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Drug permeation and metabolism in *Mycobacterium tuberculosis*: prioritising local exposure as essential criterion in new TB drug development

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Abstract

Anti-tuberculosis (TB) drugs possess diverse abilities to penetrate the different host tissues and cell types in which infecting *Mycobacterium tuberculosis* bacilli are located during active disease. This is important since there is increasing evidence that the respective "lesion-penetrating" properties of the front-line TB drugs appear to correlate well with their specific activity in standard combination therapy. In turn, these observations suggest that rational efforts to discover novel treatment-shortening drugs and drug combinations should incorporate knowledge about the comparative abilities of both existing and experimental anti-TB agents to access bacilli in defined physiological states at different sites of infection, as well as avoid elimination by efflux or inactivation by host or bacterial metabolism. However, while there is a fundamental requirement to understand the mode of action and pharmacological properties of any current or experimental anti-TB agent within the context of the obligate human host, this is complex and, until recently, has been severely limited by the available methodologies and models. Here, we discuss advances in analytical models and technologies which have enabled investigations of drug metabolism and pharmacokinetics (DMPK) for new TB drug development. In particular, we consider the potential to shift the focus of traditional pharmacokinetic-pharmacodynamic analyses away from plasma to a more specific "site of action" drug exposure as an essential criterion for drug development and the design of dosing strategies. Moreover, in summarizing approaches to determine DMPK data for the "unit of infection" comprising host macrophage and intracellular bacillus, we evaluate the potential benefits of including these analyses at an early stage in the preclinical drug development algorithm.

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INTRODUCTION

Tuberculosis (TB) remains a leading cause of death owing to an infectious disease despite the existence of multiple front-line and second-line drugs that are active against *Mycobacterium tuberculosis* (*Mtb*), its etiological agent (1,2). A critical limitation of the current anti-TB regimen is that it involves a minimum six months combination therapy, a requirement thought to reflect the inability of the existing drugs to sterilize bacilli located in different host micro-environments and in variable metabolic states, often resulting in clearance of bacilli from sputum followed by subsequent disease relapse (3,4). Consequently, rational approaches to addressing this problem are urgently needed as they might offer the prospect of elucidating (at least partially) the reasons for the often described difficulty in translating compound potency *in vitro* into drug efficacy *in vivo* (5) – and, in turn, could inform the choices of chemical properties and screening assays to be prioritized in the critical-path algorithms that drive medicinal chemistry efforts as part of new TB drug discovery (6).

A seminal review (7) highlighted the absence of knowledge about the distributions of widely used anti-TB agents into the pulmonary lesions in which infecting bacilli are sequestered and, therefore, the need to understand how individual compounds distribute into the different host cell environments (8,9). In order to reach its mycobacterial target, an anti-TB antibiotic must penetrate complex host lesions and lesion compartments comprising multiple cell types (6,7,10). For intracellular bacilli, the drug must also overcome the barrier of the host cell membrane and, in many cases, an encapsulating vesicular membrane, as well as potential sequestration by the different organelles and intracellular bodies within different cell types (11,12). The occupation of discrete host loci (13–15) presents an additional confounder (Figure 1), in that individual bacteria from a clonal infecting population are characterized by different physiological states, and this can impact drug susceptibility (11,16,17) and the ability of the drug to penetrate the complex mycobacterial cell wall (18), as well as subverting the active compound's activity against a metabolic target that might be essential only under specific conditions (19,20). As if that weren't already sufficiently complex, host-mediated (21,22) and/or mycobacterium-mediated (23,24) biotransformation might further complicate the passage of drug from ingestion by the patient to its intrabacillary target (7). Although some guiding principles have been inferred from both preclinical and clinical observations in TB as well as other diseases, the physico-chemical and pharmacological properties which enable drugs to navigate this complex delivery pathway, avoid host metabolism, and penetrate the bacillus, remain poorly understood (6,7,25). It is not surprising, therefore, that active compounds selected (and optimized) for potency in a handful of *in vitro* assays often fail to demonstrate activity *in vivo* in the infected host (26).

