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Short communication

In vivo evaluation of isolated triterpenes and semi-synthetic derivatives as antimalarial agents

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ABSTRACT

The triterpenes balsaminoside B (**1**) and karavilagenin C (**2**) were isolated from the African medicinal plant *Momordica balsamina* L. Karavoates B (**3**) and D (**4**) were synthesized by diacylation of **2** with acetic and propionic anhydrides, respectively. In previous work, derivatives **3** and **4** exhibited submicromolar median inhibitory concentrations (IC_{50}) *in vitro* against *Plasmodium falciparum* Welch (human malaria parasite) strains 20 to 25 times lower than those of natural product **2**. The main objective of the present study was to explore structure-*in vivo* antimalarial activity relationships (SAR) for compounds **1**–**4** in *Plasmodium berghei* Vincke and Lips NK65-infected mice in the 4 day suppressive test. Semi-synthetic derivatives **3** and **4** exhibited greater *in vivo* antimalarial activity than isolates **1** and **2**. Orally and subcutaneously administered karavoate B exhibited the greatest *in vivo* antimalarial activity (55.2–58.1% maximal suppression of parasitemia at doses of 50 mg kg^{-1} day^{-1}). Diacylation of natural isolate **2** with short chain carboxylic acid moieties yielded derivatives with enhanced maximal *in vivo* parasitemia suppression for both routes of administration. Maximal *in vivo* parasite suppression by diacetyl derivative **3** was roughly double that of natural precursor **2**.

1. Introduction

Malaria is still a major global threat particularly in many of the poorest tropical and subtropical countries in the world. Although the World Malaria Report 2014 showed a decrease in mortality rates by 47% globally and by 54% in the World Health Organization (WHO) African Region (between 2000 and 2013) most malaria-endemic countries are still far from achieving universal coverage with life-saving malaria interventions. An estimated 278 million people in Africa still live in households without a single insecticide-treated bed net and about 15 million pregnant women remain without access to preventive treatment for malaria. Malaria is still responsible for over 430,000 child deaths in Africa every year [1]. Of the five species of *Plasmodium* parasites that infect humans, *Plasmodium falciparum* Welch is the most lethal and contributes significantly to malaria mortality and morbidity [2]. In the absence of an effective vaccine, treatment and control of malaria is more complex due to the emergence of drug-resistant parasites, especially *P. falciparum* [3]. *P. falciparum* resistant to both components of multiple artemisinin combination therapies (ACTs) has appeared along the western Cambodia–Thailand border [4]. This situation requires the discovery of new therapeutic agents exhibiting novel mechanisms of action and chemical structures unrelated to existing antimalarial agents [5,6].

Natural product scaffolds are the basis for most of the current anti-malarial drugs. Natural antimalarial compounds from traditionally-used plants such as quinine and artemisinin gave rise to the development of the synthetic quinolines and artemisinin

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derivatives that are currently the mainstay of antimalarial therapy [7,8]. Traditionally-used plants continue to be a rich source of secondary metabolites that exhibit activity against malaria parasites [9,10] and mosquito vectors [11,12]. Among plant derived compounds, terpenoids of natural, semi-synthetic and synthetic origins are considered important leads for the development of new antimalarials as they offer new mechanisms of action compared to those of traditional drugs [13–16].

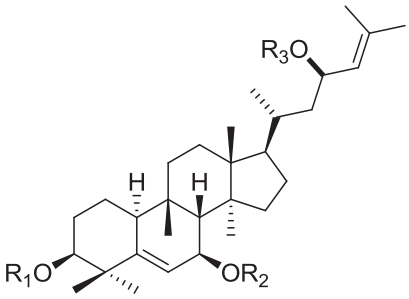
Momordica balsamina L., also known as balsam apple or African pumpkin, is a traditionally used antimalarial plant whose extracts exhibit *in vitro* and *in vivo* antimalarial activity [17,18]. Bioassay-guided fractionation of extracts of the aerial parts of *M. balsamina* led to the isolation of several cucurbitane triterpenoids [19–23]. Using criteria for *in vitro* antimalarial activity adopted by Batista et al. [24], most of these compounds and semi-synthetic derivatives exhibited excellent/good inhibitory effects ($IC_{50} \leq 1$ μM/$IC_{50} = 1 - 20$ μM) against blood schizonts of chloroquine-sensitive and resistant strains of *P. falciparum* [21–23]. Some of these terpenoids exhibited promising *in vitro* activity against liver stages of *Plasmodium berghei* Vincke and Lips infections, emphasizing their dual-stage antimalarial activity [23,25].

