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DRUG DISCOVERY AND RESISTANCE

Pharmacokinetics and dose response of anti-TB drugs in rat infection model of tuberculosis

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SUMMARY

Robust and physiologically relevant infection models are required to investigate pharmacokinetic −pharmacodynamic (PK/PD) correlations for anti-tuberculosis agents at preclinical discovery. We have validated an inhalation-based rat infection model of tuberculosis harbouring mycobacteria in a replicating state, that is suitable for investigating pharmacokinetics and drug action of anti-tubercular agents. A reproducible and actively replicating lung infection was established in Wistar rats by inhalation of a series of graded inocula of *Mycobacterium tuberculosis*. Following an initial instillation of $\sim 10^5$ $\log_{10}$ CFU/lung, *M. tuberculosis* grew logarithmically for the first 3 weeks, and then entered into a chronic phase with no net increase in pulmonary bacterial loads. Dose response of front-line anti-TB drugs was investigated following pharmacokinetic measurements in the plasma of infected rats. Rifampicin, Isoniazid, and Ethambutol dosed per orally exhibited bactericidality and good dose response with maximal effect of 5.66, 4.66, and 4.80 $\log_{10}$ CFU reductions in the lungs, respectively. In contrast, Pyrazinamide was merely bacteriostatic with 1.92 $\log_{10}$ CFU/lung reduction and did not reduce the bacterial burden beyond the initial bacterial loads present at beginning of treatment in spite of high Pyrazinamide blood levels. Rat infection model with actively replicating bacilli provides a physiologically distinct and pharmacologically relevant model that can be exploited to distinguish investigational compounds in to bacteriostatic or bactericidal scaffolds. We propose that this rat infection model though need more drug substance, can be used in early discovery settings to investigate pharmacology of novel anti-tubercular agents for the treatment of active pulmonary tuberculosis.

1. Introduction

Improved therapies for the treatment of tuberculosis are urgently required to combat the global problem that accounts for ~1.5 million deaths annually with rapidly emerging multiple drug resistant (MDR) and extensively drug resistant (XDR) strains [1]. In this context, animal models of infection have greatly facilitated screening and preclinical characterization of newer anti-tubercular compounds [2–4]. Following the first ever demonstration of tubercular infection in Guinea pigs by Robert Koch [5], several animal species including mice, rabbits, guinea pigs, marmosets, macaques etc. have been experimentally infected [6–9] to validate biologically and pharmacologically relevant experimental tuberculosis models. The choice of animal models for in vivo testing is largely dictated by economics, route of infection, and the question that is being addressed. Mice have been a model of choice for pharmacology studies for the practical reasons of size and cost [3,8,10]. In contrast, larger animals like rabbits, guinea pigs, and monkeys are considered best for understanding disease biology, immunology and tubercular pathology because they present a similar spectrum of in vivo phenotypes and pathology to that encountered in the human host [11–13]. In larger animals like Guinea pigs, rabbits, and nonhuman primates mycobacterial infection invariably leads to the formation of heterogeneous, granulomatous necrotic lesions, a hallmark of TB infection in the lung, whereas murine granuloma are less-organized collection of macrophages and lymphocytes without central caseous necrosis and are not hypoxic

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[9,11]. In recent years, rats have been used to model tubercular pathology [9,14], immunology [15,16], host pathogen interactions and mycobacterial latency [16] with low level chronic bacillary infections. Rats are also widely used in pharmacokinetic studies in general. Rat pharmacology model, though need more drug substance for testing but may provide a viable option to progress compounds that suffer with problem of high metabolic clearance in mice. We have previously validated a chronic rat infection model harbouring non-replicating bacteria for investigation of antimycobacterial activity in preclinical settings [17]. Here, we report the pharmacological relevance of a "replicating" rat tuberculosis infection model for investigating preclinical pharmacology. This rat infection model may help identifying compounds active on replicating bacilli that may be relevant to the treatment of active human tuberculosis.

2. Materials and methods

2.1. Drugs and reagents

Isoniazid (INH), rifampicin (RIF), ethambutol (EMB) pyrazinamide (PZA), hydroxypropyl methyl cellulose (HPMC), and carboxymethyl cellulose (CMC) were purchased from Sigma Chemical Co. USA.

2.2. Ethics statement and animals

The Institutional Animal Ethics Committee (IAEC), registered with the Committee for the Purpose of Control and Supervision of experiments on animals (CPCSEA), Government of India, approved all animal experiment protocols and usage. Male Wistar rats were purchased from Bioneeds, (Bangalore, India). Rats (7–8 weeks old) were randomly assigned into groups of three per cage, and were allowed one weeks acclimatisation before experimentation. Feed and water were given *ad libitum*. Infected rats were maintained in individually ventilated cages (Allentown Technologies, USA) in biosafety level 3 facilities, and all procedures on infected rats were performed under strict biocontainment.