Fortunately, the last decade has seen considerable efforts towards developing accurate methods (Table 1) to measure drug levels directly in *Mtb*-infected tissues (27–29). Through the application of advanced chromatographic, mass spectrometry, and imaging techniques including HPLC coupled to tandem mass spectrometry (LC–MS/MS) and MALDI mass spectrometry imaging (MALDI–MSI), the quantification of tissue and lesion distribution of known and experimental anti-TB drugs in animal models and clinical samples has become increasingly attainable – primarily through the work of Veronique Dartois and colleagues

(4,7,8,28,30) – thus offering the prospect of informing a systems pharmacology approach to the design of antimycobacterial therapy (31). In addition to the well-established appreciation that TB drugs can be differentially active against bacilli in different metabolic states (32–34), these new analytical techniques have demonstrated that those same drugs can also possess distinct lesion-penetrating abilities (6,7,9,18). Moreover, in many cases, tissue penetration appears to correlate well with the demonstrated activity of specific drugs *in vivo* (9); for example, the ability of pyrazinamide to diffuse through the necrotic areas at the center of mature granulomas is consistent with its sterilizing ability in the TB regimen (7). This is a critical observation, which prompted the prediction that "the next major step towards curing TB and preventing the development of resistance will come from a combination of complementary drugs, each of which preferentially distributes in the lesion or lesion compartment where its most vulnerable target bacterial population resides" (7). That is, future combination therapies for TB should be based on multidrug regimens optimized according to the specific activities and tissue distributions of each constituent drugs.

Probing the unit of infection: the *Mtb*-infected macrophage

Developing comprehensive rules for targeted TB drug delivery necessitates concerted research efforts to elucidate the various factors that influence host tissue penetration and bacillary permeation. From a drug discovery perspective, the ability to assess rapidly whether a putative hit compound possesses the required characteristics would profoundly impact the efficiency of development pipelines by ensuring that medicinal chemistry, pharmacology, and biology resources and expertise are applied only to the most promising candidates. However, as noted elsewhere (7,35), this is critically dependent on the development of medium-throughput *in vitro* assays for intralesional (e.g., necrotic foci or caseum; (11), intrabacillary (33), and intra-macrophage (36)) pharmacokinetic (PK) determinations that can be readily integrated into lead-compound discovery and lead-compound optimization campaigns.

Mtb is sequestered in multiple different host compartments during infection (3,9,29), including cellular granulomas, in which bacilli are located predominantly within macrophages but also in some extracellular niches; necrotic granulomas, where they are extracellular in the caseous centres and considered metabolically quiescent (37); and the inner surface of open cavities, where bacilli occur within multiple cell types while some are extracellular (38,39), an environment in which they are protected from the immune system and able to replicate freely (40). There is even evidence that bacilli can reside in cells in distal loci of the lung (41) and in other organs (42–44). These observations corroborate the suggestion that TB disease should be treated as a polymicrobial infection (45), and simultaneously reinforce the complexities inherent in determining the factors that might be essential to ensure optimal drug exposure for extended durations. From a drug development perspective, they also highlight a major obstacle to defining the optimal pre-clinical assays to be used in selecting compounds for advancement through the discovery pipeline.

How does the micro environment in which *Mtb* persists influence compound penetration and permeation? Current anti-TB chemotherapy may cause bacilli to revert to a drug-tolerant

phenotype, which may explain the difficulty in achieving complete bacterial clearance (46,47). Therefore, more research is needed to understand the mechanism(s) by which exposure to these compounds might force the exposed bacilli into a different metabolic state (48). It is well-established that a biphasic reduction in bacterial load is observed under current TB therapy (32), a phenomenon which has been explained by two models: (i) the killing of actively dividing bacilli in an initial rapid killing phase, followed by the gradual decrease in the persistent population of bacteria, or (ii) the lack of treatment of bacterial populations due to the inaccessibility of bacteria within granulomas (49). Adding a further layer of complexity is the fact that the bacteria exist within both an intracellular and extracellular environment (50), an observation which is exacerbated by the knowledge that these lesions are very heterogeneous, often differing even within the same patient (4,14).

