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β-Lapachone activity in synergy with conventional antimicrobials against methicillin resistant *Staphylococcus aureus* strains

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ABSTRACT

The aim of this study was to evaluate the antimicrobial activity of lapachol, α-lapachone, β-lapachone and six antimicrobials (ampicillin, amoxicillin/clavulanic acid, cefoxitin, gentamicin, ciprofloxacin and meropenem) against twelve strains of *Staphylococcus aureus* from which resistance phenotypes were previously determined by the disk diffusion method. Five *S. aureus* strains (LFBM 01, LFBM 26, LFBM 28, LFBM 31 and LFBM 33) showed resistance to all antimicrobial agents tested and were selected for the study of the interaction between β-lapachone and antimicrobial agents, busing checkerboard method. The criteria used to evaluate the synergistic activity were defined by the Fractional Inhibitory Concentration Index (FICI). Among the naphthoquinones, β-lapachone was the most effective against *S. aureus* strains. FICI values ranged from 0.07 to 0.5, suggesting a synergistic interaction against multidrug resistant *S. aureus* (MRSA) strains. An additive effect was observed with the combination β-lapachone/ciprofloxacin against the LFBM 33 strain. The combination of β-lapachone with cefoxitin showed no added benefit against LFBM 31 and LFBM 33 strains. This study demonstrated that, in general, β-lapachone combined with beta lactams antimicrobials, fluoroquinolones and carbapenems acts synergistically inhibiting MRSA strains.

Introduction

Staphylococcus aureus is the common etiological agent of different kinds of infections, affecting several tissues and organs and that has been frequently isolated from community acquired and nosocomial infections. The increased prevalence of methicillin resistant *Staphylococcus aureus* strains (MRSA) poses a serious problem to hospitalized patients and their care providers (Guzmán-Blanco et al. 2009; Coombs et al. 2011).

This pathogen has developed numerous strategies to resist the action of several antimicrobial agents mainly by an array of efflux pumps. These mechanisms have been identified as one of the main contributors to multi resistance in *Staphylococcus aureus* (Gibbons 2004; Johari et al. 2012). Thus, the search for new antimicrobial agents or resistance modifying agents is important as well as the establishment of alternative therapies in order to handle this complex infection (Mohtar et al. 2009).

The screening of secondary metabolites of different sources such as microorganisms, algae and higher plants are promising to provide diverse bioactive compounds with different pharmacological activities, including antimicrobials (Chung et al. 2011).

The therapeutic potential of phytochemicals for the development of anti-MRSA agents has been progressively recognized. Several studies have been conducted using phytochemicals combined with antimicrobial agents. These interactions can enhance the efficacy of the antimicrobial agents and are an alternative to treat infections caused by multi-drug resistant microorganisms, especially MRSA strains for which an effective therapy is limited and expensive (Lee et al. 2010; An et al. 2011; Celenza et al. 2012).

Naphthoquinones are phytochemicals with a large pharmacological activity and have been widely used for industrial purposes (Silva et al. 2003; Hussain et al. 2007). β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) and its isomer, α-lapachone are natural naphthoquinones extracted from the bark of the Lapacho tree (*Tabebuia avellanedae*) or synthesized from lapachol or lomatiol. They are known to have a variety of pharmacological properties, including antitumor, anti-inflammatory, anti-trypanosome, anti-malarial, antimicrobial activities and healing action (Ferreira et al. 2010; Fu et al. 2011; Kumagai et al. 2012). They can also act as inhibitors of the transcriptase reverse on HIV-1 and topoisomerases in eukaryotes and prokaryotes cells (Kobayashi et al. 2011).

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Few studies have been focused on the growth inhibitory activity of the lapachol and its derivatives (α and β-lapachone) against MRSA strains (Pereira et al. 2006; Machado et al. 2003). Moreover, studies on effect of β-lapachone combined with antimicrobial agents against MRSA have not been published so far.

Based on the above description, the aim of the present study was to determine the *in vitro* interaction of β-lapachone and conventional antimicrobials against MRSA strains, as well its ability to improve these antibiotic action.

Materials and methods

Bacterial strains and inocula standardization

The cultures of *Staphylococcus aureus* ($n = 12$) were obtained from stock cultures and maintained at our laboratory (Laboratório de Fisiologia e Bioquímica de Micro-organismos-LFBM). They were isolated from clinical specimens (LFBM 05, LFBM 08, LFBM 16, LFBM 26, LFBM 28, LFBM 29, LFBM 30, LFBM 31, LFBM 32, and LFBM 33) and food (LFBM 01). *S. aureus* ATCC 25923 was used as the standard strain. All bacterial strains were cultured on sheep blood agar and they were stored in brain heart infusion (BHI) plus glycerol 20% (v/v) after being identified (Koneman et al. 2008). All *S. aureus* strains used in this study showed a resistance phenotype to several antimicrobial agents such as beta-lactams, aminoglycosides, macrolides, fluoroquinolones, tetracycline, chloramphenicol, and lincosamides by the disk method assay. These strains were cultured onto Mueller-Hinton Agar (MHA) (Acumedia Manufacturers, Baltimore, USA) and incubated at 37 °C for 18 h. Single colonies were selected and inoculated into Mueller-Hinton broth (Acumedia Manufacturers, Baltimore, USA) to turbidity comparable to that of 0.5 McFarland standard, which is equivalent to a bacterial count of approximately 10^8 CFU/ml. After that, the bacterial suspension was diluted in saline to obtain a final inoculum (10^7 CFU/ml).

