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Activity and interactions of levofloxacin, linezolid, ethambutol and amikacin in three-drug combinations against *Mycobacterium tuberculosis* isolates in a human macrophage model

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ABSTRACT

Multidrug resistance is a problem in the management of tuberculosis, creating an urgent need for new regimens including currently available drugs. Macrophage models allow an evaluation of the effect of drugs against intracellular bacilli. The effect of the following different drug combinations against six multidrug-resistant and six drug-susceptible clinical isolates of *Mycobacterium tuberculosis* multiplying inside the human macrophage THP-1 cell line was studied: levofloxacin/linezolid/ethambutol; levofloxacin/amikacin/ethambutol; and levofloxacin/linezolid/amikacin. Macrophages were lysed, seeded onto Middlebrook 7H11 plates and CFU were counted after 21 days of incubation. The interaction of the drugs in combination was interpreted by the effect of the combination compared with the most active single drug alone. The antimicrobial activity of the drugs was evaluated comparing the $\log_{10}$ CFU/well of the isolate with and without the drug. Drug concentrations within infected macrophages and in extracellular medium were simultaneously determined by chromatography. The levofloxacin/linezolid/amikacin and levofloxacin/linezolid/ethambutol combinations showed antagonism against most of the isolates (91.7%) after a 4-day protocol, whereas levofloxacin/amikacin/ethambutol displayed indifference. Levofloxacin alone and levofloxacin/amikacin/ethambutol were the most potent antimicrobials, presenting reductions up to 5.49 $\log_{10}$ and 5.86 $\log_{10}$, respectively. The drug penetration percentages ranged from 5.46% to 11.10%. Intracellular concentrations for the drug alone compared with those for the drugs in combination were not significantly different. All of the combinations tested against *M. tuberculosis*-infected macrophages showed antimicrobial activity, with combinations including linezolid and levofloxacin showing an antagonistic effect that may be explained by efflux transporters or changes in the macrophage environment.

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1. Introduction

Tuberculosis (TB) remains a global threat worldwide, with nearly nine million incident cases of TB being estimated in 2010 according to the World Health Organization (WHO). Among the 12 million prevalent cases of TB reported worldwide in 2010, the WHO estimated 650 000 cases to be multidrug-resistant (MDR) TB [1]. The regimen currently used to treat drug-susceptible TB consists of 2 months of an initial intensive phase of isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) followed by 4 months of INH and RIF. Multidrug resistance is defined as resistance to at least INH and RIF, with only a maximum of two drugs of the standard regimen remaining available. Inclusion of the following drugs is recommended for the treatment of MDR-TB: PZA or EMB, one injectable agent [kanamycin, capreomycin, amikacin (AMK) or streptomycin], one fluoroquinolone [ofloxacin, levofloxacin (LEV), gatifloxacin or moxifloxacin], and cycloserine or p-aminosalicylic acid if cycloserine cannot be used [2]. Indeed, other drugs such as linezolid (LNZ), imipenem and clarithromycin have been proposed, depending on the drug resistance pattern, to treat some cases of MDR and extensively drug-resistant (XDR) TB [3–5]. Treatment of drug-resistant TB requires an individualised regimen depending on the drug susceptibility of the isolate as well as interactions and toxicity to the patient. Indeed, few drugs are available to treat drug-resistant TB, especially MDR-TB. The length of treatment along with drug toxicity makes patient compliance

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difficult in the treatment of drug-resistant TB. Despite drug susceptibility testing being done individually, the different drugs used in TB treatment act in combination. Few studies have been performed to assess the efficacy and synergy of drug combinations against *Mycobacterium tuberculosis* [6–8].

Following inhalation of infected aerosols into the lungs of the host, the first cells to respond to *M. tuberculosis* are the alveolar macrophages and tissue dendritic cells. In contrast to other pathogens, *M. tuberculosis* survives and replicates inside macrophages [9]. Thus, macrophage models have previously been used to test the efficacy of drugs against *M. tuberculosis* inside macrophages. Drug distribution throughout the tissues as well as cellular accumulation of the drugs have been shown to be important in the killing of intracellular bacteria, whilst also being relevant in the final outcome of treatment. Fluoroquinolones show good penetration to the tissues, whereas the low solubility of aminoglycosides makes their tissue distribution poor [10,11]. Our model allows evaluation of the penetration of the drug inside macrophages and consequently the intracellular efficacy of drugs. Indeed, evaluation of combined drugs within macrophages may provide useful information regarding drug penetration into macrophages in the presence of more drugs and on the effect of drug interactions on the intracellular activity against *M. tuberculosis*.

The specific objectives of the present study were: (i) to study the effect of the three-drug regimens (LEV–LNZ–EMB; LEV–AMK–EMB; and LEV–LNZ–AMK) against drug-susceptible and MDR-TB in a human macrophage model; and (ii) to determine drug penetration alone and in combination inside *M.-tuberculosis*-infected-human macrophages.