As noted elsewhere (51), the immunological lifecycle (52) of TB disease involves multiple stages at which the interaction between host macrophage and invading pathogens might be critical to the outcome of infection (3,37,53). That is, the ability of *Mtb* to survive and replicate within the macrophage is a defining feature of mycobacterial pathogenesis (54). This observation has motivated a longstanding interest in understanding the dynamics of the host-pathogen interaction within this phagocytic cell (30,50,53,55), as well as efforts to understand the factors which might undermine therapeutic outcomes (17,56). It has also underpinned the use of alternative screens to identify compounds active against bacilli in this intracellular environment (57–60), as well as approaches to understand the impact of the intracellular environment on drug partitioning and how this knowledge might be exploited for rationale drug and drug regimen design (36). The perceived centrality of the host macrophage in infection outcomes (61–63) has also been key to the use of standard mouse models as distinct from the “Kramnik” or C3HeB/FeJ model; (29,64) in pre-clinical efficacy assessments (65). Therefore, while cultured cells *in vitro* do not fully recapitulate the specialist functional properties of differentiated macrophages *in vivo* (3,66) – with their diverse ontogenies and differential trajectories of activation and development (67),– the utility of the macrophage model in inferring disease-relevant mycobacterial physiological and metabolic adaptations, as well as innate host defence strategies, seems convincing (3,51,68). This notion is perhaps best summarized in the concept that the *Mtb*-infected macrophage represents the “minimal unit of infection” (50), a term which encapsulates critical concepts in immunometabolism (69) and pathometabolism (70).

The concentration which any drug achieves within its target (myco)bacterial cell is a function of multiple factors, including passive or active uptake, pathogen-mediated metabolism, active drug efflux, and cell growth (71). Mycobacterial drug uptake is generally encapsulated in the concept of “permeation”. The complex mycobacterial cell wall is thought to function as an impermeable barrier to most compounds and undergoes dynamic architectural modifications during infection that correlate with a switch from active replication to a persistent state (18,72). Moreover, the mechanisms which enable many of the known anti-TB drugs (in particular, small hydrophilic compounds like isoniazid, ethambutol, and cycloserine) to permeate the lipid-rich mycobacterial cell wall remain unknown (73). *Mtb* also possesses an expanded complement of efflux pumps (25,74,75) which have been implicated in intrinsic resistance to applied drugs *in vitro* (76,77) and in experimental models of infection (78). Moreover, growth within THP-1 and J774 macrophages has been

shown to induce *Mtb* efflux pump activity (79), resulting in tolerance to RIF and other drugs mediated by the efflux transporter, Rv1258c (79,80). As highlighted elsewhere (7), this is an important observation since it supports the need for *in vitro* assays to determine (and, ultimately, predict) drug distributions within immune cells such as macrophages, as well as the sub-cellular organelles in which the bacterium might be contained.

The prevailing drug discovery paradigm

It is generally acknowledged that, in order to understand how a drug will respond in the human body, the pharmacokinetic/pharmacodynamic (PK/PD) parameters in various tissues and cells must be understood fully (4,30,45). The propensity for *Mtb* to occupy different microenvironments – and at the same time – within an infected host (13–15), makes it essential that new combination regimens comprise partner drugs that are active in the various micro environments and, potentially, have the ability to modulate permeability of the mycobacterial cell (81).

Anti-TB drug discovery often begins with a basic screen against replicating *Mtb*, followed by subsequent cytotoxicity screens to determine the compound selectivity against the pathogen (82). This allows high-throughput screening whilst identifying potential candidates with initial activity against replicating bacteria (82), but neglects aspects such as intracellular compound activity, membrane permeability, involvement of efflux, and metabolism of the compound (83). The use of a standard growth medium alone ignores the metabolic changes that *Mtb* undergoes when in the host cell environment, and could influence compound efficacy (84): the evidence is strong that the metabolic status of the bacterium is a function of the host environment (51,85). Therefore, the active compounds that emerge from *in vitro* screens are likely to be active only under specific conditions (86). For example, amikacin displayed potent activity in initial screens against extracellular bacteria, but little to no activity against intracellular *Mtb* (87).

