

Antimalarial activity of betulinic acid and derivatives in vitro against *Plasmodium falciparum* and in vivo in *P. berghei*-infected mice

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Abstract Malaria is one of the most important tropical diseases and mainly affects populations living in developing countries. Reduced sensitivity of *Plasmodium* sp. to formerly recommended antimalarial drugs places an increasing burden on malaria control programs as well as on national health systems in endemic countries. The present study aims to evaluate the antimalarial activity of betulinic acid and its derivative compounds, betulonic acid, betulinic acid acetate, betulinic acid methyl ester, and betulinic acid methyl ester acetate. These substances showed antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* parasites in vitro, with IC₅₀ values of 9.89, 10.01, 5.99, 51.58, and 45.79 μM, respectively. Mice infected with *Plasmodium berghei* and treated with betulinic acid acetate had a dose-dependent

reduction of parasitemia. Our results indicate that betulinic acid and its derivative compounds are candidates for the development of new antimalarial drugs.

Introduction

Malaria is one of the most important tropical diseases due to its high morbidity and mortality rates, affecting over two billion people in endemic countries. In a public health problem in countries where transmission occurs regularly, this disease mainly afflicts populations living in developing countries, constituting an obstacle to the efforts toward economic development (WHO 2005).

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The causative parasite of the malignant form of malaria, *Plasmodium falciparum*, frequently presents resistance to chloroquine, the cheapest and most utilized antimalarial drug (WHO 2008). Another problem concerning drug resistance in *P. falciparum* is the occurrence of cross-resistance among drugs belonging to the same chemical family (WHO 2001). Drug resistance plays an important role in the occurrence and severity of epidemic diseases in the world (Majori 2004). Moreover, there is a great disparity between the public health importance of tropical diseases, such as malaria, and the amount of resources invested in the development of new drugs to combat these diseases (WHO 2001).

As a consequence of drug resistance, drugs like quinine, chloroquine, primaquine, and mefloquine are ineffective in treating malaria in many endemic regions of the world (Schlitzer 2007). Thus, the development of new antimalarial pharmacotherapy is necessary. Many different classes of natural products, such as terpenes (Meshnick 2002), flavonoids (Auffret 2007; Froelich et al. 2007), and alkaloids (Chea et al. 2007), have been studied regarding antimalarial activity. This study aimed at evaluating the antimalarial activity of betulinic acid and its derivatives against chloroquine-resistant *P. falciparum* parasites in vitro and *Plasmodium berghei* parasites in vivo.

Materials and methods

Purification of betulinic acid and synthesis of derivatives

Betulinic acid was isolated from *Zizyphus joazeiro* Mart. (Rhamnaceae) (Barbosa-Filho et al. 1985). The derivatives were obtained by standard and straightforward chemical procedures already related in literature. The betulinic acid acetate was obtained by the acetylation reaction of betulinic acid with an acetic anhydride/pyridine mixture (Kim et al. 2001). Betulonic acid was obtained in the chromic acid oxidation from betulinic acid (Urban et al. 2007). The treatment of betulinic acid and the betulinic acid acetate with excess ethereal diazomethane solution rendered the corresponding methyl ester and derivatives (Urban et al. 2005; Uzenkova et al. 2005). All compounds were purified

by column chromatography and showed $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra in accordance with literature previously cited.

The structures of betulinic acid and its derivatives used in this study are shown in Fig. 1.

