

Drug resistance in nontuberculous mycobacteria: mechanisms and models

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Simple Summary: Recently, there has been a considerable rise in infections caused by nontuberculous mycobacteria (NTM). These mycobacteria, which comprise a large and diverse range of species, have developed resistance to most conventional antibiotics, rendering their treatments unsatisfactory. This review summarizes the mechanisms and strategies adopted by NTMs to evade the action of antimicrobial drugs and techniques that can be used to develop better therapies against them. We also suggest some ways to accelerate the drug development pipeline by utilizing a combination of computational, laboratory and animal testing methods.

Abstract: The genus *Mycobacteria* comprises a multitude of species known to cause serious disease in humans, including *Mycobacterium tuberculosis* and *M. leprae*, the responsible agents for tuberculosis and leprosy, respectively. In addition, there is a worldwide spike in the number of infections caused by a mixed group of species such as the *M. avium*, *M. abscessus* and *M. ulcerans* complexes, collectively called nontuberculous mycobacteria (NTMs). The situation is forecasted to worsen because, like tuberculosis, NTMs either naturally possess or are developing high resistance against conventional antibiotics. It is, therefore, important to implement and develop models that allow us to effectively examine the fundamental questions of NTM virulence, as well as to apply them for the discovery of new and improved therapies. This literature review will focus on the known molecular mechanisms behind drug resistance in NTM and the current models that may be used to test new effective antimicrobial therapies.

Keywords: nontuberculous mycobacteria; drug resistance mechanisms; antimicrobial testing; drug discovery

1. The Rise of Nontuberculous Mycobacteria

Mycobacteria are a large group of non-motile, rod-shaped bacteria that tend to grow mold-like pellicles on liquid culture media. Out of the 150 species known to this genus, nearly 25 are known to cause disease in humans. The most well-known mycobacteria species are the *M. tuberculosis* and *M. leprae* complexes, with an estimated prevalence rate of 130 (the year 2020) and 2 (the year 2018) cases per 100,000 population, respectively [1,2], while all others are collectively called nontuberculous mycobacteria (NTMs) [3]. Despite that NTMs are less widespread pathogens for humans than M. tuberculosis, they have proven to be an emerging threat to the immunocompromised population [4], with an estimated 4.1–14.1 cases per 100,000 population worldwide (2013) [5]. NTMs are ubiquitous and can survive in a wide range of environmental conditions, and their infections are difficult to diagnose [6]. The most common NTM-related pathologies are pulmonary infections (pulmonary nontuberculous mycobacterial disease) caused by strains from the *M. avium* complex and *M. abscessus* [6,7], but NTMs can also cause skin and soft tissue infections (e.g., M. marinum infection and Buruli ulcer caused by M. ulcerans), lymphadenitis in immunocompromised children, and even invasive disseminated disease eventually leading to death.



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According to Runyon, NTMs can be classified based on the growth rate and pigment formation (Table 1) [8]. Types I, II, and III strains are classified as slow-growers because they take seven or more days of growth for forming visible colonies on a subculture plate [9]. They are differentiated on their ability to produce pigments only on exposure to light (type I or photochromogens) or also in the dark (type II or scotochromogens), or not being strongly pigmented (type III or non-photochromogens) [10]. Type IV strains are regarded as rapid-growers as they take less than seven days to form visible colonies on a subculture plate [10]. Generally, slow-growing mycobacteria are much more prevalent than fast-growing ones [11] and present higher ratios of drug resistance (with the fast-growing *M. abscessus* being a notable exception) [12]. It has been suggested that all mycobacteria evolved from a common ancestral rapid growing mycobacterial strain [13–15].

Table 1. Summary of the nontuberculous mycobacteria (NTMs) mentioned in this review, their classification according to Runyon, and their reported pathogenesis in humans.

Runyon Classification	NTM Species	Pathogenesis in Humans
Photochromogens Runyon type I	M. kansasii [16], M. simiae [17]	Pulmonary infections Skin infections Disseminated infections
	M. marinum [18]	Skin and soft tissue infections Disseminated infections
Scotochromogens Runyon type II	M. gordonae [19]	Pulmonary infections Skin infections Disseminated infections
	M. scrofulaceum [20]	Cervical lymphadenitis among children Pulmonary infections Disseminated infections
Non-photochromogens Runyon type III	<i>M. avium</i> complex (<i>M. avium</i> and <i>M. intracellulare</i>) [21]	Pulmonary MAC infections Disseminated infections (mostly in AIDS patients) MAC associated lymphadenitis (in young kids and people with normal immune systems)
	M. malmoense [22]	Pulmonary infections Disseminated infections
	M. ulcerans [18]	Skin diseases (Buruli ulcers)
Rapid growing Runyon type IV	M. abscessus [23]	Pulmonary infections Skin and Soft tissue disease Central nervous system infections Disseminated infections
	M. chelonae [24]	Skin and soft tissue infections Pulmonary infections Disseminated infections
	M. smegmatis	Widely regarded as nonpathogenic

Recently, there has been a considerable increase in the number of reported NTM related diseases, including respiratory infections caused by various strains from the *M. avium* complex, *M. kansasii* and *M. abscessus* [25]. This is partly because of the awareness of the symptoms caused by these infections and improvements in detection techniques, but also because of an increase in the number of susceptible individuals and that NTM can form biofilms in common household and hospital sources of infection (such as showerheads, faucets, water distribution systems, plumbing systems, etc.) [26,27]. The situation is worrying because, just like tuberculosis, these bacteria have developed high resistance against conventional antibiotics [28]. However, these pathogens are still considered opportunistic since they require a combination of constant exposure as well as host susceptibility to infection, and these infections have mainly remained limited to patients with pre-existing lung diseases [25,29].