Next, the PK parameters of the compounds are assessed using *in vitro* or *in vivo* absorption, distribution, metabolism, and excretion (ADME) studies. In the simplest sense, the ADME parameters for a compound in combination with the dose of the drug determine the time course and concentration in serum and, consequently, the tissues and fluids. The pharmacodynamic (PD) parameters relate drug concentration with the observed antimicrobial effect. The PK/PD characteristics of a drug have implications for dosing, clinical efficacy, and the combinatorial drug classes which can be used to treat TB (88). Summary exposure parameters, such as area under the concentration-time curve (AUC) and peak concentration (C_{max}), are often used in combination with PD parameters, such as MIC (BOX 1). Importantly, drugs display varied and distinctive patterns of PK/PD relationships which can influence their activity (89,90). The main patterns include bacterial killing based on maximum drug concentration (C_{max}/MIC), total exposure over a certain time period (AUC/MIC), or time above MIC (T>MIC) (91). Notably, these parameters have been experimentally determined using multiple approaches, including the *ex vivo* hollow fibre model, which allows for the recapitulation of the different bacterial populations and their corresponding PK/PD responses. Applying this technique, Gumbo and colleagues were able

to predict the inability of moxifloxacin (MXF) used at WHO recommended doses to improve clinical outcomes significantly in a series of drug trials (92–97).

Potential for a paradigm shift?

Many of the anti-TB drugs were discovered before the routine use of PK/PD analyses, a problem that continues to impair correct drug dosage today. This is exemplified by rifampicin (RIF) (98), for which recent studies have highlighted the benefits of using a larger dose than has been employed for decades. Although plasma and tissue PK analyses are routinely used in drug discovery (99), they may not reveal the entire picture owing to the complex nature of TB lesions and the difficulty faced for a drug to access, and then penetrate, the infecting bacilli. This is further complicated by the avascular nature of necrotic lesions, where the relationship between plasma and target site drug concentrations is even more difficult to predict (7). PK within the human setting can be spatially unique and the heterogeneous nature of both the bacterial and lesion phenotypes leads to increased complexity (100).

This complexity is shown in the use of RIF in first-line anti-TB therapy. The current dosing of RIF (10 mg/kg daily) is sub-optimal for TB, with some studies suggesting that significantly higher doses are needed for more effective treatment (101). Moreover, the problem persists in vulnerable population groups, with Sub-Saharan individuals (primarily in South Africa and Malawi) displaying reduced drug exposure (102,103). This often places individuals below the recommended target concentrations, potentially exposing them to increased burden of disease, the development of resistance, and risk of mortality (104,105). Many of the problems associated with RIF stem from a poor underlying understanding of dosing requirements in combination with other first-line treatments. Interestingly, MXF has been shown to be antagonistic with most antibiotics, including RIF and pretomanid (106,107). This knowledge, in conjunction with the deficiency of MXF in penetrating different lesions types (27), could explain the recent failed drug trials (108,109). Given the cellular concentrations of these first-line drug combinations, it may have been possible to predict their efficacy before commencement of the trials.

A series of studies conducted between 1950 and the 1980’s showed that concentrations of isoniazid (INH) and RIF are far lower in TB lesion homogenate than in the plasma of infected patients (110,111), a finding which was confirmed in MALDI studies (27). Despite the increasing appreciation that the activity of antibiotics depends on their ability to reach and accumulate in lesions, there is a continued reliance on blood and plasma levels to drive drug discovery (112,113). In various other diseases, target areas and plasma levels are often quite closely related, which leads to predictable and reliable plasma PK/PD values, even when using plasma exposure as PK target (114). However, owing to the complex nature of TB pathology, blood supply is often absent and free drug has difficulty in entering the different lesion compartments homogeneously. Drug PK/PD relationships are therefore extremely difficult to determine, particularly for newer drugs. So, while plasma PK measures still offer some value for many diseases, these determinations need to be repositioned in the TB drug discovery pipeline and factored in with new PK analyses at the target site.

