In vitro synergic effect of β-lapachone and isoniazid on the growth of *Mycobacterium fortuitum* and *Mycobacterium smegmatis*

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Nontuberculous mycobacteria are ubiquitous and saprophytic organisms that have been implicated in a wide spectrum of diseases due to an increasing number of immunocompromised patients. The natural resistance of atypical mycobacteria to classical antituberculous drugs has encouraged research into new chemotherapeutic agents and drug combinations. The aim of this study was to determine the in vitro antimycobacterial activities of β-lapachone alone and in combination with isoniazid against Mycobacterium fortuitum *and* Mycobacterium smegmatis *via the Time-Kill Curve method. A 2 log_{10} CFU/mL reduction in the* M. smegmatis *culture was observed 72 h after adding β-lapachone at its minimum inhibitory concentration. This drug sterilised the culture in 120 h. For* M. fortuitum, *a reduction of 1.55 log_{10} CFU/mL occurred in 24 h, but regrowth was seen in contact with β-lapachone. Both microorganisms were resistant to isoniazid. Regrowth of* M. fortuitum *and* M. smegmatis *was observed at 48 h and 72 h, respectively. In combination, these two drugs had a bactericidal effect and sterilised both cultures in 96 h. These results are valuable because antibiotic-resistant bacteria are a major public health problem.*

Key words: β-lapachone - nontuberculous mycobacteria - isoniazid - antimycobacterial activity

Nontuberculous or atypical mycobacteria are opportunistic pathogens frequently found in water sources, soil, dust, air and animals. These organisms are implicated in infections of the skin, bones and soft tissues. Immunocompromised patients are the most susceptible to disseminated infection caused by nontuberculous mycobacteria. Furthermore, immunological status is important to the spread of the disease (Dodiuk-Gad et al. 2007, Porat & Austin 2008, Prendiki et al. 2008).

The treatment of infections caused by *Mycobacterium* species is difficult, lengthy and often unsuccessful. This is a result of multi-drug regimens, long periods of administration, a small selection of drugs, significant side effects and intrinsic resistance to a wide range of medications (Nuermberguer & Grosset 2004). These challenges have motivated the research of novel antimycobacterial agents (Brendan et al. 2007).

Most atypical bacteria are resistant to isonicotinic acid hydrazide (isoniazid), one of the most used therapeutic agents for the treatment of tuberculosis. Isoniazid is a prodrug that is activated by an endogenous mycobacterial catalase-peroxidase enzyme using molecular oxygen (Zhang et al. 1992, Chung et al. 2006).

Some published articles have demonstrated that quinones are active against *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and *Mycobacterium avium*. The mechanism of these compounds is still being investigated, although some reports have suggested that quinones stimulate oxidative stress in biological systems (Tran et al. 2004, Akhtar et al. 2006, Silva et al. 2008).

β-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol [1,2-*b*]pyran-5,6-dione) is a natural quinone extracted from the bark of the Lapacho tree (*Tabebuia avellanedae*) or synthesised from lapachol or lomatiol. β-lapachone is known to have a variety of pharmacological effects, including trypanocidal, moluscicidal, antifungal, antibacterial, antiviral and anticancer actions. Quinones have been reported to stimulate isoniazid activity, most likely by increasing intracellular superoxide production (Tran et al. 2004, Silva et al. 2008).

The present study compares the in vitro antimycobacterial activities of β-lapachone and the combination of isoniazid and β-lapachone against *Mycobacterium fortuitum* and *M. smegmatis*. Currently, there are no reports regarding the activity of β-lapachone in combination with isoniazid against *M. fortuitum* and *M. smegmatis*.

MATERIALS AND METHODS

Strains - The *M. fortuitum* and *M. smegmatis* strains used in this study were clinical isolates obtained from AIDS patients. They were scraped from Lowestein-Jensen slants and recultured in Mueller-Hinton broth (Oxoid) containing 0.01% tween 80.

Drugs - Isoniazid (LAFEPE) was dissolved in sterile distilled water. β-lapachone was provided by the Departamento de Antibióticos-Universidade Federal de Pernambuco and dissolved in a propyleneglicol/sterile distilled water solution (1:9). All drug solutions were extemporaneously prepared. Appropriate solvent controls were included in the test to exclude the possibility that the solvent concentration used would have toxic effects on the microorganisms.

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Determination of viable counts - Samples of culture were collected and 10-fold dilutions were made in sterile saline. Then, six 10-µL aliquots from each tube were plated onto Mueller-Hinton agar. Colonies were counted using a colony counter (Biomatic) after incubation at 37°C for 72 h.

Minimum inhibitory concentration (MIC) and Minimum Bactericide Concentration (MBC) determinations - The MIC values for the two drugs were determined by a standard twofold serial dilution method (NCCLS 1990) in Mueller-Hinton broth (Oxoid) containing 0.01% of tween 80. The inocula of *M. fortuitum* and *M. smegmatis* were grown at 37°C for 72 h. The microorganisms were then diluted into fresh broth and adjusted to obtain final inocula of approximately 10^6 CFU/mL. Finally, 4.5 mL of each culture was combined with 0.5 mL of the drug. The final concentration of each drug ranged from 0.5-128 µg/mL.

