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In vitro pharmacokinetic/pharmacodynamic models in anti-infective drug development: focus on TB

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Abstract

For rapid anti-tuberculosis (TB) drug development *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) models are useful in evaluating the direct interaction between the drug and the bacteria, thereby guiding the selection of candidate compounds and the optimization of their dosing regimens. Utilizing *in vivo* drug-clearance profiles from animal and/or human studies and simulating them in an *in vitro* PK/PD model allows the in-depth characterization of antibiotic activity of new and existing antibacterials by generating time–kill data. These data capture the dynamic interplay between mycobacterial growth and changing drug concentration as encountered during prolonged drug therapy. This review focuses on important PK/PD parameters relevant to anti-TB drug development, provides an overview of *in vitro* PK/PD models used to evaluate the efficacy of agents against mycobacteria and discusses the related mathematical modeling approaches of time–kill data. Overall, it provides an introduction to *in vitro* PK/PD models and their application as critical tools in evaluating anti-TB drugs.

According to the WHO report on global TB control 2009, the incidence of TB cases was 9.27 million in 2007. With half a million cases of multidrug resistant TB (MDR-TB) and approximately 55 countries and territories reporting at least one case of extensively drug resistant TB [1], there is a definite need for more effective and better tolerated anti-TB agents to go into optimized, less complicated and shorter dosage regimens. The path to new antibiotic drug discovery and development is a long and expensive process with very few compounds making it to the market. Development of anti-TB drugs in particular was halted during the last decades of the 20th century owing to a lack of commercial incentive, which has only recently been addressed after a hiatus of almost 40 years through extensive governmental and nongovernmental organizational support.

In recent years, the US FDA has suggested numerous approaches to optimize the drug-development process. **Pharmacokinetic/pharmacodynamic** (PK/PD) modeling and simulation is one such innovative tool intended to help in early go or no-go decisions and

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significantly improve development efficiency. For example, in preclinical as well as clinical development, PK/PD modeling and simulation can be used to integrate independent measures of potency, safety, bioavailability and clearance, including their inter-individual variability and measurement uncertainty, to develop optimal dosing regimens with regard to safety and efficacy, and to explore different treatment algorithms and trial designs with regard to their likelihood of being successful [2–4]. There is often only limited PK/PD information available for molecules under development owing to a lack of inexpensive suitable animal models to predict efficacy, a poor understanding of the relationship between PK and PD for novel compounds, and challenges by complex disease processes, such as the phenomena of latency and drug tolerance in TB infections [5]. Hence, improved tools are needed to evaluate the PK and PD properties and optimize dosing regimens for drug candidates early in the development process.

The PD effect of an antibiotic can be characterized by measuring the bacterial growth and death following its administration. Since these measures are difficult to quantify in humans, preclinical models in animals and *in vitro* systems play a pivotal role in understanding the concentration–effect relationship of antibiotics. Although animal models can provide similar growing conditions for bacteria as the human host and thus imitate the characteristics of a human infection, the potential differences in PK such as rate and extent of drug metabolism and in drug delivery to the species-specific tubercular lesions, such as to organized granulomas found in the human lung limit the extrapolation of information from animals to humans [6,7].

In vitro experiments are useful in evaluating the direct interaction between the drug and the bacteria, which enables the selection of candidate compounds and the determination of the target drug concentrations [8]. ***In vitro* PK/PD models** cannot incorporate all variables observed *in vivo* but they do provide valuable information for the drug-development process and the determination of optimal dosing regimens. Utilizing the *in vivo,* drug clearance profiles, *in vitro* models can help determine the PD information that is otherwise only accessible in expensive clinical trials [9].

PK/PD parameters of anti-TB agents

Pharmacokinetics describes the time course of concentration of a drug resulting from administration of a dosage regimen and accounts for its absorption, distribution, metabolism and excretion in the body. Protein binding plays an important role when extrapolating results from an *in vitro* experiment to humans or animals. It is the free drug concentration or the unbound fraction (fu) that is relevant to pharmacological action, can distribute into target tissue and undergoes metabolism and excretion. Most drugs bind to plasma proteins such as albumin, α_1-acid glycoprotein, lipoproteins and sometimes also to cellular blood components such as erythrocytes. Since these are absent in an *in vitro* setup, it is necessary to incorporate free rather than total concentrations as a PK input function for PK/PD modeling and simulation approaches [10].

Another caveat in an *in vitro* experiment is that there is no distribution involved and the drug has direct access to the bacteria. Hence the concentrations chosen should preferably reflect the concentrations in the specified compartments where the antibiotic of interest exerts its pharmacological effect. In that respect, use of unbound concentrations has proven to be useful as a surrogate correlate [11].

Pharmacodynamics describes the intensity of drug effect in relation to its concentration. In the context of anti-infective therapy, PD defines the effect of drug on the pathogen residing in the host organism. In order to quantify the *in vitro* anti-TB activity of drugs against the

infectious pathogen, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are the most commonly used parameters to date.

Minimum inhibitory concentration is defined as the lowest concentration that prevents growth of bacteria within a defined time period of incubation, typically approximately 7 days for *Mycobacterium tuberculosis* (Mtb) with a standard inoculum (~10^5 colony forming units [CFU]/ml) [12]. MBC is defined as the drug concentration that produces 99.99% killing of bacterial cells in the initial inoculum. Although MIC and MBC appear to be good measures of the potency of a drug and its interaction with a pathogen, they do not provide any information on the time course of the antimicrobial activity.

Pharmacokinetic/PD indices are composites of a PK parameters, such as area under the plasma concentration–time curve (AUC) or maximum plasma concentration (C_{max}) and a microbiological parameter such as MIC. Most frequently used PK/PD indices of antibiotics based on MIC, shown in Figure 1, are time above MIC (T >MIC), ratio of peak concentration to MIC (C_{max}/MIC), ratio of AUC to MIC (AUC/MIC) and area under the inhibitory curve (AUIC), which is calculated as the area under the curve where the plasma concentration exceeds MIC ($AUC_{T>MIC}$/MIC). If drug concentrations remain above MIC at all times, then AUIC and AUC/MIC are identical [13].

