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A Medicinal Chemists' Guide to Lead Optimization for Tuberculosis

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Abstract

Tuberculosis is a bacterial disease which predominantly affects the lungs and results in extensive tissue pathology. This pathology contributes to the complexity of drug development as it presents discrete microenvironments within which the bacterium resides, often under conditions where replication is limited and intrinsic drug susceptibility is low. This consolidated pathology also results in impaired vascularization that limits access of potential lead molecules to the site of infection. Translating these considerations into a target-product profile to guide lead optimization programs involves implementing unique *in vitro* and *in vivo* assays to maximize the likelihood of developing clinically meaningful candidates.

Globally tuberculosis (TB) ranks as the tenth most common cause of death in the world, killing 18 of every 100,000 humans annually, placing it second only to HIV/AIDS among lethal infectious diseases¹. An astounding 2 million people perish every year despite the existence of curative chemotherapy. TB is widely viewed as a disease of the developing world and death rates are often attributed to failure of overburdened public health systems to deliver appropriate care to infected individuals². While this is certainly a contributing factor, it is worth noting that standard TB treatment for simple, uncomplicated disease consists of combination chemotherapy with four agents (isoniazid, rifampicin, pyrazinamide and ethambutol) for two months followed by an additional four months of isoniazid and rifampicin³. The logistics of delivering such complex regimens, and ensuring patient compliance with the full course of chemotherapy, challenge the resources of the public health sector even in most developed countries⁴. Inevitably as this complex therapy is administered under less than ideal conditions, drug-resistant forms of the disease have become increasingly common, leading the World Health Organization to declare multidrug-resistant (MDR) TB a global threat⁵. While it is easy to ascribe this to a failure of governments to mobilize sufficient resources to deliver curative treatment, it is equally reasonable to blame the lead optimization programs that resulted in candidates that require such extended durations of treatment.

The current investment in TB drug discovery is unprecedented and last year saw the approval of the first new agent for the treatment of TB in three decades⁶. Many other agents are in preclinical development and clinical trial results are beginning to emerge for agents that were developed using current thinking about criteria adopted in lead optimization. How successful has our current paradigm been in predicting the results emerging from these clinical trials? How might we amend our strategies to improve our chances of actually shortening therapy? In this *Digest* we will review current assays used in lead optimization, how contemporary agents fare in these and other more exploratory assays, and how these results square with the emerging clinical trial data. We will focus this manuscript on TB-specific considerations and not attempt to cover all aspects of lead optimization.

The current paradigm

According to a recent survey of 25 institutions involved in preclinical drug development for TB, most current strategies largely center on assessing minimal inhibitory concentrations (MIC), which is typically defined as the concentration of an agent that inhibits growth of 99% of a standardized inoculum of a laboratory strain of *Mycobacterium tuberculosis* (*Mtb*)⁷. Most laboratories use standard rich liquid growth media containing glucose, glycerol, a detergent (Tween-80) and bovine serum albumin, and score growth visually. Most investigators utilize minimum bactericidal activity (MBC) measurements to assess hit compounds, where “cidal” activity is enumerated by measuring the drug concentration required to effect a 1 or 2 log reduction in bacterial counts after a defined exposure period. Other assays such as activity of compounds against TB-infected macrophages or activity under non-replicating conditions are rarely employed and seldom used to prioritize leads.

Potent compounds are often developed based solely on MIC/MBC criteria and subjected to optimization for traditional *in vitro* ADME parameters with the goal of achieving proof of concept in an animal model. Although historically other species such as Guinea pigs were employed, currently nearly all groups working in this area utilize mouse models of disease and then progress directly to clinical development. While this simple and linear progression strategy – often referred to as the “three Ms” for MIC, Mouse and Man (Figure 1) – is attractive in its simplicity, it ignores many of the important subtleties of the disease and promises to deliver only more of the same kind of agents we've unearthed in the past. Moving beyond this to a regimen that would potentially really shorten treatment duration and have a large public health impact requires a more deliberate and thoughtful approach. We'll consider the TB specific subtleties from two viewpoints; first from the perspective of the physiology of the pathogen and its impact on the intrinsic susceptibility of the bacterium to drug killing, and second from the perspective of the complex pathology generated by the host that is required to contain the infection within discrete lesions.

