Pantothenamides Are Potent, On-Target Inhibitors of Plasmodium falciparum Growth When Serum Pantetheinase Is Inactivated

Christina Spry1*, Cristiano Macuamule3, Zhiyang Lin1, Kristopher G. Virga4,5, Richard E. Lee5,6, Erick Strauss3, Kevin J. Saliba1,2*

1 Research School of Biology, College of Medicine, Biology and Environment, The Australian National University, Canberra, Australian Capital Territory, Australia, 2 Medical School, College of Medicine, Biology and Environment, The Australian National University, Canberra, Australian Capital Territory, Australia, 3 Department of Biochemistry, Stellenbosch University, Matieland, Stellenbosch, South Africa, 4 Department of Pharmaceutical and Administrative Sciences, Presbyterian College School of Pharmacy, Clinton, South Carolina, United States of America, 5 Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, 6 Department of Chemical Biology and Therapeutics, St. Jude Children’s Research Hospital, Memphis, Tennessee, United States of America

Abstract

Growth of the virulent human malaria parasite Plasmodium falciparum is dependent on an extracellular supply of pantothenate (vitamin B₅) and is susceptible to inhibition by pantothenate analogues that hinder pantothenate utilization. In this study, on the hunt for pantothenate analogues with increased potency relative to those reported previously, we screened a series of pantothenamides (amide analogues of pantothenate) against P. falciparum and show for the first time that analogues of this type possess antiplasmodial activity. Although the active pantothenamides in this series exhibit only modest potency under standard in vitro culture conditions, we show that the potency of pantothenamides is selectively enhanced when the parasite culture medium is pre-incubated at 37°C for a prolonged period. We present evidence that this finding is linked to the presence in Albumax II (a serum-substitute routinely used for in vitro cultivation of P. falciparum) of pantetheinase activity: the activity of an enzyme that hydrolyzes the pantothenate metabolite pantetheine, for which pantothenamides also serve as substrates. Pantetheinase activity, and thereby pantetheinamide degradation, is reduced following incubation of Albumax II-containing culture medium for a prolonged period at 37°C, revealing the true, sub-micromolar potency of pantothenamides. Importantly we show that the potent antiplasmodial effect of pantothenamides is attenuated with pantothenate, consistent with the compounds inhibiting parasite proliferation specifically by inhibiting pantetheinase and/or CoA utilization. Additionally, we show that the pantetheinamides interact with P. falciparum pantetheinase kinase, the first enzyme involved in converting pantetheine to coenzyme A. This is the first demonstration of on-target antiplasmodial pantetheinamide analogues with sub-micromolar potency, and highlights the potential of pantetheinase-resistant pantetheinamides as antimalarial agents.


Copyright: © 2013 Spry et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Aspects of this work were supported by grants from the South African Malaria Initiative (SAMI) to ES and KJS and the American Lebanese Syrian Associated Charities (ALSAC), St. Jude Children’s Research Hospital, to REL. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kevin.saliba@anu.edu.au

Introduction

Every day approximately half of the world’s population is at risk of contracting malaria, a lethal infectious disease estimated to have claimed 655,000 lives [1] (if not more [2]) in 2010. New chemotherapeutic agents are desperately needed to combat malaria as Plasmodium falciparum, the most virulent of the parasites that cause the disease in humans, has developed resistance to all antimalarial agents in clinical use [3].

Plasmodium falciparum has an absolute requirement for exogenous pantothenate (vitamin B₅; Figure 1) [4,5,6], a precursor of the essential enzyme cofactor coenzyme A (CoA). Analogues of pantothe-
ination that interfere with the utilization of pantothe-
ination in Plasmodium parasites in vitro and in vivo [5,7,8], raising interest in pantothe-
ination utilization as a potential antimalarial drug target, and pantothe-
ination analogues as the chemical entities to strike this target [6,9]. The structure of pantothanol, one antiplasmodial pantothe-
ination analogue identified previously, is shown in Figure 1. Pantothenamides, pantetheinamide analogues in which the car-
boxyl group of pantothanol is replaced with an amide group (Figure 1), have been shown to possess antibacterial activity in vitro [10,11,12,13,14]; Escherichia coli and Staphylococcus aureus are among the bacteria demonstrated to be susceptible to inhibition by these compounds. Pantothenamides have been shown to serve as substrates of pantetheinase kinase (PanK), the first enzyme in the CoA biosynthesis pathway, and as a consequence inhibit PanK-catalysed pantetheinamide phosphorylation [15,16]. The resultant 4’-phosphopantothenamides are further metabolized by...
the CoA biosynthesis pathway of bacteria to yield analogues of CoA [17]. Such CoA analogues have been shown to be incorporated by, and inhibit the function of, acyl carrier protein [16,19], a protein involved in fatty acid biosynthesis that requires the 4′-phosphopantetheine moiety of CoA for activation. Whether the mechanism that ultimately results in bacteriostasis is inhibition of CoA biosynthesis [20], fatty acid biosynthesis [19] or another CoA-utilizing process, or a combination of the above, remains to be resolved.