The most common approach to antibiotic dosing is to adjust the doses to obtain antibiotic plasma concentrations that are above the MIC for a given pathogen throughout the dosing interval. Two distinct groups can be differentiated on the basis of the pattern of antimicrobial activity. The first pattern is characterized by concentration-dependent killing over a wide range of concentrations, where higher concentrations lead to a greater rate and extent of killing. Intracellularly acting drugs such as aminoglycosides, rifamycins and fluoroquinolones fall under this pattern. The second group involves time-dependent killing, where the time of exposure governs the bactericidal activity. Antibiotics that act on cell wall targets such as penicillins and cephalosporins exhibit such killing patterns [14]. AUC/MIC and C_{max}/MIC explain the bactericidal activity of concentration-dependent killing while T>MIC explains the time-dependent killing pattern. Table 1 provides a compilation of the PK/PD indices and related PK parameters of existing anti-TB drugs. It should be noted that, with the exception of the first-line agents for which some dose optimization in the TB indication has been performed, the calculated PK/PD parameters indicated in Table 1 may not necessarily be the most optimal for driving efficacy in TB patients.

Classification of *in vitro* PK/PD models

In vitro PK/PD models have many favorable characteristics, such as flexibility, adaptability, relatively low cost, good correlation with human and animal data and no ethical concerns as compared with animal experiments, which make them excellent experimental platforms [15]. Especially with anti-TB drugs, the drug–mycobacterial interaction can be measured more precisely than *in vivo* since the influence of the immune system is absent in such models. On the basis of drug exposure and bacterial concentration these *in vitro* models can be classified into static models and dynamic models.

Static models

As the name suggests, static models evaluate how a bacterial culture responds to a constant environment with a fixed antibiotic exposure. Since there is no exchange of medium in these systems and bacteria utilize the same medium during the course of the experiment, their growth is limited by nutrition, space, aeration and toxic metabolites in addition to the effect of the antibiotic [13]. However, MIC determined from such a setup is the most prevalent and best-understood *in vitro* PD parameter for antibiotics, which is used routinely to track

antibacterial resistance in clinical isolates [8]. Although these models have been extensively used [16–20], they cannot provide information on the time course of antimicrobial activity under varying antibiotic concentrations as observed *in vivo*, and since MIC determination depends on the number of bacteria at a given time point, many different combinations of growth and kill rates can result in the same MIC [13]. However, these models when studied with a range of constant antibiotic concentrations over a period of time, can provide useful information on the kill kinetics, which approximates a constant infusion scenario of an intravenous antibacterial agent *in vivo*.

The application of such models for studying the PK/PD relationships of the anti-TB agents rifampin (RIF) and isoniazid (INH) and fluoroquinolones such as moxifloxacin (MXF), ofloxacin, sparfloxacin and ciprofloxacin (CIP) has been reported by Jayaram *et al.* [21–23] and is presented in Figure 2A. The kill kinetics were determined in BACTEC 7H12B medium, followed by plating on 7H11 agar plates to obtain colony counts. The Mtb culture was inoculated into BACTEC vials that were monitored daily by a radiometric detection method using the BACTEC TB 460 system, where the mycobacterial growth is determined by the utilization of ^{14}C with release of $^{14}CO_2$ by the multiplying bacteria. Fixed drug concentrations ranging from high to low were added to the vials and samples from the cultures with each concentration along with a drug-free control were plated over 9 days to determine the CFU per milliliter. **Time–kill curves** were plotted and analyzed for the rate and extent of bacterial killing. Rate of killing was determined from the start of experiment to the time of maximal reduction in the $\log_{10}$ CFU/ml, and *in vitro* dose–response curves were obtained by plotting the $\log_{10}$ CFU/ml against the ratio of the concentration to its MIC in broth (C_{broth}/MIC). Each fixed concentration of the drug was multiplied by the time of exposure to get AUC, which was then divided by MIC in broth to obtain the AUC/MIC index. The results from these studies allowed the authors to quantify PD parameters that could describe the *in vitro* bactericidal efficacy and indicated AUC-dependent killing for RIF, concentration-dependent killing for INH and time-dependent killing for the fluoroquinolones.

Dynamic models

Any typical dosage regimen for anti-TB drugs involves periodic administration of the antibiotic with varying drug concentrations at the effect site owing to *in vivo* absorption, distribution and particularly clearance mechanisms. As bacteria react to these changing concentrations differently than to exposure to constant concentrations, it is important to mimic similar conditions *in vitro* to understand the rate and extent of bacterial kill by the respective antibiotic under these conditions. Dynamic models utilize time–kill curves, which follow the microbial killing and growth as a function of both time as well as antibiotic concentration. These dynamic models used to study the PK/PD of anti-TB agents can be further differentiated as follows.

Type I: models without filters involving bacterial loss, where the mechanism of drug loss involves dilution—

In order to simulate *in vivo* drug clearance, a systematic loss of drug from the model becomes essential. This was usually achieved in early models via dilution performed in a stepwise or continuous manner. To a culture vessel containing actively growing bacteria, a known volume of culture was either substituted with fresh medium lacking the antibiotic at fixed intervals or it was infused using a peristaltic pump where the excess was directed into waste. Although these models mimicked a one-compartment PK model, they suffered from dilutional loss of bacteria [9,24]. Loss of bacteria from the model leads to a bias that can be corrected using mathematical equations [25,26]. However, for slow-growing organisms, the resulting bias may be substantial and

inadequate for mathematical correction. Therefore, no such models have been reported so far for studying anti-TB agents.

