Published in final edited form as:

Tuberculosis (Edinb). 2009 September ; 89(5): 378–385. doi:10.1016/j.tube.2009.08.002.

A Simple *in vitro* PK/PD Model System to Determine Time-Kill Curves of Drugs against Mycobacteria

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Abstract

In vivo tuberculosis is exposed to continually changing drug concentrations for which static minimum inhibitory concentration (MIC) testing may be a poor surrogate. While *in vitro* approaches to determine time-kill curves for antibiotics have been widely applied in assessing antimicrobial activity against fast growing microorganisms, their availability and application for slow growing microorganisms including *M. tuberculosis* has so far been scarce. Thus, we developed a novel simple *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model for establishing time-kill curves and applied it for evaluating the antimicrobial activity of different dosing regimens of isoniazid (INH) against *Mycobacterium bovis* BCG as a surrogate for virulent *M. tuberculosis*. In the *in vitro* model *M. bovis* BCG was exposed to INH concentration-time profiles as usually encountered during multiple dose therapy with 25, 100 and 300 mg/day in humans who are fast or slow INH metabolizers. Bacterial killing was followed over time by determining viable counts and the resulting time-kill data was analyzed using a semimechanistic PK/PD model with an adaptive IC₅₀ function to describe the emergence of insensitive populations of bacteria over the course of treatment. In agreement with previous studies, the time-kill data suggest that AUC₀₋₂₄/MIC is the PK/PD index that is the most explanatory of the antimicrobial effect of INH. The presented *in vitro* PK/PD model and associated modeling approach were able to characterize the time-kill kinetics of INH in *M. bovis* BCG, and may in general serve as a potentially valuable, low cost tool for the assessment of antibacterial activity in slow growing organisms in drug development and applied pharmacotherapy.

Keywords

In vitro model; time-kill analysis; isoniazid; PK/PD; tuberculosis

1. Introduction

In spite of the fact that bacteria are exposed to dynamically changing drug concentrations *in vivo*, antibiotics are frequently dosed based on point estimates of efficacy derived from exposure to constant concentrations. Until last decade, it was a common practice to select antimicrobial dosing regimens resulting in concentrations above the minimum inhibitory concentration (MIC) of the pathogen being treated. This approach, however, has several disadvantages as it does not accurately reflect the complex interaction among the three major

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Competing Interests: None.

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players, the drug, the microorganism and the host. Constant (static) drug levels used in MIC determinations hardly reflect the actual *in vivo* situation where the pharmacodynamic effect is the result of dynamic exposure of the organism to the unbound drug fraction in the target tissue. Furthermore, the MIC provides only limited information on the kinetics of bactericidal activity as it is used as a threshold value for the activity of the antibiotic. In reality, however, antimicrobials usually have already some activity at concentrations below the MIC and may not have the maximum activity just above the MIC. Thus, complete time courses of the drug effect in response to concentration-time courses encountered *in vivo* seem to be more adequate and informative for selecting optimal dosing strategies than the use of point estimates such as MIC.¹⁻⁴

In recent years, integrated pharmacokinetic/pharmacodynamic (PK/PD) approaches have been developed as a more sophisticated methodology to assess the efficacy of antimicrobials based on dynamic kill curves. These PK/PD-based approaches are being used to guide the selection of potential drug candidates and to provide a scientific rationale for selecting dosing regimens that increase the efficacy and reduce the probability for the emergence of drug resistance⁵. The US Food and Drug Administration and the European Medicines Evaluation Agency have been advocating the use of various *in vitro* and *in vivo* models to characterize the PK/PD properties of antibiotics and to predict the efficacy of candidate compounds in the early stages of the drug development process.^{6,7}

In vitro PK/PD models can be used to study the antibacterial effect of single and combination drug compounds and dosing regimens before *in vivo* efficacy studies.⁸ The advantage of these models is that the appropriate human/animal pharmacokinetic profiles can easily be simulated and the effect of these changing drug concentrations on bacterial growth (i.e. via kill curves) and emergence of drug induced tolerance and resistance can be assessed. Different species of microorganisms and clinical isolates of the same bacteria that vary in sensitivity to drug treatment can be exposed to different dosing regimens, such that the PD parameters can be identified that best explain the activity/efficacy of the drug and allow the optimization of dosage regimens for maximum efficacy. Since the influence of the immune system is absent in these *in vitro* models, the drug-bacterium interactions can be measured more precisely. Thus, *in vitro* models offer a safer and more ethical way of assessing the PK/PD relationships of antibiotics compared to animal or human studies.^{1,2,9,10}