Using the technologies available today (Table 1), it is possible to evaluate PK/PD properties at cellular level (100). The use of *in vitro* macrophage culture to represent *Mtb's* intracellular environment has shown some success with the identification of compound Q203 (86). Similar assays have been undertaken in various cell types including mouse-derived macrophages and epithelial cells, with a limited number of studies having used cell lines such as the human-derived THP-1 macrophage-like cells (60,115,116). Progress in the use of metabolomics to obtain PK/PD information from treated mycobacterial cells (117,118) has been especially useful in determining the systems-level impact of drug treatment on *Mtb* physiology, and has the capacity to provide key insights into the mechanisms of action of new (117,119) and even known (118,120,121) anti-TB drugs. However, while the effective intrabacterial concentration is critical to efficacy, for most drugs the identity of the active metabolite(s) remains unknown. It is likely, therefore, that, in addition to revealing intracellular modes of action of known drugs, analyses of active drug metabolites generated via host- or *Mtb*-mediated biotransformation could identify new targets (7).

Metabolism alone does not always account for the disappearance of drug during PK/PD analyses: instead, this is can be a multifactorial phenomenon, involving the binding of drugs to macromolecules such as plasma proteins (122) and binding to lower molecular weight targets such as the oxidative stress protectants, glutathione and mycothiol (123). The phagolysosome in which these molecules must function itself undergoes significant pH changes in response to IFNγ-dependent macrophage activation, dropping from a pH of 6.2 to approximately 4.5 (124,125), a shift which has the potential to limit the activities of acid-labile drugs while elevating the efficacy of drugs such as pyrazinamide which function optimally at low pH (126).

Drug discovery at the target site

The importance of determining lesion-specific drug concentrations stems from various sources. Mitchison and Coates (2004) described a model which explained the relationship between the different microenvironments in TB and current first-line TB drug efficacy. In their model, actively growing bacilli were killed by INH, semi-dormant bacilli were killed by RIF, intracellular bacilli were targeted by pyrazinamide (PZA) in the acidic phagolysosome, while dormant persisters were found in hypoxic environments, making them harder to treat using standard therapies (127,128).

Pioneering work by Dartois *et al.* in 2012 allowed scientists in the TB drug discovery environment to start looking at the target site of pulmonary TB in far more detail (8). Using a combination of New Zealand White rabbits, imaging mass spectrometry, three first-line anti-TB drugs (INH, RIF, and PZA), and the fluoroquinolone MXF, the group demonstrated that drug plasma concentration was indeed a poor proxy for drug concentration in TB lesions (8). The lack of data on how this might translate into humans combined with the absence of cellular protein binding data were acknowledged as weaknesses by the authors; nevertheless, this work prompted a growing appreciation of the need to include permeation studies in their drug development pipeline (16,129,130).

In a later study, Prideaux *et al.* (2015) provided a compelling extension of this work by taking advantage of a small set of resected lung samples from drug-resistant TB cases (131). In this case, the patients received a steady-state dose of RIF, INH, MXF, and PZA at intervals ranging from 2-24 hours pre-surgery, and lung samples were analysed using imaging mass spectrometry. This revealed that RIF and PZA were able to penetrate lesions, with RIF accumulating to steady-state levels in the caseum (27). Although multiple dosing was not investigated, patients had been receiving these drugs for several weeks/months. A controlled trial involving infected, drug-naïve patients would be necessary to confirm these findings and control for drug levels. This work could potentially correlate clinical trial data with lack of efficacy and motivate earlier inclusion of such studies in a pre-clinical setting (132).

The influence of caseum binding on the permeation of compounds into the deeper recesses of the granuloma was the next object of study (11). Exploiting a very large sample set which covered the molecular space using 64 parameters and over 200 compounds, it was observed that the compound's ClogP value best describing penetration into the granuloma (11). The use of surrogate caseum was also investigated: while it was acknowledged that the surrogate failed to reproduce the full composition of the *in vivo* material, and so required further development to mimic patient-derived lesions more accurately, the results supported the prospect for this assay to be standardised, thereby allowing more in-depth *in vitro* assays to take advantage of this methodology.