Type II: models with filters involving no bacterial loss, where the mechanism of drug loss involves dilution—The use of filters or membranes that do not allow the bacteria to be washed out are alternative approaches to overcome the issue of bacterial loss. A modified version of type I models involving a filter to prevent bacterial loss and a stirrer to prevent blockage of the filter membrane and to maintain the homogeneity of the culture has become popular to study the effects of antibiotics. One such model to evaluate time–kill curves of anti-TB drugs against mycobacteria has recently been reported by Budha *et al*. [27].

The *in vitro* system, as shown in Figure 2B, consisted of a two-armed, water-jacketed spinner culture flask, where a filter unit consisting of a prefilter (5 μm) and filter membrane (0.22 μm) was used to prevent leakage of bacteria during the dilution process. One of the arms covered with silicone septa was used for dosing and repeated sampling. The other arm was connected to a reservoir containing antibiotic free sterile medium. The whole filter unit was suspended into the media from the top via a hollow steel tube whose outlet was connected to a peristaltic pump to continuously withdraw the medium at a constant rate. The flask was placed on a magnetic stirrer, which ensured homogeneity of the culture and also prevented membrane pore blockage. The temperature in the flask was maintained at 37°C for bacterial growth by attaching a thermostatic water circulator to the water jacket of the flask. In this model, mycobacteria were exposed to INH concentration–time profiles as encountered during multiple dose regimens with 25, 100 and 300 mg/day in humans who are either fast or slow INH metabolizers and the resulting time–kill curves were obtained. The different elimination half-lives ($t_{1/2}$) of INH in slow versus fast metabolizers were simulated in the model by varying the flow rates of the medium. The results from this study indicate that $AUC_{0–24}$/MIC is the most explanatory **PK/PD index** for the antimicrobial effect of INH.

Type III: hollow-fiber models involving no bacterial loss, where the mechanism of drug loss involves diffusion—In spite of the modifications in type II models, membrane pore blockage could be a problem that becomes more pronounced when antibiotics with short half-lives are studied, since the flow rate across the filter is higher [28]. In order to overcome this problem, a diffusion or dialysis of the drug to create the desired antibiotic concentration profile in a separate bacterial compartment has been employed by several groups [29–31].

Gumbo *et al.* published several studies of the anti-TB agents RIF, INH, MXF and pyrazinamide (PZA) utilizing a hollow-fiber bioreactor system, which is an example of a diffusion model. Hollow-fiber systems represent a two-compartment model. The central compartment of the hollow-fiber system is composed of the central reservoir, the inner lumina of the hollow fiber capillaries, and the oxygen-permeable flow path connecting the central reservoir to and from the hollow fibers. The peripheral compartment is the space outside the hollow-fiber capillaries that is enclosed by an impermeable plastic encasement. The hollow-fiber bioreactor system allows Mtb to grow in the peripheral compartment of a hollow-fiber cartridge. This peripheral compartment is separated from the central compartment by semi-permeable hollow fibers, with pore sizes that selectively allow transfer of nutrients, drugs and bacterial metabolites but restrict bacteria from leaving the peripheral compartment, as shown in Figure 2C. In these experimental systems, sophisticated computer controlled peristaltic pumps are used to administer drugs via a dosing port and fresh 7H9 broth is pumped into the afferent port of the central compartment of the hollow-fiber system, while drug-containing media is isovolumetrically removed from

the efferent port of the system at rates programmed to simulate the drug half-life encountered in humans. These *in vitro* models were used to study a wide variety of issues such as selection of MXF doses that suppress drug resistance [32], understanding reasons for failure of CIP [33] and INH treatment [34], predicting efficacy of INH in different ethnic populations [35] and evaluating the PK/PD parameters of RIF [36] and PZA [37]. Dose-effect and dose-scheduling studies were also performed with ethambutol to identify exposures and schedules linked to optimal kill and resistance suppression [38].

In spite of these advancements, there are limitations associated with the use of the hollow-fiber bioreactor models. These bioreactors are complex and difficult to sterilize between experiments and, hence, new hollow-fiber cartridges are recommended for every study. This makes these studies very costly. Furthermore, nonspecific drug binding to the hollow-fiber capillaries has been reported to result in potentially substantial errors [27].

Perspectives relevant to drug development

The use of *in vitro* models in drug development has increased tremendously recently. Several scenarios for evaluating the activity of new molecules can be studied using these models. Since MIC does not provide any information on the persistent effects of antibiotics, inhibitory effects that persist after exposure to an antibiotic can be studied. In addition, anti-TB therapy is a combination of several antibiotic agents that is further complicated when treating patients co-infected with HIV, and it is challenging to study the different drug combinations *in vivo* from a drug-development perspective. Whole-blood bactericidal assays, serial sputum colony counting and liquid culture have been used in Phase II studies to examine different combinations of drugs, albeit not exhaustively and only with specific regimens of particular interest. *In vitro* models are reasonably simple to quantitatively assess the efficacy of such combinations and to perform more comprehensive experiments addressing these questions. Since it is relatively easy to study antimicrobial activity of drugs against different strains of bacteria in these systems, these models may also serve as useful tools to study the emergence of resistance and help identify novel drugs or combinations thereof to treat drug-resistant strains.

Owing to the fact that these *in vitro* PK/PD techniques are relatively new in the area of anti-TB drug development and tedious to work with because of the long doubling times of mycobacteria, few or no studies have been reported pertaining to the following three sections. Hence, we provide an insight into how these scenarios can be explored in the future using such *in vitro* models.

Post-antibiotic effect

The rate of bacterial killing and time before regrowth of surviving bacteria influences the design of an optimal dosing interval. Post-antibiotic effect (PAE) refers to the continued suppression of bacterial growth following limited exposure of organisms to an antibacterial agent [39]. It can be demonstrated *in vitro* by observing bacterial growth after a drug is removed. The standard method to quantitate PAE is to calculate the difference in time required for drug-exposed and control cultures to increase one $\log_{10}$ above the number present immediately after withdrawal of the antibiotic [40]. The PAE using bacterial counts as a parameter is calculated by PAE = T–C, where T is the time required for bacterial counts of drug-exposed cultures to increase one $\log_{10}$ above the counts observed immediately after washing/dilution and C is the corresponding time required for counts of untreated cultures [41].