Factors associated with the pathogen's physiology and metabolism

The lifecycle of an infection – variable microenvironments

Even under conditions where the causative agent is maintained in a non-replicating state *in vitro*, a greater than 90% kill can be achieved by standard drugs in less than a week⁸. Why then does it require six months of multiple antibiotics to sterilize a patient's lungs? One potential explanation lies in the variety of microenvironments within which the bacterium resides during the infectious cycle. Initial infection results when *Mtb* is engulfed by pulmonary macrophages residing on the airway surface⁹. The bacilli arrest development of the phagosome within which they arrive, blocking the normal acidification of these degradative vacuoles at pH 6.3. For this initial round of replication, lung macrophages are largely unactivated and mount only a weak burst of reactive oxygen and nitrogen species, but as the innate immune system engages during subsequent rounds of infection and replication, these defensive host molecules become increasingly abundant¹⁰. During this initial period, the bacteria have access to glucose, probably triacylglycerides, and normal lung concentrations of oxygen to drive respiration. As immune activation proceeds, bacterial replication slows in response to increasing stress from the host resulting in a phenotypic form of drug tolerance where specific processes such as cell wall synthesis become much less sensitive to drug action¹¹.

Immune activation triggers signaling pathways that recruit lymphocytes and more macrophages to the infection site that ultimately assemble into the complex structure known as a granuloma (*vide supra*). This multicellular structure serves to wall off and contain the bacteria, slowing their replication further and reducing their vulnerability to drug-mediated

killing even further. The interior of the granuloma ultimately becomes necrotic (often referred to as 'caseous') and severely hypoxic with pO_2 values down to less than 2mm Hg¹². Under these conditions, normal aerobic respiration becomes impossible for the bacterium which reverses its TCA cycle to enable fermentative secretion of succinate in order to maintain an energized membrane^{13,14,15}. Analysis of the lipid composition of this caseous material has revealed that the most abundant materials are cholesterol, triacylglycerides and glycosphingolipids¹⁶ (Figure 2).

Alternative assays to develop leads specific to relevant bacterial subpopulations

In vitro Mtb can be made to grow (or simply survive) on a wide variety of carbon sources, across a range of pH values, in the presence of oxidative or nitrosative stress, and under an array of oxygen tensions. All of these variables affect the growth rate of *Mtb* and result in broad remodeling of bacterial metabolism that directly impacts bacterial susceptibility to various classes of drugs. At the extremes of these micro-environmental conditions, no bacterial replication occurs at all and this has been used to create models of "non-replicating persistence" such as the Wayne hypoxic culture model where the bacteria are allowed to fully consume all available oxygen, completely arresting their growth¹⁷. Standard MIC measurements capture the activity of a compound only against organisms that are rapidly replicating under aerobic conditions on a rich mixture of carbon sources, a highly non-physiological situation. These observations have motivated some complex screening campaigns against organisms within macrophages¹⁸ or grown under conditions that more closely resemble that within a lesion¹⁹. Such screens are highly informative but are challenging to conduct on large numbers of compounds because they either involve outgrowth dilution assays, or high-content imaging and detailed data analysis.

Pursuing a hit that is specifically active under a particular screening condition, especially one that involves non-replicating bacteria, is rarely done because of long cycle times required to generate assay data. Although one could question whether this approach was worth such an effort, it is notable that the only example of a condition-specific drug currently in use for TB therapy is pyrazinamide. Pyrazinamide has no measurable activity under standard rapidly replicating culture conditions but when the media is adjusted to a pH of 5.5, the drug becomes weakly active and this is further accentuated under hypoxic conditions²⁰. Pyrazinamide has the distinction of being one of the most "sterilizing" drugs ever introduced into TB regimens. Addition of this drug dramatically reduced the relapse rate and, in combination with rifampicin, allowed for treatment to be reduced from 2 years to six months²¹. Pyrazinamide made it into clinical use simply because there was nothing else that could be paired with isoniazid and streptomycin at the time. This incredibly valuable drug would not be discovered in today's screening programs or lead optimization campaigns, yet this may be precisely what is needed to achieve significant treatment shortening. Pyrazinamide activity at pH 6.3 (the pH of an arrested phagocytic vacuole in macrophages) is extremely poor and the site of infection where this low pH is achieved remains elusive. Nonetheless, pyrazinamide illustrates the value of a condition-specific lead molecule with treatment-shortening potential. While it remains a formal possibility that pan-active molecules will be identified in the future, thus far most broadly active agents inhibit macromolecular synthesis while most macromolecular processes are slowed, or fundamentally altered, under many clinically relevant environmental conditions.