The major NTM that is infecting such individuals suffering from chronic diseases like cystic fibrosis is *M. abscessus*, which is a rapidly growing, intrinsically multidrug-resistant species [30]. These infections are often impossible to treat despite prolonged antibiotic therapy, and the therapy may even be contraindicated with lung transplantation, leaving no effective options for treatment [31]. While NTM infections were earlier thought to be independently acquired by susceptible individuals, the recent consensus is that such infections are frequently transmitted indirectly from an infected to a healthy individual, for instance, via contaminated hospital equipment [32]. Some opportunistic infectious NTM species tend to cluster in specific geographical distributions, and there may be a genetic basis for the susceptibility to their infection in particular patients [11,33,34]. Finally, relapse and reinfection is a major problem with some NTM infections, like the ones caused by *M. avium* complex [35], although it is less so for other species like *M. kansasii* [36].

Currently, the treatment for almost all NTM infections is based on macrolide-based antibiotics, such as clarithromycin or azithromycin. For NTM infections caused by the slow-growing group, the regime also includes ethambutol and rifampicin [37], while for fast-growers, it includes an aminoglycoside and either cefoxitin, imipenem or tigecycline [38]. These treatments are largely empirical, derived from years of clinical practice, can last for as long as 18 months, are costly, and are often associated with drug-related toxicities and side-effects [39]. Cure rates range from 80–90% with *M. malmoense* infections to just 30–50% with *M. abscessus* infections [40]. Thus, the discovery of new and more efficient therapies against NTMs is an important topic of research. However, a major bottleneck is the low susceptibility of mycobacteria to most antibiotics, including the ones used against tuberculosis [41]. A better understanding of the underlying mechanisms behind this drug resistance by improving the available models to study their infection could significantly help in accelerating the drug discovery process.

2. Mechanisms of Drug Resistance in Nontuberculous Mycobacteria

Drug Resistance can be either intrinsic (natural) or acquired [42]. Intrinsic resistance describes a situation where an organism possesses a set of special features that allows it to tolerate a particular drug or survive in an otherwise hostile chemical environment [42]. Mechanisms by which NTMs are intrinsically resistant to antibiotics include their thick, impermeable cell walls or their presence in biofilms and granulomas, which effectively decrease drug uptake, as well as the expression of proteins that specifically target clinically used antibacterial compounds.

On the other hand, acquired resistance refers to the case where a resistant strain emerges from a population that was previously drug-sensitive [42]. These events are usually related to the prolonged antibiotic treatments required to cure NTM infections. The acquired resistance is particularly severe for NTMs that only have a single copy of genes encoding common target proteins such as ribosomes, thus increasing the risk of acquiring protective mutations with single-drug treatments [4,43]. Here, we will focus on the mechanisms of mycobacterial physiology that make them naturally resistant to antimicrobial treatments since Nasiri et al. recently reviewed the mutations that may cause resistance to certain antibiotics in NTM [44].

Conceptually, resistance to antimicrobial drugs can be a result of one or more of the following mechanisms: decreased drug uptake, increased drug efflux, increased drug metabolism, or reduced drug sequestration (Figure 1) [45].



Figure 1. Schematic representation of the intrinsic drug resistance mechanisms in bacteria.

2.1. Drug Uptake

One of the most important factors responsible for the natural resistance of mycobacteria is its thick impermeable cell wall, which has an unusual structure: the peptidoglycan contains N-glycolyl muramic acid instead of the usual N-acetyl muramic acid, and the most abundant lipids are long-chain saturated fatty acids containing up to 90 carbons [46]. This causes an exceptionally high degree of hydrophobicity in comparison to the cell wall of other bacteria and therefore affects the uptake of compounds from the environment. For example, the rate of uptake of charged compounds in NTM can be as low as 1% of the rate of uptake in *E. coli* [46].

The outer membrane constitutes nearly a third of the total mycobacterial cell weight [47], and thus a great part of the energy generated by mycobacteria is used in cell wall synthesis and repair [48]. Consequently, mycobacteria with thick cell walls have less available energy for the production of new cells and thus show slower growth. This may help in explaining why slow-growing NTMs are generally more drug-resistant and persist more easily in a dormant state than their fast-growing counterparts [12,49]. The importance of the composition and permeability of the mycobacterial cell wall in its homeostasis is evidenced by the fact that the inactivation of genes traditionally related to metabolism also selectively affect the cell wall structure. For example, the inactivation of AsnB, which encodes an asparagine synthetase that is responsible for amino acid metabolism, disrupts the cell wall structure, thereby conferring hypersensitivity towards hydrophobic drugs in *M. smegmatis* [50]. Similarly, PknG plays an important role in imparting intrinsic resistance in *M. smegmatis* to multiple antibiotics by controlling the cell envelope structure, in addition to its role in cell metabolism [51].

In addition to proteins related to metabolism, the activity of proteins directly involved in the cell wall structure such as MtrAB [52,53], Pks12 and Maa2520 [54], and Fbpa [55] greatly affects the sensitivity of mycobacteria to hydrophilic drugs. Interestingly, deletion of the gene encoding Fbpa renders *M. smegmatis* particularly susceptible not only to hydrophilic antibiotics but also to hydrophobic ones because of the resulting increased fluidity of the envelope [56].