Post-antibiotic effect has been used to determine the optimal interval between dosing and is the basis of twice or thrice weekly therapy for TB. Administration of drugs at less frequent

dosing intervals may not only improve patient compliance, but may also reduce toxicity and costs involved in the treatment. Studies performed using static models with INH, RIF, streptomycin, ethambutol, rifapentine and MXF have each demonstrated post-antibiotic effects against Mtb [42,43]. Prolonged PAEs are usually observed with antibiotics that are inhibitors of protein synthesis or nucleic acid synthesis. In other words, concentration-dependent antibiotics tend to have larger PAEs [14]. For drugs with concentration-dependent bactericidal action, the rate of bactericidal activity is maximal at the C_{max} and higher doses of the drug not only increase the rate of reduction of bacteria, but also the length of time of drug exposure to bactericidal concentrations. Therefore, the higher the drug concentration, the longer the duration of the PAE for these drugs, and the smaller the size of the residual bacterial population at the time of the next dose [43]. Hence, from a PK/PD standpoint, large doses can be administered at longer dosing intervals because prolonged PAE protects against the bacterial re-growth when concentrations fall below the MIC.

Bacteria exposed to sub-MIC concentrations of antibiotics may undergo adaptive changes with modifications in cell wall structure, changes in ribosome density or formation of filaments. In addition to morphological changes, sub-MICs may also have a direct inhibitory effect on the bacterial growth *in vitro* [44]. Post-antibiotic sub-MIC effect (PA SME) is another useful parameter that measures such effects of sub-MIC drug concentrations on bacterial growth following serial exposure to drug concentrations exceeding the MIC. This can be calculated as PA SME = $T_{pa}-C$, where T_{pa} is the time taken for the cultures previously exposed to antibiotics and then exposed to a sub-MIC to increase by one $\log_{10}$ above the counts observed immediately after washing/dilution and C is the corresponding time for the unexposed cultures [41]. Ginsburg *et al*. reported PAE properties of fluoroquinolones against Mtb using an *in vitro* PK/PD model [45]. By simulating PK parameters in humans, they determined the PAE of a single 400 mg dose of MXF on Mtb to be over 1 week compared with a PAE of greater than 15 days in a traditional static *in vitro* study of MXF after an exposure period of 24 h. Thus, such models can be effectively used to evaluate the post-antibiotic effect of existing antimycobacterial agents by simulating a particular dosing regimen *in vitro* and studying the growth and kill rates after cessation of that specific therapy.

Evaluation of combination therapy

Simultaneous administration of antibiotics is always practiced in anti-TB therapy owing to the high potential for the development of TB drug resistance using monotherapy and the differing drug sensitivities of active and latent populations of the TB bacilli found within an established infection. There has also been substantial interest in novel combinations of new compounds with existing drugs to block the emergence of drug resistance and to shorten the duration of therapy. Testing new drugs or drug combinations for activity against TB *in vivo* is a highly expensive and time-consuming process in animal models, and is largely prohibitive in humans for ethical reasons. *In vitro* models provide an inexpensive and rapid alternative for optimizing drug combinations before they proceed to preclinical or clinical testing. They also allow a high degree of standardization to study drug–receptor interactions without other interfering biological processes.

There are several existing methods to study antibiotic combinations *in vitro*. These include checkerboard techniques with tube dilutions or microtiter apparatus, the agar dilution method and various forms of disc proximity tests and paper strip methods [12,46–48]. However, all these methods involve constant concentrations of antibiotics to evaluate their activity.

When studying the combination of drugs and their time–kill curves in *in vitro* PK/PD models, differences in their PK properties can be taken into account. One of the major

setbacks in this approach, however, is the complexity involved in effectively simulating concentration–time profiles of drugs with different half-lives. In spite of these intricacies, *in vitro* PK/PD models for simultaneous simulation of serum kinetics of two or more drugs with different half-lives have been developed against fast growing organisms such as *Streptococcus pneumonia* and *Staphylococcus aureus* [49–53]. Although no examples have yet been reported for mycobacteria, similar models could likely be used to evaluate the synergistic potential of novel combinations of anti-TB agents.

Resistance

Emerging resistance threatens the usefulness of available treatment options against the multidrug-resistant mycobacteria. For example, MDR-TB takes approximately 18–24 months of therapy for complete cure as compared with a 6-month therapy against a susceptible strain. This prolonged duration of therapy is oftentimes also associated with severe toxicity related to the applied second-line agents leading to poor patient compliance. Thus, it is desirable to develop novel drugs or drug combinations that are safe and effective against MDR strains. *In vitro* PK/PD models can be effectively used to study such resistant clinical isolates for developing new molecules or new regimens with existing drugs. Conditions that can lead to resistant strains can be simulated and such resistant strains can be further studied.

Tam *et al.* used a hollow-fiber model to examine the relationship between garenoxacin (a desfluoroquinolone) and the likelihood of selecting resistant strains with different and escalating free AUC over 24 h (AUC_{24})/MIC exposures of the drug for 48 h [54]. The authors suggested that low AUC_{24}/MIC ratios between 10 and 35 were optimal for maximally amplifying the pre-existing resistant subpopulation. Exposures in excess of these ratios amplified the resistant subpopulation suboptimally, until an exposure was achieved that kept the number of resistant clones at or below the number present at the initiation of the therapeutic pressure resulting in an ‘inverted-U’ shape for the function linking drug exposure to suppression of the resistant subpopulation.