In this study, the effect of a series of pantothenamides (see Figure 2) on the growth of erythrocytic stage \( P. falciparum \) parasites was investigated. We show for the first time that under standard \textit{in vitro} culture conditions pantothenamides inhibit parasite growth, albeit with modest potency. Serendipitously, however, we discovered that the antimalarial potency of pantothenamides is enhanced considerably when the parasite culture medium used for growth assays (which contains the commonly used serum substitute Albumax II [21] or human serum) is pre-incubated at 37°C for a prolonged period. Consequently, sub-micromolar concentrations of pantothenamides that have no effect in freshly supplemented medium are growth inhibitory in the pre-incubated medium. We present evidence that links this finding to the presence in parasite culture medium of pantetheinase activity, the activity of an enzyme that catalyzes the hydrolysis of pantetheine (Figure 1) to pantothenate and cysteamine. In animals, pantetheinase activity is typically linked with the Vanin proteins [22], soluble or membrane bound proteins that belong to the nitriase superfamily, the members of which share an invariant Glu-Lys-Cys catalytic triad [23]. We show, using an \textit{in vitro} primary amine detection assay, that a pantothenamide selected from the series tested here is hydrolyzed in the presence of Albumax II, demonstrating Albumax II to be a source of pantetheinase activity. Furthermore, we show that recombinant human pantetheinase (Vanin-1) reduces the antimalarial potency of the pantothenamide in the pre-incubated medium \textit{in vitro}, and, that the attenuating effect of the pantetheinase is alleviated by incubation of the pantetheinase-supplemented medium at 37°C. Together these data are consistent with pantetheinase-mediated pantothenamide degradation occurring in medium freshly supplemented with Albumax II or serum under \textit{in vitro} culture conditions, lowering the effective pantothenamide concentration, and thereby masking the sub-micromolar antimalarial potency of pantothenamides.

Importantly, we demonstrate that the potent antimalarial effect of the pantothenamides in the medium pre-incubated at 37°C is alleviated with pantetheinase, and therefore results specifically from inhibition of pantetheinase and/or CoA utilization. We also show that all of the pantothenamides in this series inhibit \( P. falciparum \) PanK-catalysed pantetheine phosphorylation (and hence serve as substrates or inhibitors of \( P. falciparum \) PanK).

The data presented here provide additional validation of pantothenate and CoA utilization as potential antimalarial drug targets.

**Materials and Methods**

**Reagents**

The pantothenamides tested were synthesized and purified as described previously [18]. Each pantothenamide was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 400 mM before being diluted in the solution pertinent to the experiment. For \textit{in vitro} growth assays, the concentration of DMSO introduced into cultures never exceeded 0.05% (v/v), in phosphorylation assays the DMSO concentration present was 0.025% (v/v), and in fluorescamine assays the concentration of DMSO never exceeded 0.15% (v/v). Albumax II was purchased from Life Technologies (Mulgrave, Victoria, Australia), dissolved to a concentration of 20% (w/v) in water, filter sterilized and stored frozen (−20°C). SYBR Safe DNA gel stain (10 000× stock concentration) was also from Life Technologies. Recombinant human pantetheinase (Vanin-1) was purchased from Novoprotein Scientific Inc. (Short Hills, New Jersey, USA) and was reconstituted to a concentration of 200 μg/mL in sterile phosphate-buffered saline and stored at −20°C for no longer than 3 weeks before use. \([^{14}C]\)Pantothenate (55 mCi/mmol) was purchased from American Radiolabelled Chemicals, Inc., and pantetheine and fluorescamine were from Sigma-Aldrich.

**Cell Culture**

Erythrocytic stage \( P. falciparum \) parasites (strain 3D7) were maintained within human erythrocytes in continuous culture essentially as described previously [24,25]. \( P. falciparum \)-infected erythrocytes were routinely cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 20 mM D-glucose, 200 μM hypoxanthine, 24 mg/L gentamycin and Albumax II (0.6%, w/v), which, hereafter is referred to as Albumax-complete RPMI. For several days prior to carrying out the assays testing the effect of compounds on the growth of \( P. falciparum \) in medium supplemented with human serum (instead of Albumax II), the parasites were cultured in the same medium mentioned above, except that human serum (10%, v/v, pooled from different blood donors) was used rather than Albumax II. This medium is hereafter referred to as human serum-complete RPMI.

**In vitro Growth Assays**

The \textit{in vitro} antimalarial activity of test compounds was determined using the malaria SYBR Green I-based fluorescence assay described by Smilkstein et al. [26], with minor modifications. Briefly, \( P. falciparum \)-infected erythrocytes were incubated in culture medium containing two-fold dilutions of the test compounds in 96-well microtiter plates. Assays were initiated...
Figure 2. Effect of pantothenamides on proliferation of *P. falciparum* and *P. falciparum* lysate-catalysed [1-14C]pantothenate phosphorylation. The 50% inhibitory concentrations (IC50 values) measured against *P. falciparum* parasites cultured (for 96 h) in Albumax-complete RPMI containing 1 μM pantothenate, as determined using the SYBR Green I-based growth assay are shown. Assays were performed using Albumax-