On the other hand, the thick mycobacterial cell wall not only provides a barrier for stressors but can also make it difficult for the bacilli to take up nutrients from the environment. Often, mycobacteria overcome this problem by the synthesis of porins–proteins that provide a narrow channel for the uptake of nutrients [57]. The expression of these porins in NTM has been linked to their growth rate [58]. Importantly, these porins provide a channel through which some antimicrobial compounds can enter into the mycobacterial cell [59]. For example, *M. smegmatis* mutants lacking porins have higher

survival rates inside phagocytic cells, presumably by evading the inflow of antimicrobial peptides and lysosomal enzymes [57,60]. Likewise, these porins can be entry points for small hydrophilic drugs like norfloxacin, chloramphenicol and β -lactam antibiotics. The loss of specific porins in *M. smegmatis* reduces the permeability of hydrophobic drugs (like vancomycin, erythromycin and rifampicin [61]) and significantly decreases the bacterial sensitivity to these antibiotics without altering the activity of their targets, thus causing a significant rise in the resistance to these antibiotics [61,62].

In addition to the cell wall, two of the most characteristic mechanisms to promote antimicrobial resistance in NTMs are related to their colony behavior: the formation of biofilms and granulomas.

NTMs are efficient biofilm producers, as evidenced by their frequent recovery from surfaces of, for instance, water pipes, showerheads and healthcare equipment. [63–65]. Some studies indicate a link between biofilm formation and pathogenicity [66–68]. Biofilms enable NTMs to tolerate high dosages of antibiotics in their immediate environment: cells in biofilms are at least 10 times more tolerant than suspension-grown (planktonic) bacteria [69]. The precise reason behind this remains elusive, although it is speculated that the waxy lipid-rich extracellular matrix of the biofilm creates a strong physical barrier that blocks the penetration of drugs [41]. The potential increased horizontal gene exchanges between the closely interacting bacteria in the biofilms may also help with the spread of drug resistance [70]. Moreover, several species undergo actual cellular changes during biofilm formation that may be linked to the development of adaptive resistance, which is reversed when the bacteria are removed from the biofilm [71]. This may be due to the fact that some genes are expressed differently when the bacteria are grown in biofilms than in suspensions [72]. For instance, it has been suggested that the increased chlorine resistance of *M. avium* and *M. intracellulare* cells grown in biofilms is attributable to the changes in the cell wall, which in turn results from alterations in mycolic acid structures [73].

The formation of granulomas is the immunological hallmark of most mycobacterial infections. Essentially, a granuloma is a microenvironment comprising a variety of different immune cells that entrap the infecting bacilli to contain its spread [74]. Structurally, it mainly comprises macrophages, epithelioid cells and multinucleated giant cells, surrounded by a layer of T-lymphocytes [75,76]. Although NTM infections are more commonly associated with alveolar granulomas, disseminated NTM diseases sometimes result in granulomas in other parts of the body like the liver, especially in people with a history of tumors [77]. These structures present a major challenge to NTM drug therapy in two ways: they limit the penetration of the drugs into the bacteria inside the immune cells, and the anoxic conditions in the granuloma center promote physiological and morphological states that make them more tolerant [41]. Although granulomas can be viewed as a host defensive structure intended to eliminate the pathogen, they can also provide a niche for the prolonged survival of mycobacteria in the body: mycobacteria can survive for years in a latent state within the granuloma [78]. Eventually, the death of the infected cells in the granuloma creates a necrotic zone that disintegrates, thus providing an exit route for the latent bacteria to release back into the lung [79].

2.2. Drug Efflux

In addition to the cell wall's restricting capacity for entry of potentially harmful molecules into the cell, mycobacteria utilize efflux pumps to remove unwanted molecules that may still get inside [44]. From a biological perspective, efflux pumps are essential for physiological processes like cell-to-cell communication, cellular homeostasis, detoxification of intracellular metabolites and intracellular signal trafficking [80]. However, they also extrude drugs from the periplasm to the outside of the cell, rendering them ineffective. As a consequence, deletion of specific efflux pumps in *M. smegmatis* increases its drug sensitivity by as much as two to eight times [81]. Many efflux pumps have limited substrate specificity and can expel a wide range of structurally dissimilar substrates, thus conferring resistance to multiple drugs at once [44]. This efflux-mediated resistance has been

reported for a variety of drugs as fluoroquinolones [82], tetracyclines [83,84], erythromycin and rifamycins [81].

2.3. Drug Transformation and Sequestration

Enzymatic biotransformation of drugs into compounds having much lower antimicrobial activity on several mycobacterial species has been described for penicillin, fluoroquinolones, aminoglycosides, and rifampicin. Due to the presence of β -lactamase enzymes in mycobacteria, most β -lactam antibiotics such as penicillin and cephamycin cannot be used in the treatment of mycobacterial infections [85], although some exceptions like cefoxitin and imipenem with modified structures resistant to β -lactamase activity are still used [86,87]. Because of this reason, most β -lactams only show significant activities when used in combination with β -lactamase inhibitors [88,89].

