



Teaser From crisis to cures – a review on status, knowledge gaps and major obstacles in NTM drug discovery and development, and how to move forward.



NTM drug discovery: status, gaps and the way forward

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Incidence of pulmonary diseases caused by non-tuberculous mycobacteria (NTM), relatives of *Mycobacterium tuberculosis*, is increasing at an alarming rate, surpassing tuberculosis in many countries. Current chemotherapies require long treatment times and the clinical outcomes are often disappointing. There is an urgent medical need to discover and develop new, more-efficacious anti-NTM drugs. In this review, we summarize the current status of NTM drug development, and highlight knowledge gaps and scientific obstacles in NTM drug discovery. We propose strategies to reduce biological uncertainties and to begin to populate a NTM drug pipeline with attractive leads and drug candidates.

Introduction

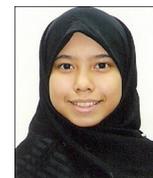
Whereas the incidence of tuberculosis (TB) is decreasing, a new health concern has been raised by non-tuberculous mycobacteria (NTM). Different from their relative: *Mycobacterium tuberculosis* (the etiologic agent of TB), NTM are opportunistic pathogens, causing mostly TB-like pulmonary diseases largely in immunocompromised patients or patients with pre-existing lung conditions, such as cystic fibrosis (CF), bronchiectasis or chronic obstructive pulmonary disease (COPD). The annual prevalence of NTM pulmonary disease (NTM-PD) varies in different regions, ranging from 0.2/100 000 to 9.8/100 000 [1,2] with an overall alarming growth rate [3,4]. The situation is worse among vulnerable populations. Large-scale epidemiological studies from several countries and regions reported a high prevalence of 3.3–22.6% in CF patients [5], whereas COPD patients treated with inhaled corticosteroid therapy are associated with a 29-fold increased risk of NTM-PD [6]. In developing countries, misdiagnosis of NTM as TB is common, owing to their similar appearance under microscopic examination of sputum smears [7,8]. This is problematic in many ways: NTM incidence is vastly underestimated, it unnecessarily drains resources dedicated to the global fight against TB and it leads to mistreatment of patients because NTM infections do not respond to classic TB drug regimens.

NTM represent >160 species commonly found in soil and water, including municipal and household water supply systems. The species show varying degrees of virulence leading to diverse

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Véronique Dartois is a Principal Investigator at the Public Health Research Institute of Rutgers University. Initially trained as a molecular biologist, she has more than 10 years' experience in the pharmaceutical and biotech industry, acquired through her previous position as Pharmacology Unit Head at the Novartis Institute of Tropical Diseases, and biotech companies in Southern California. Her current research interests include the pharmacokinetics, pharmacodynamics and imaging of antimycobacterial drugs in pulmonary lesions, and the optimization of predictive animal models and *in vitro* assays to study these questions. Specifically, lesion-specific analysis of drug exposure and bacterial killing help identify and characterize lung granuloma compartments in which organisms are not eliminated.



Thomas Dick has 20 years' experience in mycobacteriology, antibacterial drug discovery and R&D program management, in industry as well as in academia. His research focuses on the discovery of new medicines for the treatment of tuberculosis (TB) and non-tuberculous mycobacterial (NTM) lung disease. Since 2017 he has been Director of Antimicrobial Drug Discovery at the Public Health Research Institute and Associate Professor at the Department of Medicine, New Jersey Medical School, Rutgers University. Dr Dick holds a Toh Chin Chye Visiting Professorship at National University of Singapore where he manages the TB drug discovery project portfolio of the Medical School's SPRINT TB program which he co-founded in 2014.



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clinical features. Within this group of bacteria, the *Mycobacterium avium* complex (MAC: *M. avium*, *Mycobacterium intracellulare* and *Mycobacterium chimaera*) and *Mycobacterium abscessus* are the most frequently encountered pathogens associated with NTM-PD, accounting for >90% of the total cases reported [5,9]. Until recently, it was thought that the majority of NTM patients were infected with genetically unrelated strains acquired independently through exposure to soil or water [10,11]. However, recent whole-genome analysis of a large global collection of *M. abscessus* clinical isolates uncovered that most patients were actually infected with genetically clustered strains [12,13]. Although these data are compatible with infection from a common environmental source, studies on transmission events suggest that indirect human-to-human transmission, presumably through fomite spread or infectious aerosols, could have also contributed to the spread of NTM infections [12,13].

Treatment for NTM-PD as recommended by the American Thoracic Society and the British Thoracic Society is largely empirical [14,15]. Despite significant NTM interspecies variability in drug susceptibility, treatment is often lumped together. Therapeutic regimens tailored for specific species are only available for a few commonly encountered pathogens including for instance MAC, *M. abscessus*, *Mycobacterium kansasii* and *Mycobacterium xenopi*. In general, macrolide-based (clarithromycin or azithromycin) multidrug regimens are prescribed. For infection with MAC, the standard regimen includes ethambutol and rifampicin. Whereas, in the case of *M. abscessus* a macrolide is usually given with parenteral antibiotics, an aminoglycoside and either cefoxitin, imipenem or tigecycline. Standard of care calls for 12 months of negative sputum cultures while on therapy [14], which usually results in 18–24 months of treatment with a minimum of three antibiotics [16,17]. Despite this, treatment outcomes remain poor. Although ~50–88% MAC-PD patients achieve sputum conversion (with 4–12% true relapse rate, as opposed to reinfection) [16,18,19], the cure rate among patients with *M. abscessus* pulmonary infection is only 25–58% [17,20,21]. Thus, *M. abscessus* is often referred to as the ‘incurable nightmare’. In addition, the prolonged treatment for NTM-PD not only induces severe adverse events in patients but also creates a high burden to society. It has been estimated that a total of US\$815 million was spent in relation to NTM-PD in the USA in 2010 [22].

Clearly, owing to the poor treatment outcomes and lengthy treatment duration accompanied by drug toxicity, there is an urgent medical need to develop more-effective and safe regimens consisting of ideally orally bioavailable drugs with broad-spectrum anti-NTM activities for the treatment of NTM-PD. However, this target product profile is not easy to achieve. In this review, with a focus on NTM-PD caused by MAC or *M. abscessus*, we summarize the current state of NTM drug discovery and development, present our perspective on the underlying knowledge gaps and challenges in NTM drug discovery and discuss how to focus research efforts to accelerate building a NTM drug pipeline, based on lessons learnt during the past decades in the TB field.

Current status of NTM drug discovery and development

Since the critical shift in the 1990s from anti-TB regimens toward macrolide-based multidrug therapy, not much has been accomplished in the treatment of NTM diseases owing to limited research

efforts. Most agents in the current treatment recommendations are derived from clinical practice or *in vitro* drug susceptibility testing results. The only new antibiotic that has been introduced to the chemotherapy with clinical evidence is tigecycline [23]. Based on literature and information from NIH ClinicalTrials.gov (<https://clinicaltrials.gov>), we have summarized agents in development for the treatment of NTM infections in Fig. 1. Unsurprisingly, in comparison with the TB drug pipeline where >35 chemical entities are in the discovery stage and ~30 interventions are currently in clinical trials (Working Group on New TB Drugs: <https://www.newtbdrugs.org>), the NTM drug pipeline is nearly empty. Most of the current candidates and leads are derived from repurposing and reformulation of existing antibiotics or ‘cross-testing’ of a few TB active compounds.

Clofazimine

Clofazimine is an orally administered drug approved for the treatment of leprosy, currently repurposed as an anti-TB drug. Clinical uses against *M. avium* infections since the 1990s have demonstrated some efficacy in combination with other drugs [24,25], but its use remained limited owing to lack of demonstrable clinical utility and higher mortality rates reported in a trial among HIV patients where it was added to clarithromycin and ethambutol [26]. Research interest was revived in 2012 after a retrospective review reported that a significantly greater proportion of MAC-PD patients (HIV-negative) treated with clofazimine converted to negative cultures, although relapse still occurred [27]. *In vitro*, its MIC ranges from 1–4 mg/l against *M. avium* and is ≤1 mg/l against the majority of *M. intracellulare* isolates [28]. Currently, a Phase II clinical trial of clofazimine is in progress to evaluate its efficacy for the treatment of MAC-PD (ClinicalTrials.gov identifier: NCT02968212; Table S1, see Supplementary material online). Clofazimine is also active against *M. abscessus* and other rapidly growing mycobacteria: a majority of clinical isolates had clofazimine MICs of ≤1 mg/l [29,30]. A retrospective study in Korea reviewed the clinical outcomes of 27 refractory *M. abscessus* lung disease patients after addition of clofazimine for 12 months: 15% of the previously nonresponsive patients achieved sputum culture conversion [31]. Additional safety and efficacy data of clofazimine within a bigger group of patients have recently been documented in another retrospective study by Martiniano *et al.* [32]. Among a total of 112 patients with NTM infections (MAC, *M. abscessus* or other species), 80% tolerated treatment with clofazimine for >6 months; and out of 60 patients with pulmonary infections who failed previous treatment 29 (48%) converted to negative cultures within 1 year [32]. These results further imply a contributing effect of clofazimine in the treatment of NTM-PD. In line with clinical observations, *in vitro* studies have demonstrated additional merits of clofazimine in multidrug regimens for NTM infections: the drug is synergistic with amikacin against *M. avium* and *M. abscessus* [29,30] and it significantly prevented regrowth of NTM strains after clarithromycin and amikacin exposure [33].