In light of previous *in vitro* results, herein the *in vivo* antimalarial potential of some representative active triterpenoids isolated from *M. balsamina* (**1** and **2**) and semi-synthetic diacyl derivatives (**3** and **4**) was investigated in *P. berghei*-infected mice (Fig. 1).

2. Results and discussion

2.1. Chemistry

The air-dried powdered aerial parts of *M. balsamina* were exhaustively extracted with methanol as previously described [20]. Fractionation of the methanol extract led to the isolation of triterpenoids with the cucurbitane skeleton, namely balsaminoside B (**1**) and karavilagenin C (**2**) [20,23]. Further phytochemical study of other fractions of the methanol extract of *M. balsamina* was performed and resulted in the isolation of compounds **1** and **2** in larger quantity for *in vivo* assays. Similarly, acylation of compound **2** with acetic and propionic anhydrides yielded karavoates B (**3**) and D (**4**), respectively, in large enough quantity for *in vivo* animal studies. The structures of compounds were identified based on ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectral (MS) data.



1: R_1=H, R_2=glucosyl, R_3=H
2: R_1=H, R_2=CH_3, R_3=H
3: R_1=$COCH_3$, R_2=CH_3, R_3=$COCH_3$
4: R_1=$COCH_2CH_3$, R_2= CH_3, R_3= $COCH_2CH_3$

Fig. 1. Structures of compounds **1**–**4**.

2.2. Biology

The *in vivo* antimalarial potential of triterpenes **1**–**4** was investigated in *P. berghei*-infected mice. Each dose of 50 and 25 mg kg^{-1} day^{-1} of compound dissolved in 10% dimethyl sulfoxide (DMSO) in water was administrated for 4 consecutive days by oral and subcutaneous routes. Untreated (blank) control mice received only vehicle (10% DMSO in water). Chloroquine was used as positive control.

The *in vivo* effects of compounds (**1**–**4**), expressed as % parasitemia suppression compared to untreated controls, are summarized in Table 1 along with information on the overall survival measured in days post-infection. The most significant *in vivo* suppressive effects were obtained through oral treatment with compounds **1**–**4**.

Interestingly, the *in vivo* antimalarial activity observed herein and the *in vitro* antiplasmodial activity reported previously are in general agreement. Diacyl derivatives **3** and **4** exhibit superior *in vivo* and *in vitro* antimalarial effects when compared to the parent compound karavilagenin C (**2**). Thus, orally administered semi-synthetic derivatives **3** and **4** exhibited higher *in vivo* suppression (58.1 and 45.5%, respectively) on day 5 than natural compounds **1** and **2**, although these latter compounds exerted significant *in vivo* suppressive effects on *P. berghei* (36.2 and 33.1%). Similarly, derivatives **3** and **4** were approximately 20–25-fold more active *in vitro* than their natural precursor **2** against the erythrocytic stages of the chloroquine-sensitive 3D7 and the chloroquine-resistant clone Dd2 of *P. falciparum* [22]. Moreover, karavoates B (**3**, $IC_{50} = 0.5$ μM, for 3D7 and Dd2) and D (**4**, $IC_{50} = 1.5$ and 0.4 μM, for 3D7 and Dd2, respectively) exhibited the lowest IC_{50} values (largest parasite inhibition) among all natural compounds isolated from *M. balsamina* and corresponding derivatives [21–23].

Karavoates **3** and **4** are also active *in vitro* against the liver stages of *P. berghei* [25]. While isolated compounds from *M. balsamina* were active, semi-synthetic derivative karavoate B (**3**) exhibited the strongest effects including a concentration-dependent decrease in the *in vitro P. berghei* infection rate [23,25]. Even at the lowest concentration tested (1 μM) this diester exhibited significant liver stage antiplasmodial activity [25].

The average survival times of untreated (control) animals and animals treated with compounds **1**–**4** were essentially the same (Table 1). This is most likely due to the incomplete suppression of parasitemia, rapid metabolism and or excretion of terpenoid compounds **1**–**4** by the mice. Also, none of the mice exhibited any sign of intoxication as a result of treatment with compounds **1**–**4**. In general, the lack of *in vivo* toxicity observed is consistent with the results from previous *in vitro* evaluation of these compounds against MCF-7 and Huh-7 cells [22,23,25]. Among the compounds isolated from *M. balsamina* and their semi-synthetic derivatives, only balsaminoside B (**1**) exhibited toxicity against Huh-7 cells (at the highest concentration tested, 15 μM) [22,23,25]. Indeed, derivatives **3** and **4** exhibited low cytotoxicity ($IC_{50} > 133$ μM against MCF-7 cells) and good selectivity indices (SI, where $SI = IC_{50\ MCF\text{-}7\ cells}/IC_{50\ P.\ falciparum}$; **3**, SI > 151.2 and SI > 126.0, for 3D7 and Dd2, respectively, and **4**, SI > 89.0 and SI > 349.9, for 3D7 and Dd2, respectively) [22]. Despite this good selectivity, toxicological evaluation of these compounds should be carried out in the future.

