

Recent Advances in Methodologies for the Discovery of Antimycobacterial Drugs

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Abstract: Mycobacteria, especially *M. tuberculosis*, have remained a worldwide dominant cause for human morbidity (~10 million annual cases) and mortality (3 million deaths annually) since ancient times. An estimated one-third of living humans are latently infected with *M. tuberculosis*. Despite this, there have been no new drugs specifically developed against mycobacteria since the 1960s. Because of a slow growth rate, biosafety concerns, and other issues, drug discovery by *in vitro* screening of natural and synthetic compounds has been limited in the past. However, a number of new techniques and technologies for whole cell antimycobacterial screening have been developed recently. This review examines and compares these methods, discusses common issues in screening with mycobacteria, and considers potential future developments in the field.

Keywords: Mycobacterium, drug discovery, resazurin, alamar blue, nitrate reductase, luciferase, green fluorescent protein

INTRODUCTION

There is a great need for new therapeutics against mycobacteria. *M. tuberculosis* (MTB) kills an estimated 2-3 million people annually, with a global economic toll of ~\$12 billion each year [1]. The rise of multidrug resistance (MDR) in MTB has complicated and extended treatment regimens. The current therapeutic regimen is long, with 6 months considered as "short course" for fully sensitive strains of MTB. Correspondingly, one of the three main goals in the Scientific Blueprint for TB Drug Development [2] is that a new treatment should shorten or simplify the current therapy, which involves four drugs. Many second-line drugs used against MTB were developed against other organisms, and adapted for use with MTB. In fact, no new drugs have been developed specifically against mycobacteria since the 1960s. Current drugs have a number of toxicity problems, such as the marked hepatotoxicity of isoniazid (INH), thus some patients cannot tolerate them. Even worse, therapy for patients with MDR infections often lasts a year or more. The second goal in the Blueprint is to develop a new therapeutic effective against MDR strains. While approximately 8 million active cases of tuberculosis (TB) occur each year, fully one-third of the human population is latently infected (~2 billion), thus >99% of patients have a latent infection, which can reactivate at a later time. Thus, the final major goal of the Blueprint is to improve treatment of latent TB.

In addition to MTB, the environmental mycobacteria (EM) cause infections in humans and agricultural animals [3-5]. Among the EM, species of the *M. avium* complex (MAC) are the most serious cause of disease in humans. MAC is intrinsically resistant to most antibiotics, including even some used against MTB (especially INH), and is thus challenging to treat [6]. Like MTB, a multiple drug combination is required to slow the development of resistance (4 drugs for MTB, 2-3 for MAC). The majority of MAC-infected patients have some predisposing condition, such as cancer chemotherapy, kidney dialysis, advanced age, and most notably AIDS. The continually rising percentage of immunocompromised within the population means more people will be predisposed to EM infections in the future. However, there is also an increasing number of infections in patients without predisposing conditions [7-9]. Clarithromycin is the primary component of anti-MAC therapy [10], and resistant strains are often fatal [6]. HIV-infected patients with a CD4+ count below 50 generally stay on indefinite maintenance therapy for MAC. Current therapies are thus lacking in efficacy, and additionally often interact negatively with anti-HIV

drugs. Finally, while current anti-retroviral therapies have decreased the MAC caseload, the inevitable rise of resistant strains of HIV will lead to a return of MAC in that patient population.

Many new methodologies have been developed recently for drug screening against mycobacteria, yet discovery methods have not been reviewed. There have been a large number of publications on susceptibility testing, and reviews on that subject [11-13], thus clinical susceptibility testing will not be the focus of this review. This review is a discussion of methods utilized for *in vitro* screening of compounds against whole cells as antimycobacterial agents, a comparison of those methods, common issues in screening and suggested solutions, and possible future developments in the field. *In vitro* assays against specific protein targets will not be discussed.

SCREENING METHODS

The dominant initial method for compound screening and susceptibility testing of clinical isolates was growth on Lowenstein-Jensen (LJ) slants or the plate proportion method using Middlebrook 7H11 agar. These methods are inexpensive and fairly reliable, but consume a lot of compound and require 3-4 weeks for results with MTB, 2-3 weeks for MAC. Thus the major driving force for new methodologies is to preserve precious compounds, speed results, and provide a high throughput screen (HTS).