Linezolid and other new oxazolidinones

Linezolid, the first FDA-approved oxazolidinone, has been shown to exhibit some – but relatively weak – *in vitro* activity against *M. avium* and *M. abscessus*, with MICs ranging from 16 to 64 mg/l [28,34,35]. Substantial toxicity associated with prolonged use of linezolid is a major concern in clinical practice [36]. A Phase IV

Discovery		Phase I/II	Phase III	Phase IV
LCB01-0371 - Target 50S ribosome - For <i>M. abs</i>	Clofazimine* - Target NDH-2 - For <i>M. abs</i>	Clofazimine - Target NDH-2 - For <i>M. avium</i> PD	Liposomal amikacin for inhalation (LAI) - Target 30S ribosome - For refractory MAC PD	Linezolid - Target 50S ribosome - For NTM disease
PIPD1 - Target MmpL3 - For <i>M. abs</i>	Tedizolid* - Target 50S ribosome - For NTM	Liposomal amikacin for inhalation (LAI) - Target 30S ribosome - For <i>M. abs</i> PD	Clarithromycin vs azithromycin - Target 50S ribosome - For MAC PD	
Indole-2-carboxamides - Target MmpL3 - For <i>M. abs</i>	Bedaquiline* - Target ATP synthase - For NTM	Nitric oxide - Enhance host defense - Produce reactive nitrogen intermediates - For CF patients with NTM (especially <i>M. abs</i>) - From AIT therapeutics	Clarithromycin vs moxifloxacin - Target DNA gyrase - For <i>M. xenopi</i> PD	
Thiacetazone derivatives - Target FAS-II dehydratase - For <i>M. avium</i> and <i>M. abs</i>	β-lactams with avibactam* - Target penicillin-binding protein - For <i>M. abs</i> and <i>M. avium</i>	Gaseous nitric oxide (gNO)^a - Enhance host defense - Produce reactive nitrogen intermediates - For NTM - Thiolanox [®] from Novoteris		
	Rifabutin* - Target RNA polymerase - For <i>M. abs</i>			

Mechanism of action

- Inhibition of cell wall synthesis
- Inhibition of protein synthesis
- Inhibition of nucleic acid synthesis
- Other mechanisms

Drug Discovery Today

FIG. 1

Non-tuberculous mycobacteria (NTM) drug pipeline. Agents currently in discovery or development for the treatment of NTM pulmonary disease are shown. Compounds in the 'Discovery' column are from literature. The asterisks (*) indicate repurposed drugs in the discovery stage. A 'Pre-clinical' column is not included because no NTM candidates are currently in preclinical development. Drugs under 'Phase I–IV' are from ClinicalTrials.gov (<https://clinicaltrials.gov>) and more details are shown in Table S1 (see Supplementary material online). Phase I and II are combined in one column: the first three trials are in Phase II and the last one is a Phase I/II trial. ^aGaseous nitric oxide is composed of 0.5% NO and 99.5% nitrogen. Abbreviations: *M. abs*, *Mycobacterium abscessus*; MAC, *Mycobacterium avium* complex; PD, pulmonary disease; CF, cystic fibrosis; FAS-II, type II fatty acid synthase; NDH-2, type II NADH-quinone oxidoreductase.

clinical trial is underway in Bangkok to study the efficacy and tolerability of linezolid in the treatment of NTM diseases (ClinicalTrials.gov identifier: NCT03220074). Recently, two other oxazolidinones were considered based on their potency against NTM. Tedizolid, which has an improved safety profile [37], exhibits an MIC 4- to 16-fold lower than linezolid [38,39]. LCB01-0371, a novel oxazolidinone currently in Phase II clinical development for pulmonary TB, was found to be potent against *M. abscessus*, with linezolid-like activity *in vitro* and *in vivo* [40].

Nitric oxide and aerosolized amikacin

There are two inhaled drugs in clinical development: nitric oxide (NO) and liposomal amikacin for inhalation (LAI). NO is thought to be promising in eliminating NTM infections because of its roles in immune defense and its antimicrobial functions. Two formulations of NO from two companies (AIT Therapeutics and Novoteris) are currently in clinical trials with the objective of evaluating their safety in patients. LAI, a novel formulation of amikacin, seems to be associated with reduced toxicity and improved efficacy in patients with refractory NTM lung disease especially those infected with MAC, as suggested by its Phase II clinical trial results [41]. Its efficacy and safety profiles are being further evaluated in a Phase II trial against *M. abscessus* and a Phase III trial for recalcitrant MAC lung disease.

Bedaquiline

Bedaquiline, being the only TB drug approved by the FDA during the past 40 years, has low MIC values against a collection of *M. avium* and *M. abscessus* clinical isolates [42–45]. However, despite being an excellent growth inhibitor at low doses, it lacks bactericidal activity against NTM *in vitro* [44,46]. The *in vivo* efficacy of bedaquiline seems to be limited as well. Although it reduced colony-forming units (CFU) in the lungs by 1 log in an acute *M. abscessus* mouse model [47], it failed to prevent death of the infected mice in another chronic study [48]. Similarly, bedaquiline was shown to exhibit bacteriostatic activity in a *M. avium* late-infection mouse model [46]. Preliminary results of bedaquiline as salvage therapy for NTM-PD (infected with either MAC or *M. abscessus*) treatment suggested that it might have some clinical activity because six out of ten patients had a microbiological response, but its efficacy appears to be relatively moderate as suggested by a low sputum culture conversion rate at 6 months [49]. Furthermore, relapse caused by the emergence of strains resistant to bedaquiline has been reported [50]. Therefore, the clinical utility of bedaquiline remains to be further investigated. Of note, similar to clofazimine which targets type II NADH-quinone oxidoreductase (NDH-2) [51], a key component in the electron transport chain, bedaquiline also targets oxidative phosphorylation via inhibiting ATP synthase [52]. Compared with other TB drugs, such as isoniazid and rifampicin, which lose their potency against most NTM strains,

the fact that both oxidative phosphorylation inhibitors exhibit relatively broad-spectrum antimycobacterial activities suggests that targeting this energy-generating pathway could be a useful strategy in anti-NTM drug discovery.

β -lactams in combination with β -lactamase inhibitor avibactam

Owing to the presence of β -lactamases, NTM exhibit high levels of natural resistance to most β -lactams, with the exceptions of cefoxitin and imipenem, which are currently in use for the treatment of *M. abscessus* infections [53,54]. The recent discovery of the potent inhibitory effect by avibactam against β -lactamases in *M. abscessus* and *M. avium* has brought research attentions back to β -lactams. Treatment with avibactam significantly lowered MICs of several β -lactams 4–32-fold in *M. abscessus* and *M. avium* [54–56]. Reported combinations are summarized in Table 1.

Rifabutin

Rifampicin has long been known to be inactive against *M. abscessus*. Surprisingly, a recent screen of 2700 FDA-approved drugs identified rifabutin, belonging to the same drug class as rifampicin, as active against the bacteria *in vitro* [57]. It has an MIC of \sim 2.5 mg/l against a collection of *M. abscessus* strains and kills 90% of the bacteria at 5 mg/l. A better understanding of the underlying differences of these two drugs (such as differences in intrabacterial metabolism and pharmacokinetic properties) could guide the development of more-potent rifamycins against *M. abscessus*.

PIPDI and indole-2-carboxamides

Two recent screens of TB-active hits against *M. abscessus* resulted in two new leads: piperidinol-based compound 1 (PIPDI) and indole-2-carboxamides [58,59]. Coincidentally, these two structurally distinct chemical entities both target MmpL3, a transporter crucial for the export of trehalose monomycolates to the periplasmic space and outer membrane of mycobacteria. Disruption of *mmpL3* leads to defects in mycolic acid synthesis and thus is fatal for mycobacteria [60]. PIPDI and indole-2-carboxamides (lead compounds 6 and 12) have excellent activities against *M. abscessus* with MICs of 0.0625–1 mg/l. They are bactericidal *in vitro*, reducing CFU 100-fold at 1–2 \times MIC. Macrophage assays demonstrated that these compounds could arrest intracellular bacterial growth, but at much higher concentrations. Moreover, treatment of infected zebrafish with PIPDI at 24 \times MIC for 3 days decreased bacterial load by 1 log unit and improved survival of the infected embryos.

Thiacetazone derivatives

Although thiacetazone (TAC) – a former (toxic) TB drug – is inactive against NTM, a few derivatives of TAC synthesized for TB evaluation were found to be effective against *M. avium* and *M. abscessus*. SRI-286 and SRI-224 inhibited a panel of *M. avium* isolates at 2 mg/l or lower, and SRI-286 could reduce the bacterial loads in livers and spleens by 1 log [61]. D6, D15 and D17, second-generation TAC analogs, are active against *M. abscessus* with MICs ranging from 3.1–12.5 mg/l against the type strains. Similar to TAC in *M. tuberculosis*, these compounds require cellular activation by the monooxygenase EthA in *M. abscessus* [62]. These results suggest that modifications of TAC could be an approach to develop new chemical entities active against NTM.

Besides the abovementioned compounds that have been studied extensively for their effect against NTM, TP-271 (a novel fluorocycline antimicrobial related to tetracycline) and some salicylanilide esters and carbamates were also found to exhibit potent activity *in vitro* against *M. abscessus* [63,64]. Several TB actives in development, such as SQ109 (a 1,2-ethylenediamine that can target MmpL3 [65,66]), DC-159a (a novel fluoroquinolone), SQ641 (a capuramycin analog), ACH-702 (a new isothiazoloquinolone) and mefloquine (a quinoline used for malaria) are active against some NTM strains [67–71]. In addition, owing to a lack of new active chemical entities, synergy combinations especially with clarithromycin, amikacin, tigecycline or imipenem were explored *in vitro*, whereas only a few have been studied *in vivo* (Table 1). Overall, *de novo* drug discovery efforts have been very limited so far. A few attempts have been made to develop novel therapeutic approaches for NTM disease via repurposing or repositioning of existing antibiotics. Although some candidates exhibit promising activity *in vitro*, most are at an early stage awaiting *in vivo* and clinical evaluations. Clearly, more drug discovery efforts are necessary to fill the NTM antibiotic pipeline.

