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Antimalarial Activity of Physalins B, D, F, and G

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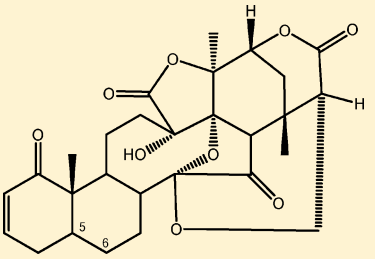
Supporting Information

ABSTRACT: The antimalarial activities of physalins B, D, F, and G (**1**–**4**), isolated from *Physalis angulata*, were investigated. In silico analysis using the similarity ensemble approach (SEA) database predicted the antimalarial activity of each of these compounds, which were shown using an in vitro assay against *Plasmodium falciparum*. However, treatment of *P. berghei*-infected mice with **3** increased parasitemia levels and mortality, whereas treatment with **2** was protective, causing a parasitemia reduction and a delay in mortality in *P. berghei*-infected mice. The exacerbation of in vivo infection by treatment with **3** is probably due to its potent immunosuppressive activity, which is not evident for **2**.



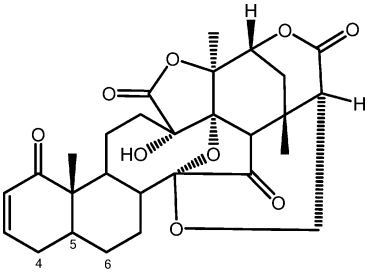
Physalin D 5α-OH, 6β-OH (**2**)

Physalin F 5β,6β-epoxy (**3**)

Malaria is a sometimes fatal disease caused by parasites of the genus *Plasmodium* that affects over 100 countries around the world.[1] Although antimalarial drugs play a central role in the control and elimination of the disease, their activity against *Plasmodium* spp. has shown a reduced efficacy because of drug-resistant parasites, leading to an increase in treatment failure rates.[1] This makes the development of new antimalarial therapeutic agents a matter of great relevance to public health.[2]

Physalis angulata L. (Solanaceae) is an annual herb distributed widely throughout tropical and subtropical regions of the world.[3,4] This plant has been used in popular medicine as a treatment for a variety of disease conditions,[5] including malaria.[6] It has been shown previously that various physalin derivatives have antileishmanial,[7] immunomodulatory,[3,8,9] and anti-inflammatory activities.[10]

In the present work, the similarity ensemble approach (SEA) was used, the principle of which is based on the concept that dissimilar molecules can have equivalent types of interactions with a protein target. This procedure evaluates the similarity between the entry structure from a database of over 65 000 small molecules annotated for protein drug targets, where the majority of such annotations contains hundreds of ligands.[11] On the basis of the SEA analysis performed, which predicted the antimalarial activity of selected physalins, the activity of four physalins (**1**–**4**) purified from *P. angulata* against chloroquine-resistant *Plasmodium falciparum* parasites was evaluated in vitro. Two these compounds (**2** and **3**) were evaluated subsequently against *Plasmodium berghei* parasites in an in vivo model.



1 Δ^5
2 5α-OH, 6β-OH
3 5β,6β-epoxy
4 Δ^4, 6α-OH

The results from the SEA webserver indicated varied activities for one or more of the physalins B (**1**), D (**2**), F (**3**), and G (**4**),[11] including antitumor and anti-inflammatory related activities (Table S1, Supporting Information). However, antimalarial activity was predicted for all four physalins, with *E* values ranging from 8.55×10^{-6} (for **1**) to 3.15×10^{-5} (for **3**)

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(Table S1, Supporting Information). Although none of the physalins gave the highest predicted biological activity score for antimalarial activity, this was the only activity common to all four compounds found by this computerized analysis. Furthermore, the Tanimoto coefficient (T_c) was analyzed for all four compounds and ranged from 0.67 (for **3**) to 0.73 (for **1**). These results also suggest that these molecules exhibit antimalarial activity, since the nearer to 1.0 for the T_c value, the greater the similarity between the molecule analyzed with other compounds that share a particular pharmacological activity.