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC50 against <em>P. falciparum</em> (μM) Fresh medium 1 μM pantothenate</th>
<th>IC50 against <em>P. falciparum</em> (μM) Aged medium 1 μM pantothenate</th>
<th>Inhibition of pantothenate phosphorylation at 100 μM (%) 0.2 μM pantothenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (N5-Pan)</td>
<td><img src="image1.png" alt="R1" /></td>
<td>124 ± 15</td>
<td>2.0 ± 0.2</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>2 (N7-Pan)</td>
<td><img src="image2.png" alt="R2" /></td>
<td>&gt; 200</td>
<td>7.7 ± 3.5</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="R3" /></td>
<td>72 ± 24</td>
<td>0.020 ± 0.002</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="R4" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="R5" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="R6" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="R7" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8.png" alt="R8" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9.png" alt="R9" /></td>
<td>&gt; 200</td>
<td>50 ± 3</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10.png" alt="R10" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td><img src="image11.png" alt="R11" /></td>
<td>134 ± 22</td>
<td>0.34 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td><img src="image12.png" alt="R12" /></td>
<td>133 ± 12</td>
<td>1.1 ± 0.1</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>13</td>
<td><img src="image13.png" alt="R13" /></td>
<td>66 ± 4</td>
<td>150 ± 5</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td><img src="image14.png" alt="R14" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td><img src="image15.png" alt="R15" /></td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td><img src="image16.png" alt="R16" /></td>
<td>&gt; 200</td>
<td>73 ± 15</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td><img src="image17.png" alt="R17" /></td>
<td>44 ± 5</td>
<td>0.067 ± 0.002</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>18</td>
<td><img src="image18.png" alt="R18" /></td>
<td>76 ± 12</td>
<td>0.23 ± 0.06</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>19</td>
<td><img src="image19.png" alt="R19" /></td>
<td>49 ± 8</td>
<td>0.27 ± 0.06</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>20</td>
<td><img src="image20.png" alt="R20" /></td>
<td>&gt; 200</td>
<td>49 ± 8</td>
<td>94 ± 6</td>
</tr>
<tr>
<td>21</td>
<td><img src="image21.png" alt="R21" /></td>
<td>166 ± 18</td>
<td>1.9 ± 0.5</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>22</td>
<td><img src="image22.png" alt="R22" /></td>
<td>162 ± 20</td>
<td>1.4 ± 0.1</td>
<td>100</td>
</tr>
</tbody>
</table>
complete RPMI prepared within 48 h of the assay, stored at 4°C, and incubated at 37°C for a maximum of 1 h (fresh) or Albumax-complete RPMI incubated continuously at 37°C for 40 h immediately after preparation (aged). The IC50 values shown for parasites cultured in fresh Albumax-complete RPMI are averages from between two and eight independent experiments, each performed in duplicate or triplicate. Where the IC50 values determined were below 200 μM, they are presented as the mean ± SEM from between two and eight independent experiments. The IC50 values shown for parasites cultures in aged medium are averages from between two and three independent experiments each performed in triplicate. Where the IC50 values determined were below 200 μM, they are presented as the mean ± range/2 or SEM as appropriate. The percentage inhibition of [14C]pantothenate phosphorylation by PanK in P. falciparum lysate caused by pantothenamides (when tested at a concentration of 100 μM) in the presence of 0.2 μM pantothenate are also shown. The percentage inhibition was calculated from the measured amounts of [14C]pantothenate phosphorylated during a 10 min incubation in the presence of pantothenamide and in the presence, instead, of the corresponding concentration of DMSO only. Data are averages ± range/2 from two independent experiments, each performed in duplicate. A value of 100 indicates complete inhibition of [14C]pantothenate phosphorylation was observed in both independent experiments. The amount of [14C]pantothenate phosphorylated by P. falciparum lysate was significantly lower in the presence of all pantothenamides (P < 0.0001, ANOVA).

doi:10.1371/journal.pone.0054974.g002

with ring-stage P. falciparum-infected erythrocytes at a hematocrit and parasitemia of 1%. Wells containing infected erythrocytes in the presence of chloroquine (0.25–2 μM) served as zero growth controls and wells containing infected erythrocytes in the absence of chloroquine or test compounds served as 100% parasite growth controls. Plates were incubated at 37°C under an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen for 96 h, before 100 μL from each well was mixed with 100 μL of SYBR Safe DNA gel stain (0.2 μL/mL) in 20 mM Tris, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.008% (v/v) saponin, 0.08% (v/v) Triton X-100, in a second 96-well microtitre plate. Fluorescence was measured using a FLUOStar OPTIMA multi-detection microplate reader from BMG LABTECH with excitation and emission wavelengths of 490 and 520 nm, respectively. The 50% inhibitory concentration (IC50) of each test compound under each test condition was calculated by fitting the data to a sigmoidal curve (typically y = α/(1 + x/IC50)6) using nonlinear least squares regression (SigmaPlot, Systat Software) and averaging the IC50 estimates from independent experiments.

Preparation of Culture Medium for in vitro Growth Assays

The antimalarial activity of test compounds was assessed in media that had been subjected to different pre-incubations. “Fresh” medium refers to medium that was stored for a maximum of 48 h at 4°C, and incubated at 37°C for a maximum of 1 h, prior to use. “Aged” medium refers to medium that was stored for a minimum of one week at 4°C and incubated intermittently at 37°C, or medium incubated continuously at 37°C for 40 h soon after preparation. As indicated at the relevant positions within the “Results” section, for some experiments the fresh medium used was, more specifically, medium used immediately following preparation, and the aged medium used was, more specifically, medium that was incubated at 37°C for 40 h immediately prior to use.

[14C]Pantothenate Phosphorylation Assays

To assess the effect of the pantothenamides on pantothenate phosphorylation by P. falciparum PanK, the phosphorylation of [14C]pantothenate by P. falciparum lysates was measured in the presence and absence of a single concentration of each pantothenamide. P. falciparum lysate was prepared from trophozoite stage parasites “isolated” from their host erythrocyte by treatment with 0.05% (w/v) saponin (essentially as described by Saliba et al. [27]), except that 10 mM potassium phosphate, pH 7.4 (rather than 10 mM Tris, pH 7.4), was used to lyse the cells. Aliquots of lysate were stored at −20°C.

The concentration of cell lysates was determined from cell counts made using an improved Neubauer hemocytometer.

[14C]Pantothenate phosphorylation was assayed using the Somogyi reagent [29] essentially as described by Saliba et al. [27], except that pantothenate phosphorylation was terminated by heat denaturation (rather than acid denaturation) of the protein in reaction aliquots. Briefly, P. falciparum lysate was added to solutions (at 37°C) of 50 mM Tris, 5 mM ATP, 5 mM MgCl2 at pH 7.4, containing [14C]pantothenate (at a final concentration of 0.01 μCi/mL, or 0.2 μM), and a pantothenamide (at a final concentration of 100 μM) or an equivalent volume of solvent. Typically, lysate prepared from 5.4–6.5 × 107 parasites was present in each mL of reaction solution. Zero phosphorylation control reactions were prepared by adding a corresponding volume of 10 mM potassium phosphate instead of parasite lysate. Following a ten minute incubation at 37°C (a period during which pantothenate phosphorylation increased linearly with time in the absence of inhibitors) 200 μL aliquots of the [14C]pantothenate phosphorylation reactions were transferred in duplicate to microcentrifuge tubes, and immediately incubated at 95°C for 10 min to terminate phosphorylation. Terminated reaction samples were centrifuged at 15 800g for 10 min to pellet the denatured protein, before two aliquots of each supernatant (typically 80 μL) were transferred to new microcentrifuge tubes. To one aliquot, 500 μL Somogyi reagent was added, and to the other, 500 μL water was added. The samples were processed for determination of phosphorylated [14C]pantothenate as described previously [27].