Mutant prevention concentration (MPC) defines the antimicrobial drug concentration threshold that would require an organism to simultaneously possess two resistance mutations for growth in the presence of the drug. For *M. bovis* BCG, the MPC is estimated by determining the minimal antibiotic concentration that results in recovery of no mutants when large numbers of cells are applied to antibiotic-containing agar plates. The use of large numbers of cells on the order of 10^{10} ensures that the restrictive antibiotic concentration blocks the growth of first-step mutants [55].

In one of the studies, the association between MPC-based PK/PD parameters (AUC/MPC, C_{max}/MPC or T > MPC) and emergence of resistant mutants of *Streptococcus pneumoniae* was evaluated for fluoroquinolones using an *in vitro* PK/PD model. The results from this study identified $AUC_{0–24}$/MPC and C_{max}/MPC indices to be associated with emergence of resistance [56]. Although no such studies have been performed using mutant Mtb for emergence of resistance against antimycobacterials, it is plausible to evaluate these scenarios using *in vitro* PK/PD models.

Population analysis profiling is a specialized technique initially developed to detect vancomycin resistance, but it can be applied to study drug-resistant Mtb strains. This technique compares the AUC of viable counts versus the concentration profile of a strain under study against a control-resistant strain after a given period of incubation. Based on this ratio, one can determine whether the test strain is a resistant one or not.

For example, in case of vancomycin-resistant *Staphylococcus aureus*, a ratio of AUC of a test strain against AUC of a hetero-resistant control strain Mu3 is calculated. This ratio exceeding 0.9 is used as a criterion to establish a vancomycin hetero-resistant *S. aureus* [57,58]. *In vitro* PK/PD models can be used to perform such analyses. They can also be used to study the population dynamics of the susceptible and resistant strains over time following a particular dosage regimen [15]. Although no such reports have been published so far for anti-TB drugs, similar studies can be performed using MDR-TB and XDR-TB in order to establish effective dosing regimens against drug-resistant strains.

PK/PD data analysis

Pharmacokinetic/PD modeling and simulation techniques are increasingly used in drug development in a variety of indications. Such techniques allow characterizing the time course of the effect intensity resulting from a certain dosing regimen [2] and have been widely applied to antibiotics for evaluating their PDs (their initial rate of the bacterial killing and the regrowth of bacteria) and to obtain a relevant PK/PD index that correlates with a therapeutic outcome.

Mathematical modeling of drug effects maximizes the information gained from an experiment, provides further insight into the mechanisms of drug effects and allows for simulations in order to design studies or even derive clinical treatment strategies [59]. Modeling approaches can be classified into mechanism-based or empirical.

Mechanism-based modeling for estimating PK/PD parameters

A mechanistic model is by definition a mathematical model based on known or hypothesized mechanisms of behavior of a biological system. The parameters are in accordance with PK, physicochemical, biophysical, physiological and pathophysiological principles, and have direct identifiable biological or biophysical interpretations [60]. Mechanism-based models for antimicrobials utilize drug concentrations and relate them to their effect. They are commonly applied to estimate the PK/PD parameters of antibiotic drugs and have to include at least a submodel of microorganism replication, a submodel of antimicrobial drug effects and a submodel for changing drug concentrations (PK submodel) [59]. Since these models are limited to *in vitro* data, a host defense submodel is usually not included.

The submodel for microorganism replication is most commonly modeled using Equation 1:

$$\frac{dN}{dt} = N \cdot k_{replication} - N \cdot k_{death} \qquad \text{Equation 1}$$

where N is the number of microorganisms and the first-order rate constants $k_{replication}$ and k_{death} describe natural replication and death of microorganisms in the absence of antibacterial agents. This model, however, is based on the underlying assumption that the number of microorganisms, N, that can replicate is the same as the number subjected to death. In addition, this model cannot adequately describe the decrease in the net growth rate as the system approaches the stationary phase of bacterial growth. k_{growth} or $k_{apparent}$, a first-order rate constant for observed growth, which is the difference between $k_{replication}$ and k_{death} is often applied because of difficulties in separating microorganism replication and death by a traditional kill curve analysis [59].

Increased knowledge regarding the production and the nature of persister cells, for example, cells with reduced growth rates and reduced antibiotic susceptibilities, can be efficiently

applied to build mechanism-based PK/PD models [61]. Nielsen *et al.* described a semimechanistic PK/PD model for assessment of antibacterial agents where the total bacterial population was divided into growing (*S*) and resting (*R*) population, allowing a transfer from the growing population to the resting population as the total population approaches the stationary phase. Mathematically this can be modeled using Equations 2 & 3:

$$\frac{dS}{dt} = S \cdot k_{growth} - S \cdot k_{death} - S \cdot k_{SR} \quad \text{Equation 2}$$

$$\frac{dR}{dt} = S \cdot k_{SR} - R \cdot k_{death} \quad \text{Equation 3}$$

where k_{SR} is the transfer rate constant [62]. To incorporate the antimicrobial effect, a submodel of antimicrobial drug effect relating the drug concentrations to the microbial survival is often described using Equation 4:

$$\frac{dN}{dt} = E_{replication} \cdot N \cdot k_{replication} - E_{death} \cdot N \cdot k_{death} \quad \text{Equation 4}$$

The E_{max} model can be used to describe drug concentration and its effect on replication and death rate of the bacteria. The maximum effect obtained when determining a concentration–effect relationship (E_{max}) and the concentration required to produce half-maximal effect (EC_{50}) are PD parameters used in defining the E_{max} model. The E_{max} model can also be used to describe the relationship between a PK/PD index and the effect. Drug effect decreasing the replication rate ($E_{replication}$) can be modeled using an inhibitory sigmoid E_{max} model shown in Equation 5:

$$E_{replication} = 1 - \frac{I_{\max} \cdot C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}} \quad \text{Equation 5}$$

where I_{max} is the maximum inhibitory effect, C is the drug concentration, γ is the Hill coefficient and IC_{50} is the concentration that produces half-maximal inhibition.