Collectively, the *in vitro* and *in vivo* results for compounds **1**–**4** reveal qualitative structure-antimalarial activity relationships (SAR). Compounds **3** and **4** are alkanoyl esters of **2** that have acetyl or propanoyl moieties at C-3 and C-23, respectively. Both **3** and **4** exhibited increased *in vivo* and *in vitro* antiplasmodial activity compared to **2**. Furthermore, from the *in vivo* results herein (Table 1) and the *in vitro* antiplasmodial results for the esters of

Table 1
In vivo suppression of *Plasmodium berghei* NK65 strain in infected mice and survival after oral and subcutaneous treatment with triterpene compounds **1**–**4**.

Dose (mg kg^{-1} day^{-1})	% Parasite inhibition[a]				Average survival time ± SD (days)	
	Oral		Subcutaneous		Oral	Subcutaneous
	Day 5	Day 7	Day 5	Day 7		
Balsaminoside B (**1**)						
50	36.2	8.6	37.1	5.5	23 ± 4	21 ± 2
25	23.9	0	12.5	0	21 ± 2	20 ± 3
Karavilagenin C (**2**)						
50	33.1	3.4	18.6	1.0	22 ± 3	22 ± 4
25	22.7	0	21.3	0	21 ± 4	23 ± 3
Karavoate B (**3**)						
50	58.1	25.6	55.2	28.4	23 ± 3	24 ± 4
25	41.8	13.2	39.2	10.1	21 ± 2	20 ± 4
Karavoate D (**4**)						
50	45.5	11.5	42.5	10.5	24 ± 3	24 ± 2
25	12.3	0	11.9	0	23 ± 4	22 ± 3
Chloroquine						
5	100	98	99	99	>40	>40
Blank/control	–	–	–	–	22 ± 3	23 ± 4

[a] Parasitemia reduction compared to untreated control mice. Mean of two independent experiments. SD: standard deviation.

karavilagenin C [22,25], it appears that the number of carbon atoms in the acyl groups affects the antimalarial activity.

Semi-synthetic terpene derivatives **3** and **4** exhibit *in vivo* antimalarial activity that is largely similar to that of other antimalarial natural products and their derivatives. Compounds **1**, **3** and **4** exhibited *in vivo* suppression of *P. berghei* comparable to that of the well-studied, antimalarial triterpenoid/limonoid natural product gedunin, 7-*O*-modified derivatives of this compound and a natural 6α-acetoxy derivative isolated from *Carapa guianensis* Aubl [27,28]. On a molar basis, compounds **3** and **4** (MW 592 and 620, respectively) exhibited *in vivo* suppression of *P. berghei* on day 5 comparable to that of the broadly studied, lower molecular weight alkaloid cryptolepine (MW 233, isolated from *Cryptolepis sanguinolenta* (Lindl.) Schltr.) and a synthetic analog of cryptolepine (MW 331) [26].

Plant terpenes [15,29,30], semi-synthetic derivatives [16,31] and analogs containing open-chain terpenoid sub-structures such as those found in triterpenes **1**–**4** exhibit antiplasmodial activity and several are known to inhibit the biosynthesis of isoprenoid compounds in *P. falciparum*. Farnesol, nerolidol, and linalool are terpenes that exhibit significant *in vitro* activity against *P. falciparum* and also have been shown to inhibit the biosynthesis of several intermediates and end products (such as dolichols and ubiquinones) of the isoprenoid pathway in these parasites [13,16]. Inhibition of isoprenoid biosynthesis may be the mechanism of action of triterpenoids **1**–**4** studied herein; however, metabolic studies are necessary to confirm this.

3. Experimental section

3.1. Chemistry

3.1.1. General procedures

NMR spectra were recorded on a Bruker ARX-400 NMR spectrometer (^{1}H: 400 MHz; ^{13}C: 100.61 MHz), using acetone-d_6 as solvent. Electrospray ionization mass spectra (ESI-MS) were taken on a Micromass Quattro Micro. Atmospheric pressure ionization mass spectra (API-MS) were recorded on a Micromass Autospec spectrometer. Thin-layer chromatography (TLC) was performed on pre-coated silica gel F_{254} plates (Merck 5554 and 5744), with visualization under UV light and by spraying with sulphuric acid-methanol (1:1), followed by heating. Column chromatography (CC) was carried out on silica 320 gel (Merck 9385). The purity of the compounds was >95% based on high performance liquid chromatography (HPLC) and NMR analyses.