2.3. Aerosol infection

Mycobacterium tuberculosis H37Rv ATCC 27294, a strain sensitive to all the standard antimycobacterial agents was used in animal infection experiments. Bacterial cultures for animal infections were prepared as described previously [18]. Rats were infected with *M. tuberculosis* via inhalation procedure to achieve acute respiratory infection as described previously [17]. A series of bacterial inocula with increasing bacterial strength were aerosolised for 30 min cycle to deliver bacilli in to lungs to find the optimum inoculum size that delivered $\sim 10^5$ CFU/lung. Infected animals were randomly distributed in to groups of three and housed for variable periods of time to establish the course of infection. The course of mycobacterial infection was monitored by enumeration of colony forming units (CFU) from excised lungs at 3, 7, 14, 28, 46 and 70 days postinfection as described previously [17].

2.4. In vivo dose–response studies

Drug treatment started three days postinfection. Front-line anti-TB drugs INH, RIF and EMB were administered orally, to rats as suspensions in 0.5% (w/v) HPMC and 0.1% Tween 80 while PZA was in 0.25% CMC. INH and RIF(3–30 mg/kg), EMB (30–300 mg/kg) and PZA (75–300 mg/kg), were administered for four weeks on a 6/7 day dosing format. At the end of dosing period animals were euthanized with CO_2, lungs were removed, and processed for CFU estimation. Lung homogenates were serially diluted in 10-fold steps and plated onto Middlebrook 7H11 agar supplemented with 10% albumin dextrose catalase (Difco Laboratories). Plates were incubated at 37 °C with 5% CO_2 for 3 weeks to obtain isolated colonies.

2.5. Statistical analysis

The colony counts obtained from plating were transformed to $\mathrm{Log}_{10}^{(x+1)}$, where x equals the total number of viable tubercle bacilli present in a given sample. Prism software version 4 (GraphPad Software, Inc., San Diego, California) was used for ploting PK and PD effects and statistical analysis by ANOVA.

2.6. Pharmacokinetics of INH, RIF, PZA and EMB in infected rats

INH, RIF (3 and 30 mg/kg), PZA (75 and 300 mg/kg) or EMB (30 and 300 mg/kg) were given orally once daily to male Wistar rats. INH, RIF and EMB were suspended in 0.5% HPMC and 0.1% Tween 80, whereas PZA was suspended in 0.25% CMC and administered at a dose volume of 10 mL/kg. Animals infected with *M. tuberculosis* were dosed with the drug substance for eighteen days, and on the nineteenth day, the pharmacokinetics were determined in infected animals. Blood samples (50 μL) were collected from animals into Lithium-Heparin microvette tubes (Sarsted, Germany) at pre-dose, 0.083, 0.25, 0.5, 1, 2, 4, 6 and 24 h following administration by puncturing the saphenous vein using a sparse sampling protocol. Plasma (25 μL) was separated by centrifugation of blood samples at 10,000 rpm for 5 min at 4 °C. Plasma proteins were precipitated with acetonitrile, and the resulting samples were subjected to LC-MS/MS analysis.

A 1 mg/mL stock solution of each drug was prepared in Dimethylsulfoxide (DMSO) and diluted three-fold with blank rat plasma to prepare standards ranging from 8 to 20,000 ng/mL. Samples were mixed on a plate shaker and centrifuged at 4000 rpm for 20 min at 15 °C. An aliquot (25 μL) of the supernatant was mixed with 225 μL of mobile phase containing an internal standard. A 10 μL aliquot of the extracted sample was injected into reverse phase C-18 analytical column on an HPLC system coupled to a triple quadrupole Mass spectrometer. A positive ion mode with turbo spray and an ion source temperature of 450 °C were utilized for mass spectrometric detection. Quantitation was performed using multiple reaction monitoring (MRM) mode. Linear regression plots of compounds to internal standard peak area ratios vs compound concentrations were fitted with $1/x$ or $1/x^2$ weighting.

2.7. PK analysis

Pharmacokinetic (PK) analysis of the plasma concentration–time relationships were performed with WinNonlin Phoenix Software (version 6.2; Pharsight, USA). A Non-compartmental analysis program, model 200, was used to calculate PK parameters. The maximum concentration of drug in plasma (C_{max}), time to C_{max} (T_{max}), elimination half-life ($t_{1/2}$), and AUC from time zero to infinity ($AUC_{0-\infty}$) were estimated. AUC was computed using trapezoidal rule (linear up and log down) and $AUC_{0-\infty}$ value was considered only when AUC extrapolated was not more than 20%. The estimation of terminal slope in order to calculate half-life was made only when there were at least three sample points in the terminal phase. C_{max}/MIC, AUC/MIC in infected rats was calculated by dividing these parameters by MIC values obtained from in vitro assays.