The value of cidal activity in prioritizing leads

"Cidal" activity, where a compound actively kills bacteria, is often employed as a criterium for lead selection yet even the most casual examination of the clinical utility of TB chemotherapeutics fails to support this use. Pyrazinamide would, of course, fail to meet the standard as a cidal agent. Amongst agents more recently developed, the fluoroquinolones

have potent cidal activity *in vitro*²² yet when substituted for either ethambutol in drug sensitive disease, or when added to MDR regimens, there is no evidence that they improve patient outcomes at all²³. The converse is equally true; linezolid is a static drug *in vitro* yet when added to failing regimens of patients suffering from untreatable chronic extensively drug resistant (XDR) disease, it has a profound effect²⁴. Mechanisms of cell death in general, and specifically in TB, are much more complex than previously appreciated. In the absence of host immune pressure, under laboratory conditions that resemble no micro-environment known to exist within the host, the cidal potential of a given lead series is not a robust criterium for prioritization.

Factors associated with lung pathology and multiple sites of infection

TB pathology is diverse and complex

The six-month duration of TB chemotherapy is dictated by the rate of disease recrudescence following the discontinuation of therapy, commonly referred to as the relapse rate. With the current four-drug standard of care, relapse rates are generally less than 5% in controlled trials. Analysis of factors that contribute to this relapse rate across large cohorts of patients points to two dominant features: the rate of clearance of bacteria from the sputum (generally at two months) and radiographic extent of disease, in particular the presence of cavities (hollowed out lesions in the lung where the tissue has been destroyed and surrounded by a thick, often fibrotic, wall)²⁵. The question of drug exposure at the multiple sites of pulmonary TB disease has recently emerged as a likely contributing factor to the long duration of therapy and failure to sterilize specific infection sites.

The pathology of TB is very complex and driven by the immune response (rather than by damage directly inflicted by the pathogen). The hallmark of TB pathology is the granuloma, a dynamic collection of immune cells that organize themselves in an attempt to wall off the infecting pathogen (Figure 2). In the majority of infected individuals, this attempt to contain the infection is somewhat successful in that the pathogen remains confined to primary lesions in a quiescent state, leading to latent TB infection (LTBI). Globally, approximately 10% of infected individuals progress to post-primary reactivation at some stage in their lives leading to active and chronic disease. Radiology and autopsy findings collected over many decades have been collectively interpreted to provide a reasonable understanding – with knowledge gaps yet to be filled – of active disease progression and corresponding histopathology^{26,27}. In the current view, lesions evolve from fully cellular granulomas made of lymphocytes, macrophages and neutrophils, to necrotizing or ‘caseous’ granulomas. The necrotic core, also called ‘caseum’ due to its cheese-like appearance, results from the combined lysis of both host cells and pathogens. It is initially formed in the very center of the granuloma, and spreads outward compressing the surrounding lung tissue and vasculature. As they age and develop, granulomas often develop extensive layers of fibroblasts, which further surround the pathogen and heal inflamed or damaged lung tissue. A cavity develops when an expanding granuloma comes in contact with an airway, leading to the formation of a fibrotic wall filled with liquefied caseum directly connected to the luminal surface of the respiratory tract. Rupture of these cavities, which contain high numbers of bacteria, enables aerosol transmission, an essential step in the infection cycle of *Mtb* as an obligate pathogen (Figure 2). There is experimental evidence that live bacilli reside (i) in fully cellular granulomas, predominantly but not exclusively inside macrophages, as well as in extracellular niches, (ii) in the caseous center of necrotic granulomas where they are thought to be quiescent and extracellular²⁸, (iii) at the luminal surface of cavities²⁹ either intracellular or extracellular and within multiple cell types^{30,31}, and (iv) in lung tissue which might appear ‘uninvolved’ macroscopically but hosts a complex spectrum of inflammation and repair^{32,33}. Though there are certainly more variations – including sclerotic or calcified granulomas in which mineralization has replaced