Several *in vitro* PK/PD models for antibiotics have been reported in the literature and can be broadly classified as either dilution models or diffusion models based on the mechanism by which the dynamic concentration-time profiles are simulated.^{8,11} However, most of the available *in vitro* models have been applied for fast growing bacteria and are not suitable for studies on slow growing microorganisms such as *Mycobacterium tuberculosis*. The previously described dilution models without a filter membrane¹²⁻¹⁴ are not suitable for slow growing organisms since the bacteria are washed out during the dilution process and the bacterial replication rate is not sufficient to offset this loss. The reported dilution models with filter membrane have so far not been utilized for slow growing organisms in the published literature. More recently, several reports have been published using hollow fiber bioreactors (diffusion models) as *in vitro* models for testing antibacterial activity against *M. tuberculosis*.¹⁵⁻¹⁸ However, there are severe limitations associated with the use of hollow fiber bioreactors for *in vitro* culturing of bacteria. As these bioreactors are complex and difficult to sterilize between experiments, new hollow fiber cartridges are recommended for every study which makes a broad-based application of these experiments cost-prohibitive. Further, nonspecific drug binding to the hollow fiber capillaries, especially of the highly lipophilic compounds frequently tested for anti-mycobacterial activity, can produce erroneous results. In view of these limitations of the existing *in vitro* models, we decided to develop a novel *in vitro* PK/PD model for studying the pharmacologic properties of antibiotics against slow-growing microorganisms

that is simple, inexpensive and easy to construct and operate. Herein, we illustrate the application of this model to determine time-kill curves of slow growing microorganisms by characterizing the effect of isoniazid (INH) dosing regimens on *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG).

2. Materials and methods

2.1. Culture, media and antibiotics

Mycobacterium bovis BCG was grown to mid-log phase in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) supplemented with 10% albumin dextrose complex and 0.1% v/v Tween 80. Aliquots were stored at -80°C in Middlebrook 7H9 broth. For each experiment, cultures were inoculated 1:100 from a frozen stock and incubated at 37°C in Middlebrook 7H9 broth, with shaking at 225 rpm, to an OD_{600nm} of 0.5. The antibiotic isoniazid (INH) was obtained from Sigma-Aldrich (St. Louis, MO). A stock solution of antibiotic was prepared in DMSO and stored at -80°C. Working solutions of INH were prepared from the stock solution using sterile water.

2.2. In vitro Model Design

The *in vitro* model system consisted of a two-armed, water jacketed spinner culture flask (1965 series spinner flask, Bellco Glass, Vineland, NJ) as depicted in Fig.1. A 25 mm diameter filter unit (Pall Corporation, East Hills, NY) was fixed to one end of a custom made hollow steel tube. The other end of the steel tube was connected to plastic tubing (PharMed tubing, Cole-Parmer, Vernon Hills, IL). One end of the hollow tube included a screw head for easy connection with the filter unit. The other end was cone shaped to allow easy attachment of the plastic tubing. A prefilter (5µm, Millipore, Billerica, MA) and filter membrane (0.22µm, Millipore, Billerica, MA) were placed in the filter unit to prevent leakage of bacteria during the dilution process. The whole filter unit was suspended into the media from the top. The flask was placed on a magnetic stirrer; a magnetic stir bar in the flask ensured homogeneity of the culture and prevented membrane pore blockage. One of the side arms covered with silicone septa was used for repeated sampling. The other sidearm was connected to a reservoir containing antibiotic free sterile medium through a thin plastic tube (PharMed tubing, Cole-Parmer, Vernon Hills, IL). The outlet tubing from the filter unit was connected to a peristaltic pump (Masterflex L/S, Cole-Parmer, Vernon Hills, IL) to continuously withdraw the medium from the flask at a constant rate. Fresh sterile medium (Middlebrook 7H9, Becton Dickinson, Sparks, MD) was pumped from the reservoir into the flask at the same rate. The solution of the antibiotic INH was added through the sidearm. INH concentration in the flask was decreased exponentially according to the equation,