TAACF Methods

Methods utilized by the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF, an NIH-funded initiative, at www.taacf.org) will serve as a starting point for discussions, as they are well accepted, and to exemplify how methods are utilized in combination for drug discovery.

MTB Protocols

The primary screen used is the Microplate Alamar Blue Assay (MABA, see section I) in Middlebrook 7H12 media with compounds at one fixed concentration, 6.25 µg/ml, against *M. tuberculosis* strain H37Rv. This strain is an excellent model for MTB as the genome is sequenced [14] and well annotated (available at <http://genolist.pasteur.fr/TubercuList>), it is genetically transformable, and it retains virulence in animal models. The minimal inhibitory concentration (MIC) of hits (≥90% inhibition in primary screen) is determined, again using MABA. Follow-up on lead compounds involves cell and animal work. First, eukaryotic cytotoxicity (IC₅₀ value) is measured in VERO (African green monkey kidney cell line) cells via the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) dye viability assay. MTS (Promega) is used instead of MTT

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because reduced MTS is water soluble. Secondly, bacterial growth inhibition (EC₉₀ and EC₉₉ values) against MTB strain Erdman in primary murine bone marrow-derived macrophages is determined to confirm compound penetration into phagosomes. The Erdman strain is aggressively virulent in most murine infection models [15]. Finally, MABA is again utilized with a panel of clinical mono-resistant MTB strains to insure no cross-resistance, and bactericidal concentrations are determined by subculturing MABA wells onto 1.5 mL 7H11 agar plugs in 24-well plates.

For confirmation of leads in an animal model, first the maximum tolerated single dose of compound is determined in C57BL/6 mice. Then, for short term treatment, interferon-gamma knockout mice infected with H37Rv (infection is established 18d before treatment begins) are treated for nine days, with compound results compared to an INH control with respect to lung cfu. A longer term 45-day treatment of aerosol low-dose Erdman-infected wild-type mice is lastly determined. A common shortfall of animal infection models is that if the compound pharmacokinetics are not determined, interpretation of the animal data is uncertain, i.e. did treatment fail because the compound is not orally absorbed or is it too rapidly metabolized and/or excreted? TAACF addresses bioavailability by taking serum samples over time from mice dosed with candidate compounds and determining residual activity by bioassay against MTB using MABA or the visual microbroth method [16].

MAC Protocols

The TAACF protocol with MAC also begins with MABA at the same set concentration, but utilizing five strains of *M. avium*. Multiple strains are needed because *M. avium*, unlike MTB, is highly genetically heterogeneous. Strains can contain up to 6 plasmids, both linear and circular, with a total extrachromosomal content of 1 million bp (personal communication, J. Falkinham, Virginia Tech). MIC of active lead compounds is determined using 30 strains of MAC, including clarithromycin-resistant clinical isolates. For follow-up, activity against 3 MAC strains within U937 cells (human monocyte cell line) is measured, and compounds are examined for synergy with ethambutol (EMB) by co-treatment. Finally, a long term 4-week treatment of IV-infected beige C57BL/J mice is performed, comparing organ burden and survival following treatment. TAACF does not perform screening against EM other than MAC.

Literature Summary

Guidelines

This summary of the literature will focus on each major method currently utilized for compound screening, starting with suggestions for all methods. In general, rapid assay methods are not needed with *M. smegmatis*, as the simple visual microbroth (see section X) only takes 3-4 d for results. *M. smegmatis*, as a non-BSL3 rapid grower, has served as a model organism for MTB. The new diarylquinoline drug candidate R207910 was discovered using *M. smegmatis* [17]. While extremely useful in genetic and biochemical studies, *M. smegmatis* is taxonomically distant (soil-dwelling rapid grower, non-pathogenic) from MTB (obligate pathogen, slow grower, no significant environmental reservoir) and studies have reported differences in susceptibilities between the two species [18-20], potentially related to differences in cell wall structures and efflux systems. Since *M. smegmatis* does not grow in macrophages or animals, it cannot be used for secondary screens. Thus most research screening focuses on MTB. MTB strain H37Ra is a good non-BSL3 surrogate for H37Rv, with the genetic differences mostly well determined [21, 22]. The vaccine strain *M. bovis* BCG can serve that purpose as well.