An important class of deactivating enzymes include transferases that modify the drug in such a way that it becomes ineffective. Prominent among them are acetylating enzymes, which are responsible for imparting resistance in most mycobacterial species against a variety of drugs, including fluoroquinolones [90], isoniazid [91] and aminoglycosides [92,93]. Similarly, enzymes that modify drug compounds by transferring nitroso and phosphate residues have been identified for fluoroquinolones and aminoglycosides, respectively [90,93]. For NTMs like *M. smegmatis* and *M. abscessus*, the major determinant of innate resistance towards macrolide and rifampicin are Erm methyltransferase and ADP-ribosyltransferase (Arr), respectively [94–96]. The resistance to macrolides is particularly significant, given that most NTM therapies involve the use of macrolides as first-line drugs [97]. The erm genes cause methylation of the 23S ribosomal RNA, which in turn prevents the binding of macrolides to their target, the ribosomes. However, this is not the only mechanism that confers macrolide resistance. Mutations in the 23S ribosome itself often render macrolides ineffective [98,99]. These mutations are frequent in mycobacteria because they possess only one or two rRNA operons, and mutation in any one of them can sufficiently alter the ribosome in a way that the macrolide can no longer bind to it [100].

Although rifampicin remains a front-line drug for the treatments in most NTM infections, instances of acquired resistance in *M. avium* complex and *M. kansasii* are reported [44]. Mutations in the target of rifampicin (rpoB gene) are generally held responsible for this [101–103]. It also has been observed that the preference of rifampicin to inhibit one of the two rpoB promoters over the other facilitates increased rpoB expression from the latter, leading to the growth of more resistant lines [104]. However, recent studies suggest that some other mechanisms might be at play. It has been suggested that RNA polymerase binding protein A (RbpA) can shield the target from rifampicin by either overlapping with its binding site or causing a conformational change to prevent any interaction [105].

3. Models for Drug Discovery against NTM

There is a wide range of techniques that can be employed in the development of new potential antimicrobial therapies against NTMs. These techniques can be classified depending on the tools employ between in silico, in vitro, or in vivo. In general terms, in silico techniques are useful to generate new leads and narrow the search of potential candidates based on prior information at the start of a study or to optimize compounds based on specific targets via virtual simulations. These leads can then be tested for efficacy using standardized in vitro analysis, which allows the determination of their potential antimycobacterial activity. Finally, in vivo animal models can be used to recreate infection environments and are therefore interesting for preclinical evaluation of potential compounds. We summarized the main attributes for each category in Table 2. A recent review by Rampacci et al. explains in detail the different techniques, assays, and preclinical models against NTMs that have been developed, with an emphasis on the newer models [106]. We direct the reader to that review for an in-depth description of these methodologies and their read-outs. Here, we will give a brief outline of the most common techniques

implemented in the lab and how they complement each other to create an integrated pipeline for drug discovery.

Table 2. Summary of the current methods available for the discovery of new antimicrobial therapies in NTMs.

	In Silico	In Vitro	In Vivo
Methods employed	Structure-activity relations, Molecular simulations, Comparative genomics	Antimicrobial effect tests on cultured cells	Tests on live infected animals
Main insights	Molecular basis for drug action	Molecular and cellular effect of drug action	Whole-organism level of drug action
Advantages	High throughput, low-cost, no need for actual chemical synthesis of compounds or bacterial growth	Relatively simple systems and lower cost and time involvement, easy to handle, scalable	Closer to the actual physiological environment
Limitations	Requires prior information and complicated models to simulate molecular events such as docking and drug-target interactions	Needs a high level of standardization and careful experimentation for reproducibility, may not reproduce clinical situations	Requires careful model selection, large organism response is less predictable, ethical considerations, high economical costs
Best-fit stage in drug discovery	Primary (for narrowing the search of potential candidates) or secondary (for optimizing compounds to species-specific targets)	Secondary (for screening initial targets and efficacy determination)	Tertiary (for preclinical evaluation)

3.1. In Silico Predictions

Computational methods are commonly used in the drug discovery process for the identification of suitable drug targets. The targets can be identified at different levels, ranging from molecular to cellular to whole-organism levels [107]. Once these targets are identified, suitable molecules that interfere in its working can be identified [108]. In silico methods can facilitate faster drug development by making predictions for a large set of drug candidates without the need of chemically synthesizing each of the compounds [109]. Moreover, they can bring in-depth molecular level insights that can allow for even more targeted drug development [110].

These methods can be especially useful in the case of strains that are not culturable or have long cultivation periods. For instance, the tuberculous mycobacteria *M. leprae* has an extremely slow doubling time in almost all available growth media and can only be inoculated in cold-like environments like the body of armadillos or hind footpads of mice [111]. Recent studies have demonstrated the importance of computer simulations in understanding drug resistance in *M. leprae*. Using molecular docking simulations, it was shown that certain drugs like rifampicin and ofloxacin bind less effectively to the drug-resistant mutant *M. leprae* strain as compared to the native strain due to loss of a hydrogen-bonding site in the target of the drug [112–114].

This approach to drug discovery is generally divided into two categories depending on if the 3D structure of the target is known or not: structure-based drug discovery (SBDD) and ligand-based drug design (LBDD), respectively [115]. SBDD methods are useful to discover the molecular basis of drug action or to optimize compound derivatives for a specific species. Usually, the target 3D structure—identified either from experimental data such as nuclear magnetic resonance (NMR) or X-ray diffraction spectroscopy or through homology modeling—is used to identify potential binding pockets [116,117]. On a molecular level, these simulations can elucidate how a point mutation on a target can lead to structural variation, which ultimately influences the effectiveness of a drug [118]. This approach was successfully used to evaluate 11 tetrahydropyridine compounds as antimicrobials for *M. abscessus* [119]. Since the mechanism of action of THP is known–inhibiting the efflux

pumps MmpL5 and Tap–, the binding sites for the drugs were identified on the pumps by docking simulations. Another method, molecular dynamics simulations [120], was recently employed to understand how the "predisposing" proteins present in certain populations make them susceptible to *M. avium* subsp. *paratuberculosis* infections [121]. The simulations could identify the exact residues where binding of mycobacterial and host proteins take place, which may open possibilities to target it specifically by suitable drugs.