3. Results

3.1. Course of M. tuberculosis infection

Inhalation of three different bacterial inocula resulted in instillation of lung bacterial loads as follows: An inoculum of 10^7 CFU/ml delivered $<10^4$ CFU/lung while both 10^8 CFU/mL and 10^9 CFU/mL inocula delivered respectively 4.6×10^6 CFU/lung and 4.8×10^6 CFU/lung (Data not shown). In the subsequent experiments, reproducible infections were achieved with 10^9 CFU/mL inocula. Therefore, 10^9 CFU/mL inoculum was considered optimum for further experiments. In a time course study, mycobacterial load in the lungs increased logarithmically for the first four weeks and attained 7.24 ± 0.15 $\log_{10}$ CFU/lung. Thereafter, bacterial replication slowed down and the net bacterial load remained constant till 12 weeks (7.26 ± 0.33 $\log_{10}$ CFU/lung) postinfection (Figure 1).

3.2. Dose response

The dose response of four front-line TB drugs was determined in the replicating rat infection model following daily doses for 4 weeks. Three drugs, INH, RIF and EMB, exhibited good pharmacodynamic dose response in the replicating infection model, and achieved bactericidality (>2 $\log_{10}$ CFU/lung reduction from the bacterial load at the time of beginning of treatment). While PZA did not achieve bactericidality, INH achieved bactericidality at 30 mg/kg (2.44 $\log_{10}$ CFU/lung reduction), and RIF at 30 mg/kg (3.45 $\log_{10}$ CFU/lung reduction), and EMB at 300 mg/kg (2.6 $\log_{10}$ CFU/lung reduction) as depicted in Figure 2. In the acute model, INH, RIF and EMB exhibited good dose responses, while PZA was largely ineffective and did not exhibit even bacteriostasis across the dose range tested (Figure 2).

3.3. Pharmacokinetics of INH, PZA, RIF and EMB upon multiple dosing

Blood concentrations of all the four drugs were estimated in the plasma obtained from *M. tuberculosis* infected rats. INH exhibited linear pharmacokinetics with C_{max} of 8.3 μg/mL and exposure (AUC) of 14.4 h μg/mL at 30 mg/kg. PZA achieved high C_{max} (112.6 and 218.6 μg/mL), and exposures, (164.5 and 753.8 h μg/mL at 75 and 300 mg/kg) respectively. INH and PZA showed dose proportional increase in exposure. In contrast, RIF and EMB exposures were more than dose proportional. RIF exhibited C_{max} of 0.15 and 8.7 μg/mL, and AUC was 1.0 and 54.7 h μg/mL at 3 and 30 mg/kg respectively. EMB C_{max} was 1.5 and 30.1 μg/mL, whereas AUC was 5.7 and 137.3 h μg/mL at 30 and 100 mg/kg respectively. Pharmacokinetic parameters and time–concentration profiles of INH, RIF, ETM and PZA are summarized in Table 1.

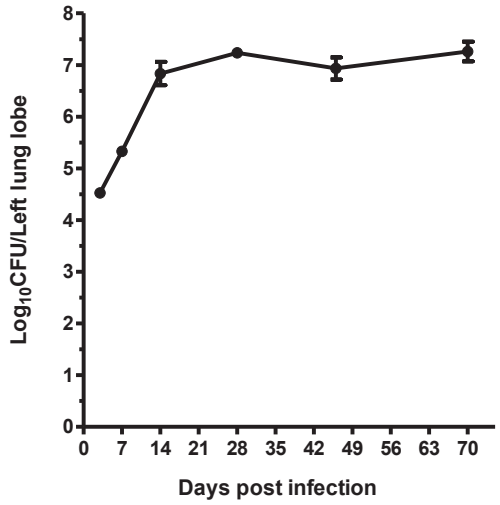

Figure 1. Course of infection of *M. tuberculosis* H37Rv in the lungs of Wistar rats following high dose aerosol infection (10^9 CFU mL^{-1} inoculum), monitored over a period of 10wks.

