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Dual-stage triterpenoids from an African medicinal plant targeting the malaria parasite

Cátia Ramalhete [a], Filipa P. da Cruz [b], Silva Mulhovo [c], Inês J. Sousa [d], Miguel X. Fernandes [d,e], Miguel Prudêncio [b], Maria-José U. Ferreira [a,*]

[a] iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal
[b] IMM, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisboa, Portugal
[c] CEMEC, Universidade Pedagógica, Faculdade de Ciências Naturais e Matemática, 21402161 Maputo, Mozambique
[d] CQM, Universidade da Madeira, 9020-105 Funchal, Portugal
[e] BioLab, Instituto Universitario Antonio Gonzalez, CIBICAN, Universidad de La Laguna, 38206 La Laguna, Spain

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ABSTRACT

Sixteen triterpenoids (**1**–**16**), previously isolated from the aerial parts of the African medicinal plant *Momordica balsamina* or obtained by derivatization, were evaluated for their activity against liver stages of *Plasmodium berghei*, measuring the luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, *Pb*GFP-Luc$_{con}$. Toxicity of compounds (**1**–**16**) was assessed on the same cell line through the fluorescence measurement of cell confluency. The highest activity was displayed by a derivative bearing two acetyl residues, karavoate B (**7**), which led to a dose-dependent decrease in the *P. berghei* infection rate, exhibiting a very significant activity at the lowest concentration employed (1 μM) and no toxicity towards the Huh-7 cells. It is noteworthy that, in previous studies, this compound was found to be a strong inhibitor of blood-stages of *Plasmodium falciparum*, thus displaying a dual-stage antimalarial activity.

1. Introduction

Despite recent efforts to eradicate it, malaria remains a major global health problem, particularly in many of the poorest countries in the world.[1] According to the WHO, half of the world's population is at risk of contracting malaria since the disease is endemic in 104 countries and territories.[2] In 2011, approximately 80% of clinical cases of malaria and 90% of deaths attributable to this disease were estimated to have occurred in sub-Saharan Africa, children under five years of age and pregnant women being the most affected groups.[2]

Malaria infection is triggered by parasites of the *Plasmodium* genus, transmitted by female *Anopheles* mosquitoes.[3,4] During the bite, sporozoites are injected with the mosquito's saliva into the bloodstream and then travel to the liver, where they infect hepatocytes and develop into exoerythrocytic forms (EEFs), formed by thousands of merozoites. This clinically silent phase of the parasite life cycle takes approximately 7–14 days, for human *Plasmodium* and 2–3 days for rodent *Plasmodium* parasites. Merozoites are then released from the hepatocytes into the bloodstream, where they will invade erythrocytes, initiating the asexual erythrocytic cycle, which is responsible for disease symptoms.[3,5]

During the past three decades, *Plasmodium falciparum*, the most lethal of the five *Plasmodium* species that infect humans, has developed resistance to most available antimalarial drugs, including recent evidence of reduced parasite clearance with artemisinin combinations, the most relevant modern treatment.[6]

Drug discovery and development has traditionally focused on the blood stage parasites, mainly due to the lack of simple in vitro and in vivo tests for studying drugs acting on the liver stages of infection.[7,8] In spite of all the limitations, several compounds have been found to be active as liver stage inhibitors.[9] However, at present, the only drug clinically approved by FDA to eliminate *Plasmodium* liver stages, including hypnozoites, the dormant forms of the *Plasmodium vivax* parasite that can cause relapsing malaria, is the 8-aminoquinoline, primaquine.[10,11] This antimalarial agent is also able to kill gametocytes, the sexual forms responsible for transmission of parasites from human blood to the mosquito vector.[12] However, primaquine causes haemolytic anaemia in people with glucose-6-phosphate dehydrogenase deficiency, a common condition in Africa. It also presents low oral

* Corresponding author. Tel.: +351 21 7946475; fax: +351 21 7946470.
E-mail address: mjuferreira@ff.ul.pt (M.J.U. Ferreira).

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bioavailability, making it a poor option for malaria therapy.[13] Atovaquone, a naphthoquinone, is also a potent inhibitor of *Plasmodium* liver stages, but it is not active against *P. vivax* hypnozoites, which contribute substantially to malaria morbidity.[11] Thus, the development of new drugs targeting *Plasmodium* liver stages represents an important and under-exploited site of intervention.

