Journal of Pharmaceutical and Biomedical Analysis xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Physicochemical characterization by AFM, FT-IR and DSC and biological assays of a promising antileishmania delivery system loaded with a natural Brazilian product

Franciane Marquele-Oliveira[a,*], Elina Cassia Torres[a], Hernane da Silva Barud[b,c], Karina Furlani Zoccal[d], Lúcia Helena Faccioli[d], Juliana I. Hori[e], Andresa Aparecida Berretta[a,f,*]

[a] Laboratório de Pesquisa, Desenvolvimento e Inovação, Apis Flora Indl. Coml. Ltda., Ribeirão Preto, SP, Brazil
[b] Instituto de Química, Universidade Estadual Paulista, UNESP, Caixa Postal 355, 14800-900, Araraquara, SP, Brazil
[c] Grupo de Química Medicinal e Medicina Regenerativa (QUIMMERA)—Centro Universitário de Araraquara/UNIARA, Araraquara, SP, Brazil
[d] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas. Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil
[e] Departamento de Biologia Celular, Molecular e Bioagentes Patogênicos, Universidade de Sa˜o Paulo, Faculdade de Medicina de Ribeira˜o Preto, FMRP/USP, 14049-900, Ribeira˜o Preto, SP, Brazil
[f] Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, FCFRP/USP, 14049-900, Ribeirão Preto, SP, Brazil

ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form 16 January 2016
Accepted 19 January 2016
Available online xxx

Keywords:
Solid lipid nanoparticle (SLN)
Anti-leishmanial activity
Sustained drug release
Ocotea duckei
Macrophage cytotoxicity

ABSTRACT

The control and treatment of Leishmaniasis, a neglected and infectious disease affecting approximately 12 million people worldwide, are challenging. *Leishmania* parasites multiply intracellularly within macrophages located in deep skin and in visceral tissues, and the currently employed treatments for this disease are subject to significant drawbacks, such as resistance and toxicity. Thus, the search for new Leishmaniasis treatments is compulsory, and *Ocotea duckei* Vattimo, a plant-derived product from the biodiverse Brazilian flora, may be a promising new treatment for this disease. In this regard, the aim of this work was to develop and characterize a delivery system based on solid lipid nanoparticles (SLN) that contain the liposoluble lignan fraction (LF) of *Ocotea duckei* Vattimo, which targets the *Leishmania* phagolysosome of infected macrophages. LF-loaded SLNs were obtained via the hot microemulsion method, and their physical and chemical properties were comprehensively assessed using PCS, AFM, SEM, FT-IR, DSC, HPLC, kinetic drug release studies, and biological assays. The size of the developed delivery system was 218.85 ± 14.2 nm, its zeta potential was −30 mV and its entrapment efficiency (EE%) was high (the EEs% of YAN [yangambin] and EPI-YAN [epi-yangambin] markers were 94.21 ± 0.40% and 94.20 ± 0.00%, respectively). Microscopy, FT-IR and DSC assays confirmed that the delivery system was nanosized and indicated a core–shell encapsulation model, which corroborated the measured kinetics of drug release. The total *in vitro* release rates of YAN and EPI-YAN in buffer (with sink conditions attained) were 29.6 ± 8.3% and 34.3 ± 8.9%, respectively, via diffusion through the cellulose acetate membrane of the SLN over a period of 4 h. After 24 h, the release rates of both markers reached approximately 45%, suggesting a sustained pattern of release. Mathematical modeling indicated that both markers, YAN and EPI-YAN, followed matrix diffusion-based release kinetics (Higuchi's model) with an estimated diffusion coefficient (D) of $1.3.10^{-6}$ cm^2/s. The LF-loaded SLNs were non-toxic to murine macrophages (20–80 μg mL^{-1} range) and exerted a prominent anti-leishmanial effect (20 μg mL^{-1}). These data suggest this new and well-characterized lipid nanoparticle delivery system safely and effectively kills *Leishmania* and warrants further clinical investigation.