Because of the long incubation times with mycobacterial culture, desiccation of both solid and liquid media is a major hazard. When screening compounds, volume loss from media by

evaporation also changes compound concentrations. To minimize culture desiccation, use sealed plastic bags around plates, add sterile water to peripheral wells in 96-well microplates, and/or use a humidified incubator. Sealed dry incubators can be humidified by maintaining a flask full of deionized water within. Because mycobacteria are slow growing, contamination is also a major issue. To prevent culture contamination, perform periodic acid fast staining on mycobacterial cultures, and maintain strains on selective LJ slants. Most strains of EM and MTB can survive on LJ slants for months at room temperature, although freezing cultures at -80 C provides genetic stability and is the optimal long-term storage method to prevent strain variation over time. Because of the inherent hydrophobicity of mycobacteria, cellular aggregation and adherence to surfaces are also issues to consider. Adherent and clumped cells are likely more tolerant of antimicrobial compounds, as has been seen with cells in biofilms. Inclusion of a detergent, most commonly Tween-80, is a routine measure to limit aggregation and adherence. Finally, care must be taken to screen cells in exponential growth phase, as stationary phase cells are more stress tolerant (see section on non-replicating bacilli).

To understand how new compounds may complement the current treatment regimen, check lead compounds for synergy/interaction with front line drugs for MTB, and with clarithromycin and EMB for MAC. Always screen with multiple *M. avium* strains because of high genetic heterogeneity in that complex. The genomic strain MAC104 (sequenced but not annotated by The Institute for Genomic Research, www.tigr.org) should be included for reference along with clinical isolates. Rapid growers are also heterogeneous, thus examining multiple clinical isolates is likely a good practice for them as well. *M. fortuitum* is the most commonly isolated clinical rapid grower. Few publications in the literature screen against pathogenic rapid growers.

Current Methods

Redox-Based Methods

I. Microplate Alamar Blue Assay, MABA

MABA is clearly the standard in the field for HTS of compounds against mycobacteria, and is the most widely cited (see Table 1). The primary reference for the method is Collins and Franzblau in 1997 [23]. In that study, MABA was effective with MTB H37Rv, H37Ra and *M. avium* strain ATCC 25291, a relatively virulent isolate [24, 25]. MABA is reliable with clinical isolates of MTB [26] and MAC [27]. MABA has also been applied to *M. kansasii* and *M. malmoense* [28], as well as *M. leprae* [29]. In addition to screening and sensitivity applications, MABA has also been used in patient treatment follow-up [30, 31]. MABA can operate in simple colorimetric mode with visual reading (blue to pink change indicates viability, with the MIC recorded as the lowest compound concentration in wells which remain blue), or a more quantitative spectrophotometric determination (A_{570} minus A_{600}). The most sensitive readout is fluorescence, with excitation at 530 and emission at 590 nm. With both spectrophotometric and fluorometric modes, MIC is recorded as the lowest concentration of compound which reduces signal by $\geq 90\%$ relative to untreated cultures. In addition, MBC can be determined by plating onto 7H11 from replicate wells before dye addition [32]. MABA has been extensively examined using receiver operating characteristic analysis and demonstrated high reliability when performed with specific controls [33, 34]. Based on a redox active dye, MABA is likely to work with all species and strains of mycobacteria. However, different morphotypes of *M. avium* may give variable results with colorimetric MABA [35], but fluorescence was not reported in that study. This could be due to lowered permeability in the rough morphotype, which also exhibited lowered uptake of the dye neutral red and radiolabeled glycerol. However, while MABA in other studies involves an incubation followed by endpoint addition of dye, Kansal *et al.* performed a constant incubation with