Another recent study investigated the mystery of ethambutol's (EMB) clinical efficacy despite its modest potency against non-replicating *Mtb* (30). Using the TB-infected rabbit model, along with microdissection and LC-MS/MS, the group determined that EMB partitions into the caseous lesions with great efficiency, potentially explaining its efficacy and again motivating for permeation studies to be conducted in early pre-clinical drug development (30). This work was followed up by a further study using the infected rabbit model, where *ex vivo* MBC measurements were taken in caseum (MBC_{caseum}) for several first-line TB drugs (16). The Wayne (133) and Loebel (134) models for non-replicating persistence failed to predict the extent to which resistance was being generated in the caseous environment, whilst also indicating that PZA has activity in the caseum, a fact which in the current non-replicating models would have missed. This motivates for the use of an *in vitro* assay which closely mimics the *in vivo* situation.

Challenges and perspectives—Incorporating findings from these recent studies into the model of Mitchison and Coates (32) has revealed interesting correlations with, and departures from, their original ideas. For example, Prideaux *et al.* (2015) suggested that drug accumulation within granulomas may not simply follow the binary 'in/out' dichotomy (131). Instead, four distinct patterns of drug accumulation were identified including: (i) rapid and homogeneous distribution with no accumulation appearing over time (INH/linezolid), which may explain the predominant killing of extracellular bacteria by INH; (ii) rapid and heterogeneous distribution with accumulation in the cellular rim rather than the caseum (fluoroquinolones and oxazolidinones); (iii) slow distribution with gradual accumulation of drug over time (RIF) explaining the intracellular killing of *Mtb*; and (iv) rapid distribution

with massive accumulation in the cellular layers and poor diffusion into the caseum (clofazimine and bedaquiline) (27).

From a translational perspective, knowledge about the permeation of current first-line drugs and novel drug candidates into TB microenvironments is clearly essential: in addition to elucidating reasons for therapeutic failure that do not simply invoke patient non-adherence, the potential to inform combinations inferred from high-order drug interactions (107) with these data promises a route to mitigate the criticisms often levelled at *in vitro*-derived compound synergies. That is, coupling penetration and potentiation might enable design of new regimens that incorporate information about drug permeation into the heterogeneous TB microenvironments. Towards this end, a number of factors may have to be considered in ensuring clinical relevance (BOX 2). In this context, it is worth noting the parallel development and application to *Mtb* infection studies of increasingly sophisticated systems for three-dimensional cell culture (135,136) since these might offer a useful intermediate in bridging the *in vitro*/*in vivo* divide.

In summary, there is increasing evidence that the incorporation of the efficacy of drugs in the microenvironments must be assessed early on in pre-clinical development to allow correct dosing in further *in vivo* experiments. A better understanding of target site drug concentrations, rather than plasma concentrations, is needed, particularly for a complex, multi-faceted disease such as TB. The aim of novel drug discovery programs should be to target populations of bacilli with the most effective drugs, which should be most proficient at reaching relevant sites in the human body. In driving this concept of target site drug concentrations, it is hoped that the translational link between the lab bench and clinic can be strengthened, allowing expedited and “smarter” drug discovery and, by implication, enhanced therapeutic regimens.

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Box 1

PK/PD relationship of TB drugs

The PK/PD relationship for TB drugs is established in the literature between AUC or Cmax (adjusted for MIC) and/or time above a certain concentration threshold (T>MIC), with many of the first-line drugs described by AUC/MIC and some of the newer compounds best described by T>MIC (28,29).

Convincing arguments can be made to justify using AUC>MIC to allow for penetration in the lesions or other “hard to reach” compartments/tissues in the body, or saturation of efflux pumps. Similar arguments can be made for the use of T>MIC to avoid the concentration falling below the threshold at which *Mtb* is able to start replicating, possibly leading to resistance if low drug concentrations are present.