2. Materials and methods

2.1. Mycobacterium tuberculosis isolates

Twelve non-clustered *M. tuberculosis* clinical isolates (six MDR-TB with *katG* and *rpoB* mutations and six drug-susceptible isolates) from the Hospital Clinic of Barcelona (Barcelona, Spain) were selected for this study.

2.2. Preparation of antimicrobial agents

AMK, EMB, LEV and LNZ were obtained from Sigma–Aldrich (St Louis, MO). Stock solutions of AMK (2500 mg/L) and EMB (500 mg/L) were prepared in sterile distilled water. Levofloxacin (600 mg/L) was dissolved in NaOH (0.1 M), and LNZ (1300 mg/L) was dissolved in dimethyl sulphoxide (DMSO) (final concentration of DMSO in LNZ ranging from 0.0002% to 0.01%) and sterile distilled water was then added. All of the stock solutions were sterilised by filtration and were stored at −20 °C.

2.3. Determination of the minimum inhibitory concentration (MIC)

The MICs for AMK, EMB, LEV and LNZ were determined for each isolate using the following ranges of drug concentrations: 0.125–4 mg/L for LEV and LNZ; and 0.31–7.5 mg/L for AMK and EMB. All of the experiments were performed in duplicate with the proportions method [12] in Middlebrook 7H11 solid medium supplemented with 10% OADC (oleic acid–albumin–dextrose–catalase) (Soria Melguizo SA, Madrid, Spain) and incubated for 21 days at 37 °C in 5% CO_2.

2.4. Cell culture

A modification of a previously described protocol [13] using the THP-1 cell line was used. The human acute monocytic leukaemia cell line THP-1 (ATCC-TIB-202) was grown in supplemented RPMI 1640 (with L-glutamine, 25 nM HEPES buffer) containing 10% heat-inactivated foetal calf serum (FCS) (Lonza, Basel, Switzerland) and a 100 U/mL penicillin/streptomycin mixture (Sigma) at 37 °C in 5% CO_2. When exponential growth was achieved, a concentration of 200 000 cells/mL was seeded in 24-well plates and was incubated until reaching macrophage differentiation by adding 100 nM of phorbol-12-myristate-13-acetate (PMA) for 10 h at 37 °C in 5% CO_2. The supernatant was then discarded and PMA was added again and the culture was incubated for an additional 4-day period.

2.5. Experimental design of Mycobacterium-tuberculosis-infected macrophages

The experiment design for the isolates studied is shown in Fig. 1. Each isolate with its respective control and all of the drug conditions were tested in the same experiment to ensure accurate measurement of antimicrobial activity and drug–drug interactions.

2.6. Mycobacterium tuberculosis inoculum preparation

Isolates were grown in 7H9 MGIT medium (Becton Dickinson, Franklin Lakes, NJ), supplemented with 0.25% Tween 80 (Merck, Darmstadt, Germany) to minimise clump formation. When the MGIT was positive, the sample was centrifuged adding 5 mm glass beads and the tube was shaken for 45 s and sonicated for 1 min. Lastly, possible clumps were disaggregated by 14 passages through a syringe (needle 20 G × 1″; 0.9 × 25 mm) (Becton Dickinson) and 4 passages through an insulin needle (27 G × 0.5″; 0.40 × 13 mm) (Becton Dickinson). Disaggregation of clumps was checked by Ziehl–Neelsen staining before and after the process of disaggregation. The inoculum density was measured using a nephelometer and making dilutions up to 400 000 bacteria/mL. Then, 50 μL of this inoculum was plated onto Middlebrook 7H11 plates and was incubated at 37 °C in 5% CO_2 for 21 days. Quantitative counts were made thereafter.

2.7. Infection protocol

After the 4-day growth and differentiation, changes in morphology were checked through microscopy. Afterwards, macrophages were washed once with supplemented RPMI 1640, adding then 1 mL of supplemented RPMI 1640 with 10% heat-inactivated FCS without the penicillin/streptomycin mixture. After 2 h of incubation, the number of cells was calculated by Trypan blue exclusion. The inoculum of *M. tuberculosis* was added into the macrophage culture for 3 h at a multiplicity of infection of 1:1. Macrophages were then washed three times with supplemented RPMI 1640 followed by 1 mL of supplemented RPMI 1640 with 10% heat-inactivated FCS added. The preparation was incubated afterwards for 72 h in order to allow bacteria to grow inside the macrophages.

2.8. Drug activity against Mycobacterium tuberculosis/macrophage culture

After the 72 h of incubation, the wells corresponding to Day 0 (Fig. 1) were washed again with supplemented RPMI 1640 and lysis was performed by adding 0.5% NP40 detergent (Roche, Basel, Switzerland). The remaining wells were washed with supplemented RPMI 1640, thereafter adding 1 mL of supplemented RPMI 1640 with 10% heat-inactivated FCS containing the following drug concentrations: 25 mg/L for AMK; 5 mg/L for EMB; 6 mg/L for LEV; and 13 mg/L for LNZ. The drug concentrations studied were selected according to maximum concentrations in human serum as reported previously [14]. As a control, drug-free macrophages were

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