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Antimicrobial activity of β-lapachone encapsulated into liposomes against meticillin-resistant *Staphylococcus aureus* and *Cryptococcus neoformans* clinical strains

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ABSTRACT

The aim of this study was to determine whether encapsulation of β-lapachone (β-lap) into liposomes interferes with its in vitro antimicrobial activity against meticillin-resistant *Staphylococcus aureus* (MRSA) and *Cryptococcus neoformans* clinical strains. Liposomes (β-lap:lipo or β-lap:HPβ-CD-lipo) were prepared using the hydration of thin lipid film method followed by sonication. The in vitro antimicrobial activities of β-lap-loaded liposomes against MRSA and *C. neoformans* were evaluated using the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI). The liposomes presented a mean particle size ranging from 88.7 ± 1.5 nm to 112.4 ± 1.9 nm with a polydispersity index ranging from 0.255 to 0.340, zeta potential from −0.26 ± 0.01 mV to +0.25 ± 0.05 mV and drug encapsulation efficiency from 97.4 ± 0.3% to 98.9 ± 0.4%. β-Lap and β-lap:HPβ-CD had minimum inhibitory concentrations (MICs) ranging from 2 mg/L to 4 mg/L, whereas the MICs of β-lap-lipo or β-lap:HPβ-CD-lipo ranged from 4 mg/L to 16 mg/L for the MRSA strains tested. β-Lap and β-lap:HPβ-CD were able to inhibit fungal growth [MIC = 2–8 mg/L and minimum fungicidal concentration (MFC) = 4–8 mg/L]. However, β-lap-lipo and β-lap:HPβ-CD-lipo were more efficient, with MICs and MFCs of <4 mg/L. These findings suggest that the liposomal formulations tested do not interfere significantly with β-lap antibacterial activity against MRSA and improve its antifungal properties against *C. neoformans*.

1. Introduction

Global health data have identified increasing levels of multi-resistant bacteria and opportunistic invasive *Cryptococcus neoformans* infections. Infections that two or three decades ago were treated with routine therapeutic protocols now appear with high mortality rates [1,2]. The pandemic of multiresistant pathogens and their continuous distribution are irrefutable. Currently, research into antibacterial agents does not provide a new arsenal of antibiotics, although various initiatives for the development of new antibacterial drugs have shown encouraging results through the collaboration of academia and the pharmaceutical industry, in association with government policies. According to the literature, *Staphylococcus aureus* is a major pathogenic bacteria related to hospital- and community-acquired human infections worldwide [2].

Another infection that has grown in importance is cryptococcosis. This disease is an opportunistic invasive fungal infection caused by *Cryptococcus* spp., particularly *C. neoformans*, and its major neurological manifestation is meningitis. This mycosis has assumed an important role in the causes of morbidity and mortality among immunocompromised patients [3]. However,

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cryptococcosis has raised additional concern to the community in general, since cases of cryptococcosis in immunocompetent patients have already been reported [4].

The therapeutic treatment of microbial infections is difficult and has become a challenge. In recent years, vancomycin has been successfully used for the treatment of infections caused by meticillin-resistant Gram-positive bacteria. However, owing to the increased resistance of these micro-organisms, some strains are now resistant to vancomycin, resulting in inefficient treatments [5]. Unfortunately, for cryptococcosis infections there are few drugs available for treatment and some of them still have limitations, such as toxicity and an unfavourable pharmacokinetic profile, which restrict their therapeutic use [6]. A remarkable increase in Gram-positive bacterial resistance to the long-established antimicrobial agents and the limiting factors in the treatment of cryptococcosis have become a worldwide concern. Thus, emphasis is placed on the need for the development of new antimicrobial drugs or therapeutic strategies for treating these infections.

β-Lapachone (β-lap) is a natural naphthoquinone extracted from the bark of *Tabebuia avellanedae*, a native tree to South America abundantly found in Brazil, or is synthesised from lapachol. β-Lap has attracted increasing attention due to its antibacterial [7], antifungal [8], antischistosomal [9] and anti-tumour properties [10]. Although β-lap presents pharmacological activity, its low water solubility (0.16 mM) [11] may limit its therapeutic use. However, β-lap solubility has been increased more than 300-fold by forming inclusion complexes with 2-hydroxypropyl-β-cyclodextrin (β-lap:HPβ-CD) [11]. In addition, drug delivery systems such as liposomes can encapsulate antibiotics improving their therapeutic efficacy as well as reducing toxicity [12], thereby providing new therapeutic options for the treatment of infections. Liposomes are aqueous vesicles surrounded by a phospholipid bilayer used to carry both hydrophilic and lipophilic drugs. They can be classified as conventional and stealth systems. Conventional liposomes are the first generation of liposome delivery systems, and stealth liposomes are coated with polyethylene glycol that prevents their uptake by the mononuclear phagocytic system [13]. More recently, engineered artificial liposomes were used to sequester bacterial toxins and protect from severe invasive infections in vivo [14].

Based on these findings, encapsulation of β-lap into liposomes may improve its microbiological properties, target to bacterium/yeast and reduce its toxicity. Thus, the present study was set out to determine whether encapsulation of β-lap into liposomes interferes with its in vitro antimicrobial activity against meticillin-resistant *S. aureus* (MRSA) and *C. neoformans* clinical strains.