Challenges in NTM drug discovery: from a bacteriology point of view

NTM are naturally resistant to a wide spectrum of antibiotics, including most TB drugs. This poses a major challenge for drug discovery. Hit rates in primary screens for *M. abscessus* can be lower than 0.1%. Thus, generation of attractive chemical starting points for lead finding presents a bottleneck. The low level of susceptibility of NTM to a wide range of drugs and compounds is attributed to their ‘intrinsic’ drug resistance.

Intrinsic drug resistance

Considering that NTM reside mostly in soil and water, the selection pressure from their antimicrobial-producing neighbors could have driven these bacteria to develop a wide array of resistance mechanisms to allow their survival in hostile environments. Our current understanding of the mechanisms underlying intrinsic drug resistance in NTM has been reviewed [72,73] and is therefore only briefly summarized here. The thick hydrophobic, double-membrane cell envelope of mycobacteria acts as a major permeability barrier. Studies from the 1990s have demonstrated that *Mycobacterium chelonae*, a species that had not been differentiated from *M. abscessus* at that time, has a cell envelope that is about 10–20-times less permeable than *M. tuberculosis* [74]. Morphotypic antibiotic resistance, a phenomenon of varying degrees of drug resistance in *M. avium* associated with a reversible colony morphology switch (white/red on Congo red containing agar, transparent/opaque), is also attributed to changes in permeability owing to cell-wall modifications [75]. Hence, antibiotics that target the cell envelope are likely to potentiate other drugs with intracellular targets, as implied by the synergistic effect observed between ethambutol and rifampicin in *M. avium* [76] or vancomycin plus clarithromycin in *M. abscessus* [77]. Efflux pumps are additional elements that prevent intracellular accumulation of drugs such as fluoroquinolones and macrolides [78]. For drugs that can accumulate inside the bacterial cells, several mechanisms have been identified that render the molecules inactive. Some NTM species harbor polymorphisms in the target gene contributing to natural resistance to the drug, for example amino

TABLE 1

Synergistic antibiotic combinations in *Mycobacterium avium* and *Mycobacterium abscessus*^a

No.	Drug combination	Type of study	No. strains tested	Reported outcome ^b	Refs
(a) <i>M. avium</i> complex					
1	Amikacin + clofazimine	<i>In vitro</i>	16	100% synergy	[29]
2	Avibactam + ceftazidime ^c	<i>In vitro</i>	1	Synergy	[56]
		In macrophage	1	Synergy	
(b) <i>M. abscessus</i>					
1	Clarithromycin + linezolid	<i>In vitro</i>	2	Synergy	[177]
2	Clarithromycin + tigecycline	<i>In vitro</i>	20	65% synergy for <i>Mycobacterium massiliense</i>	[178]
			20	25% synergy for <i>M. abscessus</i>	
		<i>In vitro</i>	31	80.6% synergy	[179]
3	Clarithromycin + vancomycin	<i>In vitro</i>	12	100% synergy	[77]
4	Clarithromycin + moxifloxacin	<i>In vitro</i>	28	39.3% synergy for <i>M. massiliense</i>	[180]
			26	3.8% synergy and 65.4% antagonism for <i>M. abscessus</i>	
		In macrophage	15	33.3% synergy for <i>M. massiliense</i>	
			15	6.6% synergy and 66.7% antagonism for <i>M. abscessus</i>	
		In mice	6	50% synergy for <i>M. massiliense</i>	
			7	71.4% antagonism for <i>M. abscessus</i>	
		<i>In vitro</i>	20	85% synergy for <i>M. massiliense</i>	[178]
			20	5% synergy and 45% antagonism for <i>M. abscessus</i>	
5	Azithromycin + moxifloxacin	<i>In vitro</i>	28	35.7% synergy for <i>M. massiliense</i>	[180]
			26	3.8% synergy and 46.2% antagonism for <i>M. abscessus</i>	
		In macrophage	15	20% synergy for <i>M. massiliense</i>	
			15	6.6% synergy and 40.0% antagonism for <i>M. abscessus</i>	
		In mice	6	50% synergy for <i>M. massiliense</i>	
			7	71.4% antagonism for <i>M. abscessus</i>	
6	Clarithromycin + linezolid + moxifloxacin/gatifloxacin/levofloxacin	<i>In vitro</i>	2	Synergy	[177]
7	Clarithromycin + ciprofloxacin + rifabutin	<i>In vitro</i>	2	Synergy	[177]
8	Imipenem + clarithromycin	<i>In vitro</i>	21	43% synergy	[181]
9	Imipenem + levofloxacin	<i>In vitro</i>	21	29% synergy	[181]
10	Amikacin + clofazimine	<i>In vitro</i>	40	100% synergy	[30]
		<i>In vitro</i>	77	80.5% synergy	[29]
11	Amikacin + linezolid	<i>In vitro</i>	32	53.1% synergy for <i>M. massiliense</i>	[182]
			32	37.5% synergy for <i>M. abscessus</i>	
12	Tigecycline + clofazimine	<i>In vitro</i>	19	42% synergy	[183]
13	Tigecycline + linezolid	<i>In vitro</i>	32	31.3% synergy for <i>M. massiliense</i>	[182]
			32	21.9% synergy for <i>M. abscessus</i>	
		In fruit fly	1	Synergy (dramatically improved survival of infected flies and reduced bacterial population per fly)	[184]
14	Rifampicin + doripenem/biapienem	<i>In vitro</i>	1	Synergy	[185]
15	Avibactam + amoxicillin ^c	<i>In vitro</i>	17	100% synergy	[54]
		In macrophage	1	Synergy	
		In zebrafish	1	Synergy (increased larva survival and reduced the proportion of embryos with abscesses)	
16	Avibactam + ceftaroline ^c	<i>In vitro</i>	1	Synergy (increased kill)	[186]
		In macrophage	1	Synergy (increased kill)	

TABLE 1 (Continued)

No.	Drug combination	Type of study	No. strains tested	Reported outcome ^b	Refs
17	Avibactam + imipenem (+amikacin) ^c	<i>In vitro</i>	1	Synergy (no significant MIC shift, but increased kill)	[187]
		In macrophage	1	Synergy (increased kill)	
		In zebrafish	1	Synergy (increased larva survival)	
18	Avibactam + tebipenem/ Ertapenem/panipenem ^c	<i>In vitro</i>	29	100% synergy	[55]
19	Avibactam + doripenem/ Faropenem/meropenem/ biapenem ^c	<i>In vitro</i>	29	55–75.9% synergy	[55]
20	Avibactam + cefalotin/ Cefuroxime/cefamandole/ ceftriaxone ^c	<i>In vitro</i>	1	Synergy	[54]
21	Clavulanic acid + meropenem ^c	<i>In vitro</i>	1	Synergy	[185]

^a Synergy combinations tested before the year 2000 are not included. *In vitro* synergy is defined as a combination with a fractional inhibitory concentration index (FICI) ≤ 0.5 , unless otherwise stated.

^b The percentage in 'Reported outcome' column indicates the percentage of strains tested that showed synergy (or antagonism in some cases). Percentage is not reported when fewer than five strains were tested. Antagonism (a FICI score > 2 as defined by Choi *et al.*) is indicated when the percentage was significantly larger than that of synergy. Almost all combinations reported here were synergistic except for macrolide (clarithromycin or azithromycin) + moxifloxacin which showed a synergistic effect for 30–50% of *M. massiliense* but an antagonistic effect for 40–70% of *M. abscessus* strains. '*M. abscessus*': *M. abscessus* subsp. *abscessus*; '*M. massiliense*': *M. abscessus* subsp. *massiliense*.

^c β -lactam and β -lactamase inhibitor combinations are defined as synergistic if the potency of the β -lactam is significantly improved (e.g., at least fourfold shift in MIC) in the presence of the β -lactamase inhibitor.

acid alterations in the arabinosyl transferase EmbB of *M. abscessus* make ethambutol inactive by preventing drug binding [79]. Upon exposure to the drug, some NTM species induce the expression of certain genes resulting in the modification of the target binding site of the drug. A well characterized example for this strategy is the inducible macrolide resistance in *M. abscessus* mediated by the *erm* (41) gene, which encodes a ribosomal methylase. Exposure to clarithromycin or azithromycin increases the expression level of *erm*(41) dramatically within 24 h. Erm(41) methylates A2058 in the 23S rRNA, leading to reduced binding of macrolides to their target site, thus rendering the drugs inactive [80]. Because such resistance occurs in specific environments (e.g., exposure to antibiotics) and does not involve any genetic alterations, this type of resistance is called adaptive – as opposed to acquired – resistance. Furthermore, NTM possess a large collection of enzymes capable of metabolizing drugs to a less active form. Knockout of these modifying genes such as *bla_{Mab}* (encoding a β -lactamase) and *eis2* (encoding a GNAT-acetyltransferase) restored the activity of β -lactams and aminoglycosides in *M. abscessus*, respectively [81,82]. More examples of these intrinsic resistance mechanisms are shown in Table 2. Interestingly, a conserved transcription factor in mycobacteria: WhiB7, acts as a regulator for many intrinsic drug-resistance mechanisms [83]. In *M. abscessus*, 128 genes including *erm*(41) and *eis2* have been identified in the WhiB7 regulon (i.e., they are induced via a *whiB7*-dependent mechanism). Deletion of *M. abscessus whiB7* sensitized the bacteria to drugs like clarithromycin, amikacin, erythromycin and tetracycline 2–8-fold [84]. Altogether, the abundant intrinsic resistance mechanisms form an elaborate network leaving behind only a few antibiotics to exhibit inhibitory activity against NTM. A better understanding of these molecular mechanisms might provide insights into overcoming or bypassing some resistance pathways. Development of a tool box to measure – and to understand – compound uptake, metabolism and excretion by the bacteria (a 'bacterial cell pharmacokinetics platform') might not only greatly facilitate specific lead finding and optimization projects but also

enable rational repositioning programs to improve the potency of poorly active antibiotics targeting pharmacologically validated pathways.