As discussed, for TB the PK/PD relationship is complicated by the fact that the PK is generally measured in plasma in clinical trials, but the majority of bacilli are sequestered in other compartments into which the drugs may not readily penetrate. Therefore, the PK profile in these regions is likely to be different from that measured in plasma. A reasonable PK/PD modelling approach in such cases is to use a hypothetical “effect compartment” (30,31) mimicking the concentration at the site of action which, while dependent on the concentration in plasma, is “delayed” by the drug traversing through the numerous layers of biological tissue, thus significantly smoothing out peak and trough concentrations. Also, the time over a certain concentration would be very different in this effect compartment. The best proxy for the concentration at the site of action is plasma AUC since the ratio between the average concentrations would remain the same, and AUC is closely related to average concentrations. In the literature, some results from clinical data report the superiority of Cmax>MIC *versus* AUC>MIC for prediction of clinical efficacy, however this should be interpreted with care since it is very difficult to discriminate which of these PK exposure parameter is most closely related to outcome - especially when the only available data is derived from observational clinical studies where all patients are on a similar dosing regimen. Without targeted studies of dose fractionation (when the same total dose is given in a single or multiple dosing events), Cmax and AUC are generally strongly correlated and difficult to separate (32). Comparing the two parameters, AUC data can also be variable, even on a daily basis, and this should also be considered when interpreting PK/PD results. The variability will result in a Cmax which fluctuates considerably as the determination of this parameter is based on a single sample. AUC may also change but is generally more stable due to the parameter being determined by an entire PK profile. Therefore, the use of AUC/MIC might better predict the outcome of clinical efficacy in diseases such as TB which have sites of action that are disparate from the plasma. Although the best PK/PD values would be derived from target site concentrations, these are often difficult to access.

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Box 2

Determining the clinical relevance of *in vitro* and *in vivo* assays

1. Is there a quantifiable and agreed measure of "cure" that can be evaluated in an *in vivo* model?
2. What host-pathogen relationships can be effectively modelled in a non-clinical setting?
3. Is there a way to model bacterial physiology, pathogenesis, and drug susceptibility effectively to allow better understanding of these aspects in patients?
4. What critical PK/PD relationships can be modelled using *in vitro*/*in vivo* systems and how might these be applied in clinical trial settings?
5. Can dosing regimens be altered according to data emerging from *in vitro*/*in vivo* experiments employed in the pre-clinical setting?