Cytotoxicity assay

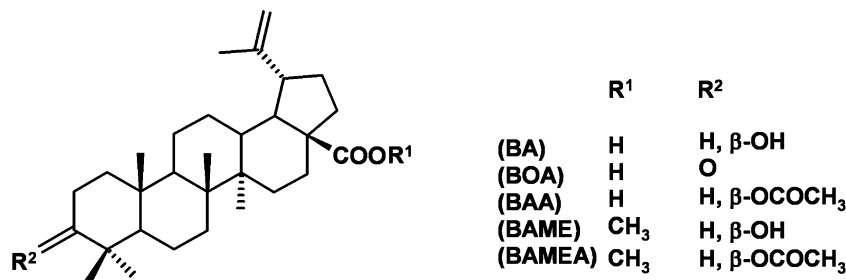
To determine the cytotoxicity of betulinic acid (BA) and the derivative compounds betulonic acid (BOA), betulinic acid acetate (BAA), betulinic acid methyl ester (BAME), and betulinic acid methyl ester acetate (BAMEA), BALB/c mice splenocytes were cultured in 96-well plate (6×10^5 cells/well) in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Cultilab, Campinas, São Paulo, Brazil) and 50 $\mu\text{g/ml}$ of gentamycin (Novafarma, Anapolis, Goiás, Brazil). The pure substances were tested in five concentrations, in triplicates. One microCi/well [$\text{methyl-}^3\text{H}$]-thymidine (Amersham, Little Chalfont, England) was added to the cultures which were incubated during 24 h at 37°C and 5% CO_2 . After this period, plates were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD, USA) to determine the ^3H -thymidine incorporation using a β -radiation counter (Multilabel Reader, Hidex, Turku, Finland). The viability of the cells was determined by ^3H -thymidine incorporation and the cytotoxicity was calculated in relation to the ^3H -thymidine incorporation of untreated cultures.

Anti-*P. falciparum* in vitro assay

Betulinic acid and the derivative compounds were tested for antimalarial activity in vitro using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive (Junior 1999). All the parasites were maintained in continuous culture of human erythrocytes (blood group A^+) using Rapid Prototyping and Manufacturing Institute (RPMI) medium supplemented with 10% human plasma (Trager and Jensen 2005).

Parasites grown at 1% to 2% parasitemia and 2.5% hematocrit were incubated with the pure substances tested at various concentrations, diluted with 4% dimethyl sulfoxide (DMSO) in culture medium (RPMI 1640) without

Fig. 1 Structures of betulinic acid and derivatives



hypoxanthine. Mefloquine was used in each experiment as a control for *P. falciparum* drug response. Cultures containing parasites were harvested using a cell harvester to evaluate the $^3\text{[H]}$ -hypoxanthine incorporation in a β -radiation counter. Inhibition of parasite growth was evaluated by comparison with $^3\text{[H]}$ -hypoxanthine uptake in drug-treated versus untreated wells. All the assays were performed in triplicates as described previously (Andrade-Neto et al. 2004; Zalis et al. 1998).

Assessment of antimalarial activity

The antimalarial activity of betulinic acid acetate was evaluated in mice infected with *P. berghei*, strain NK65 (Andrade et al. 2007). Parasites were maintained by weekly blood passage 10^6 infected red blood cells per mouse by intraperitoneal (i.p.) route. The animals were randomly sorted into groups of five and treatment was administered daily, during four consecutive days, beginning on the first day of infection. BAA was suspended in saline solution plus 10% DMSO immediately before use in doses of 10, 50, and 100 mg/kg. Each mouse received 200 μl , by oral or by intraperitoneal route. Experiments included a control group treated with the standard antimalarial drug chloroquine at 50 mg/kg and a mock-treated group (10% DMSO in saline). The antimalarial activity was evaluated by counting parasitemia in blood smears at days 4 and 7 after parasite inoculation, by optical microscopy, after fixation with methanol and staining with fast panoptic (Laborclin, Pinhais, Brazil). Inhibition of parasite growth in drug-treated groups was calculated in relation to the control (mice treated with saline solution plus 10% DMSO) group. The results were expressed as the percentage of parasitemia reduction.

IC₅₀ and LC₅₀ calculations and statistical analyses

The lethal concentration of 50% of BALB/c mice splenocytes (LC₅₀) and the inhibitory concentration of 50% (IC₅₀) of *P.*

falciparum were calculated based on a nonlinear regression (curve fit). The statistical analyses were made by one-way analysis of variance and Newman–Keuls multiple comparison tests using Graph Pad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when *p* values were <0.05.

Calculation of the selectivity index

The selectivity index (SI) was defined by calculating the value of LC₅₀ versus IC₅₀ (Bézivin et al. 2003). The SI was considered as significant for values higher than three.

Results

Cytotoxicity of betulinic acid and derivatives

In order to determine the cytotoxic potential of the compounds studied, BA, BOA, BAA, BAME, and BAMEA were tested in five different concentrations. As shown in Table 1, all substances had lower cytotoxicity compared to mefloquine, used as the standard antimalarial drug. BOA was the compound with lower cytotoxicity (higher LC₅₀ value) when compared to the other substances tested, while BAMEA was the most toxic.