Table 1. Major Screening Methods

Primary references	Species examined	Media used	Inoculation	Format	Readout	Time
MABA microplate alamar blue assay, 49 PubMed hits						
26, 27, 28, 33, [@]	Rv, MTB clinical isolates, Ra, Ma, M. leprae, M. kansasii, M. malmoense	7H9GC ± 0.05% Tween 80	Rv 1x10 ⁶ to 5x10 ⁴ Ma 3.25x10 ⁵	200 µl/well 96 well plate	colorimetric or fluorescent	7-10 d
REMA resazurin* microtiter assay, or MRA microdilution resazurin assay, 14 PubMed hits						
37, 38, 39	Rv, MTB clinical isolates	7H9S	1/20 dilution of no.1 McFarland [†] or 1/10 dilution	100 µl/well 96 well plate	colorimetric or fluorescent	8 d
NRA nitrate reductase assay, 16 PubMed hits						
71, 72, 73, 74	Rv, MTB clinical isolates	tubes-7H11 or LJ + 0.1% KNO ₃ microplate-7H9 + 10% ADC + 0.05% Tween80 + 0.1% KNO ₃	tubes-200 µl of a 1/10 dilution of no.1 McFarland microplate-100 µl/well of a 1/50 dilution of no.1 McFarland	slant tubes or 96 well plate	colorimetric	tubes 9-14 d microplate 8 d
MTT, TEMA tetrazolium microplate assay, 21 PubMed hits						
41	Rv	7H9GC	100 µl of a 1/25 dilution of no.1 McFarland	200 µl/well 96 well plate	colorimetric	6-15 d
Microbroth dilution method, no associated keyword for PubMed search						
19, 75	Rv, MTB clinical isolates, Ma, Ms, <i>M. fortuitum</i>	7H9-ADC, ver.2	150 µl of 0.001 OD at 650 nm (~10,000 CFU/well)	96 well plate	visual growth	MTB 21-28 d Ms 3-5 d Ma 7-10 d
Luciferase assays, 10 PubMed hits						
51, 52, 53, 54	Rv, BCG, <i>M. intracellulare</i> , <i>M. aurum</i>	7H9-ADC, ver.1	5x10 ⁵	520 µl/well 96 well plate	luminescence	5 d
GFPMA green fluorescent protein microplate assay, 5 PubMed hits						
48, 49	Rv, Ra, MTB Erdman	7H12G	5x10 ⁵ to 5x10 ⁴	200 µl/well 96 well plate	fluorescence	5-7 d

Rv is MTB H37Rv, Ra is MTB H37Ra, Ma is *M. avium*, Ms is *M. smegmatis*. The number of PubMed hits is articles which utilize to the particular assay in the NCBI database on July 19, 2006. [@]TAACF webpage, <http://www.taacf.org/Process-text.htm#assays> accessed 5/30/06. 7H9GC is Middlebrook 7H9 base with 0.2% glycerol plus 0.1% casitone plus 10% OADC enrichment. * note that resazurin is the active component of Alamar Blue [29]. 7H9S is Middlebrook 7H9 base with 0.5% glycerol plus 0.1% casitone plus 10% OADC enrichment. Version 1 of 7H9-ADC is Middlebrook 7H9 base with 10% ADC enrichment (BBL, Cockysville, MD) plus 0.05% Tween-80. Version 2 of 7H9-ADC is the same as version 1 with 0.1% glycerol added. 7H12G is Middlebrook 7H9 base with 4 µg/ml catalase, 0.5% BSA, 5.6 µg/ml palmitate, 0.1% casitone and 0.2% glycerol. [†]note that suspensions of MTB growth from LJ slants in 7H9 media equivalent to a no.1 McFarland standard have ~3x10⁷ CFU/ml [80].

1% Alamar Blue in media over the entire 8 day period. Since resazurin can be further reduced past pink to non-colored endproducts [36], the results are unclear. In another study with 56 clinical isolates of MAC, MABA and BACTEC 460 susceptibility results agreed by 86% [27], but morphotypes of the isolates were not reported. Thus, the MAC morphotype issue is yet unresolved. As the current conventional method, any new screening methods should be examined in reference to MABA.

II. Resazurin Microtiter Assay, REMA, or Microdilution Resazurin Assay, MRA

Resazurin is the active compound in Alamar Blue [36]. Thus, REMA and MABA are based on the same chemistry. Like MABA, an overnight incubation is required to develop color or fluorescence

change [37-39]. REMA has also be used for MTB susceptibility testing [37-39]. Resazurin (Sigma-Aldrich) is less expensive than the proprietary Alamar Blue (Invitrogen, Biosource Division).