all but the periphery of the lesion – this brief overview clearly underscores that the granuloma constitutes a friend/foe structure which both protects the host from overwhelming infection but also contributes to spreading the infection and makes it extremely difficult to eradicate using pharmacotherapy, due to sequestration of the bacilli within remote lesion compartments.

Drug distribution at the sites of infection is largely heterogeneous

A series of small clinical studies published in the 1950s to the 1980s showed that concentrations of INH and RIF, the two pillars of anti-TB therapy, in the diverse and sequestered lesions of TB patients may be substantially lower than plasma concentrations³⁴⁻³⁶. These and other studies also indicate that the extent of distribution is both lesion- and drug-specific, as was convincingly demonstrated in abscesses^{37,38}. The abscess analogy is highly relevant to TB pathology given the structural similarities between abscesses and necrotic granulomas: an outer fibrotic wall, inner layers of leukocytes and a central area of necrotic debris³⁹. Results obtained by our group have confirmed that RIF and INH concentrations in granulomas are consistently and significantly lower than in plasma, while the fluoroquinolone moxifloxacin accumulates into lung and lesion tissues relative to plasma⁴⁰. Despite increasing awareness that the activity of antibiotics depends on their unbound concentration at the site of infection, current pharmacodynamic analyses mostly rely on published blood or plasma pharmacokinetics compared to the MIC determined against replicating cultures in rich medium⁴¹. In the case of TB where the heterogeneity of infection sites has a dramatic impact on drug penetration and bacterial physiology, this approach is likely to result in poorly predictive PK-PD indices. Table 1 highlights the pharmacological conundrum of tuberculosis. For a range of anti-TB agents, we have compiled the classical PK-PD indices (i.e. the ratio between peak plasma concentration and broth MIC (C_{max}/MIC), ratio between Area Under the plasma concentration-time Curve and broth MIC (AUC/MIC), and the percentage of the dosing interval during which plasma concentrations are above the broth MIC ($T > MIC$)) corresponding to a 100-fold reduction of bacterial load in infected lungs, as measured in efficacy studies with the standard mouse model. All PK parameters were corrected for plasma protein binding to reflect the free drug fraction in plasma (indicated by “f”). Without dwelling into sophisticated PK-PD considerations, this exercise clearly shows that simple rules of thumb cannot be used to guide the discovery and development of novel TB drugs, since efficacious drugs and drug candidates display PK-PD indices spanning a 3-Log range. In contrast, the PK-PD indices of antibiotics used against most other infectious diseases usually fall within relatively narrow and reasonably predictive windows⁴².

Further impairing the drug delivery process, vascular supply is extremely heterogeneous among lesion compartments. The long standing observation that cavities are most difficult to treat and have a negative prognostic impact has led to speculation that the fibrotic outer wall of the granuloma creates a barrier to drug penetration and therefore impairs the effectiveness of pharmacotherapy. Yet prominent vascularity can be demonstrated within such fibrosis, suggesting that drugs have adequate access to the outer layers of mature granulomas and cavities (Figure 3). The more problematic niches are likely the caseous center of necrotic lesions and the central caverna of cavities where all vascular architecture has been destroyed, leading to failed immunity and lack of drug supply from blood. To reach the center of necrotic granulomas where quiescent extracellular bacilli can be found in large numbers²⁶, drugs thus have to diffuse from the cellular rim across the entire necrotic center (Figure 3) without the assistance of active or facilitated transport mechanisms.