Drug effect leading to increased death rate (E_{death}) can be modeled using a stimulatory sigmoid E_{max} model in Equation 6:

$$E_{death} = 1 + \frac{E_{\max} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}} \quad \text{Equation 6}$$

where E_{max} is the maximum stimulatory effect, C is the drug concentration, γ is the Hill coefficient and EC_{50} is the concentration that produces half-maximal stimulation leading to death.

It is well known that bacteria show different growth phases and that antibiotic-induced killing often shows an initial phase with rapid killing, followed by a decline in the killing rate with time. Therefore, ignoring the replication inhibition and including stimulation of death rate in Equation 4 leads to Equation 7, as shown below:

$$\frac{dN}{dt} = N \cdot k_{replication} - N \cdot k_{death}\left(1 + \frac{E_{\max} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}\right)$$

Equation 7

with observed growth ($k_{growth} = k_{replication} - k_{death}$) and a maximum kill rate (k_{kill} *or* $k_{max} = k_{death} \cdot E_{max}$), this equation can be further transformed into the most frequently applied PK/PD model for antibacterial drugs (Equation 8) [59]:

$$\frac{dN}{dt} = N \cdot k_{growth} - N \cdot k_{\max} \cdot \frac{C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}$$

Equation 8

The PK submodel describes the relationship between dose or dosing regimen and drug concentrations with time. Depending on the *in vitro* setup, this can be modeled as a one-compartment model or a multicompartment model. Assuming dosing by an intravenous bolus administration, a general form of the model can be defined as in Equation 9:

$$C_t = A_1 e^{-\alpha_1 t} + A_2 e^{-\alpha_2 t} + \ldots.. + A_n e^{-\alpha_n t}$$

Equation 9

where A_1 to A_n are correlation coefficients, α_1 to α_n are first-order rate constants and n denotes the number of compartments. This submodel can also be applied to approximate the concentration–time profiles for orally administered drugs that are rapidly absorbed.

Budha *et al.* reported the relationship between INH exposure and mycobacterial kill using a modified version of Equation 8, as shown in Equation 10, which was initially developed for voriconazole against *Candida* isolates [27,63]:

$$\frac{dN}{dt} = \left[k_0 \cdot \left(1 - \frac{N}{N_{\max}}\right)\right] - (1 - e^{-\alpha \cdot t}) \cdot \left(\frac{I_{\max} \cdot C}{IC_{50} + C}\right) \cdot N$$

Equation 10

where N is the mycobacterial cell counts in CFU/ml, k_0 is the bacterial net growth rate constant, N_{max} is the maximum number of bacteria in the system in CFU/ml, I_{max} is the maximum kill rate, C is the concentration of INH at time t and IC_{50} is the concentration at half-maximal kill rate. A logistic growth function, $1\text{-}N/N_{max}$ was used to describe the limited growth of bacteria in the absence of INH.

The authors reported a delay in bacterial kill, which is likely due to the time necessary to achieve sufficient intracellular drug exposure to initiate the killing process that was modeled using the term, $1 - e^{-\alpha \cdot t}$, where α is the delay rate constant.

When studying PDs of antibiotics against Mtb, an adaptive IC_{50} is often used [27,35,63]. This model component is necessary to explain the change in IC_{50} over the course of treatment due to an increase in drug insensitive cell population; for example, due to latency for *Mycobacterium* species. The adaptive IC_{50} can be modeled using Equation 11 below:

$$IC_{50,A} = IC_{50} \cdot e^{\left(\frac{k_{adaptation} \cdot N_0}{N \cdot AUC_{0-24}}\right)}$$

Equation 11

where $IC_{50,A}$ is the adaptive IC_{50}, N_0 is the number of bacterial cells at time zero, N is the cell count in CFU/ml, $k_{adaptation}$ is the adaptation constant and IC_{50} is the baseline IC_{50}.

Gumbo *et al.* reported a mechanistic PK/PD model for MXF [32], which has been further applied in modeling INH [34] and RIF [36] PK/PD data. The resultant changes in the drug-resistant [*R*] and the drug-sensitive [*S*] *Mycobacterium TB* population were described using Equations 12 & 13 below:

$$\frac{dN_s}{dt} = k_{g\max -s} \cdot (1 - L_s) \cdot N_s \cdot E - k_{g\max -s} \cdot M_s \cdot N_s \qquad \text{Equation 12}$$

$$\frac{dN_R}{dt} = k_{g\max -R} \cdot (1 - L_R) \cdot N_R \cdot E - k_{g\max -R} \cdot M_R \cdot N_R \qquad \text{Equation 13}$$

where each subpopulation has an independent growth rate constant (drug sensitive, $k_{gmax\text{-}S}$, drug resistant, $k_{gmax\text{-}R}$). Bacteria at the site of infection that were in the logarithmic growth phase in the absence of drug exhibited an exponential density-limited growth rate described by Equation 14:

$$E = 1 - \frac{N_R + N_s}{POPMAX} \qquad \text{Equation 14}$$

where *E* is the logistic growth term, and the maximal bacterial density (POPMAX) is identified as part of the estimation process. The authors allowed drug to affect the growth rate independently of kill through a saturable Michaelis–Menten-type kinetic event (*L*) and the killing effect of the drug was modeled as a saturable Michaelis–Menten type kinetic event (*M*) that relates the kill rate to drug concentration, where $C_{50\text{-}g}$ and $C_{50\text{-}k}$ is the drug concentration at which the bacterial growth or kill rate is half-maximal, as described in Equations 15 & 16:

$$L = \left(\frac{X_1}{V_c}\right)^H \Big/ \left[\left(\frac{X_1}{V_c}\right)^H + C_{50-g^H}\right] \qquad \text{Equation 15}$$

$$M = \left(\frac{X_2}{V_c}\right)^H \Big/ \left[\left(\frac{X_2}{V_c}\right)^H + C_{50-k^H}\right] \qquad \text{Equation 16}$$

where *H* is the slope constant, X_1 and X_2 are the amounts of drug in the central and the peripheral compartments of the hollow-fiber system, respectively, and V_c is the volume of the central compartment of the hollow-fiber system. For drug-sensitive and -resistant populations as expressed in Equations 13 & 14, there are separate terms for *H*, $C_{50\text{-}g}$ and $C_{50\text{-}k}$.