III. Tetrazolium Dyes, Tetrazolium Microplate Assay, TEMA

Tetrazolium dyes are redox active compounds that are reduced by an active electron transport chain to give a colored product, just like resazurin. Dyes used with mycobacteria include MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide), and TTC (2,3,5-triphenyltetrazolium chloride). With MTB, TTC gives too weak of a signal for testing drug susceptibility [40]. As with resazurin, different morphotypes of *M. avium* may give variable results with MTT [35]. In one author's experience

(Primm), no significant difference was observed between multiple strains and morphotypes of MAC species using XTT. The difference may lie in the water solubility of the XTT reduction product as opposed to the insolubility of the MTT colored product, or in differential permeation of cells by MTT as compared to XTT. More investigation is required. TEMA (uses MTT) is reported to be less expensive than MABA [41], but calculations for the costs were not detailed in that study, nor was the less expensive resazurin (as opposed to Alamar Blue) considered in cost determinations. Like MABA/REMA, a 24 hour incubation is required for color development with TEMA. The colored end product of MTT is insoluble, but inclusion of 10% ethanol during the 24 hr development incubation is sufficient to solubilize the color without a cell lysis step. XTT in a liquid format has been developed for susceptibility screening of MTB [42, 43]. XTT requires an electron shuttling agent, such as menadione [44] or phenazine methyl sulfate [40], for reduction by mycobacteria. XTT does work well with MAC in 1.5 ml tubes, although microplate format was not examined [44]. MB-Redox is a commercial (heipha Dr. Muller GmbH, Germany) tube-based system using selective Kirchner media containing a tetrazolium dye for detection and susceptibility testing of mycobacteria [45-47], but has not yet been examined for compound screening purposes.

Reporter Gene-Based Methods

IV. Green Fluorescent Protein Microplate Assay, GFPMA

GFP is a highly utilized reporter with many microbiological applications, including compound screening. A major factor preventing use of GFP-expressing mycobacteria as a screening tool is a low signal-to-noise ratio. For example, a strain of *M. aurum*, as a surrogate for MTB, was engineered to express GFP from the BCG *hsp60* promoter [48]. Drug inhibitory effects were observed, but reduction in CFU and fluorescence levels did not correlate. In another study with *M. smegmatis*, GFP fluorescence declined upon compound exposure, but could not statistically predict MIC, as determined by visual microbroth [19]. However, Changsen *et al.* has overcome this limitation by using a stronger promoter [49]. While the acetamidase promoter is inducible in *M. smegmatis* with acetamide [50], in MTB it is constitutively highly active, and thus a better indicator of viability. GFPMA has the advantages of a microplate format, no reagent addition, fluorescent readout, and it correlates with MABA results in MTB. GFPMA and BACTEC are the only assays with a continuous format compatible with kinetic monitoring of growth/inhibition, all other methods are endpoint. Since results are available continuously, with clear inhibition observable in 5-7 d, GFPMA is a rapid assay, comparable to the luciferase assay (see section V). Continuous assays are also easier to calibrate. As with MABA, MBC can be determined from GFPMA by plating onto 7H11 from replicate wells [32].

V. Luciferase Assays

While several publications describe luciferase-based assays, there is no consensus in the field on the protocols for this method. An assay has been developed with H37Rv, *M. bovis* BCG, and *M. avium* subsp. *intracellulare* expressing firefly (*Photinus pyralis*) luciferase from an integrated vector and driven by the *hsp60* promoter [51, 52], sometimes termed the Bio-Siv assay. After incubation with test compounds in tubes for 3-5d, culture aliquots are examined for luciferase activity. This assay is the most rapid described in the literature suitable for HTS. *M. avium* was not available because of an inability to transform the strains, a common issue with MAC. Transformability of strains is highly variable in MAC. MIC was defined as the lowest compound concentration to give a $\geq 99\%$ reduction in luminescence. This luciferase assay agreed 99% with MABA results of 6 control drugs. Luciferase technology may also lead to screening against the extremely slow growing *M. avium* subsp. *paratuberculosis* [53]. Firefly luciferase, again driven by the *hsp60* promoter, has also been expressed in *M.*

aurum [54]. In addition to whole cell drug screening of the recombinant strain, drug activities were also determined against intracellular bacteria within macrophages (J744A.1 murine cell line), apparently without the requirement of cell lysis. This has promise as a rapid secondary screen to confirm penetration of candidate compounds into the phagosome. Bacterial (*Vibrio harveyi*) luciferase is naturally destabilized in H37Ra [55], although how this may affect compound screening has not been determined.

The same promoter that increased signal strength with a GFP reporter [49] was also applied for the same end with the *Vibrio harveyi* luciferase gene [56] and used to develop a low oxygen recovery assay (LORA). In this case, low oxygen-adapted cultures of H37Rv were prepared in a fermentor in which oxygen depletion could be monitored. Cultures were then exposed for 7-10 days to test compounds in microplate format under anaerobic conditions. Cultures were then assessed for viability by 1) the ability to recover luciferase signal following 28 hours of aerobic incubation and 2) direct aerobic subculture onto 1.5 ml 7H11 agar plugs in 24-well plates. The LORA was used to screen the Genesis Plus library (a 960 compound discovery library based on enzyme inhibitors) from MicroSource Discovery Systems, Inc., which identified mefloquine as having activity equivalent to that found against replicating cultures [57].