$$C = C_0 \cdot e^{-k \cdot t} \quad \text{Eq.1}$$

where C_0 is the initial concentration of INH in the flask, C is the concentration at any time t , k is the elimination rate constant and t is the time elapsed since the addition of INH. The elimination rate constant k is equal to F/V , where F is the flow rate of the medium and V is the volume of the medium in the flask. Different elimination half-lives ($t_{1/2}$) of INH were simulated in the model by changing the flow rate according to the equation,

$$F = V \cdot \frac{\ln 2}{t_{1/2}} \quad \text{Eq.2}$$

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. The apparatus was sterilized by autoclaving between experiments and was kept in a biological safety cabinet during operation (Fig.2).

2.3. Minimum inhibitory concentration of isoniazid

The MIC of INH was determined using the microbroth dilution method and was read by visual inspection. Two fold serial dilutions of INH in 100 µL of the Middlebrook 7H9 broth medium were prepared in round-bottomed micro titer plates (Nunc, Sigma-Aldrich, St. Louis, MO). An equivalent volume (100 µL) of broth containing $\sim 10^5$ CFU/mL bacteria was added to each well to give final concentrations of INH starting at 200 mg/L and the plates were incubated aerobically at 37°C for 7 days. The MIC was recorded as the lowest concentration of drug that prevented visible growth.

2.4. Time-kill curves of INH

The test organism *M. bovis* BCG was used to evaluate the *in vitro* model. *M. bovis* BCG was grown to mid-logarithmic growth phase 0.5 OD_{600nm} in Middlebrook 7H9 medium. The logarithmically growing culture was then used to inoculate the *in vitro* model. The final density of the culture in the *in vitro* system was $\sim 10^6$ CFU/mL. The medium was drawn from the flask via the combination of a 5 µm and a 0.22 µm filter at a predetermined flow rate that mimicked the *in vivo* elimination half-life of INH for slow and fast acetylators.¹⁹ Fresh sterile medium was pumped into the flask at the same rate through one side arm to keep the volume of the medium at the initial level. The accuracy of the dilution process was ensured by monitoring the volume of medium in the flask at the same (initial) level and measuring the outlet flow rate throughout the experiment. The content of the flask was stirred with a magnetic stirrer at a speed of 50-100 rpm to ensure homogeneity of the culture. The addition of INH doses (as bolus doses) was started after the culture had reached the logarithmic growth phase (0.2 to 0.3 OD_{600nm}) which was determined by the optical density of the culture. INH doses were added daily for 7 days to simulate INH plasma concentration-time profiles in humans treated with 25, 100 and 300 mg/day (Table1). The volume of dosing solution for all dose groups was kept below 0.2 mL. Since INH is metabolized in humans by a polymorphically expressed enzyme, NAT2 (N-acetyl transferase 2), this difference in INH metabolizing activity results in a bimodal distribution of elimination half-lives with fast and slow acetylators. Thus, elimination half-lives of either 4.5 or 2.0 hr were simulated in the model to mimic slow and fast acetylators, respectively. Approximately 200 µL of the medium was collected at time zero and every 24 hours thereafter until the end of the experiment. The number of viable bacteria in each sample was determined by plating serial dilutions of the samples on antibiotic-free Middlebrook 7H11 agar plates. The plates were incubated for 3-4 weeks at 37°C. The antibacterial activity was measured as the reduction in number of viable bacteria, expressed as CFU/mL. Each experiment was performed in duplicate for each dose level and elimination half-life. Drug-free controls were included in the study for comparison to assess the extent of killing by INH. Time-kill curves were constructed based on the time course of CFU/mL measurements.