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1. Introduction

Drug delivery systems based on lipid nanoparticles constitute an area of extensive development due to the prominent advantages of this colloidal system as a therapeutic. In general, solid

* Corresponding authors at: Triunfo street, 945, Ribeirão Preto/SP, CEP 14020-670, Brazil.
E-mail addresses: fmarqueleoliveira@gmail.com (F. Marquele-Oliveira), andresaberretta@hotmail.com (A.A. Berretta).

http://dx.doi.org/10.1016/j.jpba.2016.01.045
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Please cite this article in press as: F. Marquele-Oliveira, et al., Physicochemical characterization by AFM, FT-IR and DSC and biological assays of a promising antileishmania delivery system loaded with a natural Brazilian product, J. Pharm. Biomed. Anal. (2016), http://dx.doi.org/10.1016/j.jpba.2016.01.045

lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) are highly stable and nontoxic. They can encapsulate both lipophilic and hydrophilic drugs and have been administered via several routes [1]. Moreover, they permit controlled and sustained drug release [2] and can be employed for organelle-specific disease treatments [3].

Studies of SLNs loaded with lipophilic drugs have yielded promising results. For example, the antibacterial responses of norfloxacin were enhanced when the drug was loaded into SLNs [4]. Moreover, Bose and Michniak observed that SLN encapsulation increased the gastrointestinal absorption of quercetin, as indicated by an increase in the drug bioavailability of 571.4% [5]. Cytotoxic compounds, such as topotecan and doxorubicin, have also been loaded into SLNs and have gained broad acceptance in this formulation as cancer treatment due to increases in drug efficacy and decreases in toxicological effects [6,7]. Notably, most lipids used to manufacture these particles are GRAS (Generally Recognized as Safe) and have low toxicity concerns [8].

Leishmaniasis, one of the main neglected and infectious diseases worldwide, is caused by parasites of the genus *Leishmania*, which multiply within macrophages to cause cutaneous and visceral pathologies. Leishmaniasis affects approximately 12 million people in 88 countries and its control is an important challenge for the scientific community, government and industry initiatives [9]. Pentavalent antimonials, the antimoniate of *N*-methyl-glucamine (Glucantime®) and sodium stibogluconate (Pentostan®) [9] followed by pentamidine and amphotericin B are currently the main strategies for leishmaniasis treatment. However, these drugs are intravenously administered for prolonged periods (at least 20 days), and they are associated with off-target toxicity and occasional low effectiveness, which may lead to treatment failure.

In the search for new anti-leishmania products, *Ocotea duckei* Vattimo, a plant originating from Brazilian biodiversity, is considered a promising lignan-based drug that targets Leishmaniasis. A purified fraction (lignan Fraction—LF) from *Ocotea duckei* Vattimo is highly lipophilic and rich in yangambin lignan (YAN) and its epimer, epi-yangambin (EPI-YAN), which have demonstrated promising activity against *Leishmania amazonensis* and *Leishmania* chagasi. Monte-Neto and collaborators showed that LF inhibited the growth of the parasite *in vitro* at a higher rate than the current reference drug, Glucantime® [10]. Significant alterations in cellular morphology and physiology associated with apoptotic cell death and autophagic characteristics have been observed in yangambin-treated parasites [11]. In addition, this lignan has demonstrated low cytotoxicity in macrophages, with CC_{50} values of 246.7 $\mu g\,mL^{-1}$ in an MTT assay, and low antimitotic and teratogenic potential at doses not exceeding 500 $\mu g\,mL^{-1}$ [12].

To administer the drug to an intracellular target, this work proposes loading SLNs with LF to safely treat cutaneous and visceral leishmaniasis. In addition to permitting administration via several routes, LF-loaded SLN may also reach the *Leishmania* phagolysosome [13] of infected macrophages in deep tissues. Data from the literature indicate that SLN can penetrate cell membranes [14], which permits the sustained intracellular release of LF.