Acquired drug resistance

Besides being equipped with plentiful intrinsic resistance mechanisms, NTM also have the ability to acquire new resistance through *genomic mutations* that is inherited by offspring. The prolonged course of treatment has greatly contributed to the emergence of resistant strains, allowing the bacteria to develop mutations in the target or other related genes to confer high-level resistance. As a result, drug efficacy is abolished.

Up to now, there have been limited studies on acquired resistance mechanisms associated with NTM. Acquired resistance to clarithromycin emerged in the early 1990s soon after its introduction for NTM treatment, especially with monotherapy. Mutations at nucleotides 2058 and 2059 in the peptidyl transferase loop of the 23S rRNA (*rrl*) were found in *M. avium* and *M. abscessus* clinical isolates to confer a high level of macrolide resistance (MICs ≥ 256 mg/l) [85,86]. Of note, it has been shown recently *in vitro* that the chance of acquiring mutations in the 23S rRNA gene is higher in the absence of a functional *erm*(41) in *M. abscessus* [87]. Rifampicin is primarily used in the treatment of *M. avium* infections. Acquired resistance has been documented in clinical isolates and is associated with mutations within the *rpoB* gene encoding the β -subunit of bacterial RNA polymerase. However, introduction of the mutated *rpoB* sequence into *Mycobacterium smegmatis* did not confer resistance to rifampicin, suggesting that there might be other factors contributing to *M. avium* rifampicin resistance [88]. Acquired resistance to aminoglycosides has been demonstrated in *M. abscessus*. Mutations at position 1408 of 16S rRNA (*rrs*) in clinical isolates are associated with aminoglycoside resistance [89]. In addition, an *in vitro* study by Nessar *et al.* has shown that mutations at positions 1406, 1409 and 1491 in *M. abscessus* could also confer a high level of resistance (MICs ≥ 1024 mg/l) [90]. Recently, Alexander *et al.* reported the emergence

TABLE 2

Intrinsic drug resistance mechanisms in *Mycobacterium avium* and *Mycobacterium abscessus*^a

Agent	Target	<i>M. avium</i> (genes involved)	<i>M. abscessus</i> (genes involved)	Refs
Isoniazid	InhA	Presumably efflux pumps	Presumably efflux pumps	[73]
Rifampicin	RNA polymerase	NA ^b	Inactivation of drug (<i>arr_{Mab}</i>)	[188]
Ethambutol	Arabinosyl transferase	NA ^b	Polymorphisms in target gene <i>embB</i>	[79]
Pyrazinamide	PanD	Presumably due to active efflux of POA ^c	Presumably owing to active efflux of POA ^c	[189,190]
Aminoglycosides	16S rRNA	NA ^b	Inactivation of drug [<i>aac</i> (2'), <i>eis2-<i>whiB7</i></i>]	[82,84,191]
Fluoroquinolones	DNA gyrase	Polymorphisms in target gene <i>gyrA</i>	Polymorphisms in target gene <i>gyrA</i>	[192]
β-lactams	Penicillin-binding protein	β-lactamase with mild activity Other unknown reasons	Inactivation of drug (<i>bla_{Mab}</i>)	[81]
Thiacetazone	FAS-II dehydratase ^d	The target is not essential: redundant dehydratase present	The target is not essential: redundant dehydratase present	[193]
BTZ043	DprE1	Polymorphisms in target gene <i>dprE1</i>	Polymorphisms in target gene <i>dprE1</i>	[194,195]
BRD4592	Tryptophan synthase	NA ^b	Polymorphisms in target gene <i>trpA</i>	[196]
Macrolides	23S rRNA	Efflux pumps (MAV_1695, MAV_1406)	Modification of drug target [<i>erm</i> (41) – <i>whiB7</i>]	[78,80,84,191]

^a Cell envelope, porins and efflux pumps are likely to be involved in intrinsic drug resistance to many antimicrobials and are thus not specified in the table unless roles have been experimentally proven or suggested.

^b NA: not applicable, the drug is active.

^c POA: pyrazinoic acid, bioactive metabolite of pyrazinamide.

^d FAS-II: type II fatty acid synthase.

of *mmpT5* mutations during bedaquiline treatment in all seven patients with *M. intracellulare* lung infections who relapsed after a positive initial microbiological response [50]. *MmpT5* is a transcriptional regulator that represses the expression of the *MmpS5–MmpL5* efflux pump. Similar to *M. tuberculosis*, mutations in *M. intracellulare mmpT5* are associated with low-level resistance to bedaquiline (2–8-fold). Two out of seven patients had mutations in the primary target (ATP synthase subunit c), conferring high-level resistance (50-fold).

There is currently limited evidence of lateral gene transfer of drug resistance genes in NTM. However, whole-genome analysis of *M. abscessus* has revealed a large number of genes in common with two pathogens most frequently isolated from CF patients: *Pseudomonas aeruginosa* and *Bulkholderia cepacia* [91], indicating that acquiring drug resistance by horizontal gene exchange is likely to play a part. Although the genetic mechanisms of acquired drug resistance have been extensively studied in *M. tuberculosis*, not much information is available for NTM with regard to mechanisms, mutation frequency and how fast resistance develops. Recent studies by Ferro *et al.* have exploited a hollow-fiber model to mimic the effect of various drug therapies in the host. Their study demonstrated that *M. abscessus* could develop genetic resistance against moxifloxacin in 3 days [92], suggesting that acquired drug resistance in NTM can develop very quickly during treatment. Thus, it is of importance to understand the genetic basis for acquired resistance, and more importantly how to optimize regimens to prevent development of resistance.

Lack of bactericidal activity

To make NTM drug discovery more daunting, there is a curious lack of bactericidal activity for most drugs tested against NTM – drugs in the current regimens are either bacteriostatic (tigecycline, imipenem) or exert only weak bactericidal activity at high concentrations (clarithromycin) [93,94]. Bedaquiline, despite a very potent growth-inhibitory effect, exerted only bacteriostatic activity in *M. avium* – the same concentrations that reduced *M. tuberculosis* CFU by 5 logs resulted in only 1 log CFU reduction after 14-day exposure in *M. avium* [46]. Similar observations have been made for a wide spectrum of rapidly growing NTM [95]. Because

mycobacteria have a high metabolic rate and divide slowly [96], they are rather unique among bacteria for their ability to adapt to stress before the cells are killed. This could provide a general physiologic basis for the lack of bactericidal activity of drugs. However, the exact reasons behind the lack of bactericidal activity of anti-NTM drugs are not known and should be explored. Insights into why certain drug-induced cell death pathways are not operational in NTM could reveal ways to overcome this phenomenon. Obviously, new anti-NTM agents should be bactericidal (i.e., have sterilizing properties to improve the currently poor treatment outcomes and accelerate cure).

Challenges in NTM drug discovery: from a disease pathology point of view

A low MIC value is often a good starting point for antibacterial drug discovery because it usually predicts eradication of the infection once adequate pharmacokinetic properties have been introduced into the lead compound. However, this general rule does not appear to hold true for NTM pulmonary diseases. Clinical practice has consistently observed a lack of correlation between *in vitro* MICs and clinical outcomes (i.e., sensitivity for a particular drug as indicated by drug susceptibility testing does not necessarily translate to a positive clinical response) [72]. Why is there such a disconnect? *In vitro* MIC testing is usually performed with mycobacteria growing exponentially as a suspension under optimal conditions in aerated nutrient-rich broth. These culture conditions are very different from the environments where bacilli reside in the host. TB studies have demonstrated a wide range of lung lesion types, from cellular granuloma mainly composed of macrophages to caseous granuloma with a necrotic core. These lesions are complex and dynamic, giving rise to microenvironments of diverse features and stresses, which in turn drive the tubercle bacilli into distinct physiological and morphological states associated with increased antibiotic tolerance [97]. This increased drug tolerance is also termed ‘phenotypic drug resistance’ to distinguish this phenomenon from drug resistance due to genetic alterations. In TB, several conditions, including intracellular and caseum growth, and quiescent states linked to

oxygen or nutrient starvation, have been associated with phenotypic drug resistance of the bacilli [98,99]. Considering similarities between NTM and TB pathology in pulmonary diseases, the same factors are likely to contribute to the persistence of NTM infections despite extensive chemotherapy. Moreover, two features unique to bacterial pathophysiology in NTM-PD – growth in airway mucus and as biofilms – can also lead to physiological adaptations associated with phenotypic drug resistance. Equally important to phenotypic drug resistance is the impact of the local microenvironments on drug penetration, which can lead to subtherapeutic concentrations of antimicrobials at the anatomical sites where bacilli reside, thus affecting treatment efficacy. These factors, altered bacterial physiology associated with phenotypic drug resistance and reduced drug penetration into infection sites are likely to collectively contribute to the observed disconnect between *in vitro* MICs and clinical outcomes observed for NTM-PD.

Intracellular growth

Similar to *M. tuberculosis*, NTM can grow and survive extra- as well as intra-cellularly, for instance inside macrophages. In the context of pulmonary infection, NTM invade the mucosa and get phagocytized by macrophages. Whereas some bacilli are killed, those remaining exhibit robust growth within phagocytic vacuoles until autophagy and apoptosis are induced after a few days of infection. *M. avium* could escape macrophage apoptosis and seize the chance to spread and infect other macrophages [100]. By contrast, *M. abscessus*, especially the smooth morphotype, could restrict intraphagosomal acidification, induce less apoptosis and block autophagy flux, and thus was able to persist inside macrophages for longer periods of time [101,102]. Regardless of the distinct strategies that can be employed by different NTM species, the selective pressures of macrophages on NTM such as reactive oxygen radicals, NO and low pH as well as carbon source composition (a lipid-rich environment inside macrophages) are likely to invoke mycobacterial adaptations and induce drug tolerance. The Ramakrishnan laboratory has carried out studies on drug tolerance in macrophages [103]. The authors demonstrated that the resistance level of *M. tuberculosis* and *Mycobacterium marinum* against isoniazid increased over time inside macrophages: after a 96 h infection period ~49.5% of *M. marinum* survived isoniazid treatment whereas only 7.6% of bacilli from a 2 h infection period survived. Importantly, it was observed that macrophages harboring drug-tolerant mycobacteria could disperse from the existing granulomas that had shrunk substantially owing to treatment and could disseminate to establish a new site of infection in zebrafish.