2.5. Time-kill curve data analysis

The relationship between INH exposure and microbial kill was modeled by using a previously described time-kill curve equation,²⁰

$$\frac{dN}{dt} = \left[k_0 \cdot \left(1 - \frac{N}{N_{\max}} \right) - \left(1 - e^{-\alpha \cdot t} \right) \times \left(\frac{I_{\max} \cdot C}{IC_{50} + C} \right) \right] \cdot N \quad \text{Eq.3}$$

where N is the *M. bovis* BCG cell count 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. The logistic growth function, $1-N/N_{\max}$ was used to describe the limited growth of bacteria in the absence of antibacterial agents. The delay in bacterial kill rate was modeled using the term $1-e^{-\alpha t}$, where α is the delay rate constant. According to the model, the delay rate constant α mathematically modulates $I_{\max}$ over time and determines the duration of time needed for $I_{\max}$ to reach its value. The delay in bacterial kill is likely due to the time necessary to achieve sufficient intracellular drug exposure to initiate the killing process. The IC_{50} was modeled as adaptive constant which changes with the exposure and the ratio of the initial cell count to the cell count at time t according to equation,

$$IC_{50,A} = IC_{50} \times e^{\left(\frac{\text{Adaptation Constant} \times N_0}{N \times AUC_{0-24}} \right) * t} \quad \text{Eq. 4}$$

where $IC_{50,A}$ is the adaptive IC_{50} , N_0 is the number of bacterial cells at time zero and IC_{50} is the baseline IC_{50} . The adaptive IC_{50} explains the change in IC_{50} due to an increased drug insensitive cell population over the course of treatment, such as the frequently observed latency for *Mycobacterium* species.^{17,21-23} All time-kill data were analyzed simultaneously by nonlinear mixed effects modeling (NONMEM v.6.2, Icon, Ellicott City, MD). PK parameters were fixed in the PK/PD model and set equal to their selected values ($V=55$ mL, where V is volume of the medium in the system; half-life $t_{1/2} = 4.5$ and 2.0 hr for slow and fast acetylators respectively). The first-order conditional estimation method within NONMEM was used to simultaneously analyze the generated time-kill curves from all experiments. The measured data and model predictions were log transformed and the random residual variability was modeled using an additive error term. The between experiment variability in the parameter α was modeled as exponential error. Model performance was evaluated using goodness-of-fit plots, including observed vs. predicted, weighted residuals vs. time and weighted residuals vs. predictions plots.

For identification of the most appropriate empirical PK/PD index associated with the microbial kill of INH, cell counts on days 2, 3 and 4 were analyzed using an inhibitory $E_{\max}$ model¹⁶ expressed as

$$E = E_{\text{control}} - \left(\frac{E_{\max} \cdot \text{PKPD}}{EC_{50} + \text{PKPD}} \right) \quad \text{Eq. 5}$$

where, E is the observed *M. bovis* BCG cell counts in Log_{10} CFU/mL, E_{control} is the BCG cell count in the control experiment, and $E_{\max}$ is the maximal antimicrobial effect in Log_{10} CFU/mL. EC_{50} is the value of the PK/PD index that produces half-maximal antimicrobial effect and PKPD is one of the empirical PK/PD indices frequently used in infectious disease pharmacotherapy, AUC_{0-24}/MIC , $T>\text{MIC}$ or $C_{\max}/\text{MIC}$. The PK/PD index that best characterized the effect of INH on *M. bovis* BCG was selected based on goodness-of-fit criteria (e.g., coefficient of determination values), residual analysis and visual inspection.

3. Results

Mycobacterium bovis BCG was utilized as surrogate for virulent *M. tuberculosis* in the development of this method because of its high genetic similarity, similar drug sensitivity and growth rate. Practically, the use of *M. bovis* BCG allowed for this technology to be developed

more rapidly under biosafety level 2 conditions rather than the more stringent biosafety level 3 conditions required for *M. tuberculosis* culture.