Natural products have been the most important source of antimalarial leads, including two of the major drugs, quinine and artemisinin, used in malaria treatment.[14]

Momordica balsamina L. (Cucurbitaceae), also known as the balsam apple, or African pumpkin, is an extensively cultivated vegetable consumed in many tropical and subtropical regions of the world.[15] This species has also been widely used in traditional medicine, especially in African countries, to treat diseases like diabetes and malaria symptoms.[16–18]

Bioassay-guided fractionation of the methanol extract of the aerial parts of *M. balsamina* led to the isolation of several triterpenoids. Most of these compounds and derivatives exhibited in vitro antimalarial activity against blood schizonts of chloroquine-sensitive and -resistant strains of *P. falciparum*.[19–21] A few compounds were also evaluated against liver stages of *Plasmodium berghei* infections, showing promising activity.[21]

Other compounds with the triterpenic scaffold, namely tetracyclic triterpenes with the lanostane skeleton, and steroids were reported to exhibit in vitro antiplasmodial activity against blood-stages of malaria parasites.[22,23]

Following on previous efforts to identify effective dual-stage antimalarial hits/leads, we report herein the evaluation of the activity of sixteen triterpenoids (**1**–**16**), previously isolated from *M. balsamina*, or obtained by derivatization,[20,24,25] against *P. berghei* liver stages. The preliminary toxicity of compounds **1**–**16** toward on Huh-7 cells was also carried out.

2. Results and discussion

A limited number of compounds have been identified as inhibitors of liver-stage *Plasmodium* infection. Aiming to discover antimalarial hits/leads, targeting both the blood and the liver stages of *Plasmodium* parasite, sixteen triterpenoids (**1**–**16**) (Fig. 1), most of which were active against *P. falciparum* erythrocytic stages,[19,20] were evaluated for their in vitro activity against the liver stages of the rodent malaria parasite, *P. berghei*, using a bioluminescence quantification method.[26] This method employs a transgenic *P. berghei* parasite, PbGFP-Luc$_{con}$, expressing the bioluminescent reporter protein luciferase to quantify parasite development in Huh-7 cells, a human hepatoma cell line (Fig. 2—bars). The toxicity of compounds (**1**–**16**) was also assessed on the same cell line by fluorescence measurement of cell confluency (Fig. 2—dots). Compounds **1**–**16** were tested at 1, 5 and 15 μM. The reference liver-stage antimalarial primaquine (15 μM) was used as a positive control.

At the highest concentration tested (15 μM) all compounds (Fig. 2), except compounds **12**, **14**, and **16**, were found to be active against *P. berghei* liver stages (≅80–99% of inhibition at 15 μM). Karavoate B (**7**), a diacetylated derivative of karavilagenin C (**5**) showed the strongest effects. It is important to note that karavoate B also displayed a significant activity at the lowest concentration assayed (1 μM), decreasing *P. berghei* hepatic load by ≅60%. At this concentration (1 μM), karavoates D (**9**), E (**10**), and I (**13**) were also still active (≅35% of hepatic *P. berghei* inhibition). Most of the compounds did not display toxicity against Huh-7 cells at the highest concentration (15 μM) (Fig. 2), except balsaminoside A (**3**), bearing a sugar unit, and the monoacylated derivatives **6** and **10** (Fig. 2).

When analysing the results obtained for compounds **1**–**16**, together with those previously reported for compounds **17**–**21** (Fig. 1),[21] which share the same triterpenic scaffold, the pattern of substitution at both the triterpenic nucleus and the side chain appear to play an important role in liver stage inhibition. As illustrated by the results obtained for the parent compound karavilagenin C (**5**, 99%, 73% and 27% of inhibition, at 15, 5 and 1 μM, respectively) and its ethanoyl (**7**) and propanoyl (**9**) diesters, the acylation at C-3 and C-23 increased the activity (compound **7**, 99%, 96% and 60% and **9**, 93%, 82% and 34% of inhibition at 15, 5 and 1 μM, respectively). At 5 and 1 μM the corresponding monoacylated derivatives (C-23), karavoates A (**6**) and C (**8**) were less active (compounds **6**, 93% and 28% and **8**, 60% and 13% of inhibition at 5 and 1 μM, respectively). These results are corroborated by those previously obtained for balsaminol F (**19**) and its corresponding triacetyl derivative (**20**).[21] Conversely, compound **11** (≅82%, 64% and 11% of inhibition, at 15, 5 and 1 μM, respectively), which has two butanoyl residues, was less active than both the corresponding monoester at C-23 (**10** ≅99%, 89% and 39% of inhibition, at 15, 5 and 1 μM, respectively) and the parent compound karavilagenin C (**5**, 99%, 73% and 27% of inhibition, at 15, 5 and 1 μM, respectively), suggesting that activity is affected by the number of carbons of the alkanoyl ester. Concerning the aroyl derivatives (**12**–**16**), only those monobenzoylated were active (compounds **13**, 97%, 88% and 34% and **15**, 99%, 87%, 14% of inhibition, at 15, 5 and 1 μM, respectively). In addition, at 5 μM, karavilagenin C (**5**), with a methoxyl group at C-7, was approximately two-fold more active than compound **19**, bearing a free hydroxyl function at this position (73% and 32% of inhibition, respectively, at 5 μM).[21] Furthermore, the presence of an additional free hydroxyl group at C-12, or at C-29 seems to decrease the activity (Fig. 2). These features are highlighted by the results observed for compound **2** (47% of inhibition, at 5 μM) when compared with that of **5** (73% of inhibition, at 5 μM).