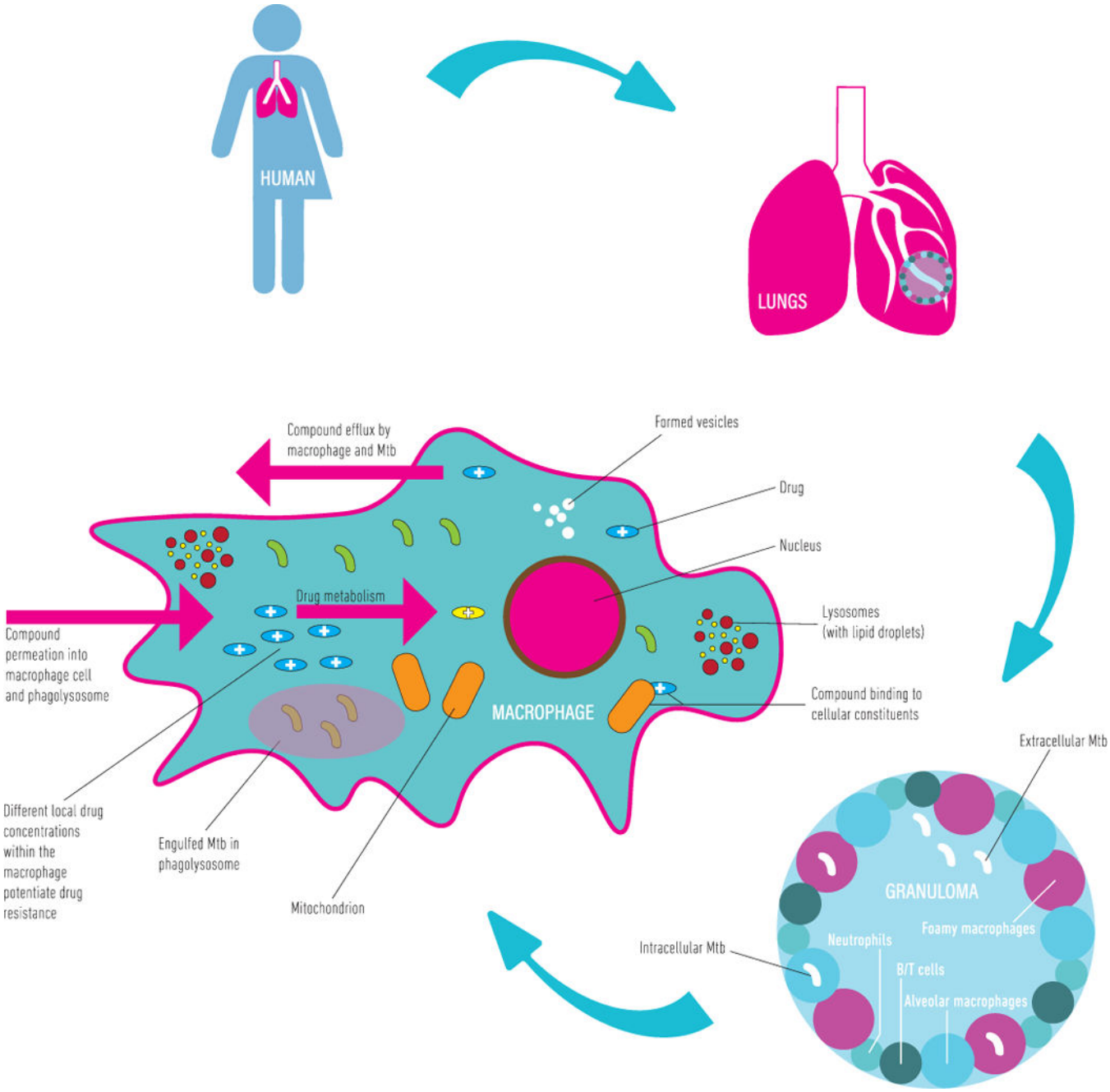

Figure 1.

The complexity of targeting *M. tuberculosis* bacilli in an infected host. After administration of drug to a patient, the drug passes from the bloodstream into the lung tissues, diffusing into the lung fluid. The drug must then penetrate the granulomatous structure and enter the various cellular microenvironments in which bacilli can be sequestered. There are a number of processes that affect drug efficacy in this microenvironment including, but not limited to: (i) drug metabolism and biotransformation, (ii) binding to various cellular components and lipids, (iii) differences in local cellular drug concentrations giving rise to potential drug resistant bacteria, (iv) compound specific differences in the permeation of drug into the

macrophage or cellular environment, (v) bacterial and cell mediated drug efflux, and (vi) differences in intracellular pH leading to in-/activation of the drug.

Table 1

Methods to measure intracellular drug concentrations

Measurement method	Detection	Advantages	Limitations
Fluorescence microscopy ([1–5])	Combination of optical imaging microscopy with computational analysis to quantify fluorescence signals from molecules or compounds	Allows temporal and physiological studies of drugs in various microenvironments	Few drugs fluoresce above background detection limit Incorporation of fluorescent tag or “click chemistry” enabled fluorescent derivatization not always feasible
Raman microscopy ([6–8])	Light scattering through change in polarisation potential or vibrational energy	Shortened data collection times for high-throughput analyses and provide indications of cellular state of *Mtb* and host cells	Can only be applied to biological systems with lower energy excitation
Nuclear microscopy ([9–11])	Uses ion microbeam with particle-induced X-ray emission	Used as a quantitative standard to complement MRI in animal or human studies where tissues may be removed after imaging	Not widely accessible technology and limited to metal containing drugs/compounds
Microautoradiography ([12,13])	Exposure or tagging using radiolabel and confocal microscopy	coupling technique to fluorescence in situ hybridisation technique allows more in depth single cell analysis	Resolution limitations; requires radioactive material and extensive processing time; often semi-quantitative
PET imaging ([14–16])	Emitted positron collides with local electron, produce photons and these are detected by γ-detectors	Can be coupled to other techniques such as micro dialysis to allow receptor site PK studies	Expensive owing to necessity of radio-labelled ligands, difficulty overcoming resolution issues
Analysis by MS or HPLC ([17–21])	Mass spectrometric (MS) analysis coupled to high performance liquid chromatography (HPLC)	Allows for intracellular drug concentrations to be calculated using pharmacokinetic approach	Long sample processing times and extensive optimisation steps, bulk analysis can cause loss of spatial information (cellular compartments)
MALDI-MSI ([22–27])	Laser desorption based ionisation technique	Allows temporal and spatial resolution of drug distribution in different organs	Difficulty in determining whether analyte truly absent or simply below limit of detection

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