SUPPORTING INFORMATION

Pharmacokinetics, metabolism, and *in vivo* efficacy of the antimalarial natural product bromophycolide A

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EXPERIMENTAL METHODS

Isolation of bromophycolide A. Bromophycolide A (BrA; **1**) was isolated from the Fijian marine red alga, *Callophycus serratus*, as previously described.[1] *C. serratus*, collection number G-0901, was collected in 2010 from the island of Yanuca on the reef slope (S18° 22.70, E177° 59.25) between 5-7 m depth. Briefly, crude lipophilic extracts from *C. serratus* were separated by liquid-liquid partitioning followed by silica gel column chromatography, from which **1** was crystallized using methanol and water.

Formulation for pharmacokinetic and *in vivo* efficacy studies. BrA (**1**) was dissolved in DMSO then serially diluted into 10% Solutol HS-15 (BASF Global, Germany) in normal saline solution to achieve a working concentration of 23 mg/kg containing 6.5% DMSO and 5% solutol HS-15 (v/v). For efficacy studies working concentration was 10 mg/kg with 2% DMSO in 5% solutol HS-15 (v/v).

Mouse serum stability. In order to determine the stability in mouse serum over 48 h, **1** in DMSO was added to mouse serum (Sigma-Aldrich, St Louis, MO USA) for a final concentration of 150 μM with 10 % DMSO (v/v). Aliquots of 10 μL were removed at t = 0 h, 1 h, 18 h, 24h , and 48 h and the concentration of **1** analyzed by HPLC (Waters 1525 binary pump connected to Waters 1585 dual wavelength detector set to 229 and 265 nm; Grace Alltima C18-silica 5 μm, 4.6 x 150 mm column; 50% aqueous MeOH to 100% MeOH over 25 min). Control samples containing normal saline plus **1** and normal saline, mouse serum, but not **1** were run in parallel. Each assay was run in duplicate.

***In vivo* efficacy.** On day one, 25-30 g Swiss-Webster female mice (Hilltop Lab Animals, Inc., Scottdale, PA USA) were inoculated by intraperitoneal (IP) injection with 10^6 *Plasmodium yoelii* infected erythrocytes. On day two, infection was confirmed by giemsa stained tail blood smear then mice were dosed daily, IP, with **1** over a subsequent four day period at 10 mg/kg or 5 mg/kg in 5 % solutol/2 % DMSO. Control animals were injected with the 5 % solutol/2 % DMSO vehicle only. Progress of infection was monitored daily by collection of tail blood. Parasitemia was traced by light microscopic analysis of giemsa stained thin smears and later confirmed by independent flow cytometry analysis. Blood samples of 4 μL were collected and fixed in 1 mL of 0.25 % glutaraldehyde (Polysciences) in PBS (pH 7.4), stained with Hoechst 33342 (Invitrogen Molecular Probes), and the average of 20,000 events collected on a Becton Dickson FACSAria. On day five, mice were terminally bled in 0.1 mM EDTA. Plasma was generated from blood by centrifugation and both blood pellets and plasma were stored at -80 °C for LC-MS analysis. Blood pellets and plasma were extracted with an equal volume of ice-cold CH_3CN and centrifuged (4 °C; 1500 × g; 10 min). Pelleted proteins were washed with an additional 100 μL of CH_3CN to assure that all **1** was extracted. The resulting supernatants from both extractions were analyzed by LC-MS for the detection of **1** using a Waters 2695 separation module connected to Waters 2996 diode array UV detector coupled to a Waters ZQ2000 mass detector. Chromatography was performed on a Grace Alltima C18-silica column (3 μm, 2.1x100 mm) with an elution method of 5 % aqueous methanol for 15 min, 5-95 % aqueous methanol over 20 min, 95 % aqueous methanol for 10 min. All solvents contained 0.1 % acetic acid. Mass spectrometry settings were optimized for the **1** isotopic cluster (m/z 661/663/665/667) in negative mode and set as follows: capillary voltage 3.0 V, cone 50 V, desolvation temperature 350 °C, source temperature 125 °C, and desolvation nitrogen flow 250 L/h. A standard curve with ten concentrations from 0.01-10.0 μM was generated in 1:1 0.1 M phosphate buffer and CH_3CN containing 0.2 % DMSO ($y = 187100x + 77520$, $r^2 = 0.97$).