The predictions of antimalarial activity based on the SEA analysis were then evaluated in an in vitro assay against the *P. falciparum* W2 clone. All four physalins exhibited antiplasmodial activity in vitro, with **1**, **3**, and **4** showing low micromolar IC_{50} values and **2** being the least active (Table 1). The cytotoxic potential against mammalian cells of the compounds was also investigated. All compounds exhibited less potent cytotoxicity compared to mefloquine, used as a standard antimalarial drug. Mefloquine had a lower IC_{50} value but higher cytotoxicity (Table 1).

Table 1. Anti-*Plasmodium falciparum* Cytotoxicity in Vitro of Physalins 1−4

compd	IC_{50} (μM)[a]	LC_{50} (μM)[a]	SI[b]
physalin B (**1**)	2.8 ± 1.20[c]	33.9 ± 9.60	12.30
physalin D (**2**)	55 ± 0.96[c]	570 ± 146.4	10.40
physalin F (**3**)	2.2 ± 1.16[c]	13.3 ± 6.01	5.94
physalin G (**4**)	6.7 ± 0.37*[c]	37.5 ± 7.10	5.60
mefloquine	0.04 ± 0.01	9.5 ± 0.46	238

[a]IC_{50} = inhibitory concentration 50%; LC_{50} = lethal concentration 50%. [b]SI = selectivity index. Values are means ± SD of three independent experiments. [c]$p < 0.05$ compared to mefloquine, a positive control.

For in vivo testing, physalin F (**3**) was selected because this compound exhibited the lowest IC_{50} value (Table 1). Treatment of *P. berghei*-infected mice (50 and 100 mg/kg) by the intraperitoneal route did not decrease the parasitemia levels in comparison to mock-treated controls (10% DMSO in saline). In fact, there was an increase in parasitemia levels upon treatment with **3**. Mice treated with chloroquine, a standard antimalarial drug, had undetectable parasitemia (Figure 1A). In addition, no reduction of mortality was observed in **3**-treated mice, which started to die by day 7, similar to the case for control animals (Figure 1B). All the mice treated with chloroquine survived during the experiment. The lack of efficacy after treatment with **3** may be explained by its potent immunosuppressive activity, as demonstrated in previous studies.[3,9]

The suppression induced by **3** may have inhibited the antimalarial immune response in infected animals, which is important for infection control.[12] Thus, although **2** gave a lower potency in vitro, its activity was tested in vivo because this physalin is the only one of the four tested that does not have immunosuppressive activity.[3,8] Treatment with **2** significantly decreased parasitemia by about 65% at the eighth day of infection (Figure 2A) and the mortality of *P. berghei*-infected animals treated by 25%, at a dose of 100 mg/kg, until day 24 after infection (Figure 2B).

Several in vitro systems were conducted to demonstrate the action of the compounds only in contact with the parasite and host cell. It is considered important to understand the effects of drug candidates on the immune system, because this may influence their therapeutic effect in vivo. Another important aspect to be considered is the route of drug administration. In the case of **3**, despite having a potent immunosuppressive action, treatment with this compound in *Leishmania amazonensis*-infected mice was beneficial topically but not orally.[7] While the positive effects of topical treatment with **3** in case of cutaneous leishmaniasis caused by *L. amazonensis* infection may result from its action on the parasite, the local suppression of the immune response may also be beneficial, since the skin lesions caused by this parasite have an inflammatory component that causes tissue destruction. In contrast, in the case of infection by *P. berghei*, systemic treatment by physalin F (**3**) seems not to be beneficial and may even cause a worsening of infection.

In addition to the antimalarial activity, the SEA analysis also predicted other activities for the physalins studied. Some of the