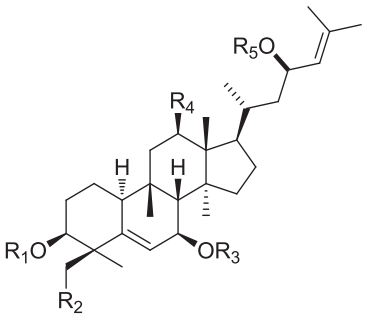
It is also noteworthy that the most active compounds were also found, in previous studies, to be strong inhibitors of *P. falciparum* blood-stages, thus having a dual stage antimalarial activity. Interestingly, from the analysis of the results obtained for compounds **1**–**21** against erythrocytic stages of the chloroquine-sensitive strain 3D7 and the chloroquine-resistant clone Dd2 of *P. falciparum*, similar conclusions concerning the effect of the substitution pattern could be drawn.[19–21] In fact, as observed for the hepatic stage, an increase in the activity was found for most alkanoyl ester derivatives of karavilagenin C (**5**) and balsaminol F (**19**).[20,21] Triacetylbalsaminol F (**20**), and karavoates B (**7**), D (**9**), and E (**10**) displayed IC$_{50}$ values similar to those obtained with chloroquine, particularly against the Dd2 resistant strain (IC$_{50}$ ⩽ 0.6 μM).[20,21] However, a decrease of activity was also observed when both positions, C-3 and C-23, of the parent compounds were acylated with aroyl moieties.[20,21]

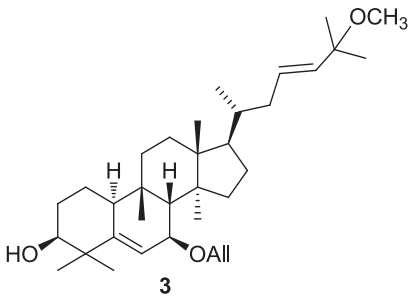
Our results demonstrate that the medicinal plant *Momordica balsamina* possesses compounds with antimalarial activity and support the traditional use of this plant for treatment of malaria. The promising dual-stage antimalarial activity of some compounds suggests their high potential as antimalarial lead scaffolds, justifying further study.

3. Experimental section

3.1. Compounds tested

Sixteen triterpenoids, whose structures are presented in Figure 1, namely, balsaminol A (**1**), balsaminol B (**2**), balsaminoside A (**3**), cucurbalsaminol C (**4**), karavilagenin C (**5**), karavoate A (**6**), karavoate B (**7**), karavoate C (**8**), karavoate D (**9**), karavoate E (**10**), karavoate F (**11**), karavoate H (**12**) karavoate I (**13**), karavoate J (**14**), karavoate M (**15**), and karavoate N (**16**) were evaluated for *P. berghei* liver-stage inhibition activity. Compounds **1**–**5** were