Factors contributing to the increased drug tolerance of intracellular mycobacteria are multifold. The tolerance might result from macrophage-induced bacteriostasis because multiple static subpopulations of *M. avium* have been found in human macrophages after quinolone treatment [104]. The increased resistance could also be attributed to replicating cells with adaptive physiological changes including induction of the master drug-tolerance regulator WhiB7, which has been shown to be strongly induced (14-fold) in *M. tuberculosis* in macrophages [105]. Upregulated along with WhiB7 are efflux pumps and drug-modifying genes in a WhiB7-dependent or -independent manner. Transcriptome studies on internalized *M. tuberculosis* suggested that macrophage residence triggered dramatic differential gene expression, among which a large group of efflux

pumps (19 out of 55 annotated) were induced [106,107]. Further studies by Adams *et al.* [103] showed that the increased drug tolerance to isoniazid and rifampicin of *M. tuberculosis* in macrophages can be reverted by addition of the efflux pump inhibitor reserpine. The same has been observed with *M. marinum* in macrophages. In addition, two *M. tuberculosis* transposon mutants defective in Rv1258c – a multidrug efflux pump – were hypersensitive to rifampicin specifically in macrophages, suggesting that the macrophage-induced tolerance in mycobacteria could be mediated by efflux pumps [103]. Besides the abovementioned reasons, drug activity could also be limited intracellularly owing to restricted uptake and accumulation inside macrophages.

Caseum growth and nonreplicating state of persistence

In advanced human TB, most bacteria reside extracellularly in caseum [108]. Owing to lack of vasculatures, tubercle bacilli in the center of caseum are believed to confront oxygen and/or nutrient limitation. Direct measurement with oxygen probes and *in situ* staining with the hypoxia marker pimonidazole have demonstrated a low oxygen tension in the necrotic and caseous regions of granulomas in patients and animal models [109,110]. *M. tuberculosis* isolated from lung lesions has also been found to display altered morphology and staining properties similar to bacilli grown in distilled water [111], suggesting that the bacilli are starved in lesions. These lines of direct or indirect evidence have promoted the development of several *in vitro* models for *M. tuberculosis* to mimic conditions of oxygen deprivation or nutrient starvation. Studies have shown that, upon hypoxia or nutrient starvation, the bacilli stop growth and enter a nonreplicating state with reduced metabolism [112–115]. Accompanied by such changes is phenotypic drug resistance to most anti-TB drugs. Isoniazid and moxifloxacin for instance are highly active against replicating bacteria; however, they have little or no effect on the viability of nonreplicating nutrient-starved cells [115]. Rifampicin, the remaining active agent against starved bacteria, is active only at a considerably higher concentration [115,116]. The drastic loss of drug potency could be attributed to reduced drug uptake [117] and/or change in drug target essentiality. For instance, absence of cell wall synthesis in nonreplicating starved cells might result in the lack of efficacy of isoniazid [116].

Direct evidence demonstrating drug tolerance of *M. tuberculosis* present inside caseous necrotic lesions has been provided recently by Sarathy *et al.* [118]. The authors measured the drug susceptibility of *M. tuberculosis* bacilli present in *ex vivo* caseous lesion samples collected from rabbits with active TB. No significant bacterial growth was observed in the caseum homogenate during the first 7 days of incubation, indicating that the bacilli from caseum are largely nongrowing. Intra-caseum *M. tuberculosis* was highly tolerant to most antibiotics: isoniazid, kanamycin and clofazimine had minimal to no activity. Similar to oxygen- and nutrient-starvation-induced nonreplicating bacteria, rifampicin was active but achieved tenfold killing only at 8 μ M, a concentration 100-times higher than the concentration required to kill actively replicating cells. In accordance with the data from this *ex vivo* caseum model, preclinical efficacy studies that focused on the bacterial population surviving drug treatment also illustrated that lesion compartments that were not sterilized at the end of therapy were mostly necrotic granulomas and caseous foci [119,120].

NTM lung disease shares many traits with TB: pulmonary lesions are broadly classified as nodular bronchiectatic and fibrocavitary [14], highly reminiscent of TB-induced pathology. Advanced lung histopathology of *M. avium*-infected mice is characterized by necrotizing granulomas with minimal microvessels and a hypoxic center, strongly resembling human TB lesions [121,122]. Whether *M. abscessus* and/or *M. avium* have evolved similar metabolic and physiologic adaptations to *M. tuberculosis* under hostile conditions remains to be determined. Nevertheless, earlier studies on the non-pathogenic NTM species *M. smegmatis* showed its capability to maintain long-term viability by entering a nonreplicating state under nutrient or oxygen limitation [123,124]. Reminiscent of *M. tuberculosis*, nongrowing *M. smegmatis* is characterized by lower intracellular ATP concentrations, reduced oxygen consumption and extreme tolerance to antibiotic treatment [123]. Interestingly, DosR, the dormancy survival response regulator crucial for hypoxia survival of tubercle bacilli, along with the two histidine kinases, is conserved across NTM species [125]. In a recent *M. abscessus* transcriptomic study, the entire DosR regulon was strongly upregulated upon NO-induced hypoxia [126], suggesting that there are largely conserved molecular strategies between NTM and *M. tuberculosis* that persist inside the host. A similar conclusion was drawn by Drapal and colleagues after metabolite profiling of several *Mycobacteria* species including *M. avium* under hypoxic conditions [127]. Therefore, it is tempting to hypothesize that, similar to *M. tuberculosis*, NTM are capable of entering a nonreplicating state and exhibiting phenotypic drug resistance inside lung lesions, and this could be one of the factors contributing to the persistence of NTM infection under prolonged treatment.

In addition to phenotypic drug resistance, the pharmacokinetics of drugs inside lesions also affects the drug efficacy via differential drug access. In a laboratory, *in vitro* MIC setting, bacteria are exposed to constant drug concentrations present in broth. In patients, however, for successful treatment of NTM pulmonary disease, drugs from the blood vessel need to penetrate the complex lung lesions to reach the bacteria (i.e., they must diffuse from the cellular rim that borders the necrotic center and penetrate the entire caseous region without any assistance) [128]. The Dartois laboratory has carried out several studies on drug penetration into rabbit and human TB granulomas. By visualizing drugs using MALDI MS imaging, the authors observed a heterogeneous distribution of drugs across different lesion compartments. First-line TB drugs with treatment-shortening properties such as pyrazinamide and isoniazid could reach a high accumulation in caseum and maintained therapeutic levels in this compartment throughout the dosing intervals [129]. However, for moxifloxacin, although it accumulates in lesions at relatively high concentrations, it stays predominantly in immune cells and barely diffuses to the acellular caseum [130]. This seems to explain why moxifloxacin, a strong killer of replicating and nonreplicating tubercle bacilli, failed to shorten TB treatment in clinical trials. The correlation between drug distribution into caseous foci and its efficacy in TB is likely to be applicable to NTM-PD as well. Thus, the extent of diffusion into the caseous center by current NTM drugs and new candidates needs to be investigated.

Mucus growth

Different from TB, mucus plays an important part in the development of NTM pulmonary disease, at least in selected patient

populations with preexisting chronic lung diseases such as CF or COPD. Owing to the hyper-production of mucus or defective cilia function, sticky mucus cannot be effectively swept out of the lungs. As a result, bacteria residing in mucus remain in the lung, evade the immune system and thrive in the excess of thick stationary mucus adherent to airway surfaces. Excessive mucus in the patient's airways is likely to trap the bacilli in a unique environment with varied oxygen and nutrient content, as suggested by studies on CF patients infected with *P. aeruginosa*. By inserting an O₂ electrode directly into the right upper lobar bronchi of chronically *Pseudomonas*-infected CF patients, it was shown that oxygen is depleted in the mucopurulent material obstructing the lobar bronchus possibly owing to restricted oxygen diffusion through thickened mucus, consumption by the CF epithelium or by the bacteria themselves [131]. Nutrient composition in sputum from CF patients also differs with an increased amount of amino acids [132]. Preliminary studies on *M. abscessus* toward an artificial sputum media that mimic the nutritional composition of CF sputum showed that the organism slowed its growth and induced a 'low energy' transcriptional response [126], suggesting that the bacilli indeed undergo a phenotypic switch in the mucus niche. More studies are warranted to further explore how NTM adapt in the mucus and most importantly how mucus growth affects drug susceptibility.

Biofilm growth

Being a common persistence strategy for many microorganisms, biofilms are multicellular structures in which bacterial cells stick to each other and to living or nonliving surfaces. NTM are notorious biofilm producers in nature and human-engineered environments. They are frequently recovered in biofilms from surfaces of water pipes, showerheads and even healthcare equipment, such as heater-cooler units [133,134]. Biofilms of NTM are hard to eliminate by conventional decontamination procedures, thereby raising a serious public health threat. The ability of NTM to form biofilms has been linked to their pathogenicity. This correlation was first speculated by Carter *et al.* when they observed that a *M. avium* isolate attenuated in mice happened to form a biofilm less effectively *in vitro* [135]. Yamazaki *et al.* further provided evidence by demonstrating that biofilm-deficient transposon mutants of *M. avium* showed impaired invasion in human bronchiolar epithelial cells and caused limited infection in mice [136]. Importantly, the connection between NTM biofilms and pulmonary disease pathology has been demonstrated recently. *M. abscessus* has been observed to form biofilms within thickened alveolar walls and airways of CF patients, as well as within the lung cavity in COPD patients [137,138]. One notable attribute of biofilms is their increased tolerance to antimicrobials, and this holds true for NTM biofilms [139–142]. NTM in biofilms are generally ten-times less susceptible to antibiotics than their planktonic counterparts. Even clarithromycin, the cornerstone of NTM treatment therapy, which showed some activities on the initial formation of *M. avium* biofilms *in vitro*, is completely inactive in established biofilms [143]. Thus, biofilm growth is likely to contribute to the disconnect between MIC and clinical outcome, and the persistence of NTM infection.