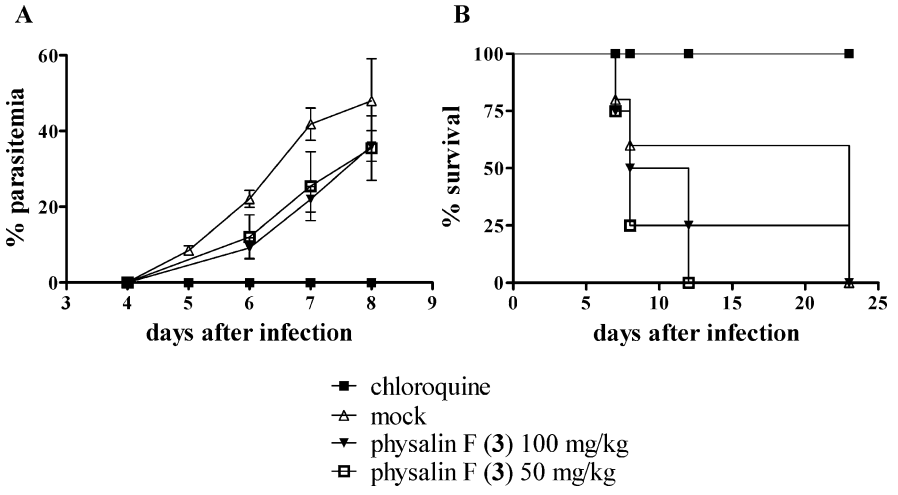

Figure 1. (A) Treatment of *P. berghei*-infected mice with physalin F (**3**): parasitemia of *P. berghei*-infected mice treated with 10% DMSO in saline (mock), **3** at 50 and 100 mg/kg, or chloroquine at 50 mg/kg, by the intraperitoneal route, administered daily, for four consecutive days. Blood parasitemia was determined for several days after infection. Values represent the means ± SEM of five mice per group in one experiment of two replicates performed. (B) Results are from one experiment of two replicates performed, carried out with five mice per group.

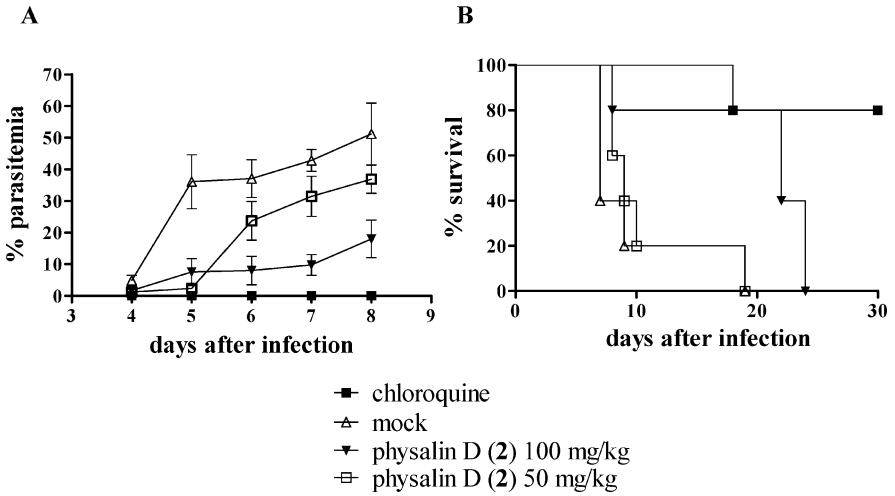

Figure 2. (A) Treatment of *P. berghei*-infected mice with physalin D (**2**). Parasitemia levels were investigated of *P. berghei*-infected mice treated with 10% DMSO in saline (mock), **2** at 50 and 100 mg/kg, or chloroquine at 50 mg/kg, by the intraperitoneal route, administered daily, for four consecutive days. Blood parasitemia was determined for several days after infection. Values represent the means ± SEM of five mice per group in one experiment of two replicates performed. (B) Results are from one experiment carried with five mice per group of two replicates performed. ($p < 0.05$ in comparison to mock-treated controls).

activities predicted for the physalins by this analysis, such as antitumor, immunosuppressive, and anti-inflammatory activities, have already been demonstrated.[3,8−10,13,14] Altogether, these results indicate that SEA analysis is a valuable tool for the investigation of putative biological activities of chemical entities.

EXPERIMENTAL SECTION

General Experimental Procedures. Physalins B (**1**), D (**2**), F (**3**), and G (**4**) were isolated from *Physalis angulata* collected in Belém do Pará, Brazil, as described previously.[8] Preparations of **1**−**4** (96%, 95.6%, 97.8%, and 95% purity by HPLC, respectively) were dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium or saline for use in the assays. The chemical structures of **1**−**4** were previously determined.[15−18] Mefloquine and chloroquine (Farmanguinhos, Rio de Janeiro, RJ, Brazil) were used as positive control antimalarials in vitro and in vivo, respectively.