The MIC was determined to be the lowest concentration that prevented visible growth after incubation at 37°C for 72 h. Samples of 10 µL were transferred from tubes, which had not grown microorganisms, to Mueller-Hinton agar plates. Once on the plates, the samples were incubated for 72 h at 37°C. The MBC was determined to be the lowest drug concentration inhibiting ≥ 99.9% of the bacterial population. All experiments were carried out in triplicate on three different days.

Bactericidal kinetic studies - The bactericidal activity of drugs was determined by the Time-kill curve method (Krogstad & Moellering 1986). The log-phase inoculum was prepared in a manner similar to the MIC and MBC. Tubes were prepared containing the inoculum and single or combined drugs at MIC. A growth tube without drugs was used as a control. The tubes were incubated at 37°C and appropriate dilutions were performed at 0, 24, 48, 72, 96, 120 and 144 h in order to determine the number of viable bacteria ($\log_{10}$ CFU/mL). Time-kill curves for the individual drugs and the combinations of drugs were constructed by plotting $\log_{10}$ CFU/mL versus time. All experiments were carried out in triplicate on three different days.

RESULTS

Determination of MIC and MBC of β-lapachone and isoniazid - For both microorganisms, the MIC and MBC values of β-lapachone were 32 and 64 µg/mL, respectively. For isoniazid, the MIC and MBC values were 8 and 16 µg/mL, respectively.

Bactericidal activity - For β-lapachone, there was no significant reduction in the number of viable bacteria during the 144 h incubation. Compared to the control, isoniazid produced a 1.54-log CFU/mL reduction in the count at 24 h. However, regrowth was observed after 48 h of incubation. Unlike β-lapachone or isoniazid alone, the combination of these drugs exhibited a bactericidal effect on *M. fortuitum* at 96 h of incubation. Moreover, this combination was able to prevent regrowth.

β-lapachone alone was more active against *M. smegmatis* than isoniazid alone. β-lapachone had a bactericidal effect at 120 h of incubation. Compared with the control, isoniazid produced a 1.46-log CFU/mL reduction in the count at 48 h. However, regrowth was observed with isoniazid after 48 h of incubation. The combination of isoniazid and β-lapachone showed a bactericidal effect against *M. smegmatis* at 96 h of incubation. In the combination samples, regrowth was not observed.

DISCUSSION

The data in the literature concerning the in vitro activity of quinones against atypical mycobacteria is limited. However, the MIC values obtained in this work for β-lapachone are in agreement with those obtained by D'albuquerque et al. (1972) against *M. smegmatis* (MIC values ranging from 40-60 µg/mL).

Tran et al. (2004) reported the in vitro antimycobacterial of quinone derivatives against *M. fortuitum* and *M. smegmatis*. Plumbagin was the most potent synthesised quinone against *M. smegmatis* and *M. avium*, exhibiting a MIC value of 12.5 µg/mL. Meanwhile, 2,3-dipropyl-1,4-naphthoquinone had a MIC value of 50 µg/mL against *M. fortuitum*.

The high resistance of nontuberculous mycobacteria to isoniazid has been attributed to (i) decreased permeability of the cell wall; (ii) reduced conversion of isoniazid to its active form and (iii) extrusion by efflux pumps. High levels of resistance may be acquired with the loss of catalase-peroxidase activity and the overproduction of superoxide dismutase (Mdluli et al. 1998, Bulatovic et al. 2002, Gupta et al. 2006).

The results obtained using the killing curve method indicate that regrowth of *M. fortuitum* and *M. smegmatis* occurs in the presence of isoniazid alone at MIC.

Some in vitro studies have used only one drug to evaluate the efficacy of new antimicrobial agents. However, the combination of two or more antibiotics is required to avoid an increase in drug-resistant mycobacteria during therapy (Maheshwari 2007).

A combination of β-lapachone and isoniazid demonstrated bactericidal activity against both microorganisms. Quinones generate reactive oxygen species that can damage lipids, proteins and deoxyribonucleic acids. Published works have postulated that the activity of β-lapachone and other quinones against bacteria and *Trypanosoma cruzi* is due to superoxide and hydrogen peroxide formation (Cruz et al. 1978, Bulatovic et al. 2002, Salas et al. 2008).

The activation of isoniazid also produces reactive oxygen species. These reactive by-products are important in the action mechanism of isoniazid. Bulatovic et al. (2002) described the synergistic activity of plumbagin (a naturally occurring naphthoquinone) with isoniazid against *M. tuberculosis* and *M. smegmatis*. This synergic effect was easily prevented by the overexpression of superoxide-dismutase.

Further studies will focus on the possibility that β-lapachone stimulated isoniazid activity by raising the amount of activated isoniazid and increasing cell damage by oxidative stress.

The effectiveness of the combination of β-lapachone and isoniazid demonstrates that quinones and their derivatives may be useful against resistant mycobacteria.

The combination therapy prevented the selection of resistant variants and consequently stopped the regrowth of resistant strains. The data from this study are valuable because antibiotic-resistant bacteria are currently a major public health problem.

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