M. bovis BCG was successfully cultured in our novel *in vitro* PK/PD model for 10 days. The theoretical INH concentrations achieved in all slow and fast acetylase dose groups are shown in Fig. 3A and 3B. The growth rate constant of *M. bovis* BCG in the absence of any antibacterial compound was found to be 0.0464 hr^{-1} and the corresponding doubling time/generation time was calculated as 14.9 hours. This doubling time of *M. bovis* BCG is similar to the range observed *in vitro* in the literature (13-20 hours).²⁴ Administration of INH dosing regimens to logarithmically growing cultures of BCG in the *in vitro* model resulted in reduced cell counts when compared to the control experiment (Fig.4A and Fig.4B). The initial kill rate during the first two days of therapy for simulated slow acetylators was 0.32, 1.36, and 1.35 $\log_{10}$ (CFU/mL)/day for the 25, 100 and 300 mg/day dosing regimens, respectively. In contrast, the initial kill rate for the simulated fast acetylators was calculated as 0.09, 0.05 and 0.78 $\log_{10}$ (CFU/mL)/day for the 25, 100 and 300 mg/day dosing regimens, respectively. The initial kill rate of INH, analogous to *in vivo* early bactericidal activity (EBA) was exposure-dependent. As INH systemic exposure in simulated slow acetylators was higher than in fast acetylators, the INH mediated kill effect was also more pronounced in slow acetylators compared to fast acetylators at each dose level (Fig.4A and Fig.4B). The bactericidal activity appeared with a delay of approximately 24 hours in the fast acetylase dose groups compared to the corresponding dose groups in slow acetylators. The INH mediated killing of bacteria ceased after three days of therapy and re-growth of bacteria appeared in both slow and fast acetylase dose groups (Fig. 4A and Fig.4B).

Different mathematical models were evaluated for simultaneously describing the *M. bovis* BCG time-kill data generated with the *in vitro* PK/PD model for fast and slow acetylators.^{1, 25-30} An adaptive IC_{50} kill curve model best described the experimental data based on goodness-of-fit criteria (see Methods section). Graphical representations showing observed and predicted BCG cell counts at all tested dosing regimens in slow and fast acetylators are displayed in Fig.4A and Fig.4B. The corresponding parameter estimates are listed in Table 2. The maximum kill rate ($I_{\max}$) was calculated as 0.852 hr^{-1} . The concentration of INH that resulted in a half-maximal kill rate, IC_{50} , was estimated as 0.244 mg/L. The adaptive IC_{50} , $\text{IC}_{50,A}$, correspondingly ranged from 0.244 to 15.4 mg/L. For comparison, the MIC for INH determined with the classic dilution approach was found to be 0.05 mg/L. The goodness-of-fit plots presented in Fig.5 and Fig.6 indicate that the selected mathematical time-kill curve model (Eq. 3) is able to describe the data well. The plots of observed vs. predicted CFU concentrations (Fig. 5) show a uniform distribution around the line of identity indicating a high correlation between model predicted and observed data and thus a good model fit of the data. The residual plots (Fig. 6) are an indicator of potential trends in the data unaccounted by the model. The largely random distribution of residuals over time and over the range of predicted CFU concentrations indicate a lack of bias in the model prediction.

Examination of the relationship between empirical PK/PD indices and microbial kill for day 3 of the treatment revealed that the PK/PD index $\text{AUC}_{0-24}/\text{MIC}$ was the most explanatory of the INH mediated microbial kill (Fig.7). The relationships between microbial kill and the other PK/PD indices, $C_{\max}/\text{MIC}$ and $T>\text{MIC}$ are also shown in Fig.7. Similar results were observed for day 2 and day 4 data (results not shown). The parameter estimates from the empirical PK/PD analysis are provided in Table 3.

4. Discussion

Identification of pharmacodynamic parameters has become an integral part of the development process of novel antibiotics.⁵ Characterization of pharmacodynamic parameters in the early