In vitro Fluorescamine-based Assay

Hydrolysis of compound 12 to pantothenate and isobutylamine was measured using a modification of a fluorescence-based assay described previously for the measurement of N-acetyl-L-D-myo-inositol-2-amino-2-deoxy-α-D-glucopyranoside deacetylation activity [30,31]. The assay utilizes fluorescamine (4-phenylspiro-[furan-2(3H),1-phtthalan]-3’-dione; a non-fluorescent molecule that reacts with primary amines to form a fluorescent product [32]) for the detection of primary amines. Briefly, recombinant human pantetheinase (at a final concentration of 100 ng/mL), Albumax II (at a final concentration 0.6%, v/v), or an equivalent volume of water, was added to solutions (at 37°C) of 100 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.001% (v/v) bovine serum albumin, 0.1% (v/v) DMSO, with or without pantothenamide (at a final concentration of 200 μM). At the appropriate time points, aliquots (30 μL) of the reaction mixtures were removed in duplicate and mixed with 10 μL 20% (v/v) trichloroacetic acid to terminate the reaction. Following removal of the precipitated protein by centrifugation (14 000g, 15 min), 25 μL of each supernatant was transferred to the wells of a black 96-well microtitre plate and 75 μL 1 M borate (pH 9.0) followed by 30 μL 10 mM fluorescamine was added to the wells. The plate was then
incubated at 37°C for 10 min before fluorescence was measured using a FLUOstar OPTIMA multidetection microplate reader from BMG LABTECH with excitation and emission wavelengths of 390 and 485 nm, respectively. The fluorescence detected was converted to the corresponding isobutylamine concentration using an isobutylamine standard curve. In each experiment, the isobutylamine standard curve was generated from aliquots (30 μL) of isobutylamine solutions (0.02–200 μM) processed in parallel with the reaction aliquots as described above.

Statistical Analysis
To test for a statistically significant difference between the means of two groups, two-tailed student t tests were performed. To test for statistically significant differences between the means of more than two groups, one-way analysis of variance (ANOVA) was performed. Pairwise comparisons were made post-hoc with Tukey’s multiple comparisons test (when comparing all means) or Dunnett’s multiple comparisons test (when comparing all means with a control mean). P values reported are multiplicity adjusted. ANOVA was performed using GraphPad 6 (GraphPad Software, Inc).

Results
Antiplasmodial Activity of Pantothenamides under Standard in vitro Culture Conditions
A series of 22 previously published [18] pantothenamides was tested in vitro for antiplasmodial activity against erythrocytic stage P. falciparum parasites (strain 3D7) in 96 h growth assays initiated with parasites predominantly in the ring stage. The series of pantothenamides (see Figure 2 for structures) was composed of 21 secondary amides of pantothenate, each with a different amide substituent, as well as a lone tertiary amide. The series included the prototypical pantothenamides N5-Pan and N7-Pan (compounds 1 and 2, respectively). All of the pantothenamides tested were stereoechemically pure, with R-configuration at the chiral carbon of the 2,4-dihydroxy-3,3-dimethylbutanamide moiety. As shown in Figure 2, when tested in “fresh” Albumax-complete RPMI (Albumax-complete RPMI stored at 4°C for a maximum of 48 h, and incubated at 37°C for a maximum of 1 h, prior to use), ten of the 22 pantothenamides inhibited proliferation of P. falciparum parasites with IC50 values below 200 μM. With the exception of compound 2 (N7-Pan; the pantothenamide with the longest linear alkylic chain), the pantothenamides with simple alkylic (linear or branched) or alkyl amide substituents were among the ten active pantothenamides. Compound 3 (with a phenethyl substituent) was the only pantothenamide containing a carbocycle with an IC50 value below 200 μM.

The potency of pantothenamides with acyclic heteroatom-containing substituents varied. Three pantothenamides with linear thioether substituents were tested for antiplasmodial activity; compounds 17 and 18 were among the most active of the series, while compound 14 had little effect even at a concentration of 200 μM. Compound 17 was also significantly more active than N5-Pan, the corresponding alkylic pantothenamide (P = 0.02, ANOVA). Among the four pantothenamides with linear ether substituents, compound 19 was the most active (P<0.0001, ANOVA), compounds 21 and 22 were also active, while compound 15 lacked antiplasmodial activity at the concentrations tested. The less polar thioether pantothenamides 17 and 18 were significantly more active (P<0.003, ANOVA) than the corresponding ether pantothenamides 22 and 21, respectively. Com- pounds 16 and 20 (both with terminal hydroxyl groups in the substituent) were without effect even at the highest concentration tested.

Albumax II-complete RPMI contains 1 μM pantothenate, a concentration close to the normal whole blood pantothenate concentration [33]. When the ten pantothenamides with IC50 values below 200 μM were tested against P. falciparum parasites in fresh Albumax-complete RPMI to which 100 μM pantothenate was added, the IC50 values were very consistent (compound 3: mean ± range/2 and 1.7±0.4 (compound 19: mean ± range/2) times higher than those measured against parasites in Albumax-complete RPMI containing 1 μM pantothenate. The minor attenuating effect (or lack of an effect) of pantothenate supplementation on the antiplasmodial activity of the pantothenamides, contrasts with the dramatic attenuating effect of pantothenate on the antiplasmodial activity of previously reported antiplasmodial pantothenate analogues such as pantotheno- nol and CJ-15,801 [5,7,8].