A report focusing on LF composition, main compounds achievement and characterization (YAN and EPI-YAN) has recently been released. A RP-HPLC-DAD method was developed and validated: specifically, the identity and purity of markers were assessed based on their UV profile, IR, ESI-IT MS, ^{13}C NMR, ^{1}H NMR and the melting point range. Additionally, preliminary cytotoxicity analyses of murine macrophages demonstrated the safety of LF, but not of a LF-loaded nanoparticle formulation [15]. As a consequence, the present work aimed to develop a new and safe LF carrier system, as well as, to fully characterize it by PCS, AFM, SEM, FT-IR, DSC, HPLC and kinetic drug release studies. The present carrier system is supposed to target not only the pathophysiology of leishmaniasis but also to examine the properties of LF to improve the *in vivo* distribution and absorption of LF. Therefore, to this end, this work also presents for the first time the *in vitro* safety and efficacy protocols to highlight the potential medicinal uses of this new approach for Leishmaniasis treatment. The development of a safe and non-toxic lignan-based nanomedicine may provide numerous benefits for patients suffering from the side effects of the current treatments for Leishmaniasis.

2. Materials and methods

2.1. Material and reagents

The lignan fraction and the isolated pure yangambin (YAN) and epi-yangambin (EPI-YAN) compounds were obtained from the dried leaves and stem bark of *Ocotea duckei* Vattimo (Lauraceae) according to a previously described methodology [10,15]. The plant material was collected in February of 2010 in the state of Paraíba (Northeast from Brazil) and identified by Prof. Dr. Maria de Fátima Agra (Cbiotec/UFPB). A voucher specimen (AGRA 4309) was deposited in the Lauro Pires Xavier herbarium at the Federal University of Paraíba (UFPB). The experimental data from the isolated compounds confirmed the YAN and EPI-YAN identity as previously described [15].

The acetonitrile and methanol used herein were of HPLC grade (JT Baker, Philipsburg, NJ, USA). Acetic acid, dichloromethane and ethanol were of analytical grade (Vetec, Duque de Caxias, RJ, Brazil). Reagent-grade methanol was used to isolate yangambin (YAN) and epi-yangambin (EPI-YAN) and obtained from Merck (Darmstadt, Germany). Ultrapure water (Type I) was used for HPLC and obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Lipid carrier preparation

SLNs were obtained via the microemulsion method as previously described [6,7] with minor modifications. Specifically, the SLNs were prepared using stearic acid (Sigma–Aldrich, St. Louis, MO, USA) as the internal phase, soy lecithin (Lipoid GMBH, Ludwigshafen, German) as the surfactant, sodium taurodeoxycholate (Sigma–Aldrich, St. Louis, MO, USA) as a co-surfactant and purified water as the continuous phase. The microemulsion for the SLNs was prepared as follows: stearic acid (20%) was first melted at 10 °C above its melting point (65.0–70.0 °C), surfactant (10%) was then added and stirred until it was completely dissolved. The aqueous phase (sufficient to prepare 1 g of microemulsion) containing the co-surfactant (2.5%) was then heated to 80 °C and added to the melted lipid mixture. The mixture was maintained stirring at 80 °C until the microemulsion formed. Subsequently, 2% LF was added to the resulting microemulsion, which was dispersed in purified water at 2–5 °C under mechanical stirring (20,000 rpm for 10 min, Ultra Turrax, AT15, IKA-Werke, Staufen, Germany). The LF-unloaded SLNs were also prepared in the absence of the lignan extract. In some cases, as described below, the SLNs were lyophilized using sucrose as cryoprotector (7.5%). The lyophilized SLNs were readily resuspended in ultrapure water when necessary.

2.3. Photon correlation spectroscopy and zeta potential

The average diameter and polydispersivity index (PdI) of the SLNs were measured by photon correlation spectroscopy (PCS) (Malvern Zetasizer Nano ZS90, Malvern instruments Ltd., Worcestershire UK) with a 50 mV laser. The SLN suspensions were diluted 1000× in purified water before analysis. The measurements were performed at 25 °C at a fixed angle of 173°. The SLN zeta potential

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