stages of development enables the design of optimal dosage regimens for animal and human studies and allows for better predictions of the success/failure of an antibiotic development program.^{1,11} *In vitro* PK/PD models are used widely for the determination of pharmacodynamic parameters of antibiotics^{25,28,29,31-34} due to a variety of advantages. One of the advantages of *in vitro* models is that human and animal pharmacokinetic profiles can easily be simulated. In addition, the lack of immune system response in these *in vitro* experiments allows for precise characterization of bacteria-drug interactions. Further, the *in vitro* models are better suited over animal models to test and identify the time- or concentration-dependant killing nature of antibacterial drugs.² The *in vitro* PK/PD model described here is a further development of previously reported dilution models and is to our knowledge the first-time use of a dilution model to assess the time-kill curves for *Mycobacterium* species. The model is easily constructed, inexpensive, prevents dilution of the bacteria without membrane pore blockage and can be used for slow growing organisms as shown with the time-kill analysis using *M. bovis* BCG. Compared to the previously reported models of similar nature,^{8,10} the model described in this paper has several advantages: 1) It is a completely closed system that easily can fit in most biosafety cabinets and is safe to work with pathogenic strains under biosafety level 3 conditions, 2) the model can be used to work with slow and fast growth rate organisms, 3) the temperature in the flask can be controlled precisely with a water jacket, 4) the system is low cost, and 5) assembling and disassembling of the components of the model is simple and straight forward, allowing for easy study conduct and equipment sterilization. In addition, the model requires only a very limited amount of drug substance, which might be advantageous in early drug discovery when drug substance supply is oftentimes limited. Although the amount of drug substance required for experiments in the model system varies dependent on a drug's *in vivo* pharmacokinetic characteristics and the number of doses and treatment period simulated, all experiments described in this manuscript required less than 5 mg INH.

Slow growth rate microorganisms such as *M. bovis* BCG pose many challenges for *in vitro* culturing using a continuous flow apparatus. Much longer periods, usually more than a week, are required to assess the efficacy of an antibacterial agent against these slow growing organisms when compared to fast growing organisms. Consequently, the integrity of the filter membrane is crucial for allowing media exchange but preventing outflow of bacteria throughout the duration of the experiment. In addition, the *in vitro* PK/PD model should not hinder natural growth of bacteria in the absence of antibacterial agents. The concordance between the observed doubling time for *M. bovis* BCG in our studies and the literature reported values suggests that the *in vitro* PK/PD model described in this report is not interfering with the normal growth of *M. bovis* BCG.

INH is one of the first line agents used in the multi drug combination therapy for tuberculosis that kills actively dividing bacteria. Early bactericidal activity (EBA) of the combination drug therapy for tuberculosis is derived mainly from INH activity on the logarithmically growing bacteria.^{35,36} EBA is defined as the rate of fall of colony forming units (CFU/mL) in the sputum of patients during the first two days of drug treatment and is expressed as $\log_{10}$ (CFU/mL)/day. PK/PD models based on *in vitro* time-kill curves have been used to assess the efficacy of antimicrobial agents. However, reports about the use of such models for anti-tuberculosis drugs are scarce. Only a few recently reported *in vitro* models characterized dynamic time-kill curves of the anti-tuberculosis agents INH, rifampicin and moxifloxacin.^{15-18,37} From the results of the time-kill experiments performed in this study, it can be seen that the kill rate of *M. bovis* BCG is different at identical dosing regimens in the slow and fast acetylator groups of INH. An initial delay in killing was observed at all the dosing regimens studied in the fast acetylator groups in contrast to the slow acetylator dose groups (Fig.4A and Fig.4B). In an *in vitro* study with ¹⁴C-labeled INH, it was shown that it takes more than 2 hours for complete intracellular uptake of INH when incubated with *M. bovis* BCG.³⁸ Since INH is eliminated with a half-life

of 2 hours in fast acetylators the observed delay in killing could be due to the rapid removal of drug from the medium in this group compared to slow acetylators (half-life 4.5 hr). Similarly, the rapid removal of drug in the fast acetylators is likely the reason for the observed differences in the initial kill rates of bacteria among the identical dose groups in both slow and fast acetylators. Whether similar differences in bacterial kill between fast and slow acetylators of INH are also seen for other mycobacteria will likely be dependent on their MIC relative to the INH concentrations encountered in the two acetylator groups.