Contrasting, LBDD methods can be employed even when the 3D structure of the target is not known, thus being excellent tools for the generation of initial leads. Essentially, from previously known ligand structures and their bioactivities, predictive models are created, which can be subsequently used to assess the viability of new ligands [122]. Most frequently, LBDD uses the structure–activity or structure–property relationship (SAR/SPR) studies, wherein the chemical structure of the ligand is correlated to its activity (or property) from a model developed from previously acquired data. The SAR approach was successfully used to evaluate a series of piperidinol derivatives [123], based on a previous finding that piperidinol efficiently works against *M. abscessus* and *M. tuberculosis* by targeting the mycolic acid transporter MmpL3 [124]. A series of similar compounds were synthesized and tested in vitro, and the data were used to create a SAR model to guide the design of subsequent derivatives, as well as to identify the molecular sites that can be effectively modulated.

Finally, comparative genetics is an important tool that should be explored further. In this technique, the genomes of pathogenic species are compared with non-pathogenic species to identify unique genes that encode potential virulence factors [125]. For example, a study of the genome of *M. abscessus* has led to the identification of several "non-mycobacterial" virulence genes that are likely acquired by the horizontal gene transfer (HGT) from other pathogens like *Pseudomonas aeruginosa* or *Burkholderia cenocepacia* [93]. These virulence factors can be an important target for potential new drugs and vaccines [126]. An important challenge in the discovery of new drugs for NTM is the lack of whole-genome information on different strains, although the situation is rapidly changing [127]. Indeed, this can mark a paradigm shift in NTM drug discovery as WGS has been shown to predict species and drug susceptibility with remarkably high accuracy [128,129].

3.2. In Vitro Susceptibility Testing

The first step towards the prediction of success or failure of a new antibiotic therapy is antimicrobial susceptibility testing (AST) in vitro. These tests measure the growth response of isolated organisms to a particular antimicrobial therapy. They are relatively cheap, easy to replicate, and scalable [130]. In addition, they are also relatively fast: the optimal incubation times for the broth microdilution method range from 7 days (at 28 to 30 °C for *M. marinum*) but can reach 6 weeks for the slower growers like *M. ulcerans*. The most widely accepted protocol for AST of NTMs is published by the Clinical and Laboratory Standards Institute (CLSI), which has recommended the use of microdilution as the gold standard for the determination of antibacterial susceptibilities [131]. Other methods are not recommended for testing antimicrobial effects against NTMs. For example, although commonly used for *M. tuberculosis*, the proportion method often yields misleading results for NTMs [132]; the agar disk diffusion method carries the inherent difficulty in the interpretation of zones of inhibition, especially when the amount of drug in the disk is near the breakpoint of the drug [133]; and the epsilometer test is quite rapid and simple, suffers from lack of reproducibility and exaggeration of drug susceptibility as determined by other techniques due to tailing of the ellipses [134,135].

There is controversy about the role of in vitro susceptibility testing for NTM diseases. This is mainly due to the unpredictable correlation between in vitro and clinical outcomes: correlation is particularly poor for *M. abscessus* and *M. simiae*, while it is reasonably satisfactory for *M. kansasii*, *M. marinum* and *M. fortuitum*, and for other species such as *M. avium* complex, the correlation holds good only for certain drugs like macrolides, but not for

others [4]. This disconnect probably stems from a multifactorial origin ranging from strain selection and testing conditions to the usual absence of host effects in the tests.

For example, in vitro, antibiotic testing using the broth microdilution method is usually performed with exponentially growing mycobacteria as a suspension under optimal conditions in an aerated nutrient-rich broth, which hardly bears any resemblance with the actual host environment [41]. In addition, there are practical considerations that must be considered. An important fact about NTM is that owing to the intrinsic hydrophobicity of their cell walls; they generally attach to the surface of the individual wells in the 96-well plates rather than staying in the aqueous suspension [136]. Therefore, merely measuring the turbidity of cell suspension can lead to inaccurate results, and results can be different from the ones from cells present in surface-attached biofilms [48]. Although microdilution continues to be the most trusted in vitro method, it does have some drawbacks, which include: a large volume of reagents, long experimental time, the possibility of false positives due to long incubation times, chances of cross-contamination, etc. [137].

Strain selection is a crucial aspect of in vitro testing. Typically, *M. smegmatis* is used for laboratory testing and modeling of NTM disease pathogenesis due to its rapid growth rate and ease of handling [138]. However, most isolates of *M. smegmatis* are derived from the same ATCC 607 strain, a strain that has become "lab adapted" by losing several unique NTM features like slow growth and the cell-wall hydrophobicity. As a result, the susceptibility to specific compounds can be seriously overestimated if only typical lab strains are taken as a reference [48]. This, however, should not completely disregard the use of these strains, as, for example, bedaquiline was discovered by using *M. smegmatis* as a model [139]. Moreover, strains of the same species obtained from different sources can have different growth rates, thus affecting the quality of the model used to study the disease. Similarly, two patients infected with the same strain can have different susceptibility to the same antibiotic because of differences in immunity [140]. It is therefore recommended to validate drug susceptibilities in a panel of NTM isolates to increase the chances of selecting strains with the closest growth profile as the isolate of interest [131,141].