At present, the exact composition of caseum and the physicochemical properties driving diffusion through caseous material remain to be established. MALDI mass spectrometry imaging⁴³, an emerging imaging modality that allows the visualization of unlabeled drugs in

tissue sections, has provided ion maps of TB drugs in infected lungs, showing that some classes of anti-TB drugs, such as the fluoroquinolones⁴⁴, do not diffuse effectively through caseum, and that selected members of the class accumulate in cellular layers to various extents (Figure 4). It is reasonable to speculate that small molecules with defined – yet unknown so far – physicochemical properties could preferentially diffuse through acellular material, thereby reaching extracellular quiescent populations present in the necrotic foci of caseous granulomas.

Anti-TB chemotherapy is combinatorial in nature, requiring long and resource intensive clinical development to treat a disease that mostly affects resource-poor countries. Until now, new and existing antibiotic regimens have not been rationally designed by taking into account the differential abilities of each companion drug to distribute into the multiple types of lesions that make up the complex TB pathology, including lung tissue which appears ‘uninvolved’ macroscopically. The next quantum leap in TB drug development will come from identifying a combination of drugs that complement each other in (i) their ability to penetrate the range of lesions found in the spectrum of TB disease, and (ii) their potential to kill all bacterial subpopulations present in these lesions.

The right animal model at the right stage of discovery and development

The complex interplay between host and pathogen inevitably implies that different animal species will reach different stages of granuloma formation, in terms of structure and composition, leading to varying abilities to contain the pathogen. Currently, not one animal model fully mimics the complete and elaborate spectrum of lesion types that we see in humans. As our understanding of the TB pathology and latent disease in humans has improved, we have come to realize that both active and latent TB are characterized by a continuum of more or less quiescent and healed lesions rather than a unique and well defined lesion type^{28,45,46}. Successful use of any model depends on our ability to identify the features and lesion types which they lack or exhibit, recognize their respective limitations, and determine whether these traits are compatible with the expected outcome of the intended study⁴⁷.

Of mice and men – the predictive value of murine models for shortening treatment

Both logistic and scientific considerations play an important role in the selection of an animal model. For practical reasons, the most popular model has been the mouse due to its low cost, modest compound requirements, availability of genetically defined mouse strains, and extensive literature describing the immune response to TB infection. The mouse is also the preferred species because all existing TB drugs have been tested at multiple doses against acute and chronic infection, generating the belief that the model is fully ‘validated’ for drug efficacy testing. This constitutes a circular argument if we recognize that TB drugs and drug candidates have been selected solely based on kill curves obtained in the mouse model, where we know pathology and disease progression are markedly different than in humans. The granulomas that develop in mice are not the well-formed structures observed in humans, but consist of aggregates of lymphocytes and macrophages that do not progress to caseation, liquefaction or cavity formation associated with poor prognosis in human TB. Within mice the predominant type of infected cell is the alveolar macrophage, while *Mtb* bacilli are mostly found within neutrophils in human sputum, with a substantial proportion of bacteria found outside of any host cell³¹. We thus have biased ourselves in believing that the mouse is a predictive model because all drug combinations which are effective against human TB have proven efficacious in the mouse, while we may have missed small molecules that could have turned into very useful anti-TB drugs but had no or poor efficacy in the mouse.

A number of recent clinical trials have highlighted the dangers of extrapolating treatment duration from mouse efficacy models^{48,49}. Several of these trials attempted to shorten therapy based on the observation that substituting moxifloxacin for either ethambutol or isoniazid resulted in a much more rapid cure of murine TB⁵⁰. In mice the combination of rifampicin, pyrazinamide and moxifloxacin was sufficient to sterilize more than half of the infected animals by three months, and all of them by four months, suggesting that treatment duration could be significantly shortened. Yet in humans four Phase II studies have been performed and showed only an inconsistent small effect on the rate of culture conversion^{51,52,53,54}. The largest two of these studies involved 300-400 subjects each and found no statistically significant difference in their primary endpoints. Similarly, rifapentine, a derivative of rifampicin with an extended half-life was found to be much more potent than rifampin in sterilizing murine disease, achieving sterilizing cures in three months⁵⁵. But in humans no significant improvement could be observed for the same substitution in over 500 subjects⁵⁶. Likewise, substitution of rifapentine for rifampicin did not shorten the time to cure in the guinea pig model of chronic TB, where necrotic granulomas with hypoxic foci are well represented, similar to the clinical trial outcome⁵⁷. Because the caseum of necrotic granulomas is thought to be the preferred niche of extracellular *M. tuberculosis* persists which are phenotypically tolerant to many drug classes⁵⁸, the guinea pig model likely includes a clinically relevant bacterial subpopulation that is absent in conventional mouse models.