VI. Beta-Galactosidase Assays

The beta-galactosidase reporter has been expressed in *M. smegmatis* and *M. aurum* for rapid (<24 hr) colorimetric drug screening [58, 59]. A tube and a microplate format were performed, but a cell permeabilization step is required before substrate addition. Interestingly, *lacZ* expression was not stable in *M. smegmatis*, with deletions and insertions causing gene inactivation [60]. How the stability issue extends to other reporter genes and other mycobacterial species is still unclear. Fluorescent substrates for beta-galactosidase detection in *M. bovis* BCG function well in microplates and with flow cytometry [61], although this has not yet been adapted for compound screening.

Other Methods

VII. BACTEC 460 TB

The BACTEC 460TB (Becton, Dickinson and Company) was the first automated system for growth of MTB. The system detects release of radioactive CO₂ generated by metabolically active MTB in a selective media loaded with ¹⁴C-palmitate. The method is reproducible and readily monitors the kinetics and growth (and inhibition), but is costly, particularly the instrumentation, is not HTS capable, involves radioactivity, and has a large 4 ml media volume, thus requiring a large amount of compound during screening. MABA gives comparable results to BACTEC 460TB [23]. The newer BACTEC MGIT 960 system (see susceptibility testing section XI) is similar, although non-radioactive.

VIII. Nitrate Reductase Assay, NRA

The NRA method detects the production of nitrite from nitrate, and is adapted from the biochemical test used in clinical labs to identify mycobacteria. MTB is positive for this test, while other slow growing mycobacteria, especially *M. bovis*, are negative (except for *M. triviale* and some *M. terrae* strains) [62]. Interestingly, *M. bovis* has much lower nitrate reduction activity not because it lacks the genes required, but because of a promoter mutation in the *narGHJI* operon [63]. In MTB, nitrate transport by NarK2 but not enzymatic reduction is inhibited by oxidizing conditions [64]. Nitrate reduction is a good readout for drug activity because the *narK2* transporter mRNA is upregulated during murine infection [65], and nitrate reductase mRNA is upregulated during the *in vitro* anaerobic Wayne dormancy model [66]. Finally, *narK2* mRNA levels rise during anaerobic culture of *M. bovis* BCG [67]. NRA has not been used with MAC, but *M. kansasii* and the common model organism *M. smegmatis* are positive. This species

specificity of NRA can be an advantage if working with MTB. *M. avium* is reported as nitrate reductase negative, but gives a negative result in the clinical biochemical test because nitrite is also reduced, and thus is not detected [68]. While NRA is colorimetric like MABA/REMA, it does not share a fluorescence mode as well. However, fluorescent probes for nitrite detection have been developed recently [69, 70]. In the solid agar tube version of the NRA, the MIC is defined as less color after reagent addition than a ten-fold dilution of the control, thus $\geq 90\%$ reduction in color [71]. With MTB, NRA and MABA agree more than 98% [72]. The tube NRA is sensitive and specific enough to be used directly after sputum decontamination for susceptibility testing of MTB [71, 73]. The NRA microplate liquid format was developed by the group of G. Qazi [72, 74], and also agrees with MABA MTB results $>90\%$. One difference between the NRA and MABA/REMA is that while both take approximately equal time to complete, development of the endpoint color following reagent addition takes 12-24 hrs with MABA/REMA but minutes with NRA.

IX. Disk Diffusion

The disk diffusion solid agar method is best used for determining the susceptibility of clinical isolates to existing drugs for which the inhibition zone sizes have been correlated to resistance or susceptibility in patients. A recently improved and commercially available version of this would be the E-test strip (AB Biodisk), which estimates MIC value as well. However, since compound diffusion is dependent on solubility and molecular weight, agar diffusion is not generally applicable for drug discovery. It is seriously limited with mycobacteria because during slow growth of the bacteria on a plate, a very soluble compound can diffuse into the entire agar volume instead of creating a gradient surrounding the disk, thus underestimating the compound activity. Also, since the mycobacterial cell wall is lipid-rich and hydrophobic, hydrophobic compounds tend to permeate better and have more efficacy. However, disk diffusion requires uniform compound diffusion into the hydrophilic agar media, and is known to underestimate the antibacterial efficacy of hydrophobic compounds. Thus, disk diffusion is not recommended for compound screening with mycobacteria, only as a tool for determining comparative toxicity of compounds with similar size and solubility.

