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The role of the time-kill kinetics assay as part of a preclinical modeling framework for assessing the activity of anti-tuberculosis drugs

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ABSTRACT

Novel treatment strategies for tuberculosis are urgently needed. Many different preclinical models assessing anti-tuberculosis drug activity are available, but it is yet unclear which combination of models is most predictive of clinical treatment efficacy. The aim of this study was to determine the role of our *in vitro* time kill-kinetics assay as an asset to a predictive preclinical modeling framework assessing anti-tuberculosis drug activity. The concentration- and time-dependent mycobacterial killing capacities of six anti-tuberculosis drugs were determined during exposure as single drugs or in dual, triple and quadruple combinations towards a *Mycobacterium tuberculosis* Beijing genotype strain and drug resistance was assessed.

Streptomycin, rifampicin and isoniazid were most active against fast-growing *M. tuberculosis*. Isoniazid with rifampicin or high dose ethambutol were the only synergistic drug combinations. The addition of rifampicin or streptomycin to isoniazid prevented isoniazid resistance. *In vitro* ranking showed agreement with early bactericidal activity in tuberculosis patients for some but not all anti-tuberculosis drugs.

The time-kill kinetics assay provides important information on the mycobacterial killing dynamics of anti-tuberculosis drugs during the early phase of drug exposure. As such, this assay is a valuable component of the preclinical modeling framework.

1. Introduction

Although the incidence and mortality of tuberculosis (TB) are declining worldwide, the actual numbers are still impressive with over 9 million new cases and 1.5 million deaths in 2014 [1]. To end the global TB epidemic, a shorter treatment duration is required [1]. Therefore, novel treatment strategies with increased sterilizing capacity are needed to improve cure rates and to reduce the emergence of anti-TB drug resistance and disease relapse. The correct (preclinical) assessment of the activity of novel drugs and drug combinations is complex. Many different preclinical models to determine the activity and therapeutic efficacy of anti-TB drugs are available, but it is yet unclear which combination of models are most predictive of clinical treatment response with an acceptable cost-effectiveness. Optimizing preclinical modeling is important, enabling rapid and reliable identification of novel potentially powerful anti-TB regimens and their translation into clinical practice.

PreDiCT-TB is a European multidisciplinary consortium focusing on anti-TB drug development (www.predict-tb.eu). The main goal of PreDiCT-TB is to find the combination of preclinical models that is most representative of response in TB patients. As a first step, PreDiCT-TB focuses on studying the most important anti-TB drug combinations in a number of preclinical models, the results of which are compared to historical clinical efficacy data. In this way, an integrated modeling framework will be constructed, serving as a reliable method to study novel anti-TB drug regimens and forming the basis for clinical trial design. In the context of the PreDiCT-TB consortium the present study determined the concentration- and time-dependent killing activity of six anti-TB drugs alone and in combination *in vitro*. The aim of this study was to establish the role of our *in vitro* time kill kinetics assay as an asset to a predictive preclinical modeling framework assessing anti-TB drug activity and therapeutic efficacy.

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2. Methods

2.1. Bacterial strain and culture

The *Mycobacterium tuberculosis* genotype strain Beijing VN 2002-1585 (BE-1585) was cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Becton, Dickinson and Company (BD), Sparks, MD, USA), 0.5% glycerol (Scharlau Chemie SA, Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co., St Louis, MO, USA), under shaking conditions at 96 rpm at 37 °C. Vials with *M. tuberculosis* suspensions were stored at −80 °C. Cultures on solid medium were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 28 days at 37 °C with 5% CO_2. Antibiotic susceptibility in terms of Minimal Inhibitory Concentration (MIC) was determined according to the guidelines of the Clinical and Laboratory Standards Institutes (CLSI) [2].

2.2. Anti-TB drugs

Isoniazid (I-3377), rifampicin (R-3501), streptomycin (S6501), ethambutol (E-4630), para-amino salicylic acid (A-79604) and pyrazinamide (P-7136) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Time-kill kinetics assay