Specific-pathogen-free, 5-week-old male Swiss mice were maintained at the animal facilities at the Gonçalo Moniz Research Center-FIOCRUZ and provided with rodent diet and water ad libitum. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

Similarity Ensemble Approach (SEA). The structure files[19] were downloaded in the SDF format for **1**−**3**. The compound **4** file was built from the structure of **3** using HyperChem software (Hypercube, Inc.) and MM+ molecular mechanics with the semiempirical AM1 method.[20] These structures were converted to the SMILES format using HyperChem software and submitted to SEA, in order to evaluate their potential activities.[11] In the present work, 10^{-4} was used as a cutoff for the *E* value, and values higher than this threshold value were not considered in the analysis made, since they indicated low statistical significance. The original proposal of the SEA application suggests also the use of the Tanimoto coefficient (T_c), with a threshold of 0.57 and 1.0 the maximum similarity between two molecules.[11] A threshold of 0.67 for T_c was adopted. Values lower than 0.67 were not considered in the analysis made.

Cytotoxicity Assay. To determine the cytotoxicity of physalins B, D, F, and G (**1**−**4**), BALB/c mice splenocytes were cultured in a 96-well plate (6×10^5 cells/well) in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal calf serum (Cultilab, Campinas, São Paulo, Brazil) and 50 μg/mL of gentamycin (Novafarma, Anapolis, Goiás, Brazil). Compounds were tested at five concentrations, in triplicate. A 1 μCi/well amount of [methyl-3*H*]thymidine (Amersham, Little Chalfont, U.K.) was added to the cultures, which were incubated for 24 h at 37 °C and 5% CO_2. After this period, plates were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD) to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Multilabel Reader, Hidex, Turku, Finland). The viability of the cells was determined by ^{3}H-thymidine incorporation, and the cytotoxicity was calculated in relation to the ^{3}H-thymidine incorporation of untreated cultures.

Anti-*Plasmodium falciparum* in Vitro Assay. Compounds **1**−**4** were tested for antimalarial activity in vitro using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive.[21] All parasites were maintained in a continuous culture of human erythrocytes (blood group O^+) using RPMI-1640 medium supplemented with 10% human plasma.[22] Parasites grown at 2−5% parasitemia and 2.50% hematocrit were incubated with the pure test substances at various concentrations, diluted with 4% dimethyl sulfoxide (DMSO) in RPMI-1640 medium without hypoxanthine. Mefloquine was used in each experiment as a positive control. Cultures containing parasites were harvested using a cell harvester to evaluate the ^{3}[H]-hypoxanthine incorporation in a β-radiation counter (Multilabel Reader; Hidex, Turku, Finland). Inhibition of parasite growth was evaluated by comparison with ^{3}[H]-hypoxanthine uptake in drug-treated versus untreated wells. All assays were performed in triplicate as described previously.[23,24]

Assessment of in Vivo Antimalarial Activity for Physalin D (2) and F (3). The antimalarial activities of **2** and **3** were evaluated in mice infected with *Plasmodium berghei*, strain NK65.[25] Parasites were maintained by weekly blood passage of 10^6 infected red blood cells per mouse by the intraperitoneal route. The animals were sorted randomly into groups of five, and treatment was administered daily, for four consecutive days, beginning on the first day of infection. The test compounds were suspended in saline solution plus 10% DMSO immediately before use in doses of 50 and 100 mg/kg. Each mouse received a volume of 200 μL, by the 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). Antimalarial activity was evaluated by counting parasitemia in blood smears at days 4−8 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 (vehicle-treated) group. The results were expressed as the percentage of parasitemia reduction.

Statistical Analyses. The lethal concentration 50% of BALB/c mice splenocytes (LC_{50}) and the inhibitory concentration 50% (IC_{50}) of *P. falciparum* were calculated on the basis of a nonlinear regression (curve fit). The selectivity index (SI) was defined by calculating the value of LC_{50} versus IC_{50}.[26] The SI was considered as significant for values higher than 3. Statistical analyses were performed 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). Differences were considered significant where p values were <0.05.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table giving novel target selectivity predictions for physalins **1**–**4** using the SEA database. This material is available free of charge via the Internet at http://pubs.acs.org.

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