In contrast to these results suggesting that agents may appear more active in mice than in humans, a recent example demonstrates that poor efficacy in a conventional mouse model does not necessarily predict clinical failure. A recent Phase II study with linezolid, an oxazolidinone antibiotic, in subjects with extensively drug-resistant TB (XDR-TB) showed that this agent, added as effective monotherapy to failing treatment regimens, was highly active in clearing bacteria from patients sputum²⁴. Yet in mice linezolid showed only limited bacterial killing at a dose selected to mirror expected human exposure⁵⁹. These failures have led to rethinking the predictive value of the mouse model of TB disease, particularly with respect to truncating the duration of therapy.

However, because the mouse is most compatible with the timelines and compound requirements that can be afforded in the early stages of drug discovery, there has been strong pressure to develop new murine models which would improve the prognostic capability of conventional models through more representative pathological signatures, including necrotic and fibrotic granulomas. The C3HeB/FeJ and Nos2^{-/-} mouse model are under validation with a panel of drugs, drug candidates and combinations thereof. Unlike conventional mouse strains, these mice develop necrotic granulomas with hypoxic foci following low-dose aerosol infection with *M. tuberculosis*^{60,61} or injection in the ear dermis⁶².

Table 2 summarizes histological characteristics of the major animal models of TB infection and disease, as well as practical considerations associated with each species.

Since the early days of anti-TB therapy, the presence and extent of cavitary disease have often been cited as correlates of poor clinical outcome, development of resistance and relapse^{85,25,86}. For drug testing in a species that proceeds to liquefaction and cavity formation, rabbits or non-human primates are required (Table 2). Why is this important? With a dozen drug candidates in various phases of clinical trials, and an increasing number or repurposed drugs showing promise against TB, a new trend in TB drug development seeks to accelerate the development of combination regimens in parallel with single drugs to accelerate the registration of shorter and more efficacious therapies. Because TB clinical trials are combinatorial, long and complex while financial resources and capacity in endemic

countries are limited, it is imperative to rationally inform the prioritization of drug combinations that have the best potential to dramatically reduce treatment duration.

In conclusion, critical steps in the optimization of predictive animal models of TB disease are to recreate (1) the proportion of necrosis present in human TB, as the niche where persisting organisms are thought to reside, (2) the histological features of clinical TB associated with poor prognosis such as the cavitating lesion which is particularly difficult to treat⁸⁶, and (3) the size of caseous necrosis lacking blood supply. Not one animal model possesses all these traits while also presenting the pragmatic attributes required in early drug discovery. Knowledge of a shortcoming in an animal model does not preclude its use at one stage or the other along the path to the clinic, but unreserved use of an animal model for historical reasons can have costly consequences down the road.

A TB target product profile?

TB drug development is obviously a difficult undertaking, requiring activity of a lead molecule against organisms under very different environmental conditions, replicating or not, and in multiple discrete compartments representing the spectrum of lesion types seen in infected humans. Any one single agent is unlikely to fulfill all of these characteristics, although obviously this would be ideal. Historically our answer to this has been polypharmacy and this may not change dramatically in the foreseeable future. What has changed is our understanding of the importance of addressing these factors systematically and during lead optimization. In the past we have combined therapies for the purpose of minimizing the emergence of resistance because the early clinical trials of isoniazid and streptomycin showed that such resistance emerged readily with monotherapy. Optimization of treatment duration with the current four-drug regimen was a strictly empirical process, the product of 40 years of controlled trials undertaken by the British Medical Research Council Tuberculosis Units⁸⁷. *In vitro* assays and animal studies were limited to very simple proof of concept endpoints and were not performed to predict the most successful regimen. While animal models can be used predictively and have been used retrospectively to recapitulate some human data - albeit with very different pharmacodynamics at least in murine infections - their strengths and limitations should be carefully considered when interpreting the results.