The exposure-dependant kill pattern for mycobacteria under INH therapy has been characterized in previously reported *in vitro* and *in vivo* animal studies.^{18,39} The initial rapid kill rate followed by a slow kill rate along with the re-growth of bacteria observed in our study is remarkably similar to that observed in an *in vitro* study by Gumbo et al., using *M. tuberculosis* H37Ra.¹⁷ Similar results were found in several other *in vitro* drug susceptibility assays. It was hypothesized that the development of drug induced tolerance and transient resistance to INH is responsible for the cessation of INH bactericidal activity.^{22,40}

We used a semi-mechanistic PK/PD model to describe the rate of killing of bacteria over time at different dosing regimens. The principle behind the analysis is that the change in CFUs over time is governed by the difference in the rate of growth and the rate of killing with INH increasing the rate of microbial kill. The limiting growth of bacteria in the absence of INH was described by a logistic function, $1 - N/N_{max}$ where, N is the number of CFU/mL at time t and N_{max} is the maximum number of bacteria (CFU/mL) in the system which corrects for the limitations of space and nutrients that are inherent to *in vitro* systems.^{20,25,30} A correction factor ($1 - e^{-at}$) was incorporated to describe the delay in kill effect after the addition of INH in the fast acetylator dose groups.²⁰ Since the numbers of INH insensitive populations of bacteria due to latency or resistance likely increased over the course of treatment, the selected PK/PD model needed to be able to describe the re-growth of bacteria. Various approaches have been reported for modeling drug resistance and/or growth of drug insensitive populations of bacteria.^{1,16,25-30} Here, we used an adaptive IC₅₀ model to describe the increase in apparent IC₅₀ due to growth of drug insensitive populations of bacteria. The increase in IC₅₀ was inversely related to the INH exposure and the ratio of cell counts to the initial cell numbers (Eq. 4). This means that low exposure to INH results in increased development of insensitivity which is manifested as an increase in apparent IC₅₀. This model was able to describe the observed re-growth of bacteria after several days of drug exposure. The IC₅₀ was estimated as 0.244 mg/L and could increase over the course of treatment up to 15.4 mg/L. The stationary concentration of INH which is defined as the concentration at which the rate of bacterial killing equals growth was calculated using IC₅₀ of INH as described by Moutin and Vinks.⁴¹ The calculated stationary concentration (0.0043 mg/L) was much lower than the MIC (0.05 mg/L), a commonly observed phenomenon for the concentration/exposure dependent killing by antibiotics.⁴¹

In the current study, INH exhibited exposure-dependent antibacterial activity on *M. bovis* BCG. The empirical PK/PD index AUC₀₋₂₄/MIC was found to be well associated with the microbial kill. In contrast, the PK/PD index C_{max}/MIC did not correlate well with antimicrobial activity (Fig. 7): The same value of the C_{max}/MIC index in slow and fast acetylators resulted in different microbial kill, likely due to the different exposures for slow and fast acetylators since INH was administered as the same bolus dose in both groups, but different elimination half-lives were simulated. Although the other considered PK/PD index, T>MIC showed some association with bacterial killing, it has previously been shown that microbial kill by INH is an exposure-dependent process.^{17,18,39} However, the parameter estimate (AUC₀₋₂₄/MIC) from a previous study, 61.6 is different from that of the current study, 36.0. The difference is likely due to the difference in the test organisms' susceptibility to INH (*M. tuberculosis* H37Ra vs. *M. bovis* BCG). In order to corroborate the findings of our empirical PK/PD analysis, further studies with mycobacteria with different susceptibilities to drug treatment (a range of MICs) are

required. Since only *M. bovis* BCG with a single susceptibility was used in this study, the results of the empirical PK/PD analysis should be interpreted cautiously.

The novel *in vitro* PK/PD model and associated PK/PD modeling approach described here can be used as a tool to evaluate and predict the bactericidal activities of novel anti-tuberculosis compounds. The underlying principle is to describe the whole time course of the events seen in the bacterial system when exposed to antibiotics. Such models, when combined with other information such as toxicology data, can be used to design improved dosing regimens of antibiotics beyond the empirical approaches. Simulations based on the mechanistically based time-kill models allow for evaluation and comparison of previously tested and hypothetical, untested dosing strategies.