2. Materials and methods

2.1. Materials

β-Lap, obtained from lapachol by the semisynthetic route, was supplied by Dr Alexandre Góes (Department of Antibiotics, UFPE, Recife, Brazil). Cholesterol (Chol), trehalose, stearylamine (SA), 2-hydroxypropyl-β-cyclodextrin (HPβ-CD), RPMI medium, morpholino propanesulfonic acid (MOPS), vancomycin and oxacillin were purchased from Sigma–Aldrich (St. Louis, MO). Soybean phosphatidylcholine (PC) (Lipoid S 100®) and distearoylphosphatidylethanolamine–polyethylene glycol 2000 (DSPE-PEG 2000) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Amphotericin B was purchased from Bristol-Myers Squibb (Princeton, NJ) and fluconazole was obtained from Pfizer (New York, NY). Sabouraud dextrose agar (SDA) medium was purchased from Difco Laboratories (Detroit, MI). Mueller–Hinton broth (MHB) and Mueller–Hinton agar (MHA) were obtained from Gibco BRL (Life Technologies, Grand Island, NY). Microtitre plates were purchased from Techno Plastic Products AG (Trasadingen, Switzerland) and 0.22 μm membranes were purchased from Millipore (Billerica, MA). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Methodology

2.2.1. Preparation of stealth β-lap- or β-lap:HPβ-CD-loaded liposomes

Initially, β-lap:HPβ-CD were prepared as previously reported [11]. Next, stealth liposomal formulations were prepared with and without SA encapsulating β-lap or β-lap:HPβ-CD, namely as follows: positively charged stealth liposomes (β-lap-SASL or β-lap:HPβ-CD-SASL); and neutral stealth liposomes (β-lap-NSL or β-lap:HPβ-CD-NSL). Unloaded and drug-loaded liposomal formulations were prepared using the thin lipid film method followed by sonication. Briefly, lipids at 117.6 mM (PC:DSPE-PEG:Chol:SA at 6.5:0.5:2:1 mol%) and β-lap (1 mg/mL in final liposomal formulation) were dissolved in a mixture of $CHCl_3$:MeOH (3:1, v/v) under magnetic stirring. The solvents were then removed under pressure for 60 min (37 ± 1 °C, 80 rpm), resulting in a thin lipid film. This film was then hydrated with 10 mL of phosphate buffer solution (pH 7.4) containing trehalose (264 mM) used as a cryoprotectant agent. Multilamellar liposomes were then sonicated (Vibra Cell; Branson Ultrasonic, Danbury, CT) at 200 W and 40 Hz for 300 s to form small unilamellar vesicles. Finally, liposomes were lyophilised (EZ-DRY; FTSS System, New York, NY; 4×10^6 bars for 48 h). β-Lap:HPβ-CD-loaded liposomes were prepared as described above, in which β-lap:HPβ-CD was dissolved in the aqueous phase (corresponding to 1 mg/mL of β-lap in the final preparation). Furthermore, for comparative purposes with a view to verifying the influence of a positive charge, in addition to PEG coating of the liposome surface, on the antimicrobial activity of β-lap or β-lap:HPβ-CD, positively charged conventional liposomes (SACL) containing β-lap or β-lap:HPβ-CD were prepared on the basis of our previous study [11].

2.2.2. Stability of stealth β-lap- or β-lap:HPβ-CD-loaded liposomes

Liposomes were characterised after preparation in the dispersed form and after redispersion of the lyophilised dosage forms in order to verify the effect of the lyophilisation process on their stability after storage at 4 °C for 0–60 days and 1 year, respectively. The following parameters were analysed: macroscopic appearance; pH variation; particle size (Ø); polydispersity index (PDI); zeta potential; and drug encapsulation efficiency (%EE). β-Lap content was determined using ultraviolet spectroscopy (β-lap standard curve ranging from 0.5 μg/mL to 3.0 μg/mL). %EE of β-lap content was determined after ultrafiltration/ultracentrifugation of liposomes using filtration units (Amicon Ultra Centrifugal Filters; Millipore, Billerica, MA) and was calculated as follows: $\%\ EE = ([\beta\text{-lap}_{filtrate}] - [\beta\text{-lap}_{content}])/[\beta\text{-lap}_{content}] \times 100$.

2.2.3. In vitro antimicrobial activity of β-lap- or β-lap:HPβ-CD-loaded liposomes

2.2.3.1. Microbial strains and culture media. MRSA ATCC 33591, meticillin-susceptible *S. aureus* (MSSA) ATCC 29213 and ten MRSA clinical strains were studied. Clinical samples were supplied by the Hospital das Clínicas da Universidade Federal de Pernambuco (Recife, PE, Brazil). These micro-organisms were cultured on MHA and were allowed to grow for 24 h at 37 °C. Identification of *S. aureus* was carried out by catalase, mannitol salt agar, DNase and tube coagulase tests. MRSA strains were confirmed as being meticillin-resistant by the disc diffusion method with cefoxitin and oxacillin as drug reference, as well as by screening using MHA supplemented with 4% NaCl and 6 mg/mL oxacillin. Furthermore, PCR was performed for *mecA* gene detection [15].