Antiplasmodial Activity in Albumax-complete Medium Pre-incubated at 37°C for a Prolonged Period
During the course of screening pantothenamides for antiplasmodial activity, it was serendipitously discovered that a prolonged 37°C pre-incubation of the Albumax-complete RPMI used for a growth assay has a dramatic effect on the antiplasmodial activity of pantothenamides. As shown in Figure 2, nine of the ten pantothenamides that inhibit growth of P. falciparum with IC50 values below 200 μM in fresh Albumax-complete RPMI were found to be more potent in Albumax-complete RPMI incubated at 37°C for 40 h prior to use (“aged” Albumax-complete RPMI) than in fresh Albumax-complete RPMI. When tested in aged medium, the IC50 values determined for these nine compounds were between one and three orders of magnitude lower than the IC50 values determined when the pantothenamides were tested in fresh medium. Additionally, four of the 12 pantothenamides that did not inhibit growth of P. falciparum with IC50 values below 200 μM in fresh Albumax-complete RPMI (compounds 2, 9, 16 and 20) did so in aged Albumax-complete RPMI. The effect of increasing concentrations of three selected pantothenamides (compounds 3, 12, and 19) on the growth of P. falciparum in fresh and aged Albumax-complete RPMI is shown in Figure 3A-C. The activity of pantothanol was, by contrast with the activity of many of the pantothenamides, comparable in aged and fresh medium (P = 0.32, unpaired t test; Figure 3D). Hence, the potency of pantothenamides is selectively enhanced in aged medium.

Among the most potent pantothenamides in aged medium was the phenethyl substituted pantothenamide, compound 3 (IC50 = 20±2 nM; mean ± SEM; n = 3). One other carbonyl-bearing pantothenamide (compound 9; a pantothenamide with a methoxy-substituted benzyl substituent) was also observed to inhibit parasite growth in aged medium with an IC50 value below 200 μM, but was significantly less potent (P = 0.0009, ANOVA). When tested in aged medium, an IC50 value below 200 μM was measured for compound 2 (N7-Pan), however the pantothenamide remained less active than the pantothenamides with shorter alkyl amide substituents (compounds 1, 11 and 12). As observed when the compounds were tested in fresh medium, in aged medium: (i) compounds 17 and 18 (two thioether substituted pantothenamides) were among the most potent pantothenamides, and the pantothenamide with the shorter thioether substituent lacked appreciable activity even at 200 μM; and (ii) the activity of the pantothenamides with ether substituents increased with increasing chain length, with compound 19 being among the most potent pantothenamides. Compounds 16 and 20 (both with terminal hydroxyl groups in the substituent) demonstrated an inhibitory
effect in aged medium, however, they remained among the least potent pantothenamides.

Not only did the potency of pantothenamides in fresh and aged Albumax-complete RPMI differ, the effect of pantothenate supplementation on pantothenamide potency differed depending on whether the compounds were tested in fresh or aged medium; the effect of supplementation with 100 μM pantothenate was greater when compounds were tested in aged medium than in fresh. For example, as shown in Figure 4 (closed bars), the IC_50 values determined for compounds 12 and 19 against parasites cultured in aged Albumax-complete medium supplemented with 100 μM pantothenate were 21±1 (mean ± SEM; n=3) and 24±1 (mean ± range/2; n=2) times higher, respectively, than the corresponding IC_50 values against parasites cultured in fresh Albumax-complete RPMI containing 1 μM pantothenate; by comparison, the IC_50 values determined for compounds 12 and
19 against parasites cultured in fresh Albumax-complete RPMI supplemented with 100 μM pantothenate were only 1.3±0.2 and 1.7±0.4 (mean ± range/2; n = 2) times higher, respectively, than the corresponding IC₅₀ values against parasites cultured in fresh Albumax-complete RPMI containing 1 μM pantothenate (Figure 4, open bars). These data are consistent with the inhibition of growth in aged medium resulting from inhibition of pantothenate and/or CoA utilization. Pantothenate was found to antagonize the antiparasoidal activity of pantothenol effectively in both fresh and aged medium (Figure 4). Taken together, these data are consistent with (i) the pre-incubation of Albumax-complete RPMI at 37°C effecting a change in the medium that specifically and reproducibly enhances the antiparasoidal activity of pantothenamides; and (ii) pantothenamides acting via an effect on pantothenate and/or CoA utilization under these conditions.

Inhibition of Pantothenate Phosphorylation

To explore the mechanism of action of pantothenamides, we investigated whether, like antiplasmodial pantothenate analogues reported previously [5,7,8], pantothenamides inhibit *P. falciparum* PanK-catalyzed pantothenate phosphorylation. As shown in Figure 2, in the presence of each pantothenamide, the amount of [¹⁴C]pantothenate phosphorylated by PanK present in *P. falciparum* lysate during a 10 min period (in which pantothenate phosphorylation increased linearly with time under control conditions) was significantly reduced relative to the amount phosphorylated in the absence of pantothenamides (P<0.0001, ANOVA). At the pantothenamide concentration tested (100 μM, a concentration 500-fold higher than the concentration of pantothenate present in the assay), each of the pantothenamides inhibited [¹⁴C]pantothenate phosphorylation by ≥94% (Figure 2). These data are consistent with all of the pantothenamides tested here interacting with *P. falciparum* PanK, either as alternate (competitive) substrates or as inhibitors of its phosphorylating activity.

