Journal of Drug Delivery Science and Technology 40 (2017) 59–65

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Assessing the performance of copaiba oil and allantoin nanoparticles on multidrug-resistant *Candida parapsilosis*

G. Svetlichny [a], I.C. Külkamp-Guerreiro [b, c, *], D.F. Dalla Lana [b], M.D. Bianchin [c], A.R. Pohlmann [d], A.M. Fuentefria [b, c], S.S. Guterres [a, b]

[a] Programa de Pós-Graduação em Ciência dos Materiais, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Prédio A, sala A 216, Porto Alegre, RS, 91501970, Brazil
[b] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av Ipiranga, 2752, Santana, Porto Alegre, RS, 90610-000, Brazil
[c] Programa de Pós-Graduação em Farmacologia e Terapêutica, Faculdade de Farmácia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, Sala 305, Porto Alegre, RS, 90050-170, Brazil
[d] Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Porto Alegre, RS, 91501-970, Brazil

ARTICLE INFO

Article history:
Received 12 December 2016
Received in revised form
29 May 2017
Accepted 30 May 2017
Available online 31 May 2017

Keywords:
Solid lipid nanoparticles
Antifungal activity
Multidrug-resistant yeast
Copaiba oil
Allantoin
Candida parapsilosis

ABSTRACT

This study investigated the chemical and morphological structure of solid lipid nanoparticles made from copaiba oil. Moreover, the influence of allantoin in the formulations was investigated, as was the mechanism of action of the nanoparticles against multidrug-resistant *Candida parapsilosis*. Formulations were prepared via high-pressure homogenisation. Photon correlation spectroscopy and laser diffraction showed nanoparticles with diameters below 150 nm, narrow size distribution and negative zeta potential values. The nanoparticles' antifungal activity was assessed using a sorbitol protection assay. The ergosterol content and the influence of sorbitol on the minimum inhibitory concentrations were determined. The nanoparticles displayed nonspecific action on fungal cells. The antifungal effect is related to a complex synergism of natural substances with deeper action on cell replication. Thermogravimetric analysis showed that the individual behaviour of each component was modified in the formulation. Transmission electron microscopy showed differences in morphology caused by the presence of allantoin. The smaller particles can penetrate deeply into fungal cells. These findings indicated that even without antifungal effects when used in isolation, the incorporation of allantoin modified the size distribution, zeta potential and morphological structure of copaiba oil nanoparticles, resulting in particles with greater antifungal effects against multidrug-resistant yeasts.

1. Introduction

During the last two decades, in the entire world, the incidence of fungaemia has increased to become the principal cause of concern in populations of at-risk patients, such as those with solid organ and haematopoietic stem cell transplants, immunosuppressed patients, premature infants, surgical patients and cancer patients [1–3]. Among all such infections, yeasts are responsible for superficial and invasive infections that have become more frequent among patients in intensive care units [2,4].

The most common type of fungal infection in humans is termed candidiasis and results in the anarchic growth, in any part of the body, of a particular genus of yeast-like fungus, *Candida*, which lives on all surfaces and mucous membranes of the body. In some circumstances, these yeasts can become invasive to the bloodstream, creating an infection termed candidaemia. When induced by different *Candida* species, these infections are recurrent and represent a significant source of morbidity and mortality in hospitalised patients [2]. Among other *Candida* species, *Candida parapsilosis* is one of the most common pathogens, causing diverse infections [5] such as monomicrobial necrotizing soft tissue infections [6] and endocarditis [7]. Candidiasis has become increasingly difficult to treat due to the widespread and long periods of use of fluconazole, other azoles, echinocandins and flucytosine [2,8,9].

* Corresponding author. Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av Ipiranga, 2752, Santana, Porto Alegre, RS, 90610-000, Brazil.
E-mail address: irene@ufrgs.br (I.C. Külkamp-Guerreiro).

http://dx.doi.org/10.1016/j.jddst.2017.05.020
1773-2247/

Lately, there are increasing numbers of yeasts resistant to antifungal drugs worldwide; consequently, the utilisation of *in vitro* laboratory tests might help in choosing an appropriate therapy [10]. The ability of *Candida* species to form drug-resistant biofilms is an important factor in their contribution to human disease. Due to the growing antifungal drug resistance and the lack of novel clinically available therapeutic options, the research into and development of novel antifungal strategies are crucial [11]. Thus, studies have been directed towards natural substances that possess either fungicidal or fungistatic activity [12].

Copaiba oil is obtained from Copaifera (*Fabaceae*), an important renewable source of natural remedy for Amazon populations [13]. This oil has been reported to possess numerous important pharmaceutical properties, such as antibacterial [14], antiparasitic [15], larvicidal [16], anti-inflammatory, analgesic [13,17], cellular proliferation-promoting [18], expectorant and diuretic activities [13,19]. Fewer works have explored the product's antifungal activity; however, oleoresin extracted from copaiba has previously been employed to treat dermatophytosis [20].