Pharmacokinetic studies. Pharmacokinetic studies were conducted under contract by Explora Biolabs (San Diego, CA) using 24 BALB/c female mice (Harlan Laboratories,

Indianapolis, IN USA). Briefly, two groups of 12 BALB/c mice were given a single dose of **1** (23.1 mg/kg) by either IV or IP injection. Blood was collected twice for each mouse (survival bleed and terminal bleed) at: pre-dose, 0 h, 1 h, 1.5 h, 3 h, 4 h, 6 h, and 24 h. After blood collection, EDTA was added as an anticoagulant and processed to obtain plasma for LC-MS analysis by centrifugation (4 °C, 5000 rpm, 3 min). Plasma was frozen and stored at -80 °C until analysis. Plasma samples were treated with 2x ice-cold acetonitrile and centrifuged (6100 × g; 30 min) before a final dilution with 3 × 0.2 % formic acid in water for LC-MS analysis.

Analysis of **1** was performed by LC-MS using a Shimadzu VP system coupled to an Applied Biosystems MDS SciEx API 3000 mass spectrometer with Turbo-Ion spray ionization in negative mode. Chromatography was achieved on a Peeke Duragel G C18-silica column (3.5 μm, 2.1 x 10 mm) with a linear gradient of 55% aq. MeOH with 0.2% formic acid to 95% aq. MeOH with 0.2% formic acid over 5 min with a flow rate of 800 μL/min following a 0.25 min pre-wash at 5% methanol with 0.2% formic acid. Standard curve y = 0.00808x0.89, r^2 = 0.9987 (eight concentrations from 5 ng/mL to 15 μg/mL). Pharmacokinetic parameters were calculated using a one-compartment model.[2, 3]

Quantitative analysis of liver metabolism of 1. Incubation of **1** with S9, microsomal, and cysotolic subcellular fractions of human liver enzymes (Invitrogen Life Technologies Corp., Grand Island, NY USA) were performed to determine the extent of metabolism and the metabolic products formed from **1**. All incubations were performed in triplicate in a total volume of 1 mL with a maximum concentration of 0.2 % DMSO and 0.8 % CH_3CN. Three separate assays were performed using the human liver subcellular fractions S9, microsomal, and cytosolic with a final protein concentration of **1** mg mL^{-1} and 10 μM **1** in 0.1 M potassium phosphate buffer (pH 7.4). Additional assays were performed as above with either microsomal or S9 subcellular fractions, adding 5 mM UDPGA as a co-factor for determination of glucuronidation. The enzymes and **1** were incubated at 37 °C prior to the addition of a pre-warmed (37 °C) NADPH regenerating solution (0.3 mM $NADPNa_2$, 3 mM glucose-6-phosphate, 0.5 units mL^{-1} glucose-6-phosphate dehydrogenase, 0.5 mM $MgCl_2$). Aliquots of 100 μL were removed after vortex mixing at t = 0 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 6 h, and 24 h and quenched with an equal amount of ice-cold CH_3CN. Each aliquot was stored at -80 °C until analysis. Samples were centrifuged (4 °C; 3,000 rpm; 5 min) and the resulting supernatants were diluted 1:1 with deionized water and analyzed as above by LC-MS with electrospray ionization in negative mode. Two separate controls were ran in parallel: a) **1** without enzyme and b) **1** and enzyme solution in the absence of NADPH regenerating solution. Apparent intrinsic clearance by S9 and microsomes *in vitro* was calculated as follows:[4] CL_{int} = (0.693/$t_{1/2}$ *in vitro*)(incubation volume/mg protein)(45 mg protein/g liver)(20 g liver/kg body weight).

Statistical analyses. Linear regression analysis was performed using GraphPad version 4.00 to determine if the concentration of **1** changed over time for the serum stability and cytosolic enzyme metabolism experiments. Data in figure 4 was analyzed by one-way analysis of variance (ANOVA) with Tukey post-hoc test using GraphPad version 4.00 at time points 6 h and 24 h.

***In vitro* assays.** Antimalarial activity was performed as previously described.[5]

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ABBREVIATIONS

BrA, bromophycolide A; DMSO, dimethylsulfoxide; MeOH, methanol; CH_3CN, acetonitrile; UDPGA, uridine 5'-diphosphoglucuronic acid tri-sodium salt; FACS, Fluorescence-activated cell sorting; IV, intravenous; and IP, intraperitoneal; Kel, first order rate constant for the elimination phase; Kab, first order rate constant for the absorption phase.

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