Empirical modeling for identifying appropriate PK/PD indices

An empirical model is a nonmechanistic model whose parameters may be adequate for describing the longitudinal data at hand, but does not take the underlying biological mechanisms into account. It can be predictive when used in conditions sufficiently similar to the ones already studied, but has usually limited predictability beyond these conditions [60]. A typical empirical modeling exercise utilizes a time-integrated measure of exposure, such as AUC or dose, from *in vitro* time–kill experiments to determine the primary PK/PD index

responsible for the antibacterial effect and to understand whether a molecule exhibits a time- or an exposure-dependent killing pattern.

AUC/MIC, C_{max}/MIC and T>MIC, as discussed in the previous sections, are the three most commonly used PK/PD indices to evaluate antibacterial effects. Using a combination of dose escalation, fractionation and different strains, one can identify the best suitable index related to the antibacterial effect for a specific compound [15]. A sigmoid inhibitory dose–response model is one of the most frequently used models to characterize antimicrobial activity. For instance, in one of the recent studies for identification of the most appropriate empirical PK/PD index associated with the microbial kill of the first-line anti-TB agent INH, viable cell counts on different days following multiple dosing of INH were analyzed using an inhibitory E_{max} model shown in Equation 17:

$$E = E_{control} - \left(\frac{E_{\max} \cdot PKPD}{EC_{50} + PKPD} \right) \quad \text{Equation 17}$$

where E is the observed *M. bovis* BCG cell counts in $\log_{10}$CFU/ml, $E_{control}$ is the cell count in the control experiment, and E_{max} is the maximal anti-microbial effect in $\log_{10}$CFU/ml. EC_{50} is the value of the PK/PD index that produces half-maximal antimicrobial effect and PK/PD is one of the empirical PK/PD indices $AUC_{0–24}$/MIC, T>MIC or C_{max}/MIC. INH exhibited exposure-dependent antibacterial activity on *M. bovis* BCG where the empirical PK/PD index $AUC_{0–24}$/MIC was found to be well associated with the microbial kill [27].

For the first-line anti-TB agents INH, RIF and PZA and second-line fluoroquinolones and aminoglycosides, a concentration-dependent killing pattern that correlates best with AUC/MIC ratio is shown to be a reliable predictor of efficacy [27,32–37].

Drawbacks of *in vitro* models

In spite of the potential advantages of *in vitro* models discussed in this manuscript, these models are obvious simplifications of *in vivo* scenarios and therefore come with certain drawbacks that cannot be ignored. These can be classified under host factors, pathogen factors and PK factors.

Host factors

The host immune system plays a major role in TB. Most infected immunocompetent individuals fail to progress to full-blown disease because the Mtb bacilli are directly killed by macrophages or walled off by the immune system inside a tissue nodule known as a granuloma. The granuloma's primary function is to contain and prevent the dissemination of the mycobacteria [64]. Human tuberculous granulomas contain an organized collection of differentiated and activated macrophages, T-lymphocytes, some B-lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components that limit nutrient delivery to the core [65]. Bacteria are often found multiplying inside macrophages around the edge of the necrotic center of a granuloma. *In vitro* PK/PD models lack the ability to mimic this complex host-defense mechanism and therefore approximate more to conditions of an immunodeficient patient where the infection is more bacteremic in nature [9]. However, during the reactivation phase of the disease, the majority of the bacilli in pulmonary cavities reside extracellularly [66] and, hence, the results obtained from *in vitro* models may be reflective of the *in vivo* killing under this condition.

Besides the lack of immune response, physiological conditions also vary between *in vivo* and *in vitro* setups leading to differences in anti-TB activity. For example, the front-line

anti-TB drug pyrazinamide is inactive in normal culture conditions and is only active at acidic pHs that are thought to mimic those experienced by bacteria in activated macrophages. The TB granuloma is also believed to be subjected to a gradient of microaerophilic/anaerobic conditions that limit growth. Since these conditions are technically difficult to simulate *in vitro*, antibacterial efficacy can differ significantly between the *in vitro* model and the *in vivo* environment.

Pathogen factors

The growth rate for most bacteria is faster *in vitro* compared with growth *in vivo* or in human serum. Since antimicrobial efficacy in an *in vitro* model relates to the rate of bacterial growth, this can be a potential limitation of *in vitro* models, especially when there are significant differences in the growth rates [67,68]. Biochemical data suggest that Mtb growing *in vivo* upon granuloma formation shifts to anaerobic metabolic pathways. It has also been demonstrated that tubercle bacilli are able to shift into a nonreplicating drug-tolerant state when nutrients are depleted. This process gives Mtb the capability to lie dormant in the host for long periods of time and this results in differences in growth conditions *in vitro* and *in vivo* [69].

Furthermore, the growth rate of Mtb within the infected host varies according to the type of lesion, such as extracellular and actively multiplying bacilli in the liquefied caseous material covering the cavity wall, semi-dormant bacilli with intermittent bursts of metabolic activity inhabiting solid caseous material and a small population of bacilli within the acidic environment around the areas of active inflammation or necrosis. The growth characteristics within each lesion result in differing susceptibility to specific anti-TB agents [70] and simulating such varying populations *in vitro* is very challenging. Only a few studies have been reported so far where different metabolic populations of Mtb growing under acidic conditions and under anaerobic/hypoxic conditions have been subjected to anti-TB drug treatment using *in vitro* PK/PD models [37].