During antibiotic susceptibility testing, it is important to remember that the growth rate of the strain can have a significant impact on its susceptibility towards the drug. For example, *M. avium* bacteria are more susceptible in media that supports faster growth than in nutrient-limited medium [142]. Similarly, it is important to note that the same strain may have different colonial variants, and these can have remarkably different susceptibilities. For instance, in *M. avium*, transparent colonies that are usually obtained from isolates of patients are more antibiotic-resistant than the opaque variant that appear during laboratory cultivation [143]. Therefore, these results must be taken with caution: high susceptibility levels in vitro may not necessarily imply an effective in vivo outcome [144].

Indeed, the discrepancy between in vitro and in vivo results is evidenced by the lack of effect of moxifloxacin in *M. abscessus* in vivo, despite encouraging in vitro results [145]. On the other side, cefoxitin and imipenem showed only moderate in vitro activity against *M. abscessus* but are nonetheless effective in vivo, possibly to the differential testing conditions [94].

3.3. In Vivo Models

Following in vitro tests, animal models are used for preclinical in vivo testing. Animal models are used essentially to understand the pathogenesis, host immune responses, and for testing potential antimicrobial compounds and vaccines [146]. Cell-based assays provide restricted information about the absorption, distribution, metabolism, excretion and toxicity of screening compounds, but results obtained from animal models often reveal such insights. Here, a persisting issue is the paucity of suitable animal models available for studying NTM pathology [147,148]. Because of the low virulence of NTM compared with *M. tuberculosis*, it is usually difficult to generate an infection in animals unless they are severely immunocompromised since, for instance, lung pathology can only be observed to some extent [149]. This, however, leads to a complication. Since NTM infection in

humans may manifest as localized lung infections (in immunocompetent persons) or disseminated infections (in immunocompromised persons), the animal models should be chosen according to the disease of interest [150]. However, only immunocompromised models can sustain low pathogenic NTM infections and therefore, it is generally very difficult to simulate chronic, localized NTM diseases [146]. The overall consequence of the above problems is inconsistent results that are difficult to reproduce. Ideally, animal models that possess hallmarks of human NTM pathology are required for better in vivo results in the testing of NTM anti-mycobacterials [41].

Apart from the traditional animal models—mice, guinea pigs, and rabbits; the recent development of cellular models as well as using the zebrafish embryos have proven to be useful alternatives [146,147,151]. Depending on various parameters, every animal model has its own advantages and disadvantages and hence, some are more valuable in testing potential antimicrobial compounds than the others [146].

Mouse models are widely used because of the abundance of reagents and their lowcost, and they have been instrumental in understanding the host immune response to tuberculosis [146]. Since there are two major categories of NTM diseases (lung disease and extrapulmonary-disseminated disease), the mouse strain that is chosen depends on the disease of interest, despite the fact that it is difficult to mimic chronic NTM infection, which is exclusively isolated to the lungs [146]. Earlier studies also revealed the most immunocompetent mouse strains, like C57BL/6, serve as excellent models for more virulent *M. avium* complex species, but are cleared when infected with *M. abscessus* [152]. In these studies, C57BL/6 and leptin-deficient (Ob/Ob) mice that were infected with M. abscessus (with low-dose aerosol inoculum) did not develop a sustained infection; while on the other hand, when infected with high-dose aerosol inoculum, these mice developed an infection that was subsequently cleared [147,153]. Using severely immunocompromised mice as a model is advantageous due to the presence of foamy cells and necrotizing as well as non-necrotizing granulomas in the lungs, 40 days post-infection, which is observed in histopathologic sections of human NTM lung disease [127]. However, one of the challenges is that most strains are able to clear infections by *M. abscessus* within the first few weeks post-infection, which makes the development and selection of the model extremely challenging [152–154].

For studying Buruli ulcers, a chronic NTM infection caused by *M. ulcerans* that infects the skin, soft tissues and bone in humans, mouse models are the most used [155]. Other models, such as guinea pigs, have also been used as models for studying Buruli ulcers and characterizing the pathogenicity of *M. ulcerans* and its mycolactone toxins [156–158]. Nevertheless, they are much less commonly used because of their resistance to *M. ulcerans* infections [155].

Zebrafish has successfully been established as an efficient model to study infectious diseases in the last decades [159]. Their embryos offer unique in vivo imaging possibilities due to their transparency, and a high number of existing transgenic reporter lines expressing fluorescent proteins permit tracking various immune cell types while they interact with pathogens [160–163]. Importantly, zebrafish larvae rely only on innate immune defenses during the first weeks, thus being attractive models to study infections that require the host to be immunosuppressed to a certain degree. Zebrafish models permit high-throughput analysis and therefore are convenient for the initial steps of preclinical evaluation. One of the most studied zebrafish infection models is the zebrafish tuberculosis model. Infections conducted on zebrafish have revised our interpretation of the mechanism of granuloma formation by allowing real-time visualization of the biological events that take place inside the host, in particular the pathogen-macrophage interactions [164]. However, it is important to mention that these models, in most cases, utilize *M. marinum* to simulate *M. tuberculosis* infection, and studies of pathogenesis on this model may be, therefore, applicable to other mycobacteria. Considering the common ancestry of M. marinum and M. ulcerans, future research could also focus on the difference of specific virulence determinants of these strains, such as the importance of mycolactones that are encoded on plasmids specific for the *M. ulcerans* lineage. In addition to *M. marinum*, pathogenesis models of *M. kansasii* [165] and of *M. abscessus* [166,167] exist in zebrafish, which can be used for high-throughput drug screen processes.