The *in vitro* time-kill curve approach described for anti-mycobacterial drug activity, however, has some limitations. The current study was performed with an assumption that the plasma concentrations are similar to the effect site concentrations. However, in most of cases *in vivo* effect site concentrations are difficult to determine and may differ from plasma concentrations dependent on protein binding and tissue uptake. Secondly, the environment for bacterial growth *in vitro* differs from that *in vivo*. Thirdly, the drug effect was measured on logarithmically growing bacteria which only describes the EBA of a drug. Thus, the approach described here cannot predict the sterilizing activity of drugs on persister populations of mycobacteria which are thought of being frequently responsible for relapses under drug therapy. Although this can be regarded as one of the major limitations of most *in vitro* and *in vivo* models currently available for evaluating drug activity in tuberculosis, the PK/PD model described herein offers at the same time the opportunity to selectively create persister populations in a controlled environment that can then be further studied, for example with regard to metabolic activity and response to other antibacterial agents.

In conclusion, we have shown that a newly developed *in vitro* PK/PD model can be used to determine time-kill curves for slow growing mycobacteria as exemplified by the determination of time-kill curves for dosing regimens of INH against *M. bovis* BCG. We have also shown that the time-kill data of INH against *M. bovis* BCG can be described using an adaptive IC₅₀ model that corrects for increased drug insensitive populations of bacteria during the course of treatment. The newly developed model can be used to determine time-kill curves of novel anti-tuberculosis compounds to aid in their development. Also, the approach can be used to evaluate the effectiveness of new dosing regimens of currently used anti-tuberculosis agents.

Acknowledgments

This study was supported by research grant R01 AI 062415 from the National Institutes of Health. Its content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1.
Schematic diagram of *in vitro* PK/PD model

Figure 2.
A picture showing two working units of *in vitro* PK/PD model

Figure 3. Concentration-time profiles of INH for all dose groups simulated in the *in vitro* PK/PD model. Panel A shows concentration-time profiles for slow acetylators of INH, panel B for fast acetylators. The horizontal line indicates the MIC (0.05 mg/L) for *M. bovis* BCG.

Figure 4.

Plots showing measured (mean $\pm$ SD) and model predicted (lines) *M. bovis* BCG CFU/mL over time for the PK/PD model. Panel A shows kill curves for slow acetylators of isoniazid, panel B for fast acetylators. Symbols for dose groups: Control experiment, ○; 25 mg/day, ■; 100 mg/day, ▲; and 300 mg/day, ●. Lines represent individual predictions based on the PK/PD Model.

Figure 5.

Goodness-of-fit plots for the PK/PD model, A) observed vs. individual predicted values (log₁₀ CFU/mL) and B) observed vs. population predicted values (log₁₀ CFU/mL).

Figure 6. Goodness-of-fit plots for the PK/PD model, A) weighted residuals vs. time (hr) and B) weighted residuals vs. population predicted values (log₁₀ CFU/mL).

Figure 7. Relationship between PK/PD indices and microbial kill on day 3 after treatment. Panels A, B and C show AUC/MIC, T>MIC and C_{max}/MIC, respectively.

Table 1

Experimental plan with initial concentrations of isoniazid (INH)

Dose Group	Elimination Half-life hr	INH Dose/day mg	Number of doses	Initial Conc. (C_0) mg/L
Control	-	-	-	-
Slow Acetylators	4.5	25	7	0.3
		100	7	1.21
		300	7	3.64
Fast Acetylators	2.0	25	7	0.3
		100	7	1.21
		300	7	3.64

Table 2

Parameter estimates of INH PK/PD model

Parameter	Units	Estimate (% CV)
k_0	hr ⁻¹	0.0464 (61.6)
IC ₅₀	mg/L	0.244 (271)
Adaptation constant	L/mg.hr	0.00224 (36.5)
I _{max}	hr ⁻¹	0.852 (87.6)
α	hr ⁻¹	0.088 (85)
Between experiment Variability (%CV) in α		95.02 (64.2)
Residual Variability (%CV)		81.3 (27.1)

Table 3

Empirical PK/PD indices

Parameter	Units	AUC ₀₋₂₄ /MIC	T>MIC (%)	C _{max} /MIC
E _{max}	log10 CFU/mL variable	4.69	6.94	4.90
EC ₅₀	dependent on index	36.01	57.33	12.33
R ²		0.991	0.988	0.988

R² is the coefficient of determination