The recent failures to achieve treatment shortening based upon studies in mice demand a radical rethinking of the process for selecting combinations of agents for testing in Phase IIb/III studies that extends back to the lead optimization phase of development. Beginning to understand where special bacterial populations reside and evaluating the ability of lead molecules to penetrate the site where they will display activity would allow a more rational approach to the design of combination chemotherapy than blind testing in clinical trials. Despite the inconvenience of employing non-standard assays for activity against these bacterial subpopulations, and developing assays to measure penetration of agents to the site of action most relevant for their sterilizing activity, these steps are critical to radically shorten TB treatment and minimize the number of agents required to achieve a sterile cure. Rather than proposing a single “target product profile” for TB, it may be more helpful to articulate a series of target agent profiles that, when combined, would achieve the goals of shortening and simplifying treatment. These target agent profiles should be based upon current understanding of the TB pathology and bacteriology, and should be validated in advanced disease models before embarking on much more expensive clinical trials. Simply repeating the “3Ms” paradigm hoping to get lucky in Phase III is a false economy. We may save time and resources in the 1st and 2nd M stages, but our knowledge will stand still while the death toll of the 3rd M and the costs of clinical trials continue to grow.

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Figure 1.

The traditional methodology for lead optimization is driven by the “three Ms”. Left represents determination of the Minimum Inhibitory Concentration (MIC) with the top panel showing a scanning electron micrograph of *Mtb* and the bottom showing a transmission electron micrograph of the same culture. The center panels show the second M, mouse efficacy is typically assessed as both single agents and as combinations of agents. The lower panel shows a Hematoxylin and Eosin (H&E) stain of murine TB lesions which are typically not well organized discrete structures but rather loose aggregates of infected cells. The right panels show the third M, man, with the top figure being a 3-dimensional reconstruction of Computed Tomography (CT) scans of a patient with extensive excess high-density lesions in the lungs. The bottom shows an H&E stain from a human lung resection surgery in which both acellular, caseous lesions (A) and necrotic lesions in the process of cavitation are present (B).

Figure 2.

The development of cavitory TB in adult humans and the implications for drug discovery. Initial infection of pulmonary macrophages is followed by migration of these infected cells into the airway interstitium where they attract tissue macrophages and migrating monocytes and other immune cells to initiate formation of a cellular granuloma. Vascularization of this structure is initially intact but as it expands and becomes caseous vascularization is minimal. TB bacteria contained within this granuloma experience a variety of stresses resulting in them becoming drug tolerant while at the same time the vasculature becomes limiting. Eventually this structure breaks down and the bacteria, completely insulated from the immune system, are released into the airway (and the bloodstream) where they can be expelled and initiate another cycle of infection in a new host. Cavity formation is a hall mark of TB disease and the organisms in cavities are the most difficult to eradicate resulting in long durations of treatment.

Figure 3.

(A) Typical well-circumscribed granuloma with central caseous necrosis devoid of vascular supply, surrounded by a layer of immune cells and a thick fibrotic rim; (B) Cavitory lesion showing softened caseum in the center (partially disintegrated due to the sectioning procedure). The cellular and fibrotic rim of the cavity is rich in blood capillaries (indicated by the white arrows), effectively bringing inflammatory cells and drugs to the cellular part of the lesion. Hematoxylin and eosin stain, 12.5x magnification.

Figure 4.

MALDI mass spectrometry (MS) images showing moxifloxacin (MXF) distribution within rabbit granulomas (A), MXF in a human necrotic nodule (B), and PA-824 in a rabbit necrotic granuloma (C). On the lower left panel, an H&E stained reference tissue section is displayed below the corresponding MALDI MS image. White circles surround a necrotic granuloma. The necrotic center containing caseating material (black arrows) is visible as a light pink center in the H&E stained reference. (B) and (C): the white contour lines highlight the necrotic center of each granuloma, surrounded by the cellular region on the lower right side in each case. The poor distribution of MXF and PA-824 within caseum relative to the cellular cuff is clearly visible in all samples. Signal intensities are shown as a fixed scale.

