 10-fold higher at 3 nM [46]. The MOA of Q203 is via disruption of ATP generation through inhibition of cytochrome b [45••]. Resistance to Q203 has been mapped to mutations in *cytochrome b* [45••]. Compound Q203 has progressed to phase I clinical trial as of August 2015 [47].

Recently, in a collaboration with GlaxoSmithKline (GSK), we performed a side-by-side comparison of luminescence-based and high-content screening methods [37••]. Two intracellular screens of a set of 158 compounds that are known to have in vitro anti-Mtb activities were performed. Both luminescence and high-content assays are found to be similar in their ability to assess intracellular efficacies of candidate compounds [37••]. As expected, nearly two thirds of the test compounds exhibited lower activity in the intracellular assays. In contrast, more than one third of the test compounds exhibited higher anti-Mtb activity inside differentiated THP-1 cells, some as much as 20-fold more potent [37••]. These results clearly illustrate the widespread problem of decreased efficacy when in vitro hit compounds are tested in an infection model. At the same time, these results also showcase the potential increase in efficacy that one may observe when the host cell is capable of enhancing inhibitory activities. This is the first evidence of its kind to justify advocating for large-scale high-throughput drug screens using intracellular Mtb model.

Targeting host immunological pathways can also be beneficial and as such adopted for high-throughput screening approaches. For example, we examined the effect of nitazoxanide (NTZ), an autophagy-stimulating compound, on intracellular Mtb. Autophagy is a homeotic cellular process mainly for recycling cellular components. It has also been shown to be an alternative process to eliminate intracellular pathogens [48, 49]. NTZ is normally used to treat intestinal parasites. Nevertheless, both NTZ, a prodrug, and its active compound tizoxanide (TIZ) were found to significantly stimulate autophagy [50••]. NTZ, TIZ and several analogs were tested in our luciferase-based screen for their ability to inhibit intracellular Mtb. NTZ (10 μM) almost completely eliminated

all Mtb inside differentiated THP-1 cells, where the same concentration showed little effect on in vitro Mtb survival [50••]. More interestingly, when NTZ activity was tested on Mtb-infected PBMCs, two significant differences from Mtb-infected THP-1 cells emerged. Firstly, NTZ appeared to be significantly less toxic to PBMCs [50••], which may be attributed by the fact that THP-1 cell line is a cancerous cell line. Since significant biological differences do exist between primary cells like PBMCs and cell lines, we expected that there would be cases where some compounds behaved differently. The second observation is also caused by the same fundamental differences between the two cell types. Since all THP-1 cells are genetically identical, we observe consistent results throughout experiment and between experiments. However, PBMCs are purified from multiple donors, and thus, we would expect genetic variations leading to variable compound behaviour. In this case, we observed difference in dose-response with PBMCs from one of the donors [50••]. Both of these observed factors can have significant impact on screening outcome as well as on future diagnostic/treatment design.

Stimulating autophagy in macrophages is an example of host signal manipulation. We have explored this phenomenon in another collaborative project to examine how mammalian kinase inhibitors affected intracellular Mtb survival. The major benefit of screening known host signal transduction manipulator is the existing medicinal chemistry knowledge that could speed up drug development. Out of nearly 800 compounds tested, none was able to achieve 90 % inhibitory concentration (IC_{90}) below 1 μM while maintaining 80 % macrophage survival threshold (unpublished data). However, many compounds from distinct chemical clusters exhibited moderate ability to inhibit intracellular Mtb with IC_{50} at or below 10 μM (unpublished data) while unable to inhibit Mtb in vitro at up to 30 μM. These data suggest that macrophage killing of Mtb can be boosted through manipulation of host signalling pathways.

Stanley et al. were also engaged in identifying host-directed therapies. They tested a library of small molecules that included some host kinase inhibitors using high-content screening monitoring Mtb survival in murine macrophages. A total of 133 unique hit compounds were identified targeting G-protein-coupled receptors, ion channels, kinases, membrane transporters, inflammatory agents, etc. [51••]. Some of the identified pathways have not been previously reported in relation to TB and thus enhance our knowledge of host processes during Mtb infection [51••]. Furthermore, this study demonstrates the abundance of pathways that can be targeted by future host-directed therapies.