In a previous work, our research group developed nanoparticles with copaiba oil (NCO) and nanoparticles with copaiba oil and allantoin (NCOA) [21]. We showed that the nanoencapsulation of copaiba oil, as well as the presence of allantoin in the formulations, increased the antifungal activity against *Candida parapsilosis*. The NCO suspension demonstrated moderate antifungal effects limited to fungistatic activity, while the NCOA suspension was found to be more efficient (lower minimum inhibitory concentration - MIC), exhibiting both fungistatic and fungicidal activity. In that previous study, we demonstrated that copaiba oil formulations displayed antifungal action on drug resistant yeasts. However, two questions remained: 1) How do the copaiba oil formulations act on fungal cells? 2) If allantoin did not have antifungal action, why did its incorporation into the formulations improve the antifungal action? Thus, the aim of the present research was to investigate the chemical and morphological structure of nanoparticles to better understand their mechanism and correlate it with the antifungal activity of the formulations. Moreover, the influence of allantoin on the structure of the formulations was investigated, as well as the mechanism of action of the solid lipid nanoparticles against multidrug-resistant *Candida parapsilosis*.

For this purpose, the formulations of previously developed nanoparticles were deeply studied and morphologically evaluated using transmission electron microscopy (TEM). Thermal and morphological analyses were performed, and antifungal action mechanisms were assessed, in this study. Additionally, the antifungal activity of these solid lipid nanoparticles (SLN) was assessed using a sorbitol protection assay. The evaluation of ergosterol's effects and a MIC assay were completed using scanning electron microscopy (SEM) morphological analysis.

2. Materials and methods

2.1. Chemicals and solvents

PEG-80 sorbitan monooleate (Tween 80) and sorbitan monooleate (Span 80) were supplied by Oxiteno (Brazil), copaiba oil by Inovam (Brazil), cetyl palmitate by Cognis (Germany), and allantoin and butyl hydroxy toluene by Viafarma (Brazil). All of these chemicals were of pharmaceutical grade and were used as received. Milli-Q water was used as the solvent.

2.2. Preparation of solid lipid nanoparticles

SLN suspensions comprised of NCO or NCOA were prepared by the hot high-pressure homogenisation technique, as previously described [22]. New batches were prepared in triplicate. The lipid phase was composed of cetyl palmitate (12 g), copaiba oil (6 g), Span 80 (2 g) and butyl hydroxy-toluene (0.1 g). The aqueous phase contained Tween 80 (4 g) that was dissolved in Milli-Q water to a final volume of 200 mL. Allantoin (2 g) was added and diluted in the aqueous phase to prepare the NCOA suspensions. The lipid and aqueous phases were heated to 85 °C and agitated until homogeneity. Then, the two phases were mixed using an Ultra-Turrax (T25, Ika), which permitted rapid stirring (11,000 rpm for 1 min, 13,000 rpm for 1 min and 16,000 rpm for 3 min). Next, the pre-emulsion was homogenised with a high-pressure homogeniser (Panda 2KNS 1001 L, Saovi Niro) applying 3 cycles of 400 bars of pressure. Finally, the recrystallisation of the lipids occurred due to the cooling of the oil/water nanoemulsion to 25 °C, which permitted the formation of NCO or NCOA suspension, obtaining colloidal dispersion.

2.3. Particle size analysis

The particle sizes were measured using Photon Correlation Spectroscopy with a Zetasizer Nano ZS® (Malvern Instruments, UK) at 25 °C after a 1/500 (v/v) dilution with ultrapure water. This instrument allows the calculation of the average diameter (z-average) and size distribution, which were measured in triplicate. The results are expressed in terms of the particles' light scatter intensity, number and volume. The determination of the Zeta potential values was realised by 3 measurements at 25 °C for each suspension after a 1/500 (v/v) dilution with 1 mM NaCl.

Laser diffraction (LD) (Mastersizer® model 2000, Malvern Instruments, UK) was also utilised to reject the presence of micrometric particles. The suspensions were not diluted and for each suspension, three measurements were performed. The results were presented as the D[4,3] value for diameter. The size distribution (SPAN) width was determined using the same technique, in which the calculation is based on the 10%, 50% and 90% quantiles (i.e., the diameter that 10%, 50% or 90% of the nanoparticles are below). This mathematical value is known as the Span value and is represented by equation (1), in which D[v, 0.9], D[v, 0.1], D[v, 0.5] are the 90%, 10% and 50% quantiles, respectively.

$$\mathrm{SPAN} = \mathrm{D[v,/0.9]} - \mathrm{D[v,0.1]/D[v,0.5]} \quad (1)$$

2.4. Transmission electron microscopy analysis

A Carl Zeiss Libra 120 TEM operating at 80 kV was utilised for the morphological examination of the SLN. Ten-microliter samples of the NCO and NCOA suspensions were diluted with 90 μL of Milli-Q water. Next, 10 μL of these solutions was deposited on a carbon-polymer grid. After 1 min, excess solution was removed, and 3 min later, 10 μL of filtered uranyl acetate (Millipore 0.45 μm) was dropped onto the grid. Again, the excess material was removed after 1 min, and the grid was finally ready for viewing.

2.5. Thermoanalytical analysis

The thermal behaviour of copaiba oil, allantoin, cetyl palmitate and both suspensions was assessed using thermogravimetric analysis (TGA). TGA can detect changes in the physical and chemical properties of a material such as decomposition, degradation, oxidation or loss of volatiles. From the TGA curve, the derivative of the mass change versus time (dm/dt) or temperature (dm/dt) of the reaction was calculated and the derivative thermogravimetry (DTG) curve obtained. The analyses were performed using a TA TGA