Table 1

Standard pharmacokinetic/pharmacodynamic indices of anti-TB agents at doses which provide a 2-log reduction of lung colony forming units (CFUs) in the acute mouse model of TB disease.

Drug	<i>f</i> C _{max} /MIC	<i>f</i> AUC/MIC	<i>f</i> T>MIC
Rifampicin	20	300	100
Isoniazid	400	800	60
Ethambutol	3	10	15
Pyrazinamide	15	31	19
Clofazimine	0.02	1	0
Moxifloxacin	10	53	80
Mefloquine	0.1	1.6	0
PA824	2	19	21
TMC207 (Bedaquiline)	0.1	1	0

Table 2
Histopathology, characteristics and applications of animal models in TB drug discovery and development

Species	Compound requirements*	Pathology	Size of necrotic core and time required for development	Most appropriate stage for drug discovery	Published drug studies
Mouse	Up to 1g	Aggregates of lymphocytes and macrophages; lack of organized granulomatous structures; macrophage as the predominant type of infected cell ⁶³	n/a	Hit-to lead	Practically all existing drugs and drug candidates; large numbers of early discovery compounds ⁶⁴
Hypoxic mouse	Up to 1g	Necrotic granulomas with caseous hypoxic center; relative proportion of cell types different than in granulomas from larger animal species and TB patients; some fibrosis; large numbers of extracellular bacilli within necrotic foci ^{60,61,62} ; cavitation rare	In Nos2 ^{-/-} mice: 2mm at 8 weeks; in C3HeB/FeJ mice: 2 to 5mm at 10 weeks p.i.; occasionally up to 10mm	Hit-to-lead	Recently initiated, including comparison with the conventional mouse model ⁶⁵
Guinea pig	15-25g	Necrotic and fibrotic granulomas with associated caseation resulting in the development of regions of low oxygen tension in the core of caseating lesions ^{66,62} ; mineralization and calcification of caseous foci in healing granulomas ⁶⁷ ; cavitation rare	Varies depending on Mtb strain used ⁶⁸	When discrepancies are observed between efficacy results in mice versus human TB ^{69,70} ; in transmission studies due to their high susceptibility to pulmonary infection ^{57,71,72}	Largely limited to standard TB drugs so far; also used to test new aerosolized formulations, adjunctive immunotherapy and vaccine candidates ⁷³
Marmoset	5-10g	Same as above but includes cavities; differentially susceptible to various clinical isolates of <i>M. tuberculosis</i>	Variable from <2mm to 6mm and occasionally larger (L. Via, personal communication)	Lead optimization; Preclinical development; selection of combinations for clinical trials	Has only been recently adopted as PK-PD tool to characterize new and existing drugs (L. Via <i>et al.</i> , submitted)
Rabbit	30-50g	Differentially susceptible to various Mtb strains leading to the development of a spectrum of pathologies from LTBI to chronic cavity disease ^{74,75,76} ; Sequential appearance of major lesion types found in human TB: solid cellular granulomas, necrotic and fibrotic lesions, calcification and cavitation	In HN878 (Beijing lineage) infected rabbits, 6 to 7 mm at 12-16 weeks post-infection ⁷⁷	Preclinical development; selection of combinations for clinical trials	Selected standard TB drugs and metronidazole ^{1278,40}
Macaque	30-50g	Spectrum of latent and active disease with most histological features found in human TB ⁷⁹ ; primary active disease, latent-like infection, and spontaneous or induced reactivation tuberculosis ^{80,81}	In Erdman infected monkeys, 5-6 mm beyond which it evolves as a cavity or a coalescence of spreading granulomas	Late preclinical development; efficacy of new drug candidates in sterilizing latent disease and preventing reactivation ^{82,83}	Standard TB drugs and metronidazole to prevent TB reactivation ⁸⁴
Human	n/a	Neutrophil as the predominant type of infected cell; substantial proportion of bacteria extracellular ³¹			

p.i.: post-infection

* Assuming one month treatment at 25mg/kg and 100mg/kg and 2 timepoints at 14 and 28 days post-treatment in the mouse and guinea pig, 10 and 50mg/kg and 1 timepoint at 56 days post-treatment in other species.