**Effect of Albumax II-supplementation on the Antiplasmodial Activity of Pantothenamides in Aged Culture Medium**

The data presented thus far are consistent with there being a heat-labile component in Albumax-complete RPMI that antagonizes the activity of pantothenamides. In an attempt to identify such a component, the activity of a selected pantothenamide (compound 12; in aged Albumax-complete RPMI supplemented immediately prior to the assay with various components of Albumax-complete RPMI), was investigated. A component of the medium that was found to antagonize the activity of pantothenamides was Albumax II, the lipid-rich bovine serum albumin preparation used as a serum substitute [21]. As shown in Figure 5A (open circles), the addition of Albumax II (0.6%, w/v) to aged Albumax-complete medium reduced the activity of compound 12. In the presence of the additional Albumax II, compound 12 had little-to-no effect on parasite growth even at a concentration of 200 μM, a concentration that inhibits parasite growth completely in aged Albumax-complete RPMI without additional Albumax. Furthermore, the activity of pantothenol was unaffected by supplementation with the additional Albumax II (Figure 5B), consistent with Albumax II specifically influencing the potency of pantothenamides. To investigate whether the attenuating effect of Albumax II could be linked to the increased potency of pantothenamides in Albumax-complete RPMI incubated for a prolonged period at 37°C, we determined whether the attenuating effect of the additional Albumax II could be alleviated by incubating the aged medium to which additional Albumax II had been added for a further 40 h at 37°C. The pantothenamide was indeed more potent (>65-fold; Figure 5A, grey circles) when tested in aged Albumax-complete RPMI supplemented with Albumax II and aged a second time. The possibility that this increased potency was due to further inactivation of an independent component of the aged medium was investigated by testing the pantothenamide in aged medium incubated for the same total length of time as the aged Albumax-complete RPMI supplemented with Albumax II and then incubated. Compound 12 was slightly more active in the Albumax-complete RPMI subjected to two 40 h incubations at 37°C than in the Albumax-complete RPMI subjected to a single 40 h incubation (Figure 5A, closed squares). This demonstrated that a component of the Albumax-complete RPMI had not been fully depleted/inactivated after the initial 40 h and hence that the pantothenamide had not reached a maximum potency in this medium after the initial 40 h incubation. Nonetheless, the increase in potency was far less than the increase in potency observed following incubation of the aged medium supplemented with additional Albumax II, consistent with the Albumax II being sensitive to heat treatment. Taken

![Figure 4](image-url)

**Figure 4. Effect of pantothenate supplementation on pantothenamide and pantothenol potency in fresh and aged Albumax-complete RPMI.** The bars represent the fold-increases in the IC₅₀ values of compound 12, compound 19 and pantothenol in Albumax-complete RPMI following supplementation with 100 μM pantothenate. The fold-increases in IC₅₀ values were calculated by dividing each IC₅₀ value measured against *P. falciparum* cultured in the presence of 100 μM pantothenate by the corresponding IC₅₀ value measured against *P. falciparum* cultured in the presence of 1 μM pantothenate. The fold-increases in IC₅₀ values were determined in assays performed using Albumax-complete RPMI stored for a maximum of 48 h at 4°C, and incubated at 37°C for a maximum of 1 h (fresh; open bar) or Albumax-complete RPMI stored for a minimum of one week at 4°C and incubated intermittently at 37°C, or, soon after preparation, incubated continuously at 37°C for up to 40 h (aged; closed bar). The fold-increases in IC₅₀ values are averaged from between two and four independent experiments in which test compounds were tested in Albumax-complete RPMI containing 1 μM pantothenate and Albumax-complete RPMI supplemented with 100 μM pantothenate in parallel. Each experiment was performed in duplicate or triplicate, and error bars represent SEM or range/2. Pantothenol bars are shown with a broken edge to indicate that only a lower limit on the fold-increase in IC₅₀ could be determined. This is because less than 50% inhibition of growth was observed at the highest pantothenol concentration tested (2 mM).

doi:10.1371/journal.pone.0054974.g004

Pantothenamides Are Potent Antiplasmodial Agents

The data presented thus far are consistent with there being a heat-labile component in Albumax-complete RPMI that antagonizes the activity of pantothenamides. In an attempt to identify such a component, the activity of a selected pantothenamide (compound 12; in aged Albumax-complete RPMI supplemented immediately prior to the assay with various components of Albumax-complete RPMI), was investigated. A component of the medium that was found to antagonize the activity of pantothenamides was Albumax II, the lipid-rich bovine serum albumin preparation used as a serum substitute [21]. As shown in Figure 5A (open circles), the addition of Albumax II (0.6%, w/v) to aged Albumax-complete medium reduced the activity of compound 12. In the presence of the additional Albumax II, compound 12 had little-to-no effect on parasite growth even at a concentration of 200 μM, a concentration that inhibits parasite growth completely in aged Albumax-complete RPMI without additional Albumax. Furthermore, the activity of pantothenol was unaffected by supplementation with the additional Albumax II (Figure 5B), consistent with Albumax II specifically influencing the potency of pantothenamides. To investigate whether the attenuating effect of Albumax II could be linked to the increased potency of pantothenamides in Albumax-complete RPMI incubated for a prolonged period at 37°C, we determined whether the attenuating effect of the additional Albumax II could be alleviated by incubating the aged medium to which additional Albumax II had been added for a further 40 h at 37°C. The pantothenamide was indeed more potent (>65-fold; Figure 5A, grey circles) when tested in aged Albumax-complete RPMI supplemented with Albumax II and aged a second time. The possibility that this increased potency was due to further inactivation of an independent component of the aged medium was investigated by testing the pantothenamide in aged medium incubated for the same total length of time as the aged Albumax-complete RPMI supplemented with Albumax II and then incubated. Compound 12 was slightly more active in the Albumax-complete RPMI subjected to two 40 h incubations at 37°C than in the Albumax-complete RPMI subjected to a single 40 h incubation (Figure 5A, closed squares). This demonstrated that a component of the Albumax-complete RPMI had not been fully depleted/inactivated after the initial 40 h and hence that the pantothenamide had not reached a maximum potency in this medium after the initial 40 h incubation. Nonetheless, the increase in potency was far less than the increase in potency observed following incubation of the aged medium supplemented with additional Albumax II, consistent with the Albumax II being sensitive to heat treatment. Taken
Figure 5. Effect of Albumax II supplementation on pantothenamide and pantothenol potency in aged Albumax-complete RPMI. The concentration-response curves show the effect of increasing concentrations of compound 12 (A) and pantothenol (B) on proliferation of *P. falciparum* parasites cultured (for 96 h) in Albumax-complete RPMI as measured using the SYBR Green I-based growth assay. Assays were performed using (i) Albumax-complete RPMI incubated immediately after preparation at 37°C for 40 h (aged; closed circles); (ii) aged Albumax-complete RPMI supplemented with additional Albumax II (0.6%, w/v) immediately prior to the assay (aged+(fresh) Albumax; open circles); (iii) aged Albumax-complete RPMI supplemented with additional Albumax II (0.6%, w/v) and heated at 37°C for 40 h immediately prior to the assay (aged+(fresh) Albumax+heat; grey circles); and (iv) aged Albumax-complete RPMI heated at 37°C for 40 h immediately prior to the assay (aged+heat; closed squares). The data presented in A are averaged from three independent experiments, each performed in triplicate, and error bars represent SEM. The data presented in B are averaged from two independent experiments, performed in duplicate or triplicate, and error bars represent range/2. For clarity, in A, the concentration-response curves represented by the closed circles are shown with negative error bars only, and the concentration-response curves represented by the grey circles are shown with positive error bars only. In B, the concentration-response curves represented by the closed circles are shown with positive error bars only, and the concentration-response curves represented by the open circles are shown with negative error bars only. Where not shown, error bars are smaller than the symbol.