PK factors

Drug distribution is technically challenging to simulate *in vitro*. Using complex *in vitro* setups, one can approximate the *in vivo* elimination process of the drug, but the model may not truly represent the *in vivo* drug distribution. Plasma protein binding of the antibiotic is absent in *in vitro* models and needs to be accounted for. Thus, free drug concentrations relevant to the site of infection must be simulated. Although this is feasible, protein binding *in vivo* is a dynamic process and can be altered due to co-existing disease conditions or the presence of other drugs, which is challenging to simulate *in vitro*.

Future perspective

With the increasing incidence of MDR-TB and XDR-TB cases, it is more than ever pressing to develop new anti-TB drugs and evaluate combinations of these new drugs with existing anti-TB agents to block the development of drug resistance and shorten TB therapy. The path to new antibiotic drug discovery and development is a long and expensive procedure and major tools are needed to expedite this process.

In vitro PK/PD models serve as useful tools to evaluate the efficacy of such new anti-TB drugs under development. Since they come with benefits of reduced cost, flexibility and adaptability, they can be used to guide the early drug development process and establish efficacy in experimental disease models. As advances in the technology associated with the currently existing *in vitro* models are made, these models will become more efficient to evaluate *in vivo* scenarios. From predicting the relevant PK/PD indices to studying the effect of novel therapeutics on resistant clinical isolates, these *in vitro* models in combination with

preclinical data can not only help in optimizing dosing regimens that can drive efficacy, but can also help in a bench to bedside translation of newer classes of anti-TB agents. In spite of the potential drawbacks associated with *in vitro* models, the wealth of information they provide will be exploited to understand mechanisms of drug resistance and develop new drugs that can target resistant strains. Associated mathematical modeling and simulation techniques will allow the prediction of outcomes for new scenarios and thus allow improving study designs for expensive preclinical and clinical studies, thereby optimizing the use of limited development resources and accelerating the project progression.

In vitro PK/PD models will also likely be more often applied in academic research and clinical settings. Utilizing such models may help clinicians optimize dosing regimens for new drugs or combinations thereof with existing anti-TB therapies and, thus, improve available treatment options for TB patients, especially those with MDR-TB, thereby producing better treatment outcomes and ultimately getting one step closer to eradicating TB.

Key Terms

Pharmacokinetics	Describes the time course of concentration of a drug resulting from administration of a dosage regimen and accounts for its absorption, distribution, metabolism and excretion in the body
Pharmacodynamics	Describes the intensity of drug effect in relation to its concentration. In the context of anti-infective therapy, it defines the effect of the drug on the pathogen residing in the host organism
***In vitro* PK/PD model**	Static or dynamic system that can mimic *in vivo* conditions of changing drug concentrations, designed to evaluate the efficacy of antibiotics *in vitro*
Time–kill curves	Profiles that follow the microbial killing and growth as a function of both time as well as antibiotic concentration
PK/PD index	Composite of a PK parameter such as area under the plasma concentration–time curve or maximum plasma concentration and a microbiological parameter such as minimum inhibitory concentration

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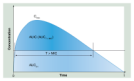

Figure 1. Commonly used pharmacokinetic/pharmacodynamic indices

AUIC: Area under the inhibitory curve; AUC: Area under the curve; C_{max}: Maximum plasma concentration; T > MIC: Time above minimum inhibitory concentration.

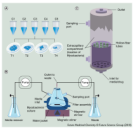

Figure 2. *In vitro* pharmacokinetic/pharmacodynamic models for anti-tuberculosis agents

(A) Static model where C1–5 denote a series of concentrations of a drug and T1–4 denote times at which aliquots of mycobacteria are plated for every given concentration of drug. **(B)** A dynamic model with filter assembly involving no bacterial loss and the mechanism of drug loss involves simple dilution by the media. **(C)** A hollow-fiber model where there is no bacterial loss and the mechanism of drug loss involves diffusion into the extracapillary compartment.

Table 1

Reported pharmacokinetic/pharmacodynamic parameters of anti-tuberculosis drugs.

Drug	Dose range	Half-life (h)	fup	C_{max}/MIC	AUC/MIC	T > MIC
First-line						
Oral agents						
Rifampin	8–12 mg/kg	2–5	0.1	24	39.9	9
Isoniazid	4–6 mg/kg	2–4.5 (slow)	0.99	40	19.2	18
		0.75–1.8 (fast)			11.6	9
Pyrazinamide	20–30 mg/kg	10–24	0.9	3.8	52	N/A
Ethambutol	15–20 mg/kg	3–4	0.7	10	23.4	13
Injectable agents						
Streptomycin	12–18 mg/kg	2–4	0.45	10	124.5	8
Second-line						
Fluoroquinolones						
Moxifloxacin	400 mg	12–14	0.5	12.3	110.5	N/A
Gatifloxacin	400 mg	7–14	0.8	9.5	85.6	N/A
Ciprofloxacin	750 mg	4	0.6	5	16.9	10.5
Ofloxacin	1000 mg	3–7	0.7	5	47.4	15.5
Injectable agents						
Amikacin	15 mg/kg	2–3	0.9	43	N/A	N/A
Kanamycin	15–30 mg/kg	2.5	0.99	20	N/A	N/A
Capreomycin	15–30 mg/kg	4–6	N/A	20	N/A	N/A
Oral bacteriostatic agents						
Ethionamide	15–20 mg/kg	2–3	0.7	1.6	1	1.5
Cycloserine	15–20 mg/kg	10	N/A	3.8	195.5	22.5
P-aminosalicylic acid	150 mg/kg	2–3	0.85	75	153.7	4
Thiacetazone	150 mg	4	0.05	1.3	1.2	5.5
Other agents						
Linezolid	600 mg	4.5–5.5	0.7	20	107.8	N/A

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AUC: MIC: Ratio of area under plasma concentration–time curve to MIC; C_{max}: MIC: Ratio of peak serum concentration to minimum inhibitory concentration; fup: Fraction of unbound drug in plasma; MIC: Minimum inhibitory concentration; T > MIC: Percentage of time during which the serum concentration remains above the MIC.

Data from [5,27,69–77].