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Potent anti-inflammatory activity of betulinic acid treatment in a model of lethal endotoxemia

José Fernando Oliveira Costa [a], José Maria Barbosa-Filho [b], Gabriela Lemos de Azevedo Maia [b], Elisalva Teixeira Guimarães [a,c], Cássio Santana Meira [a], Ricardo Ribeiro-dos-Santos [a,d], Lain Carlos Pontes de Carvalho [a], Milena Botelho Pereira Soares [a,d,*]

[a] Laboratory of Tissue Engineering and Immunopharmacology Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Rua Waldemar Falcão, 121, 40296–750, Salvador, Bahia, Brazil
[b] Laboratory of Pharmaceutical Technology, Federal University of Paraíba, João Pessoa, Cidade Universitária, s/n, 58051–900, João Pessoa, PB, Brazil
[c] Department of Life Sciences, State University of Bahia, Rua Silveira Martins, 2555, 41150–000, Salvador, BA, Brazil
[d] Center of Biotecnology and Cell Therapy, São Rafael Hospital, Av. São Rafael, 2152. São Marcos 41253–190, Salvador, BA, Brazil

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ABSTRACT

Betulinic acid (BA) is a lupane-type triterpene with a number of biological activities already reported. While potent anti-HIV and antitumoral activities were attributed to BA, it is considered to have a moderate anti-inflammatory activity. Here we evaluated the effects of BA in a mouse model of endotoxic shock. Endotoxemia was induced through intraperitoneally LPS administration, nitric oxide (NO) and cytokines were assessed by Griess method and ELISA, respectively. Treatment of BALB/c mice with BA at 67 mg/kg caused a 100% survival against a lethal dose of lipopolysaccharide (LPS). BA treatment caused a reduction in TNF-α production induced by LPS but did not alter IL-6 production. Moreover, BA treatment increased significantly the serum levels of IL-10 compared to vehicle-treated, LPS-challenged mice. To investigate the role of IL-10 in BA-induced protection, wild-type and IL-$10^{-/-}$ mice were studied. In contrast to the observations in IL-$10^{+/+}$ mice, BA did not protect IL-$10^{-/-}$ mice against a lethal LPS challenge. Addition of BA inhibited the production of pro-inflammatory mediators by macrophages stimulated with LPS, while promoting a significant increase in IL-10 production. BA-treated peritoneal exudate macrophages produced lower concentrations of TNF-α and NO and higher concentrations of IL-10 upon LPS stimulation. Similarly, macrophages obtained from BA-treated mice produced less pro-inflammatory mediators and increased IL-10 when compared to non-stimulated macrophages obtained from vehicle-treated mice. In conclusion, we have shown that BA has a potent anti-inflammatory activity *in vivo*, protecting mice against LPS by modulating TNF-α production by macrophages *in vivo* through a mechanism dependent on IL-10.

1. Introduction

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid; BA), a C-30 carboxylic acid derivative of the ubiquitous triterpene betulin, is a member of the class of lupane-type triterpenes. The molecule is abundant in the plant kingdom and has been isolated from several plant species, including *Zizyphus joazeiro* [1], *Syzigium clariflorum* [2], and *Doliocarpus schottianus* [3]. A number of reports have shown diverse biological activities of BA, such as anticancer [4], anti-HIV, anti-HSV-1 [5], anti-HBV [6], antihelmintic [6], antinociceptive [7], and antiplasmodial [1,8]. Of particular interest, in view of the large prevalence of chronic inflammatory-degenerative diseases, is the BA anti-inflammatory activity [9,10]. However, in a number of *in vitro* and *in vivo* models of inflammation, the intensity of the BA anti-inflammatory activity has been considered only moderate (reviewed by [5]).

Sepsis is one of the most frequent complications in surgical patients and one of the leading causes of mortality in intensive care units. Severe sepsis is an important cause of mortality worldwide, and is estimated as directly responsible for 9% of all deaths in the United States [11]. It is defined as an infection-induced syndrome characterized by a generalized inflammatory state and can be caused by infection with Gram-negative or Gram-positive bacteria, fungi, or viruses. Sepsis can also occur in the absence of detectable bacterial invasion and, in these cases, microbial toxins (lipopolysaccharide; LPS) and endogenous cytokines have been implicated as initiators and mediators of the condition [12].

Macrophage activation by LPS results in the release of several inflammatory mediators, including proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, and IL-12,

* Corresponding author at: Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121 – Candeal, Salvador, BA, 40296–710. Tel.: +55 71 3176 2260; fax: +55 71 3176 2272.
E-mail address: milena@bahia.fiocruz.br (M.B.P. Soares).

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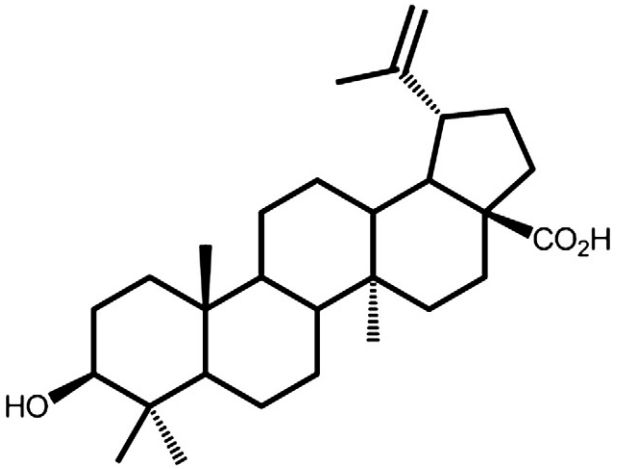