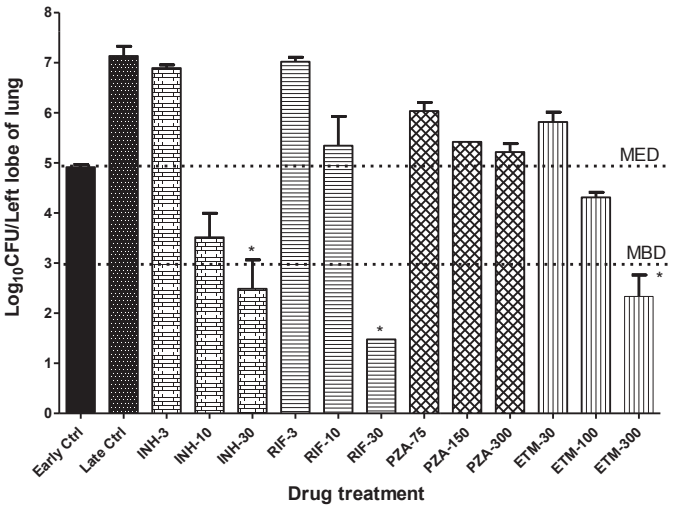

Figure 2. Efficacy and Dose-response of front-line TB drugs in replicating (high dose) aerosol infection model in rats following once daily treatment for four-weeks. Log_{10} CFU counts (left lung lobe) were plotted against the range of drug regimens. Isoniazid and Rifampicin (3, 10 and 30 mg/kg), Pyrazinamide (75, 150 and 300 mg/kg) and Ethambutol (30, 100, 300 mg/kg) were tested. Each bar represents the mean U ± U SD CFU counts from a group of three animals. * Represents statistically significant reductions ($p < 0.05$) in CFU from untreated controls. MED: Minimum effective dose. The minimum dose that holds the bacterial infection at the level of onset of treatment (stasis). MBD: Minimum bactericidal dose. The minimum dose that results in a $2\log_{10}$ CFU reduction in bacterial load in the lungs from the onset of treatment (cidal).

Further integration of pharmacokinetics data with MIC was done to derive C_{max}/MIC and AUC/MIC at different doses in infected rats vs the maximum bacterial killing (Table 2). Increase in dose of INH, RIF and ETM lead to increased C_{max}/MIC and AUC/MIC as well as net bactericidal effect in terms of CFU reduction (Table 2). However, in case of pyrazinamide, C_{max}/MIC and AUC/MIC increased with dose but net bactericidal effect did not increase much.

4. Discussion

Tuberculosis is a complex disease, and no single animal model mimics the complete spectrum of pathobiology seen in humans [2,6,9]. Nevertheless, animal models of tuberculosis have played a significant role in understanding disease biology [7–9,11–13] and PKPD of new anti-tubercular agents [3,4,11,13,17–19]. Mice have been the species of choice for in vivo efficacy testing of new anti-TB compounds due to practical reasons of size, cost and precedent of

Table 1
Oral multiple dose pharmacokinetic parameters of Isoniazid, Rifampicin, Pyrazinamide and Ethambutol in male Wistar rats infected with *M. tuberculosis*.

PK parameter	Isoniazid		Rifampicin		Pyrazinamide		Ethambutol	
Dose (mg/kg)	3	30	3	30	75	300	30	300
C_{max} (μg/mL)	0.31	8.38	0.15	8.69	112.6	218.6	1.52	30.04
$AUC_{0-\infty}$ (h μg/mL)	1.23	14.37	0.99	54.71	164.55	753.85	5.76	137.34
$t_{1/2}$ (h)	2.65	1.04	4.41	2.44	1.05	1.04	2.16	2.90

Table 2
Integration of multiple dose pharmacokinetic parameters of Isoniazid, Rifampicin, Pyrazinamide and Ethambutol obtained in male Wistar rats with minimum inhibitory concentration (MIC). C/MIC: C_{max} divided by MIC; AUC/MIC: total plasma exposure divided by MIC; Delta CFU: net bacterial colony forming unit reduction in the lungs of rats following drug treatment at respective doses.

Compound	Isoniazid		Rifampicin		Pyrazinamide		Ethambutol	
Dose (mg/kg)	3	30	3	30	75	300	30	300
MIC (μg/mL)	0.05	0.05	0.1	0.1	64	64	1	1
C/MIC	6.2	167.6	1.5	86.9	1.8	3.4	1.5	30.0
AUC/MIC	24.6	287.3	9.8	547.0	2.5	11.8	5.7	137.3
Delta CFU	0.24	4.60	0.12	5.60	1.10	1.90	1.30	4.80

reproducible experimental infections and therapeutic outcomes [3,4,10,18–22]. However, mice do not develop the characteristic human like granuloma with central necrosis and cavitary disease that is seen with TB infections in larger animals [2,8,9,11,21,23], except the recently reported C3HeB/FeJ strain of mice [24].