In contrast to host-directed therapy, some groups are working towards drugs that specifically target intracellular Mtb metabolism. It is well documented that Mtb utilizes host cholesterol as carbon source [32]; given that mammalian cells cannot degrade cholesterol, it seems like an ideal target for future TB therapy. A recent high-throughput screen study utilized a fluorescence-based assay monitoring compound effect on infected murine macrophages [52••]. The authors found that a large proportion of compounds that inhibited intracellular growth of Mtb also inhibited Mtb in vitro growth on media containing cholesterol as the sole carbon source, and no inhibiting activities were observed when Mtb was grown in vitro on rich media [52••]. Given the slow growth rate of Mtb on cholesterol as the sole carbon source, it is also possible that this phenomenon can be attributed to metabolic intermediate toxicity or the need for cholesterol for macrophage infection [53]. Nonetheless, induction of the cholesterol

degradation pathway has fundamentally altered Mtb metabolism and perhaps revealed vulnerabilities that are not present in typical in vitro culturing conditions.

Alternative methods based on LTBI models

Latent TB is a neglected aspect of the disease in terms of TB drug development, due to the lack of an efficient or relevant model to study this disease state. Historically, high-throughput assays were found to be useful at identifying compounds that target actively replicating Mtb; however, alternative methods are needed to assess their efficacy on latent persister bacilli. Persisters are Mtb bacteria that exist in a nonreplicating persistence (NRP) state, also known as dormant bacteria. Persisters exist inside granuloma of patients with LTBI; therefore, they are in a different metabolic state that may increase their tolerance to TB drugs [54]. Furthermore, the granuloma structure reduces drug penetrability and renders standard treatments ineffective. Therefore, a method to assess compound activity against persisters should create an environment that mimics that of the granuloma. Indeed, several efforts to artificially create ex vivo assays that rely on cell structures resembling granulomata have been developed recently [55•, 56•].

Silva-Miranda et al. developed a cell structure using PBMCs and infected them with Mtb. The granuloma-like structure can be formed in as little as 3 days and used thereafter to test compound efficacy. In their pilot study, authors tested several first- and second-line TB drugs in their model and observed significant shift in minimum inhibitory concentration (MIC) [55•]. This mimics the drop in compound concentration observed inside granuloma. Perhaps this method would be useful in identifying compounds capable of penetrating the granuloma. The use of PBMCs allows the authors to generate cell structures that resemble the makeup of naturally occurring granuloma. However, this method relies on healthy blood donors, which heavily restricts cell availability and thus throughput of the assay. Alternatively, Schaaf et al. have recently published a similar protocol that utilizes THP-1 cell line to create granuloma-like structures [56•], even though a cell structure created from a single cell type, such as macrophage-like cells, seems to be an overly simplified granuloma model. The metabolic states of Mtb inside both of these models remain uncertain; they seem adequate at assessing compound penetration into the granuloma and as such can be adapted to high-throughput and high-content assays.

Conclusions

Existing TB drugs, especially INH and RIF, are exceptional at inhibiting actively replicating Mtb. Based on our experience with intracellular screens, it seems that compounds that specifically target intracellular Mtb through host cell processes may not be as efficient at killing replicating Mtb. However, these compounds may be more effective at eliminating persister bacteria due to the fact that killing mechanisms provided by host cells are nonspecific. Host-directed therapy also complements traditional TB drugs since it is less likely, if at all, for Mtb to develop resistance to these new treatments. Consequently, host-directed therapies are able to complement shortcomings of traditional TB drugs.

Current TB drugs inhibit Mtb by directly targeting Mtb cellular processes. Standard WHO treatment regimens are effective for all patients suffering from drug-sensitive TB, while drug-resistant TB requires associated diagnostic tools to determine drug susceptibility and resistance pattern, which dictates the best course of treatment for each individual paving the way for personalized medicine in TB. Future personalized medicine in TB would boost discoveries of new host-directed therapies. Therapies that rely on host cell processes can be affected by genetic variations with patients responding differently to treatment based on their genotype. Therapy effective for one individual may not be for another, and adverse effects may even be observed. Therefore, new diagnostic tests must be developed to determine the set of optimal therapy for each patient. As such, the development of intracellular high-throughput and high-content screening methods marks the beginning of new era of TB drug discovery, diagnostics and treatments.

Compliance with Ethical Standards

Conflict of Interest
Xingji Zheng declares that he has no conflict of interest. Yossef Av-Gay declares that he has no conflict of interest.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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