Fig. 1. Molecular structure of betulinic acid.

as well as nitric oxide. The exacerbated production of these mediators by activated macrophages are the main cause of the deleterious consequences of septicemia or endotoxemia, and may lead to hypotension, disseminated intravascular coagulation, neutrophil extravasation to tissues, tissue hypoxia, and death. Conversely, IL-4, IL-10 and IL-13 may function as anti-inflammatory cytokines, modulating macrophage and monocyte responses and inhibiting the production of TNF-α, IL-1, and IL-8 [11–13]. The discovery of new agents capable of down-modulating the production of the inflammatory mediators that play key roles in the installation of sepsis is therefore of great interest for the development of effective treatments. Since BA has been shown to inhibit the TNF-α-induced activation of NF-κB [14], in this work we investigated the effects of BA in a mouse model of endotoxic shock and on the production of cytokines by activated macrophages.

2. Materials and methods

2.1. General experimental procedures

Melting points were determined using a Geahaka model PF1500 version 1.0 apparatus and were not corrected. The NMR spectra were acquired on a Varian System 500 spectrometer, equipped with a XW4100 HP workstation. High-resolution mass spectra were recorded on a microTOF spectrometer (LC-ITTOF model 225–07 100–34, Bruker) with positive ionization modes of the ESI source. Silica gel 60 (Merck) was used for column chromatography, and Si gel 60 PF254 (Merck) was used for purification of compounds by preparative TLC. All solvents used were analytical grade (Merck).

2.2. Plant material

Tabebuia aurea (Manso) S. Moore was collected in March 2002 in the surroundings of São João do Cariri, State of Paraíba, Brazil, and identified by botanist Dr. Maria de Fátima Agra of the Universidade Federal da Paraíba. A Voucher specimen (Agra 2337) is deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), Universidade Federal da Paraíba.

2.3. Extraction and isolation of betulinic acid

Five kg of air-dried ground bark of *Tabebuia aurea* (Manso) S. Moore were exhaustively extracted with 95% ethanol. The solvent was evaporated to yield a dark syrup residue (167 g, 3.3%), which was partitioned with water and successively treated with hexane, chloroform, ethyl acetate and n-butanol, yielding 8.5 g (0.2%), 4.1 g (0.08%), 6.2 g (0.13%) and 74 g (1.5%), respectively. The chloroform residue was also subjected to column chromatography over silica gel, and eluted with a chloroform-hexane gradient. Seventy five 100-mL fractions were collected, after analysis by TLC silica gel-60 F254 developed with either I_2 reagent. Combined fractions 41–50 were rechromatographed on CC column silica gel (with a chloroform-methanol gradient) to afford pure betulinic acid (BA) (0.015 g, 0.0003%; Fig. 1). Identification of the betulinic acid was performed by analyzing 1H e 13C NMR spectral data and high-resolution mass spectra compared with those published in the literature [1,15].

2.4. Animals

Male 4- to 6-week old BALB/c, wild-type C57BL/6, and IL-$10^{-/-}$ C57BL/6 mice were used. All mice were raised and maintained at the animal facilities of the Gonçalo Moniz Research Center, Fundação Oswaldo Cruz, Salvador, Brazil, in rooms with controlled temperature (22 ± 2 °C) and humidity (55 ± 10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (6 am–6 pm) and provided with rodent diet and water *ad libitum*. All mice, when necessary, were subjected to euthanasia and treated according to the Oswaldo Cruz Foundation guidelines for laboratory animals. The work had prior approval by the institutional Ethics Committee in Laboratory Animal Use.

2.5. Endotoxic shock model

Groups of 11 BALB/c or C57BL/6 mice were used for the lethality assays and groups of 6 BALB/c mice were used for *in vivo* cytokine studies.

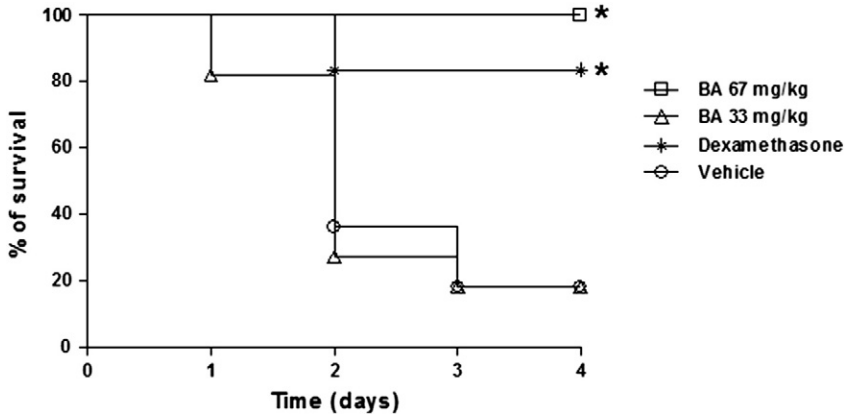

Fig. 2. Survival curve of mice treated with betulinic acid and submitted to endotoxic shock. Male BALB/c mice (n = 11) were treated with betulinic acid (33 and 67 mg/kg) or vehicle (5% DMSO in saline) and challenged with LPS 90 min later, intraperitoneally administered. Survival was monitored during 96 hours after LPS challenge. Results are from one experiment of two replicates performed. *$P < 0.05$ compared to vehicle group. Statistical analysis was carried out using Logrank (Mantel Cox).