The reason why NTM in biofilms are more prone to escaping attack by antimicrobials remains elusive. Nevertheless, it seems

clear that antibiotic tolerance promoted by biofilm growth is adaptive and temporary (i.e., rather than genetic) [139]. A few factors could contribute to the increased tolerance to antibiotics. Similar to other bacterial species, NTM in biofilms are embedded within a matrix composed of extracellular polymeric substances (EPS). Despite interspecies differences, the EPS matrix of NTM has been found to primarily comprise free mycolic acid, glycopeptidolipids, mycolyl-diacylglycerol or lipopeptides [144]. Therefore, it is likely that the waxy nature of the lipid-rich NTM EPS matrix builds a physical barrier that shields antibiotics from penetration. In addition, the presence of extracellular DNA (eDNA) in *M. avium* and *M. abscessus* biofilm matrix has also been associated with antibiotic resistance. Disruption of eDNA by DNase treatment led to a significant increase in the susceptibility of NTM biofilm to clarithromycin and moxifloxacin [144]. Besides the physical barrier provided by biofilm architecture, antibiotic resistance could also be attributed to the adaptations that NTM undergo during the biofilm development, as suggested by the transient resistance observed after resuspension of biofilm bacteria [142,145], as well as transcriptional changes observed during *M. smegmatis* biofilm formation [146].

Although the biofilm structures of NTM have been characterized to some extent, the molecular mechanisms are poorly described. Genes involved in the transition from ‘swimmers’ to ‘stickers’ have not been identified. Interestingly, the fact that exposure to a sub-inhibitory concentration of streptomycin or tetracycline resulted in increased *M. avium* biofilm formation [139] implies that some of the stress-response-related genes triggered by antibiotic exposure are linked to biofilm formation. Besides the molecular mechanisms, two more knowledge gaps of NTM biofilms remain to be filled. First, biofilms of other bacterial species are usually dynamic and complex, harboring bacterial cells at various growth stages with differential physiological activities in the same biofilm [147]. However, whether NTM biofilms are a dynamic and differentiated community or a relatively more homogenous population remains to be determined. Second, given the nature of NTM infection in patients, especially in CF patients where co-infection with other bacteria such as *P. aeruginosa* or another NTM species in the respiratory tract is common, mixed-species biofilms appear to be likely. Thus, interspecies interactions in biofilm formation as well as its impact on antibiotic tolerance are important to investigate.

Taken together, it appears that, over the course of evolution, the dual lifestyle of NTM – saprophytic in soil and water vs pathogenic – has equipped these bacteria with a large arsenal of molecular strategies that allows them to adapt swiftly and effectively to a wide spectrum of hostile environments encountered in various habitats. Owing to the complex nature of NTM pulmonary disease, the abovementioned conditions and stresses are not mutually exclusive. For instance, nonreplicating bacilli or biofilm-like structures might be present in thick mucus together with growing organisms. In fact, these factors are likely to collectively contribute to the emergence of NTM persisters. The development of reproducible persister assays and a more comprehensive understanding of molecular mechanisms employed by *M. avium* and *M. abscessus* to adjust to various conditions and exert phenotypic drug resistance will assist discovery of leads able to eradicate these physiological forms of NTM and thus increase treatment effectiveness.

Additionally, attention should be paid to the assessment of drug penetration pharmacokinetics into macrophages, caseous lesions, biofilms and mucus, because sufficient penetration into the site of infection is crucial for an efficacious treatment.

A road map to new anti-NTM drugs

To date, *de novo* drug discovery campaigns for novel anti-NTM agents are limited. To facilitate NTM drug discovery, a workflow for compound progression from whole-cell screening to preclinical development (i.e., from primary hit generation to compounds showing tolerability, exposure and efficacy in a mouse model of disease) is proposed in Fig. 2. The overall workflow corresponds to the generic compound progression cascade for antibacterial and anti-TB drug discovery that we have described previously [148,149], with a number of biological and pharmacological assays designed specifically to cater for the needs of NTM-PD regimens according to their unique bacteriological and pharmacokinetic requirements. Briefly, to discover new chemical entities active against NTM, a whole-cell-based phenotypic screening should be pursued to generate hits with antimicrobial activity. With the objective to kill two birds with one stone, double hits (i.e., hits active against *M. avium* and *M. abscessus*) with reasonable growth-inhibitory and -cidal activity and an acceptable cytotoxicity profile should be identified and further profiled and prioritized for their potency in bacterial persister models, and for their *in vitro* pharmacokinetic properties in assays that predict lesion penetration. It is important to confirm medium-independent activity of hits as well as activity against a selection of *M. avium* complex and *M. abscessus* species and subspecies early in the process. Parallel to hit-to-lead activities, target deconvolution should be carried out to understand drug mechanism-of-action and facilitate medicinal chemistry efforts during lead optimization. Regarding the choice of chemical libraries to be screened for the identification of chemical starting points, collections of TB actives generated over the past two decades should be considered, because they display higher NTM hit rates compared with ‘random’ compound collections [150]. Furthermore, this strategy increases the chance of identifying broad-spectrum antimycobacterials. A few considerations regarding compound profiling and prioritization are elaborated below.

NTM strains for MIC determinations

Different from the obligate parasite *M. tuberculosis*, which is genomically rather homogeneous, environmental NTM present a heterogeneous group of species and subspecies. This needs to be considered early in drug discovery projects because different species and subspecies do differ in their drug susceptibility profile. Using surrogates such as the model organism *M. smegmatis* instead of the actual pathogenic species in drug screening could be counterproductive. Because the most common and the most difficult to cure NTM-PD are caused by members of the *M. avium* and *M. abscessus* complexes, respectively, efforts should focus on these two species. For instance, we use the clinical isolates *M. avium* 11 (belonging to *M. avium* subsp. *hominissuis* [151]) and *M. abscessus* Bamboo (belonging to *M. abscessus* subsp. *abscessus* [152]) for primary screens. Immediately after hit confirmation, hits should be tested for activity against reference strains obtained from culture collections representing species and subspecies of *M. avium*

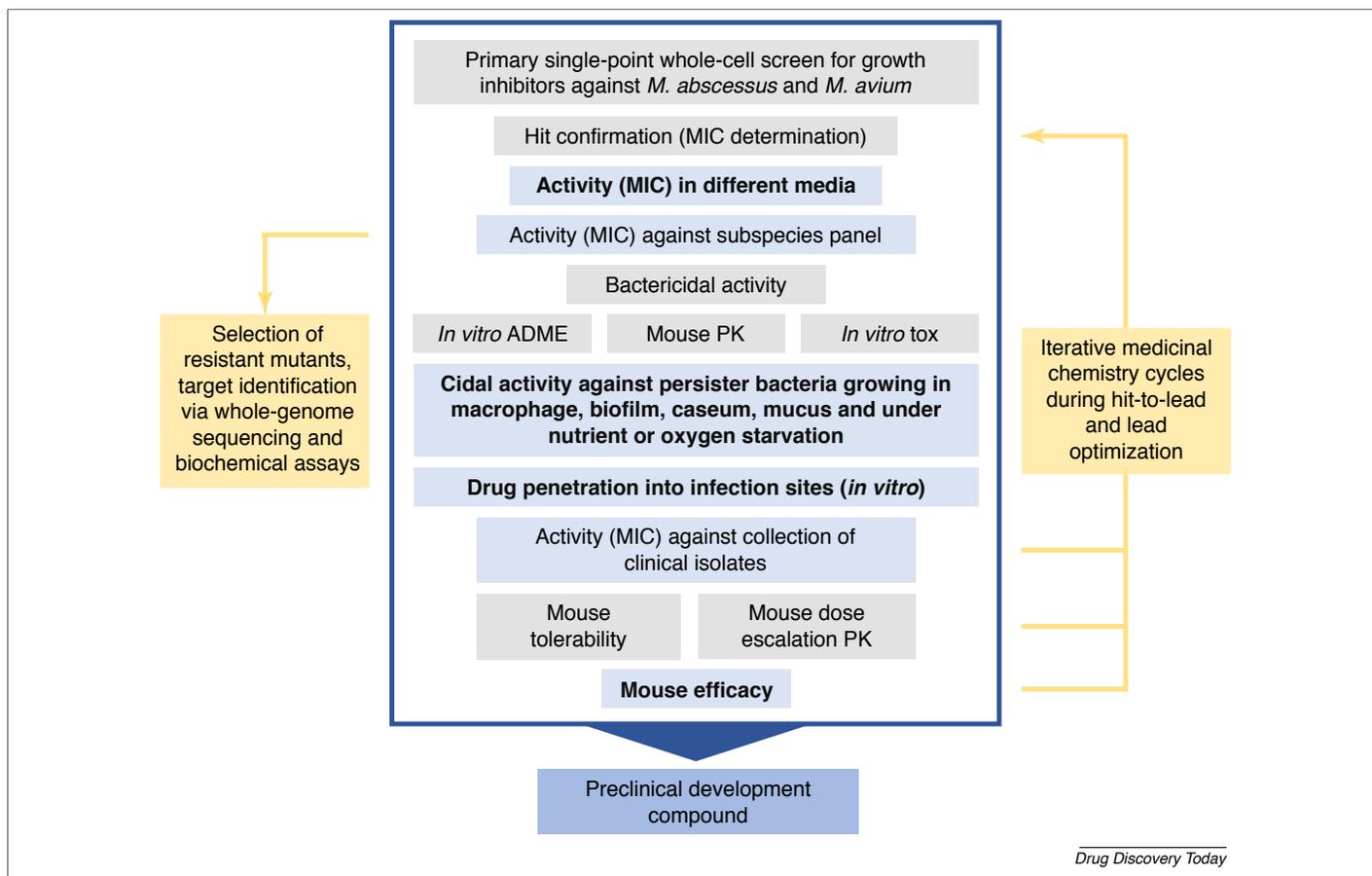


FIG. 2

Proposed workflow for non-tuberculous mycobacteria (NTM) drug discovery. Activities and assays from whole-cell library screening to the preclinical development compound are shown. Light blue boxes indicate NTM-specific assays discussed in more detail in the text. Assay and model gaps are in bold. Gray box indicates generic drug discovery activities and assays described elsewhere [148,149]. Abbreviations: PK, pharmacokinetics; tox, cytotoxicity assays.