Since most animal models develop disseminated infections instead of localized infections seen in humans, the search for a robust NTM model is not yet complete. Non-human primates have been used as models to study NTM infections because of their closer resemblance to human immunology and physiology. Rhesus macaques have been shown to develop isolated pulmonary infections that tend to persist for long, similar to NTM pathogenesis observed in humans [168]. Similar observations have also been made for marmosets [169]. Nevertheless, in addition to ethical concerns, their availability, purchase and husbandry cost present a practical limitation to their use [170]. Moreover, smaller sample sizes of these animals used in disease studies may lead to statistically insignificant results, and even small genetic changes can cause greater variance [171].

3.4. Iterative Approach to Drug Design

Despite using different tools and giving different experimental insights, the drug discovery process is usually an iterative optimization of the techniques presented previously (Figure 2). For example, the first step towards the discovery of new antimicrobial therapies is usually the screen of compounds that show potential for inhibiting mycobacterial growth. Since it is not possible to try every compound for activity against NTM, narrowing the search space is important to save time, money, and resources. This can be done in silico, based on drug-target interactions or structure–activity relationship models [172–174]. The opposite approach is also viable: libraries of bioactive compounds with unknown effects in NTM infections can be screened in vitro, and the results used to improve computational models and predictions. Similarly, information about in vivo drug pharmacokinetics is an important step during a preclinical study for a new therapy. Understanding system and drug-specific properties and modeling them with systems biology or systems pharmacology models provide important information that potentially can speed up the drug development process [175].



Effectivity and safety assessment

Figure 2. Overview of the development process for new therapies against NTMs.

Recently, artificial intelligence methods such as machine learning have been used not only to estimate the bioactivity of drug candidates [176] but also to complement existing tools across different stages in the NTM drug discovery process, for example, in automating the cell count from fluorescence microscopy imaging and identification of mycobacteria species from mass spectroscopy [177,178].

4. Considerations for the Design of Therapies against NTMs

4.1. Optimization of Known Compounds Relevant for Combatting NTM

When designing new possible therapies against NTMs, it is important to consider the mechanisms that confer drug resistance that we reviewed above. For example, the exceptionally high hydrophobicity of mycobacterial cell walls has an important bearing on drug design: the more lipophilic molecules generally show higher permeability and hence are more active. This means that a possible route to developing anti-NTM antibiotics is to synthesize hydrophobic derivatives of existing antibiotics [179]. For instance, ciprofloxacin, when modified by the addition of hydrophobic alkyl substituents, showed higher activity against *M. avium* [180]. Similarly, for *M. leprae*, the efficacy of fluoroquinolones improved by incorporating the hydrophobic cyclopropyl groups [181].

Compounds that have been identified to be active against other diseases may directly be screened by in vitro bacterial assays, and MIC values may be determined to check the efficiency of the drug [182]. However, for *M. abscessus*, the hit rates among drug libraries that are active against neglected diseases like ascariasis, Buruli ulcer, Chagas disease, and malaria is just 1% [183], highlighting the great difficulty in finding new drugs for NTMs. A way forward could be to screen the compound libraries active against tuberculosis for their effect against NTM because of the structural similarity and homology of their drug targets [184]. In a recently conducted study, 129 compounds known to be active against *M. tuberculosis* were tested against *M. abscessus* and *M. avium*, and their rates were higher than for drugs that are not active against tuberculosis [185]. Rifabutin, an antibiotic used for the treatment of tuberculosis, has recently been found to be active against *M. abscessus* [182]. Notwithstanding these positive outcomes, most existing drugs specific to tuberculosis are usually ineffective against NTM [185,186].

4.2. Synergies and Combination Therapies

In the development of a novel treatment, it should be considered that most of the successful anti-NTM drug therapies involve synergistic effects of two drugs: one antibiotic to disrupt the permeability of the outer membrane in order to ensure entry of the drug into the cell, and the another disrupting at least one vital cellular processes (such as DNA, RNA, or protein and outer membrane synthesis) for inhibiting cell growth [48]. For example, the performance of hydrophobic drugs that have intracellular targets can be improved by using them in conjunction with compounds that specifically target cell wall homeostasis. Such synergistic effects have been observed between ethambutol plus rifampicin in *M. avium* [187] or vancomycin plus clarithromycin in *M. abscessus* [188]. Similar effects can be seen using adjuvants that inhibit specific efflux pumps [189,190] or that increase the expression of the enzymes required for the biotransformation of a prodrug, thus boosting antibiotic effectivity [191].

