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Short communication

In vitro time–kill curves study of three antituberculous combinations against *Mycobacterium tuberculosis* clinical isolates

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ABSTRACT

The objective of this study was to examine the in vitro synergism of three-drug combinations against *Mycobacterium tuberculosis* (levofloxacin/linezolid/ethambutol, levofloxacin/amikacin/ethambutol and levofloxacin/linezolid/amikacin) using the time–kill curves method. In total, 8 multidrug-resistant and 12 drug-susceptible *M. tuberculosis* isolates were used. Minimum inhibitory concentrations (MICs) of the isolates for each drug were determined by the proportions method. Time–kill curves were studied for the three combinations proposed over 14 days using two different protocols. In protocol 1, 0.5× MIC for each drug was used. In protocol 2, 0.5× MIC for levofloxacin and linezolid and 0.25× MIC for amikacin and ethambutol were used. The MICs for all of the isolates studied were 0.5 mg/L for levofloxacin and linezolid and 2.5 mg/L for ethambutol and amikacin. All of the combinations displayed an additive activity compared with the most active individual drug. In conclusion, these results demonstrate that the three combinations tested were equally effective against *M. tuberculosis* isolates. The study of antituberculous combinations using in vitro methods is an excellent first step to predict their effect in clinical development phases as well as to test new regimens of the antituberculous drugs currently available.

1. Introduction

Tuberculosis (TB) remains a global health threat with nearly nine million cases of TB estimated in 2013, including 480,000 multidrug-resistant (MDR) TB cases [1]. Treatment of susceptible isolates is based on a 6-month drug schedule including four drugs, usually rifampicin, isoniazid, ethambutol and pyrazinamide [2]. On the other hand, few drugs are available to treat drug-resistant TB, especially MDR-TB. Inclusion of the following drugs is recommended for the treatment of MDR-TB: pyrazinamide or ethambutol, one injectable agent and one fluoroquinolone [3]. A number of new antituberculous drugs have appeared as promising agents that may potentially shorten the treatment for MDR and drug-susceptible TB, including bedaquiline, the oxazolidinones linezolid and sutezolid, and nitroimidazoles such as PA-824 and delamanid [4]. In addition, previously used antibiotics with antimycobacterial effect such as clofazimine, *p*-aminosalicylic acid or cycloserine might now be added to potential combination options in the treatment of MDR and extensively drug-resistant (XDR) TB.

Although drug susceptibility testing is done individually, the various drugs used in TB treatment act in combination. To determine the efficacy of these combinations, many studies have been performed against *Mycobacterium tuberculosis* (MTB), but few of them have used the time–kill curves method.

The main objective of this study was to test the interaction of four antituberculous drugs in combination in three-drug regimens (levofloxacin/linezolid/ethambutol, levofloxacin/amikacin/ethambutol and levofloxacin/linezolid/amikacin) against drug-susceptible and isoniazid/rifampicin-resistant MTB isolates using the time–kill curves method.

2. Materials and methods

2.1. Mycobacterium tuberculosis *isolates*

This study included 8 isoniazid/rifampicin-resistant and 12 drug-susceptible clinical MTB isolates from Hospital Clínic de Barcelona (Barcelona, Spain). Strain identification was done by 16S PCR sequencing. Genotyping of the studied strains using

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restriction fragment length polymorphism (RFLP) and mycobacterial interspersed repetitive unit–variable number tandem repeats (MIRU–VNTR) analysis. All of the strains were susceptible to all of the drugs tested in the present study.

Rifampicin and isoniazid resistance was determined by *rpoB* and *inhA* gene sequencing, and specific minimum inhibitory concentrations (MICs) for the two drugs were studied by the proportions method in solid medium. The MIC for rifampicin ranged from 2–8 mg/L in rifampicin-resistant strains and was 1 mg/L in rifampicin-susceptible strains. The MIC for isoniazid ranged from 0.4 to 12 mg/L in isoniazid-resistant strains and was 0.1 mg/L in isoniazid-susceptible strains.

2.2. Antimicrobial agents

Amikacin, ethambutol, levofloxacin and linezolid were obtained from Sigma-Aldrich (St Louis, MO). Amikacin and ethambutol were prepared in sterile distilled water; levofloxacin was dissolved in NaOH (0.002% final concentration) (0.1 M); and linezolid was dissolved in dimethyl sulphoxide (DMSO) (0.002% final concentration) and sterile distilled water. All of the stock solutions were sterilised by filtration and were stored at −20 °C.

2.3. Determination of minimum inhibitory concentrations

Individual MICs were determined for each isolate using a Steers-Foltz replicator and the agar dilution technique, plating a 10^5 CFU/mL inoculum on Middlebrook 7H11 solid medium supplemented with oleic acid–albumin–dextrose–catalase (OADC) (Soria Melguizo S.A., Madrid, Spain) and the following ranges of final drug concentrations: 0.125–4 mg/L for levofloxacin and linezolid; and 0.31–7.5 mg/L for amikacin and ethambutol. Replicates of the inoculum of each isolate and 1/100 dilutions were cultured on antibiotic-free Middlebrook 7H11 plates as a growth control. Plates testing the highest final concentrations of DMSO and NaOH were also added as controls. Plates were incubated at 37 °C in 5% CO_2 for 21 days before reading. Agar plates were read after 21 days. An isolate was considered resistant if ≥1% of CFU were observed in the drug-containing medium compared with the drug-free medium. The MIC was the first concentration that visually inhibited bacterial growth. Every experiment included two replicates for each isolate.