and *M. abscessus* complexes: *M. avium* subsp. *hominissuis*, *M. intracellulare* and *M. chimaera*; *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*; *M. abscessus* subsp. *massiliense* (Fig. 2). At a later stage, lead compounds are tested against a large collection of clinical isolates of the two NTM species derived from different regions of the world because geographic variabilities of antibiotic susceptibility have been described [153–156]. Moreover, considering colony phase variation (e.g., smooth vs rough, opaque vs transparent) seen in *M. avium* and *M. abscessus*, it is useful to cover all types of colony morphologies in the clinical isolates tested to identify any potential correlation between colony morphology and drug susceptibility.

Media and incubation conditions for MIC determinations

Currently, most *in vitro* drug testing studies for NTM followed either the Clinical and Laboratory Standards Institute (CLSI) recommendations for NTM susceptibility testing in clinical settings (i.e., cation-adjusted Mueller–Hinton broth and incubation at 30 °C) or standard mycobacterial drug discovery culture conditions (i.e., Middlebrook 7H9 broth and incubation at 37 °C). We and other researchers have observed that differences in these two assay systems including media composition and presence or absence of detergent (Tween[®] 80 in 7H9) can affect drug potency and lead to variabilities in the MIC results [54,77,157]. This highlights a need for further investigations

to improve the predictive value of current NTM *in vitro* drug testing assays for clinical outcome. For the time being, primary hits should be tested in at least two different media to provide preliminary evidence of medium-independent activity. Medium dependence of antimycobacterial compounds is not a new phenomenon. During the past ten years of TB drug discovery, we have encountered several cases in which compound activity was dependent on certain components present in the culture media. One well-documented example is a series of pyrimidine-imidazole compounds, which exhibited low MICs *in vitro* (and adequate pharmacokinetic properties) but had no efficacy *in vivo*. It was later realized that the activities of these compounds were dependent on glycerol – a major carbon source in TB culture media but apparently not relevant for bacterial growth in mice [158].

Besides media composition, incubation time is another crucial factor that can affect drug susceptibility results for NTM, especially in the case of macrolides when tested in *M. abscessus*. It was shown by Nash *et al.* that extended exposure (14 days) to macrolides is required to detect the inducible resistance conferred by *erm*(41): the MIC on day 3 was ≤ 0.5 mg/l, this value increased to >32 mg/l on day 14 [80]. Therefore, to better evaluate macrolides and their potential synergistic effects with other drugs in *M. abscessus*, it is recommended to perform 14-day incubation for MIC determinations. Of note, drug stability should be considered when inter-

preting susceptibility results, particularly when long incubation periods are used. As demonstrated by Rominski *et al.*, severe drug degradation observed for β -lactams (e.g., imipenem and cefoxitin) after 3 days results in misleading MIC values [81].

Persister-specific assays

As elaborated above, the existence of persisters (i.e., bacteria displaying phenotypic drug resistance) in the host might be one of the main factors that hampers the eradication of NTM infection in patients. Hence, conventional MIC alone is not a good predictor of clinical outcome; we need persister-specific assays to identify leads with improved efficacy *in vivo*. Yet, most persister-specific assays have not been thoroughly characterized, profiled or benchmarked for NTM. Research efforts are warranted to adapt and implement assays developed for either *M. tuberculosis* or other pathogens such as *P. aeruginosa* to generate a tool box for NTM drug discovery.

Macrophage infection assays

Macrophage assays have been employed to study the intracellular activity of drugs and chemicals against NTM. A wide variety of macrophages including bone-marrow-derived macrophages, J774 and THP-1 cells are being used [40,58]. Drug treatment usually starts a few hours post-infection followed by incubation for 24–96 h. Drug activity is usually indicated by a reduction in CFU or CFU per macrophage.

Nonreplicating assays

Two *in vitro* assays, namely the Loebel model (nutrient-starvation model) and the Wayne model (oxygen-depletion model), have been established and widely applied in the TB field to evaluate drug potency against nonreplicating bacilli. In the Loebel model, log-phase cultures are washed and transferred to PBS and nutrient-starved for 14 days before drug treatment [115]. In the Wayne model, hypoxic nonreplicating bacilli are generated under gentle stirring in tightly sealed glass tubes with rubber septa. Oxygen indicator methylene blue is used to monitor oxygen depletion over time in a control tube. Upon reaching a nonreplicating phase, drugs are injected into each tube via a needle through the rubber septa. Because Loebel and Wayne bacilli are nonreplicating, the potency readouts in these systems are concentrations that kill 90% of the bacteria: LCC₉₀ for Loebel cidal concentration and WCC₉₀ for Wayne cidal concentration are the counterparts of minimum bactericidal concentration, or MBC₉₀, for growing bacteria. Characterization of growth and survival kinetics of NTM in the Wayne and Loebel models is required before applying these assays for drug testing. Both models have been established for *M. smegmatis* [123,124] and should thus be adaptable to *M. avium* and *M. abscessus*.

Caseum assay

An *ex vivo* caseum assay was recently described for *M. tuberculosis* [118]. Caseum tissue obtained from cavities of TB-infected rabbits is homogenized and dispensed into 96-well plates with drugs. Owing to the nonreplicative nature of bacilli inside caseum, the readout is also the cidal concentration (casMBC₉₀). Currently, this assay might be difficult to develop for NTM because caseum-producing animal models are not well established (see below). Alternatively, the development of artificial, cell-culture-based caseum [129] could be considered.

Biofilm assays

To study NTM biofilms, several laboratory-based biofilm formation models have been developed, among which two systems

might be suitable for NTM drug susceptibility testing. The first assay system is known as the Calgary Biofilm Device employed by Bardouniotis *et al.* to evaluate the cidal activity or the minimal biofilm eradication concentration (MBEC) of biocides on *Mycobacterium phlei* [159]. The device consists of a 96-peg lid-plate that bacteria can adhere to and a ridged trough into which a standardized inoculum is added. Upon biofilm formation on the pegs, the lid can be transferred to a standard 96-well plate in which the biofilms are challenged by antibiotic treatment. Surviving bacteria in biofilms can be removed from pegs by sonication in media following washing and plated for CFU numeration. This method could be applied to the determination of biofilm MIC and cidal concentrations. The second assay system utilizes a 96-well polyvinyl chloride (PVC) microtiter plate in which NTM such as *M. avium* and *M. smegmatis* can adhere and develop biofilm on the PVC surface [141,143]. Following incubation, planktonic bacteria are rinsed away and the remaining adherent bacteria in biofilms with or without antibiotics are stained and quantified by crystal violet. This method is simple and straightforward and enables the determination of concentrations that inhibit biofilm growth. However, because crystal violet is toxic to bacteria, this is usually an endpoint measurement and the stained bacteria cannot be used for further studies.

Mucus assay

Kirchner *et al.* have described the use of synthetic mucus media or artificial sputum medium (ASM) in microtiter plates to test antibiotic efficacy against *P. aeruginosa* [160]. The ASM contains amino acids, mucin and free extracellular DNA to mimic the thick mucus within the lung of CF patients. *P. aeruginosa* and *Staphylococcus aureus* grow as biofilm-like structures (not attached to the surface) in this matrix [161]. This method can be further investigated for NTM (especially for *M. abscessus*) and exploited as an effective platform to identify antibiotics with potential activity in the lungs of CF patients. The use of patient-derived mucus should be considered to validate assays based on artificial mucus.

Lesion- or infection-site-specific pharmacokinetic assays

NTM reside in many anatomical locations where drugs might not penetrate efficiently owing to differential vascularization and cellular composition. Owing to the complexity of NTM pathology, PK/PD correlations that are made on the basis of drug exposure in plasma are not sufficient to inform drug distribution at the site of infection (inside lesions) in patients. Clinical studies with resected lung lesions have indicated that drugs with better penetration into lesions, especially the necrotic core where most of the persisters locate, tend to play a key part in pathogen sterilization and treatment shortening [130]. To this end, Sarathy *et al.* developed a medium-throughput caseum-binding assay to predict lesion partitioning of TB drug candidates [129]. In this assay, a surrogate caseum is generated by lysing oleic-acid-induced foamy macrophages followed by protein denaturation to mimic the situation in the necrotic core of granulomas. Based on the negative correlation between *ex vivo* caseum binding and *in vivo* passive diffusion into caseum, the authors could predict drug diffusion capacity based on the unbound fraction in the surrogate caseum. Combined with drug uptake into macrophages, this assay can be used to predict the partitioning of drugs and newly discovered compounds at the cellular–caseum interface of necrotic lesions [162]. Owing to the

similarities in granuloma and cavity structure between TB and NTM, these assays could be applied to predict the lesion penetration of novel anti-NTM compounds as well.