Antimicrobial agents

Lapachol was extracted from *Tabebuia avellanedae* sawdust. The extraction of lapachol and the synthesis of α and β-lapachone were carried out at the Department of Antibiotics, Federal University of Pernambuco-Brazil, according to the methodology described by Lima et al. (1962).

Ampicillin, amoxicillin/clavulanic acid, cefoxitin, ciprofloxacin, gentamicin and meropenem were provided by Eurofarma Laboratory LTDA – Brazil. Resistance was defined for each case: ampicillin (AMP, MIC ≥ 0.25 μg/ml); amoxicillin/clavulanic acid (AMC, MIC ≥ 8 μg/ml); cefoxitin (CFO, MIC ≥ 4 μg/ml); ciprofloxacin (CIP, MIC ≥ 4 μg/ml); gentamicin (GEN, MIC ≥ 8 μg/ml) and meropenem (MER, MIC ≥ 16 μg/ml) according to the criteria established by the Clinical Laboratory Standard Institute (CLSI 2010). The naphthoquinones were solubilized in dimethylsulfoxide/tween80/H_2O (1.0/0.5/8.5) while the antimicrobial agents were solubilized in water.

Antimicrobial activity

The Minimal Inhibitory Concentration (MIC) test was performed by using the microdilution broth method, following the recommendations established by CLSI (2010), with some modifications. Serial 2-fold dilutions of lapachol, α-lapachone, β-lapachone and antimicrobial agents were prepared in sterile 96-well microplates containing Mueller Hinton broth (MHB). Five microliters of the bacterial suspension were inoculated in each well to give a final concentration of 10^4 CFU. Antimicrobial concentrations ranged from 1024 to 0.03 μg/ml for all antimicrobial agents. The growth inhibition was demonstrated by optical density at 630 nm using a microplate reader (Thermo plate – TP Reader®). Considering the total growth (100%) in the control well (MHB + bacteria), the percentage of growth reduction was attributed to the remaining wells. Control solution containing dimethylsulfoxide/tween80/H_2O (1.0/0.5/8.5) was included in this experiment to exclude the possibility of toxic effects on the microorganisms.

The MIC was reported as the lowest concentration of lapachol, α-lapachone and β-lapachone or antimicrobial agents that inhibited the bacterial growth after 24 h of incubation at 37 °C. In order to determine the Minimal Bactericidal Concentration (MBC), the contents of the wells that showed higher or equal than 70% of growth inhibition were seeded onto Mueller-Hinton Agar (MHA). After 24 h of incubation at 37 °C, the number of surviving *S. aureus* was determined. The MBC value was defined as the lowest concentration of the drugs at which 99.9% of the bacteria have been killed. All experiments were carried out in duplicate on two different days. With this study, the most active naphthoquinone against MRSA strains was selected for the synergic activity assay.

Checkerboard assay

Combinations of β-lapachone and antimicrobial agents were tested by using the checkerboard method. Appropriate dilutions of β-lapachone and antimicrobial agents were performed into MHB. From these dilutions, 100 μl aliquots were added into 96-well microplates to obtain a final concentration equal to the MIC or six dilutions lower than the MIC of the β-lapachone and nine dilutions lower than the MIC of the antimicrobial agents. Each well received 5 μl of the bacterial suspensions (10^4 CFU/well). Plates were incubated for 24 h. The data were interpreted after calculating the FICI values as follows: (MIC of the β-lapachone in combination with antimicrobial agents/MIC of the β-lapachone) + (MIC of antimicrobial agents in combination with β-lapachone/MIC of antimicrobial agents). The combination was considered synergistic when the FICI was ≤0.5; additive when it was 0.5 to ≤1 indifferent when $1 \leq FICI \leq 2$ and, antagonistic when ≥2 (An et al. 2011).

Results

Antimicrobial activity

MIC and MBC values of the naphthoquinones and antimicrobial agents against twelve *S. aureus* strains are shown in Table 1.

β-Lapachone, when compared to lapachol and α-lapachone, showed the strongest anti-MRSA whose MIC values ranged from 8.0 to 32 μg/ml.

The *Staphylococcus aureus* strains revealed a resistance profile against most antimicrobial agents tested, in particular to the beta-lactam antibiotics. The MIC values for ampicillin and cefoxitin ranged from 4.0 to 512 μg/ml, which showed to be less effective against the *S. aureus* strains tested. For amoxicillin/clavulanic acid the values ranged from 4.0 to 64 μg/ml. Among all *S. aureus* strains tested, seven showed to be resistant to ciprofloxacin. For meropenem, MIC values ranged from 4.0 to 32 μg/ml. All strains showed to be sensitive to gentamicin, except LFBM 01, LFBM 26, LFBM 28, and LFBM 33 strains.

Four strains (LFBM 01, LFBM 26, LFBM 28, and LFBM 33) showed resistance to all antimicrobial agents tested. These *Staphylococcus aureus* strains and the *Staphylococcus aureus* LFBM 31 strain, which proved to be the most resistant to beta β-lapachone (MIC = 32 μg/ml), were selected for the study of the interaction between β-lapachone and antimicrobial agents. MBC values were one dilution higher than the respective MICs.