Currently, rats are widely being used in preclinical discovery settings for investigating pharmacokinetics [25], toxicokinetics [26], tubercular pathology [9,14–17,27] and immunology of latent tuberculosis [15,16]. A significant number of early discovery compounds cannot be progressed due to high metabolic clearance in mice. In the similar lines, a recent study that identified oxyphenbutazone an old anti-inflammatory drug with potent activity on non-replicating mycobacteria, reported inability to explore and progress this compound further due to high mouse metabolic clearance in mice and cost and complexity of models in higher animal species [27]. A validated rat infection model may provide an alternate pharmacology model to progress such anti-TB compounds that cannot be tested in mice. However, need for relatively large quantity of compound at early discovery stages remains the limitation. The pharmacologic relevance of rat infection model was further strengthened recently by demonstration of zones of low oxygen tension (hypoxia) in the lungs of *M. tuberculosis* infected Wistar rats by immunohistochemical methods [28].

We have previously validated and reported a rat model of TB infection, for investigating pharmacodynamics of anti-tubercular compounds in chronically infected Wistar rats [17]. The model investigated pharmacodynamic effect of drugs on chronic/non-replicating bacillary infection however, we had no information on the underlying pharmacokinetics or the utility in investigating replicating bacterial infections in rats. Here we report validation of a replicating rat infection model with feasibility of pharmacokinetic sampling in *M. tuberculosis* infected rats to better link the exposure–effect relationships (Table 2).

Replicating bacilli are hallmarks of active pulmonary tuberculosis associated with fulminating clinical outcomes and spread of air born infection in humans. Hence, replicating bacilli represent distinctive phenotype vs non-replicating persistent tubercle bacilli in vivo [32]. Evaluation of bactericidal activity of candidate drugs on various phenotypes in respective models (replicating and chronic) is needed for rational positioning of the candidate(s) in combination regimens. Pharmacodynamic studies on chronic bacillary infections with anti-TB drugs are well documented in mice [4,19,20,30,31] but not in rats [17]. Most anti-TB drugs are not equally bactericidal on the two forms in vivo [29] due to phenotypic drug tolerance exerted by immune and drug pressures in the human host. In the replicating infection model described here, drug treatment started 3 days postinfection where bacilli are presumed to be replicating either extracellularly or intracellularly within macrophages in the absence of a characteristic granulomatous environment. Replicating state of bacilli was evident from the increasing (2.2 $\log_{10}$ CFU/lung) bacterial loads in lungs of untreated animals during the four week drug treatment period similar to observations in the acute or high dose aerosol (HDA) mouse infection models [20,22,30].

Cell wall inhibitors like INH and EMB, that are presumed to work best on replicating bacteria by virtue of being cell wall inhibitors achieved bactericidal activity of >2 $\log_{10}$ CFU/lung reduction (INH 30 mg/kg–2.44 $\log_{10}$ CFU reduction/lung and EMB 300 mg/kg–2.6 $\log_{10}$ CFU/lung reduction) compared to much inferior activity (1.3 $\log_{10}$ CFU/lung and 0.94 $\log_{10}$ CFU/lung reduction) observed in the chronic rat infection model harbouring non-replicating bacilli reported by us previously [17]. In contrast, PZA had no activity on replicating bacilli in this model in spite of high plasma C_{max} (218 μg/ml) and $AUC_{0-\infty}$ exposures (753 h μg/mL) at 300 mg/kg dose vs 0.62 $\log_{10}$ CFU/lung reduction in chronic rat infection model [17]. RIF, obviously was efficacious (RIF 30 mg/kg–3.45 $\log_{10}$ CFU/lung reduction vs and 1.5 $\log_{10}$ CFU/lung reduction in chronic rat model) since it works on both replicating and NRP phenotypes due to targeting essential and central DNA machinery.

Lack of bactericidality of PZA in spite of significant systemic exposures demonstrates that rat infection model with replicating bacilli is highly suitable for identifying compounds that preferentially hit targets in replicating bacteria. The replicating rat infection model adds value in differentiating investigational compounds into bacteriostatic and bactericidal scaffolds, because the bacterial numbers in untreated animals grow beyond the numbers present at initiation of therapy similar to high dose mouse infection models [22,31,32]. Since active TB exerts massive impact on health and well being due to acute and debilitating nature of tuberculosis, animal models mimicking active disease with replicating bacilli will be – very useful in understanding disease process vis-a-vis identifying specific inhibitors that are bactericidal in nature. Rats being moderately sized animals offer a pathophysiologically relevant [9,14,17,28] and better alternative pharmacology model for testing of novel anti-TB compounds.

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