Animal efficacy

Animal models, including many mouse strains, the guinea pig, rhesus macaque, zebrafish and fruit fly, have been investigated for their potential to develop NTM disease. The details of each model have been reviewed by Chan and Bai and Bernut *et al.* [163,164], and will thus only be briefly mentioned here. Because we are dealing with NTM lung disease, we consider the use of mammalian species as advantageous for *in vivo* efficacy determinations. Overall, owing to the general low virulence of NTM compared with *M. tuberculosis*, it is difficult to generate a sustained infection with advanced human-like lung pathology in animals. Although *M. avium* can cause a relatively high level of infection in the lungs of mice, *M. abscessus* is cleared rapidly from the lungs of most mouse strains and other animal models including guinea pigs. Only when severely immunocompromised strains of mice such as GKO or SCID mice are used can characteristics of certain lung disease histology (e.g., cellular infiltration, consolidation) be observed, yet non-necrotizing and necrotizing lesions have only been shown in the SCID mouse model [47,165]. A few attempts have been made to evaluate various mouse strains for their adequacy for antimycobacterial testing. However, owing to the differences in disease progression (acute vs chronic infection stage), and disease manifestations (lung vs disseminated infection), *in vivo* studies in these mouse models have sometimes led to inconsistent results. For instance, bedaquiline treatment reduced CFU by 2 logs in a SCID mouse model but was almost inactive in nude mice [47,48]. As discussed above, the morphological structure and composition of infection sites are likely to impact bacterial pathophysiology and drug penetration. Therefore, robust and practical animal models that present hallmarks of human NTM pathology (e.g., caseous necrosis) are urgently required for improved *in vivo* assessment of novel anti-NTM compounds.

Design of clinical trials

Owing to the heterogeneity of NTM disease presentation, clinical trials that evaluate the efficacy of new drug candidates or new combinations against NTM-PD are not simple to design [166]. One major challenge lies in the great variabilities among study populations in terms of disease manifestations (e.g., fibrocavitary vs nodular-bronchiectasis type), pre-existing conditions (nil, CF, COPD, AIDS or other causes of immune-suppression), causative agents (*M. avium*, *M. abscessus* or co-infection with other NTM or bacteria) and drug susceptibility profiles. Previous studies have demonstrated that such heterogeneity could result in distinct treatment outcomes [18,167]. Thus, it should be taken into consideration in the clinical trial design whether to target a general population or a particular patient group. If working with a broad patient population, trial results should be stratified by factors such as subspecies and clinical presentations. Another difficulty encountered in NTM clinical trials is the limited choice of endpoints. Very few prospective clinical trials have been conducted for NTM treatment [23,168,169], all of which used sputum culture conversion or clinical improvement at the end of chemotherapy as a measure

of treatment success (Table S1, see Supplementary material online). In TB studies, primary endpoints such as sputum culture conversion at 2 months, time to sputum culture conversion and serial sputum colony counting, have been introduced in Phase II clinical trials as surrogates of end-of-treatment outcome [170,171]. Unfortunately, none of these markers has been evaluated for NTM pulmonary disease. As a whole, identification of accurate, NTM-specific biomarkers capable of predicting long-term treatment outcome or relapse risk are desired and would greatly accelerate the development of new NTM agents by enabling small and more-reliable proof-of-concept studies. Recently, there has been consensus achieved by international experts from the NTM-NET (international network to promote clinically oriented research in the field of non-tuberculous mycobacterial diseases; <http://www.ntm-net.org/>) committee on treatment outcome definitions for NTM-PD [172]. The critical outcome parameters proposed in the statement should be considered in future clinical trial designs to standardize outcome reports for meta-analyses of clinical data.

Concluding remarks

To combat recalcitrant NTM lung diseases with more-effective regimens, we need well-tolerated and preferably orally bioavailable compounds that: (i) are active against at least *M. abscessus* and *M. avium*, and ideally against a wider spectrum of mycobacteria including *M. tuberculosis*; (ii) are bactericidal against growing and ideally against various drug-tolerant persisters forms of the bacteria; (iii) show not only adequate standard plasma pharmacokinetic properties but also penetrate the various sites of infection; (iv) eradicate the bacteria in an animal model that presents human-like pathologies. Importantly, drug–drug interactions must be kept to a minimum because NTM treatments are based on multidrug regimens. Furthermore, NTM patients often take additional antibiotic and nonantibiotic medications. Meeting all these requirements seems to be a daunting task at the moment. NTM drug discovery is still in its infancy with many questions of resistance, persistence and pathophysiology remaining to be unveiled, and many assays and models to be developed and validated. Thus, there is an urgent need to reduce the biological uncertainties around NTM-PD by increasing research efforts. Today's state of NTM drug discovery is reminiscent of the TB situation 20 years ago. Despite a discovery void in the TB field from the 1970s to early 2000s, significant progress has been achieved during the past two decades, owing to a better understanding of the pathogen and disease pathology and – importantly – increased drug discovery efforts. Looking on the bright side, we can benefit from knowledge, bacteriological and pharmacokinetic assays, and from models developed for TB drug discovery. Furthermore, we can make use of chemical matter generated in TB screening campaigns. Libraries of TB actives are available and have been shown to deliver relatively high hit rates for NTM, offering a much-needed impetus to jump-start NTM drug discovery projects, start populating the drug pipeline and discover broad(er)-spectrum antimycobacterials. In addition to *de novo* drug discovery approaches, repositioning and repurposing efforts must be undertaken (Box 1). We believe that, with increasing research and drug discovery efforts and expanding knowledge on NTM, significant therapeutic advances for NTM diseases will be achieved in the coming years.

BOX 1

Proposed strategies for populating the non-tuberculous mycobacteria (NTM) drug pipeline*Repurposing*

In the context of drug development, 'repurposing' is the application of known drugs to treat new indications (i.e., new diseases). Because *de novo* drug discovery and development easily takes longer than a decade, the evaluation of existing and clinically used antibiotics is a low-hanging-fruit approach that can dramatically accelerate drug development, and has the potential to bring rapid relief to NTM patients. For example, the rifampicin analog rifabutin was recently shown to be active against *Mycobacterium abscessus in vitro* through a systematic screen of FDA-approved drugs [57]. Likewise, the systematic exploration of antibiotic combinations could reveal synergistic pairs that could rapidly be tested clinically in patients with very limited therapeutic options such as those with refractory *M. abscessus* lung disease. In addition, more-targeted synergistic studies based on mechanisms-of-action expected to deliver synergistic effects could yield high hit rates: (i) large-scale combinations of β -lactamase inhibitors with β -lactams; (ii) cell wall targeting drugs with drugs modulating intracellular targets; and (iii) efflux pump inhibitors with other drugs should be further explored, as suggested by several recent studies [55,56,77,78].

Repositioning

We refer to repositioning as the NTM-specific chemical optimization of antibiotic classes that act against pharmacologically validated targets but have been developed for infectious diseases other than NTM. Because these drug classes include members that are FDA-approved, attrition rates are lower and the probability of success is significantly higher than incurred through *de novo* drug discovery. Examples include the macrolide clarithromycin, the backbone of NTM therapy, but whose efficacy against *M. abscessus* is affected by inducible resistance conferred by *erm*(41). Screening of macrolide collections against *M. abscessus* is a low-risk and high-reward approach to identify analogs that are not affected by the ribosomal modifications. Likewise, the oxazolidinone linezolid is active against NTM but suffers from low potency and toxicity. A few recent studies reported oxazolidinones with improved potency [38,40,150], thus validating the proposed strategy. Finally, although rifampicin is inactive against *M. abscessus*, other rifamycins appear to exhibit adequate potency [57] (unpublished data).

De novo drug discovery

The third strategy is based on the identification of new chemical entities and targets. Whole-cell screens against mycobacteria suffer from extremely low hit rates. We propose the acceleration of NTM drug discovery by screening compound collections with known activity against *Mycobacterium tuberculosis* rather than 'random' large compound libraries – an approach that has delivered significantly higher hit rates [150]. Furthermore, novel antibacterial discovery concepts such as screening of fragment (MW <300 Da) libraries and membrane targeting molecules should be explored. Although fragment-like compounds are often filtered out in whole-cell screening campaigns, many crucial components of current TB chemotherapies such as isoniazid and pyrazinamide are 'dirty' fragments that hit multiple targets and display attractive physicochemical and pharmacokinetic properties, an asset to eradicate the notoriously robust mycobacterial diseases [173]. Targeting the *M. tuberculosis* membrane has been investigated over the past few years with the attempt to tackle the issues of resistance and persistence. Targeting membrane integrity as opposed to a specific enzyme or pathway also has a lower propensity to enable emergence of genetic resistance. In addition, as opposed to classical pathways

targeted by conventional antibiotics, membrane integrity is essential irrespective of the physiological status of the bacilli. Thus, membrane-targeting compounds should retain their activity against slow-growing or nonreplicating persisters. Indeed, several novel compounds targeting the *M. tuberculosis* membrane do overcome resistance and persistence, with some compounds showing cross-activity against NTM [174–176].

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

We thank all participants of the workshop 'Advancing Translational Science for Pulmonary NTM Infections', organized by the National Institute of Allergy and Infectious Diseases and held in Rockville, MD, September 2017, for very stimulating and enlightening presentations and discussions. We also thank the anonymous reviewers for their useful and insightful comments. We are grateful to Pooja Gopal for her comments on the manuscript. We apologize to the many authors whose work could not be discussed and cited owing to space and reference limits. T. D. holds a Toh Chin Chye Visiting Professorship at the Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI132374 and by the Cystic Fibrosis Foundation under Award Number DICK17XX00 (to T.D.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Cystic Fibrosis Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.drudis.2018.04.001>.

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