In vitro antimalarial activity

To assess the antimalarial activity of the compounds, BA and its derivatives were first assayed in vitro against W2 strain of *P. falciparum*. BA and its derivative compounds BOA and BAA had antiplasmodial activity, with IC₅₀ values below 10 μM , whereas BAME and BAMEA presented IC₅₀ values above 45 μM (Table 1). Mefloquine had an IC₅₀=0.04 μM against this strain of *P. falciparum*, a result similar to that described before for an inoculum of 1% to 2% parasitemia and 2.5% hematocrit (Andrade-Neto

Table 1 Anti-*P. falciparum* activity and in vitro cytotoxicity of betulinic acid and derivative compounds

Substance	Yield (%) ^a	IC ₅₀ (μM)	LC ₅₀ (μM)	SI
BA	90.65	9.89±0.52	62.36±32.67*	6.3
BOA	93.28	10.01±1.29	96.10±8.52*	9.6
BAA	95.08	5.99±0.06	21.98±2.71	3.7
BAME	93.74	51.58±10.85*	83.21±6.46*	1.6
BAMEA	60.33	45.79±36.26*	15.42±19.30	0.3
Mefloquine	91.69	0.04±0.01	9.53±0.46	238

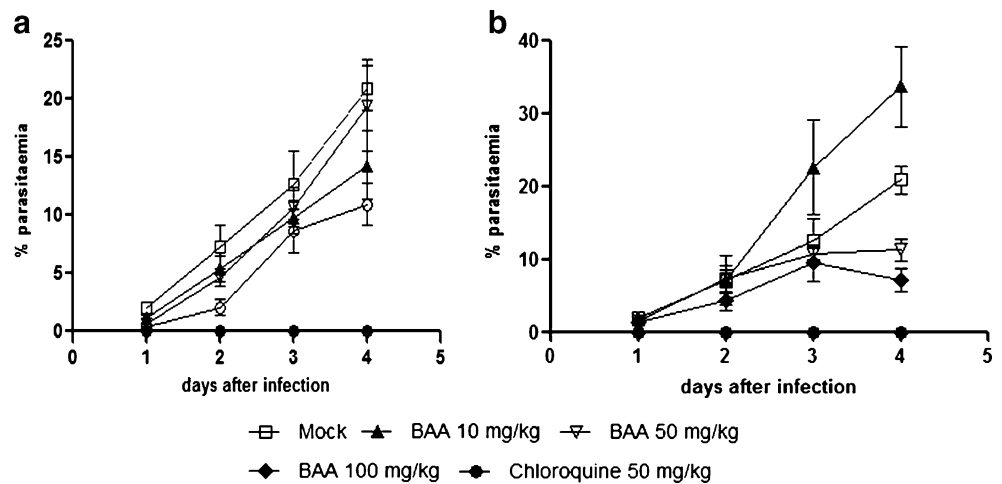
Values are mean \pm standard deviation of three independent experiments

SI selectivity index

**P*<0.01 compared to control

^aThe maximal concentration of BAA and derivatives tested was 100 $\mu\text{g/ml}$ and of mefloquine was 0.25 $\mu\text{g/ml}$

Fig. 2 Parasitemia of *P. berghei*-infected mice treated with 10% DMSO in saline (*mock*), BAA at 10, 50, and 100 mg/kg, or chloroquine at 50 mg/kg, by oral (A) or intraperitoneal (B) routes, daily, for four consecutive days. Blood parasitemia was determined on several days after infection. Values represent the means \pm standard error of five mice per group in one experiment of two performed



et al. 2004). The analysis of the selectivity index (SI) showed that BAA was the compound with higher SI, whereas BAMEA had the lowest SI (Table 1). The SI index of mefloquine, however, was significantly higher than the one found for BAA.

In vivo antimalarial activity of BAA

Since BAA was the compound with best SI, we decided to test its antimalarial activity in vivo in *P. berghei*-infected mice. Treatment with BAA by oral route did not alter the levels of parasitemia in mice infected with *P. berghei* compared to mice treated with saline solution plus 10% DMSO (Fig. 2a). In contrast, the administration of BAA by i.p. route caused a dose-dependent reduction of parasitemia of at least 70% on the seventh day following infection (Fig. 2b). Mice treated with chloroquine, a standard antimalarial drug, had undetectable parasitemia (Fig. 2a, b).