X. Visual Microbroth, or Broth Microdilution

Observing growth visually by accumulation of cells in the bottom of 96-well microplates [19, 75] is inexpensive, simple, reliable, has no compound interference with readout except precipitation, and requires no instrumentation; but is slow (3-4d for rapid growers, 10d for MAC, 3 weeks for MTB). U-bottom wells are superior to flat-bottom plates for bacterial pellet visualization. MBC is determined by plating onto 7H11 and determining CFU after an additional equivalent incubation time. Practically, this is an excellent method with rapid growers, or involving small compound libraries with slow growers, but HTS of slow growers requires a more rapid method. A modification of the microbroth, called the GIC (Growth Inhibition Concentration) assay [16], involves agitation of the plate and using a plate reader to measure daily absorbance at 600 nm. This not only gave kinetic data, but also sped sensitivity determinations of H37Rv to 7 days.

Susceptibility and Culture Methods, Potentially Applicable to Screening

Several methods reported in the literature for testing the drug susceptibility of mycobacterial strains or for detection of mycobacteria in clinical samples are described here in reference to their potential for conversion to being utilized for compound screening.

XI. Automated Systems

Becton, Dickinson and Company has developed a second generation non-radioactive detection system, the BACTEC MGIT 960. MGIT, or mycobacterial growth indicator tube, is an auto-

mated system which is very similar to the original BACTEC 460 (see section VII above), however an oxygen-based sensor gives a fluorescence signal to indicate mycobacterial growth. MGIT works well for susceptibility testing of clinical isolates of MTB against front-line [76] and second-line [77] drugs, and is comparable to the BACTEC 460 system [78]. For compound screening, MGIT shares many limitations with BACTEC, including large tube volume of 7 ml, non-HTS, and is fairly expensive per sample. There is also a MGIT manual version with the fluorescence being read visually from a UV box so no expensive instrumentation is required. This would however require more user time and be less quantitative.

The BacT/ALERT MB and MP systems from bioMerieux are another non-radioactive automated system for clinical isolation and susceptibility testing of MTB. This system employs a colorimetric sensor for CO₂ production and selective media, and was comparable to the BACTEC 460TB system for susceptibility testing with 166 MTB clinical isolates [79]. BacT/ALERT again shares the same limitations for drug discovery as the BACTEC systems. Thus, while automated systems are useful for rapid clinical susceptibility testing, they are not the best compound screening tools.

XII. Malachite Green

Malachite green dye has been utilized for many years as a selective agent for mycobacteria in Middlebrook and other media. When actively growing, mycobacteria enzymatically decolorize and detoxify the dye to clear products [80]. A tube-based method using decolorization of malachite green has been used to highlight growth of MTB from sputum samples, and was similar to Alamar Blue in tubes, although slightly less sensitive and specific [30, 31]. Whether this could be adapted to a microplate format for compound screening remains to be seen. When added to 7H11 plates at 2.5 $\mu\text{g/ml}$ (10X the typical concentration), significant decolorization of malachite green did not occur until after 3-4 d, thus dye addition did not speed colony detection (Primm, unpublished).

XIII. STC Agar

In contrast to other tetrazoliums such as MTT and XTT, STC (2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride) is stable enough to be added to the test medium prior to inoculation. The traditional agar proportion method for susceptibility has been modified by the addition of STC to the solid agar [81, 82]. Clinical MTB isolates were examined, and STC allowed colony observation in 2 weeks instead of the normal 3 weeks due to the contrast provided by the red colonies [83]. Otherwise, results with frontline drugs were the same. With *M. smegmatis*, resazurin does not indicate colonies well on 7H11 plates (Primm, unpublished). STC agar could be combined with MABA/REMA and other liquid microplate systems above, providing a more rapid MBC determination step by plating on agar media containing STC.