doi:10.1371/journal.pone.0054974.g005

Together, these data implicate Albumax II as the labile factor in fresh Albumax-complete RPMI that influences the potency of pantothenamides.

**Antiplasmodial Activity in Human Serum-complete Medium**

To investigate whether the pantothenamide-attenuating property of Albumax II was unique to this bovine serum albumin preparation or whether the property was shared with human serum, the activity of compound 12 in medium containing human serum (10%, v/v), rather than Albumax II, was determined. In human serum-complete RPMI prepared immediately prior to growth assays (fresh human serum-complete RPMI), compound 12 had no effect on parasite growth even at a concentration of 200 μM, the highest concentration tested (Figure 6A, open circles). In human serum-complete RPMI that had been incubated at 37°C for 40 h (aged human serum-complete RPMI), however, compound 12 inhibited proliferation of *P. falciparum* with an IC₅₀ value of 22±10 μM (mean ± range/2; n=2; Figure 6A, closed circles). Pantothenol, on the other hand, inhibited parasite growth with similar activity in fresh and aged human serum-complete RPMI (Figure 6B). These data demonstrate that pantothenamide potency is selectively enhanced in human serum-complete RPMI following incubation of the medium at 37°C for 40 h, as it is in Albumax-complete RPMI following incubation, consistent with Albumax II and human serum having in common a labile component that decreases pantothenamide potency.

**Pantetheinase-mediated Pantothenamide Degradation in vitro**

The activity of pantetheinases, enzymes that catalyze the hydrolysis of pantetheine to pantothenate and cysteamine, has previously been detected in human serum [34]. Since pantetheine is a secondary amide of pantothenate it more closely resembles the pantothenamides (Figure 1) than pantothenol and other previously reported [7,8] antiplasmodial pantothenate analogues do. In light of this, we considered the possibility that pantetheinases in this series are pantetheinase substrates and that Albumax II may be a source of pantetheinase activity. To explore this hypothesis we adapted a fluorescence-based assay used previously to measure N-acetyl-l-D-myo-inositol-2-amino-2-deoxy-α-D-glucopyranoside deacetylase activity [30,31] to measure any breakdown of compound 12 to pantothenate and isobutylamine (a primary amine). This assay utilizes fluorescamine, a molecule which is itself non-fluorescent, but generates a fluorescent product upon reaction with primary amines. Using an isobutylamine standard curve, fluorescence measurements were converted to isobutylamine concentrations. As shown in Figure 7 (closed circles), when compound 12 (200 μM) was incubated with recombinant human pantetheinase (100 ng/mL), primary amine was generated, and the concentration increased approximately linearly with time before reaching a maximum after ~4 h. The maximum reached corresponded to an isobutylamine concentration of ~200 μM, consistent with all of the pantothenamide present having been hydrolyzed. By contrast, pantothenate hydrolysis was not
observed when compound 12 was incubated under the same conditions in the absence of pantetheinase (Figure 7, open circles). Hence, these data are consistent with pantetheinase mediating the hydrolysis of pantothenamides in addition to pantetheine. Primary amine was also generated during incubation of compound 12 with Albumax II (0.6%, w/v) (Figure 7, grey circles), consistent with Albumax II (when present at a concentration equivalent to that present in Albumax-complete medium) mediating hydrolysis of the pantothenamide. Furthermore, incubation of Albumax II in the absence of pantothenamide did not result in primary amine generation (Figure 7, grey squares), which eliminated the possibility that the amine generated resulted from degradation of the Albumax II and not the pantothenamide. In the presence of Albumax II, over one third of the pantothenamide present was hydrolyzed during a 6 h incubation, and pantothenamide hydrolysis appeared to reach completion within 24 h.

Pantetheinase-mediated Pantothenamide Degradation under in vitro Culture Conditions

To establish whether Albumax II-mediated pantothenamide hydrolysis could explain the reduced potency of pantothenamides in fresh Albumax-complete RPMI, we compared the activity of compound 12 in aged Albumax-complete RPMI with and without recombinant human pantetheinase. As shown in Figure 8 (open circles), 100 ng/mL recombinant human pantetheinase (when added at the start of the assay) alleviated the antiplasmodial effect of compound 12 in aged Albumax-complete RPMI. This result is consistent with pantetheinase-mediated pantothenamide degradation occurring under in vitro culture conditions and, in turn, attenuating the antiplasmodial effect of pantothenamides. Importantly, the activity of compound 12 in aged Albumax-complete RPMI supplemented with recombinant human pantetheinase increased after the medium was incubated at 37°C for 40 h (Figure 8; grey circles). Moreover, the pantothenamide was more potent in this medium than in aged Albumax-complete medium incubated in parallel but to which the recombinant human pantetheinase was added only after the second incubation at 37°C (i.e. immediately prior to the assay; Figure 8; grey squares). The latter provides evidence that the increase in pantothenamide activity is largely due to inactivation of pantetheinase and not a result of further inactivation of an independent component of the medium. Taken together these data demonstrate that the antagonizing effect of pantetheinase in parasite culture medium can be alleviated by incubating the medium at 37°C, and are consistent with inactivation of pantetheinase occurring during the incubation. Hence, inactivation of Albumax II-derived pantetheinase during prolonged incubation at 37°C, can explain the increase in pantothenamide potency observed when Albumax-complete medium is incubated for a prolonged period at 37°C.