Moreover, the synergistic effects cannot only improve drug efficacy but also reduce the chances that treatments lead to drug resistance [192,193]. For instance, the combination of β -lactam antibiotics and β -lactamase inhibitors has shown to be a promising strategy against *M. avium* infections [89]. Further, synergies can also be achieved by using a combination of two or more drugs that have the same cellular target. This approach has been validated for *M. abscessus* complex, wherein a dual β -lactam drug regimen proved to be much more effective than a single-drug regimen, with or without β -lactamase inhibitors [88,194]. In such regimens, each of the β -lactams preferentially targets a different enzyme that is involved in cell wall synthesis, thereby ensuring that "overlapping" effects are minimized, and combinedly, all biochemical pathways are exhaustively targeted [194]. Typically, mutations that cause the development of resistance mechanisms can have subsequent "spill-over" effects: the same mechanism may confer resistance to the entire class of drugs that target the same biochemical pathway (cross-resistance), or it can lead to increased vulnerability to other drugs that target a different pathway (collateral sensitivity) [195]. Exploiting collateral sensitivity by either combinatorial or cyclical treatment regimens

involving multiple drugs can be an effective strategy, as recently demonstrated with drugs directed against *M. marinum* [196].

4.3. Host-Directed Therapies

A different approach towards finding more effective treatments against NTM infections is host-directed therapies (HDT), in which specific immune pathways of the host are modulated in such a way that it leads to a better clinical outcome. That is, HDTs aims to empower the host to clear the infection instead of directly targeting bacteria. The ways in which HDTs can help against NTMs are strengthening innate immunity against mycobacterial infections, preventing the growth of the bacilli by inhibiting the essential host-related growth-factors, restoring the immune response suppressed due to the infection, or reducing tissue damage due to hyperinflammation [197,198].

HDTs offer several unique advantages over conventional therapies. First, chances of drug-related resistance are considerably reduced because it is difficult for the bacteria to develop completely new mechanisms of interacting with the host quickly and while being under the same hostile immune selection [199]. They also offer the possibility of making conventional drugs more effective against already resistant strains by neutralizing pathogen defenses [200]. The synergistic role of HDT adjuvants with anti-mycobacterials was demonstrated in a study in which picolinic acid (PA) was shown to potentiate fluoroquinolones against bacteria from the *M. avium* complex [201]. Fluoroquinolones are otherwise only very weakly effective against *M. avium* infections. This was attributed to two factors- upregulation of the immune system by PA and chelation of Fe ions by PA, which deprive the bacteria of the essential ions needed for growth [202]. In a previous study, PA was shown to inhibit *M. avium* growth inside mouse macrophages by inducing apoptosis- causing morphological changes [203].

In addition, some compounds show both host-directed and bacterial-directed actions. Clofazimine, a commonly used drug in *M. leprae* infections, is a good example of a drug that simultaneously affects the host as well as the bacteria. Upon infecting the body, *M. leprae* creates a safe microenvironment for itself inside the macrophages of the host by increasing the accumulation and retarding the breakdown of macrophage lipids [204]. It was shown that clofazimine not only helps reverse these two processes but also activates immune reactions in *M. leprae* infected host cells. Hence, effectively, it not just prevents the growth of the bacteria but also actively helps to eliminate it [205]. Another example of an antibiotic with a strong effect on the host inflammatory system is minocycline that modulates the endocannabinoid signaling pathway and, in this way, might have HDT potential [206].

Finally, considering the mechanisms of defense of the host system can lead to more effective ways of delivering drugs to the target, adding value to existing treatments. An example would be precision-targeting the drug by loading them into the host cells that act as carriers. This approach was demonstrated by loading dendritic cells to deliver amikacin inside alveolar granulomas and thus enhancing the killing of residing mycobacteria [207].

However, a major challenge in the development of effective HDTs is that different patients may not have the same immune status, which may depend on factors like the stage of the disease, health of the individual, pre-existing conditions, and genetic makeup [200]. This can be a hindrance towards a universal HDT and may require an approach for personalized medicine, much like cancer immunotherapy [208].

5. Summary and Future Perspectives

The discovery and validation for new therapies against NTMs is an urgent necessity as increasing cases of these infections are being reported worldwide, and existing therapies prove to be ineffective. We discussed the reasons why the development of effective antimycobacterial drugs remains elusive: their intrinsic resistance against antimicrobial compounds. Several molecular mechanisms are used by mycobacteria to survive current antibiotic therapies, including a thick impermeable hydrophobic cell wall that acts as the first line of defense; intracellular enzymes that reduce the antimicrobial effect of the drug; efflux pumps that expel molecules from the cytoplasm; and adaptive mechanisms that prevent drugs from sequestering its target. In addition, even if the drug is effective in killing mycobacteria or inhibiting their growth, mycobacterial colonies can persist on surfaces by forming inert biofilms or enter latent states within the granulomas inside the host.

We delineated the main techniques and models that can be used for the development of new effective therapies against NTM and how they can complement each other in different stages of the drug development pipeline, thus accelerating drug discovery. For example, whole-genome sequencing can provide crucial leads in target identification based on the genetic makeup of the strains, which can be followed by in silico drug-target interaction studies to identify the potential drug molecules that can effectively dock on the target and initiate action. Validation of these drugs and the determination of their efficacy would require testing on clinical isolates, taking into account variations arising due to different colonial morphologies, and media-dependent growth rates, among others. To fully understand the mechanism of drug action, suitable animal models are very important. Moreover, insights on in vivo infection growth can help to select the relevant drug regimes that focus on the specific touchpoints of the mechanism, rather than a general broadspectrum therapy. Finally, it is important to mention that when dealing with mycobacteria, an effective drug regime would include not only one drug but must work in combination with other antimicrobials or host-directed therapies, thus improving drug activity while preventing the buildup of resistance against any single drug.

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