2.4. Mycobacterium tuberculosis *inoculum*

Isolates were inoculated in MGIT™ medium (Becton Dickinson, Sparks, MD) containing 10% polyoxyethylene stearate (POES) supplement (Becton Dickinson). When the MGIT reached positive growth, 5-mm glass beads were added to the sample, shaken for 45 s and sonicated for 1 min (Ultrasons; Selecta, Barcelona, Spain). Possible remaining clumps were disaggregated by 14 passages through a 20 G syringe (Becton Dickinson) and 4 passages through a 27 G syringe (Becton Dickinson). The inoculum was then measured using a nephelometer (CrystalSpec™; Becton Dickinson) and was adjusted to 10^7 CFU/mL.

2.5. Time–kill curves protocols

Time–kill curves were determined by incubation of a prepared inoculum in the presence of the drugs. Two different approaches were evaluated. In protocol 1, seven MGIT tubes supplemented with 10% POES were used for the drug tests (one tube for each of the four drugs and one tube for each of the three combinations). The final concentration of antibiotic in both the individual and combination tubes was 0.5× MIC. An additional tube without drug was included as a growth control. The eight tubes were inoculated with 0.5 mL of the previously prepared inoculum for a starting concentration of 5×10^5 CFU/mL and were incubated for 14 days at 37 °C. At defined time intervals (3, 6, 10 and 14 days), 0.5 mL was taken from each tube and 10^{-1}, 10^{-2} and 10^{-3} dilutions were prepared. A volume of 200 μL of each dilution was plated directly on Middlebrook 7H11 supplemented with OADC agar for CFU counting. Agar plates were incubated at 37 °C in 5% CO_2 and were read after 21 days. Protocol 2 applied the same scheme as Protocol 1 with the following changes in the final concentration of antibiotics: 0.5× MIC for levofloxacin and linezolid and 0.25× MIC for ethambutol and amikacin.

2.6. Drug interaction analysis

The results were interpreted by the effect of each combination compared with the most active drug tested individually [4]. The activity of the combinations was calculated as follows: $\log_{10}$ CFU/mL A − $\log_{10}$ CFU/mL B, where A is the combination and B is the most active single drug. Synergism was defined as a 2 $\log_{10}$ increase in killing at the time point with the combination compared with the most active single drug alone. Antagonism was defined as a 2 $\log_{10}$ decrease in killing, and additive activity or indifference was defined as a less than 2 $\log_{10}$ increase or decrease [5,6].

3. Results

All of the isolates were susceptible to the drugs tested. For levofloxacin and linezolid the MIC for all of the isolates was 0.5 mg/L (except for one strain with an MIC of 1 mg/L for linezolid). For ethambutol and amikacin the MIC for all of the isolates was 2.5 mg/L (except one isolate with a MIC of 1.25 mg/L for amikacin).

Fig. 1 shows an example of the time–kill curves obtained by both protocols. In Protocol 1, ethambutol and amikacin showed good activity at 0.5× MIC, both causing a mean 3.3 log CFU/mL decrease compared with the initial inoculum. Levofloxacin showed a mean decrease of 0.34 log CFU/mL. Linezolid showed a 0.84 log CFU/mL increase on Day 14, close to the 1 log increase shown by the growth control. The combination levofloxacin/linezolid/amikacin showed a 3.09 log CFU/mL decrease. The levofloxacin/linezolid/ethambutol combination showed a decrease of 3.22 log CFU/mL. Levofloxacin/amikacin/ethambutol was the most effective combination, causing a mean reduction of 4.24 log CFU/mL at Day 14 compared with the initial inoculum and a 1.1–1.6 log CFU/mL difference compared with the most active drug alone (Table 1).

In Protocol 2, amikacin and ethambutol showed 0.46 and 0.44 log CFU/mL decreases, respectively, compared with the initial inoculum. Levofloxacin presented a 0.17 log CFU/mL decrease. Linezolid growth curves were similar to the growth control curves with a 0.48 log CFU/mL increase. In all of the isolates the combinations were found to be more effective than the drugs given alone. Similar to Protocol 1, the combinations showed an additive activity, with levofloxacin/amikacin/ethambutol combination being the most effective (Fig. 1), albeit not statistically so. In addition, no significant differences were found among the isolates or between the isoniazid/rifampicin-resistant and the drug-susceptible strains using either Protocol 1 or 2.

4. Discussion

The most relevant result of this study is that the three-drug combinations showed an additive activity against all of the isolates tested, being equally effective against isoniazid/rifampicin-resistant and drug-susceptible isolates. Since all of the strains were susceptible to the drugs studied, the similarity in the results obtained indicates that no factors associated with isoniazid and