The concentration- and time-dependent killing capacities of isoniazid, rifampicin, streptomycin, ethambutol, para-amino salicylic acid and pyrazinamide were determined as previously described [3]. In brief, fast-growing (log phase) mycobacterial cultures were exposed to anti-TB drugs at 4-fold increasing concentrations for 6 days at 37 °C under shaking conditions at 96 rpm. In the absence of anti-TB drugs, this mycobacterial population showed an average increase from 5.4×10^5 cfu/mL to 4.2×10^7 cfu/mL within 6 days of incubation. We previously showed that this exponential growth was associated with a 6-fold increase in ATP concentration per viable *M. tuberculosis* after 3 days of incubation in contrast to the steady ATP levels as observed by the slow-growing *M. tuberculosis* population [3]. The anti-TB drug concentrations ranged from 0.01 to 40 mg/L for isoniazid, from 0.002 to 32 mg/L for rifampicin, from 0.02 to 96 mg/L for streptomycin, from 0.008 to 32 mg/L for ethambutol and para-amino salicylic acid and from 0.02 to 80 mg/L for pyrazinamide. The tested concentrations were based on the maximum free drug concentrations (*f*Cmax) of the individual anti-TB drugs ranging from 1/1024x *f*Cmax to 4x *f*Cmax comprising a representative range of clinically achievable drug concentrations for studying *in vitro* drug activity. As for rifampicin, a concentration of 16x *f*Cmax was added considering the high protein binding of this agent. When pyrazinamide was included, the medium pH was reduced from 6.6 to 5.6, which is considered to be essential for detection and evaluation of pyrazinamide activity [4,5]. At day 1, 2, 3 and 6 during antibiotic exposure, samples were collected, centrifuged at 14000*g* to avoid drug carry-over, serially diluted (10-fold, 10^0 - 10^5) and subcultured onto solid medium. Plates were incubated for 28 days at 37 °C with 5% CO_2 to determine colony forming units (cfu) counts. The lower limit of detection was 5 cfu/mL. All experiments were performed in duplicate. The bactericidal activity of the anti-TB drugs is expressed as the lowest concentration resulting in ≥99% killing of *M. tuberculosis* and as the mean daily fall in log 10 cfu/mL during the first two days and during six days of drug exposure, which is an approach similar to calculation of the early bactericidal activity (EBA) in TB patients [6]. The *in vitro* sterilizing activity of the anti-TB drugs is expressed as the lowest concentration resulting in 100% killing of *M. tuberculosis*.

In the combination experiments, the anti-TB drugs were tested using combinations of isoniazid 0.01, 0.63 and 40 mg/L, rifampicin 0.002, 0.125 and 8 mg/L, streptomycin 0.02, 1.5 and 96 mg/L, ethambutol and para-amino salicylic acid 0.008, 0.5 and 32 mg/L and pyrazinamide 0.02, 1.25 and 80 mg/L. These concentrations were based on 1/1024x, 1/16x and 4x *f*Cmax, which was considered an appropriate range of concentrations for synergy testing. In the dual combinations, isoniazid was combined with rifampicin, streptomycin, ethambutol or pyrazinamide. Rifampicin was also combined with ethambutol or pyrazinamide. In the triple combinations, isoniazid and rifampicin were combined with streptomycin, ethambutol or pyrazinamide. The other triple combinations analysed were isoniazid - streptomycin - para-amino salicylic acid and streptomycin - ethambutol - pyrazinamide. In the quadruple combinations, isoniazid, rifampicin and pyrazinamide were combined with streptomycin or ethambutol.

2.4. Selection of drug-resistant M. tuberculosis

In order to assess selection of drug resistant mutants after 6 days of drug exposure, subcultures were also performed on solid media containing anti-TB drugs [3]. The drug concentrations in the subculture plates were a 4-fold of the critical concentrations, i.e 0.8 mg/L for isoniazid, 4 mg/L for rifampicin, 40 mg/L for streptomycin, 20 mg/L for ethambutol, and 8 mg/L for para-amino salicylic acid. The critical concentration was chosen as a cut off as this is the concentration that best differentiates between wild type *M. tuberculosis* strains unexposed to drugs and resistant *M. tuberculosis* strains isolated from patients not responding to treatment [2]. Pyrazinamide resistance was not determined due to technical issues related to the reduced medium pH required for pyrazinamide activity.

2.5. Endpoints for assessment of the activity of anti-TB drug combinations

The two endpoints were 1) synergy and 2) prevention of the emergence of drug resistance. Synergistic activity was defined as a ≥100-fold ($2\log_{10}$) increase in mycobacterial killing with the 2-drug combination compared to the most active single drug (or with 3- and 4-drug combinations compared to 2-drug and 3-drug combinations, respectively). The definition of synergy was also met when a 2-drug combination achieved elimination of *M. tuberculosis* after 6 days of drug exposure which was not achieved during single drug exposure (or 3-drug and 4-drug combinations compared to 2-drug and 3-drug combinations, respectively) [7,8]. The definition of synergy was only met when those criteria were achieved in both (duplicate) experiments.

3. Results

3.1. Susceptibility

The BE-1585 strain was found to be susceptible to isoniazid (MIC 0.125 mg/L), rifampicin (MIC 0.25 mg/L), streptomycin (MIC 2 mg/L), ethambutol (MIC 5 mg/L), and para-amino salicylic acid (MIC 0.125 mg/L). The BE-1585 strain was also found to be susceptible to pyrazinamide, which was tested by the radiometric method (MIC <100 mg/L).

3.2. Concentration- and time-dependent bactericidal activity of anti-TB drugs at single drug exposure

The concentration- and time-dependent bactericidal activity of