3.1.2. Isolation of karavilagenin C (2) and balsaminoside B (1)

Dried aerial parts of *M. balsamina* (1.2 kg) were powdered and exhaustively extracted with methanol at room temperature, as previously described [20]. Briefly, the residue of the methanol extract (45 g), after removing the waxy material, was chromatographed over silica gel to obtain six crude fractions (Fr 1–6). Karavilagenin C (**2**) and balsaminoside B (**1**) were obtained from the crude fractions Fr 2 (*n*-hexane/ethyl acetate, 11:9 to 9:11), and Fr 6 (ethyl acetate/methanol, 93:7 to 9:1), respectively [20,23].

In order to isolate a larger amount of karavilagenin C (**2**), the residue (2.1 g) of a subfraction obtained from fraction Fr 2, was chromatographed on a column of silica gel (100 g) using mixtures of *n*-hexane/ethyl acetate (1:0 to 0:1) and ethyl acetate/methanol (1:0 to 0:1) as eluents. The fraction (1.7 g) that eluted with mixtures of *n*-hexane/ethyl acetate (3:2 to 11:9) was crystallized from *n*-hexane/ethyl acetate to afford compound **2** (300 mg). The mother liquors from this crystallization procedure were repeatedly re-chromatographed with mixtures of dichloromethane/acetone (1:0 to 4:1), yielding more karavilagenin C (**2**, 707 mg), after crystallization from *n*-hexane/ethyl acetate. Overall the yield of **2** was 1.01 g (0.08%, based on weight of dry plant material).

Similarly, to increase the amount of balsaminoside B (**1**), the residue (10.5 g) of a subfraction obtained from fraction Fr 6 was chromatographed twice (silica gel, 250 g) using gradients of *n*-hexane/ethyl acetate (1:1 to 0:1) and ethyl acetate/methanol (1:0 to 1:1) followed by dichloromethane/methanol (23:2 to 4:1). Further purification by crystallization from *n*-hexane/ethyl acetate afforded balsaminoside B (200 mg). Overall, the yield of **1** was 200 mg (0.02%, based on the weight of dry plant material).

3.1.3. Acylation of karavilagenin C (2)

*3.1.3.1. Karavoate B **(3)**.* Compound **2** (221 mg) was suspended in dry pyridine (2 mL) and acetic anhydride (2 mL). After stirring at room temperature for 2 h, excess reagents were removed under a stream of nitrogen and the crude product obtained was purified by column chromatography using a gradient of *n*-hexane and ethyl acetate (1:0 to 4:1) as eluents to afford compound **3** (246 mg, 95%).

*3.1.3.2. Karavoate D **(4)**.* Compound **2** (212 mg) was suspended in dry pyridine (2 mL) and propionic anhydride (2 mL) and stirred at

room temperature overnight. After evaporation as in the above procedure, the crude product was purified by column chromatography using a gradient of *n*-hexane and ethyl acetate (1:0 to 4:1) as eluents to afford **4** (236 mg, 90%).

3.2. Biology

Adult female BALB/c mice (22 ± 3 g weight) were used and received water and food *ad libitum*. *In vivo* tests were performed using Guidelines for Ethical Conduct in The Care and Use of Animals of the National Institute for Amazon Research (INPA). This work was authorized by INPA's Commission of Ethics for the Use of Animals (CEUA 062/2012).

Groups of 5 mice were inoculated intraperitoneally with 0.2 mL of infected blood suspension containing 1×10^5 *P. berghei* NK65-parasitized blood cells. After 24 h, groups of mice were treated orally (by gavage tube) or subcutaneously for 4 consecutive days with single daily doses of 50 or 25 mg kg^{-1} of each compound dissolved in 10% DMSO (in water). Untreated (blank) control mice received only vehicle (10% DMSO in water). Chloroquine was used as positive control at doses of 10 mg kg^{-1} day^{-1} over 4 consecutive days. Parasitemia was evaluated on the 5th and 7th days after inoculation based on blood smears prepared from all mice. Blood smears were stained with Giemsa and microscopically examined (1000 × magnification). Parasitemia was determined as a percentage. The percentage of parasite growth suppression (PGS) was calculated based on the difference between the average parasitemia of negative-control groups (A, 100% parasite growth) and the parasitemia of each test group (B) according to the expression $PGS = 100 \times [(A - B)/A]$. Mice survival was followed until the 40th day after infection [15,32]. Each compound was tested in two independent experiments. *In vivo* suppression of *P. berghei* growth by triterpenes **1**–**4** and average survival period for each group are presented in Table 1.

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