XIV. Flow Cytometry

Flow cytometry has been used for susceptibility testing of clinical isolates of MTB, applying the viability dye SYTO 16 (Invitrogen Corp., formerly Molecular Probes) [84]. Results matched the MGIT system, and were available in 3 days. Negative staining with propidium iodide was used as an indicator of MTB to exclude contamination. Individual bacteria can be encapsulated in agarose microspheres, and then growth detected using flow cytometry, called the Gel Microdrop method [85]. Susceptibility results with clinical MTB isolates correlated 90% with BACTEC 460TB. Gel Microdrop also worked with H37Ra, BCG, and *M. avium*. Since flow cytometry looks at sensitivity at the individual cell level instead of the population level, it delivers more information than other methods. However, the clinical significance of detection of resistance in rare clonal populations is unclear. In addition, proper interpretation of flow cytometric data requires user training and experience.

Assays for Non-Replicating Persistent *M. tuberculosis*

Latent infections which can reactivate upon immune suppression and persistence of (genetically) drug-sensitive *M. tuberculosis* in active disease despite prolonged ongoing therapy are both major issues with tuberculosis, as mentioned in the introduction above. Both phenomenon are believed to involve MTB which are in a state of reduced metabolic activity that involves replicating sporadically, very slowly or not at all. There is debate over exactly how to define dormancy (also termed non-replicating persistence or latency) and how important a clinical role it plays. As the environmental/physiological factors responsible for such phenotypic drug tolerance are not completely understood, it is not surprising that there is no consensus in the literature on how to examine compound activities against non-replicating bacilli (NR, for a recent review see [86]). The most common *in vitro* model systems for NR involve oxygen deprivation, starvation, and stationary phase culture, all of which typically result in tolerance to current drugs. None of the assays described previously are applicable for screening with NR because they are based on growth, which by definition, NR do not exhibit. Two example compounds illustrate the recent history of searching for drugs active against NR. Metronidazole was the first compound noted to kill MTB under anaerobic conditions, but have little activity against exponentially growing aerobic cells [87]. Yet metronidazole demonstrated only marginal efficacy with three studies in murine infection models [88-90]. If novel compounds are isolated in the future which kill NR but not actively growing cells, these may not have clinical utility but could still serve as excellent tools to understand dormancy *in vitro* and in animal models. On the other hand, pyrazinamide has comparatively low activity *in vitro* against growing MTB along with higher killing of stationary phase or anaerobically persisting cells [91, 92]. Pyrazinamide serves as a major front-line drug in human therapy, decreasing the treatment regimen from 9 to 6 months. A wise strategy in the future development of drugs to further shorten therapy may be to select lead compounds which have activity against both growing and NR organisms. Interestingly, the nitroimidazopyran PA-824 (based on the structure of metronidazole), which exhibits killing of both NR and actively growing MTB [93], has excellent activity in mice [94].

While *in vitro* NR models exist, little screening has been reported, including only one HTS system, LORA (see section V). Metronidazole-resistant *M. smegmatis* mutants were isolated using a system where bacteria are exposed to metronidazole while on agar plates in anaerobic jars [95]. Following several days to kill sensitive organisms, the survivors are grown to colonies after oxygen is reintroduced. This system could be used to identify compounds active against anaerobic NR. In another study, nutrient-deprived NR *M. smegmatis* were generated by suspension in 1.5 ml tubes in phosphate buffered saline (Primm and D. Sriram, unpublished). Following 4 days of starvation, cells were exposed to drugs for 24 hours, then plated for CFU determination. The killing concentration of fluoroquinolone derivatives against NR determined by this method did not statistically correlate with either the MIC or MBC against growing *M. smegmatis* (determined by visual microbroth), supporting the concept that bacterial physiology is fundamentally altered between the two states.

CONCLUSIONS

With the TB Structural Genomics Consortium releasing protein structures of potential drug targets [96], bioinformatics and molecular modeling may help generate new leads in the future [97]. Increasing understanding of host-pathogen interactions of mycobacteria may also uncover novel targets for future antimicrobials [98]. Renewed interest has appeared in natural product discovery, including usages as antimycobacterials [99, 100]. Researchers working on discovery of antimycobacterials now have a variety of assays and techniques at their disposal capable of identifying interesting compounds/compound classes. The choice of assay for a

particular study will be influenced by a variety of factors, including the readout desired and the background signal of compounds to be tested, the need for HTS, instrumentation available, and of course costs. It is a reasonable expectation to see a number of new drugs finally reach the market in the near future, after a greater than 45 year gap.

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