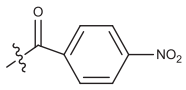




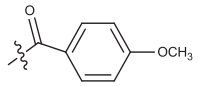
3

1: R_1 = H; R_2 = OH; R_3 = H; R_4 = H; R_5 = H
2: R_1 = H; R_2 = OH; R_3 = CH_3; R_4 = H; R_5 = H
4: R_1 = H; R_2 = H; R_3 = CH_3; R_4 = OH; R_5 =H
5: R_1 = H; R_2 = H; R_3 = CH_3; R_4 = H; R_5 = H
6: R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = $COCH_3$
7: R_1 = $COCH_3$; R_2 = H; R_3 = H; R_4 = H; R_5 = $COCH_3$
8: R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = $COCH_2CH_3$
9: R_1 = $COCH_2CH_3$; R_2 = H; R_3 = H; R_4 = H; R_5 = $COCH_2CH_3$
10: R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = $COCH_2CH_2CH_3$
11: R_1 = $COCH_2CH_2CH_3$; R_2 = H; R_3 = H; R_4 = H; R_5 = $COCH_2CH_2CH_3$
12: R_1 = Bz; R_2 = H; R_3 = H; R_4 = H; R_5 = Bz
13: R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = *p*-nitroBz
14: R_1 = *p*-nitroBz; R_2 = H; R_3 = H; R_4 = H; R_5 = *p*-nitroBz
15: R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = *p*-methoxyBz
16: R_1 = *p*-methoxyBz; R_2 = H; R_3 = H; R_4 = H; R_5 = *p*-methoxyBz
17: R_1 = H; R_2 = H; R_3 = All; R_4 = H; R_5 = H
18: R_1 = H; R_2 = H; R_3 = Glc; R_4 = H; R_5 = H
19: R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = H
20: R_1 = $COCH_3$; R_2 = H; R_3 = $COCH_3$; R_4 = H; R_5 = $COCH_3$
21: R_1 = Bz; R_2 = H; R_3 = Bz; R_4 = H; R_5 = Bz

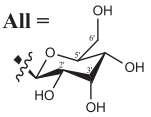
***p*-nitroBz=**



***p*-methoxyBz=**



All =



Glc =

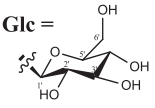


Figure 1. Structure of compounds **1**–**21**.

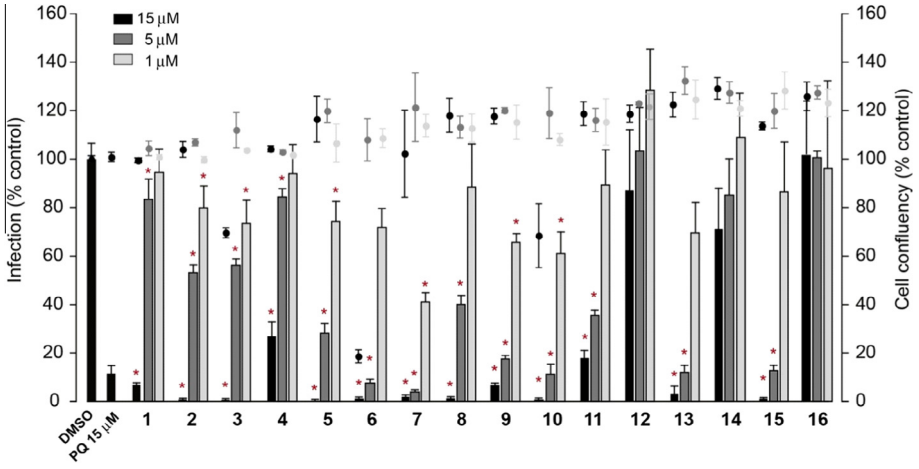


Figure 2. Luminescence-based measurement of dose-dependent effects of compounds **1**–**16** on *P. berghei* infected Huh7 cells. Bars represent infection loads and dots represent cell confluency. Results are expressed as mean ± SD (triplicate wells). PQ-primaquine, used as positive control. DMSO-solvent-treated control. $^*p$ <0.05, as compared to DMSO-treated cells.

previously isolated from *M. balsamina*.[24,25] Compounds **6**–**16** were obtained through derivatization of karavilagenin C (**5**), using several alkanoyl and aroyl acylating reagents as described.[20] The purity of the compounds was more than 95% based on HPLC analysis.

3.2. In vitro *Plasmodium* liver stage assays

Huh-7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium supplemented with 10% v/v fetal calf serum (FCS),

1% v/v non-essential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2. Huh-7 cells (12×10^3 per well) were seeded in 96-well plates the day before drug treatment and infection. Inhibition of liver stage infection was determined by measuring the luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, *Pb*GFP-Luc_{con}, as previously described.[26] Compounds were added 1 h prior to addition of sporozoites freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes. Sporozoite addition was followed by centrifugation at 1700*g* for 5 min. Inhibition of parasite development was measured 48 h after infection. The effect of the compounds on the viability of Huh-7 cells was assessed by measuring AlamarBlue fluorescence (Invitrogen, UK), using the manufacturer's protocol.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.06.019. These data include MOL files and InChiKeys of the most important compounds described in this article.

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