Treatment with BAA i.p. increased the survival of *P. berghei*-infected mice at a dose of 100 mg/kg at least until day 21 postinfection. Treatment with BAA in doses of 10 and 50 mg/kg, however, did not affect the mortality rate of *P. berghei*-infected mice, which started to die by day 10,

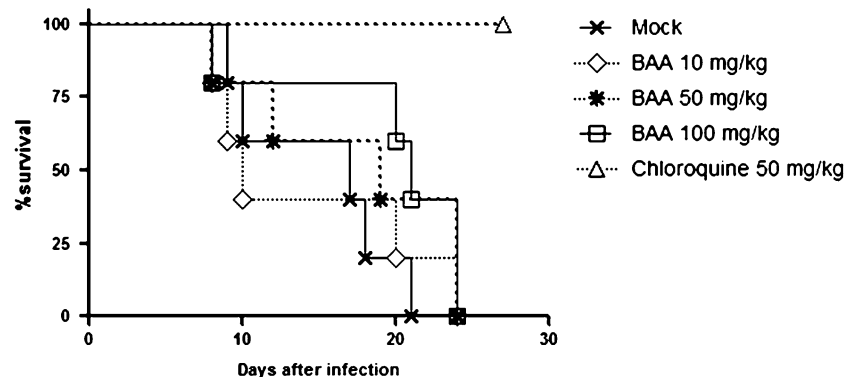
similar to control mice. All the mice in the chloroquine-treated group survived during the experiment (Fig. 3).

Discussion

In this study, we demonstrated that betulinic acid and the derivative compounds BOA and BAA have a significant antimalarial activity in vitro against *P. falciparum* chloroquine resistant (W2 strain). In addition, we showed that BAA, the most active anti-*Plasmodium* substance tested, has antimalarial activity in vivo.

The IC_{50} of mefloquine against *P. falciparum* is about 150 times lower than that of BAA, the most active betulinic acid derivative in vitro. On the other hand, this drug was more toxic than BAA, with LC_{50} two times lower than BAA. It is important to say that selectivity index is an important parameter to evaluate the potential of the drugs tested, and a SI value >3 indicates high selectivity (Prayong et al. 2008). BAA present a SI value of 3.7 which mefloquine SI was 238. Although mefloquine SI was 64 times higher than BAA SI, this drug may serve as basis for the development of new malaria pharmacotherapies able to control mefloquine-resistant parasites.

Fig. 3 Cumulative mortality rates of *P. berghei*-infected mice treated with 10% DMSO in saline (*mock*), BAA at 10, 50, and 100 mg/kg, or chloroquine at 50 mg/kg, by intraperitoneal route, daily, for four consecutive days. Mortality was evaluated several days after infection. Results are from one experiment done with five mice per group of two performed



The identification of new molecular targets in *Plasmodium* sp. may aid the design of compounds with selective activity during the process of drug development. The investigation of the mechanisms of action of BAA may help the identification of new molecular targets. With this regard, it has been suggested before that BA inhibits topoisomerases (Wada et al. 2005). Thus, BAA may also act by inhibiting topoisomerases from *Plasmodium* sp., a possibility that we are currently investigating.

BAA was also active in vivo when administrated to *P. berguei*-infected mice by intraperitoneal route, although it did not cause a reduction of parasitemia when used by oral route. This result may be attributed to a slow absorption of the compound, to the high metabolism rate of the mouse model, or to retention of the compound as lipid-bound complexes due to its lipophilic nature. These factors could lead to low bioavailability of the drug at the target site of action, the intracellular parasite.

A previous report has shown that treatment of *P. berghei*-infected mice with betulinic acid by intraperitoneal route did not cause a reduction of parasitemia (Steele et al. 1999). Our finding that the derivative betulinic acid acetate has in vivo activity suggests that the structural changes in the molecule significantly alter the anti-*Plasmodium* activity. Further studies to explore this compound as a prototype for an antimalarial drug should be carried out, especially aiming at the *P. falciparum* chloroquine-resistant parasites, which are rather frequent worldwide.

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