Discussion

That pantothenamides possess antibacterial activity has been known for some time [12]. In this study we show for the first time that pantothenamides also possess antiplasmodial activity. Additionally, we show there to be a labile serum-derived factor common to Albumax II and human serum that specifically antagonizes the antiplasmodial activity of pantothenamides (Figures 5 and 6) and thereby masks their potency. We
Pantothenamides Are Potent Antiplasmodial Agents

Figure 7. Hydrolysis of a pantothenamide in the presence of recombinant human pantetheinase and Albumax II. The time-courses show the concentration of isobutylamine (a product of compound 12 hydrolysis) detected during incubation of compound 12 with recombinant human pantetheinase (100 ng/mL; closed circles), Albumax II (0.6%, w/v; grey circles), or an equivalent volume of water (open circles), and during incubation of Albumax II (0.6%, w/v) in the absence of compound 12 (grey squares). At each time-point, the amount of primary amine was measured using a fluorescamine-based fluorescence assay. Fluorescence measurements were converted to isobutylamine concentrations using a standard curve generated using isobutylamine samples of known concentration that had been processed in the same manner as the test samples. The data are from three or four independent experiments, each performed in duplicate, and error bars represent SEM. For clarity, the time-courses represented by the open circles are shown with negative error bars only, and the time-courses represented by the grey squares are shown with positive error bars only. Where not shown, error bars are smaller than the symbol.

doi:10.1371/journal.pone.0054974.g007

Figure 8. Effect of pantetheinase supplementation on the potency of a pantothenamide in aged Albumax-complete RPMI. The concentration-response curves show the effect of increasing concentrations of compound 12 on proliferation of P. falciparum parasites cultured (for 96 h) in Albumax-complete RPMI as measured using the SYBR Green I-based growth assay. Assays were performed using (i) Albumax-complete RPMI incubated immediately after preparation at 37°C for 40 h (aged; closed circles); (ii) Aged Albumax-complete RPMI supplemented with recombinant human pantetheinase (100 ng/mL) immediately prior to the assay (aged+pantetheinase; open circles); (iii) aged Albumax-complete RPMI supplemented with recombinant human pantetheinase (100 ng/mL) and heated at 37°C for 40 h immediately prior to the assay (aged+pantetheinase+heat; grey circles); and (iv) Aged Albumax-complete RPMI heated at 37°C for 40 h before being supplemented with recombinant human pantetheinase (100 ng/mL) immediately prior to the assay (aged+heat+pantetheinase; grey squares). The data are from three independent experiments, each performed in triplicate, and error bars represent SEM. For clarity, the time-courses represented by the open circles are shown with negative error bars only, and the time-courses represented by the grey squares are shown with positive error bars only. Where not shown, error bars are smaller than the symbol.

doi:10.1371/journal.pone.0054974.g008

Five pantothenamides were found to inhibit growth of P. falciparum in aged culture medium with sub-micromolar IC50 values; for one pantothenamide (compound 3), an IC50 value as low as 20 nM was determined (Figure 2). Furthermore, the finding that the potency of at least one pantothenamide (compound 12) was greater in medium incubated for 80 h than in medium pre-incubated for 40 h (the standard incubation period employed in this study to “age” medium; Figure 5) is consistent with there being residual pantetheinase activity in medium pre-incubated for 40 h, and indicates that a maximum pantothenamide potency has not been reached in this medium. To determine the maximum (true) potency of pantetheinamides, it will be important to test the pantetheinamides under conditions where pantetheinase-mediated degradation cannot take place. This, however, is not a trivial task. Serum albumin is required for growth of P. falciparum [38,39] and so to achieve strictly pantetheinase-free conditions, an albumin preparation from which all pantetheinase activity has been removed is needed. Whether there is pantetheinase activity associated with preparations of human erythrocytes (required for growth of erythrocytic-stage P. falciparum) will also need to be

demonstrate this factor to be pantetheinase, an enzyme that hydrolyzes pantetheine to form pantetheine and cysteamine. This conclusion is based on several findings: (i) that recombinant human pantetheinase (Vanin-1) also mediates hydrolysis of an antiplasmodial pantothenamide (compound 12), a finding consistent with earlier reports of secondary amides of pantethenate other than pantetheine serving as pantetheinase substrates [35,36,37]; (ii) that the pantetheinamide is hydrolyzed in the presence of Albumax II (Figure 7), consistent with Albumax II, like serum, being a source of pantetheinase activity; and (iii) that the antiplasmodial potency of the pantetheinamide is reduced in the presence of recombinant human pantetheinase and that this attenuating effect is alleviated by incubating the pantetheinase-supplemented culture medium at 37°C (Figure 8). Taken together these results provide strong evidence that in fresh medium, pantetheinase-mediated pantetheinamide degradation masks the antiplasmodial potency of pantetheinamides. Moreover, we show that in aged culture medium, pantetheinamides inhibit growth of P. falciparum with potency unparalleled by antiplasmodial pantethenate analogues identified previously.
determined. Based on homology searches conducted using the human pantetheinase sequences, the parasite genome appears devoid of putative pantetheinase-encoding genes. This observation, and the finding that the parasite is sensitive to pantothena-
mide-mediated inhibition, suggests that the parasite is incapable of pantetheinase (and pantothenede) hydrolase. An alternative
strategy for determining the true potency of pantothamides is to test them in the presence of known pantetheinase inhibitors [36]. However, as some pantetheinase inhibitors are also reported to inhibit parasite growth [e.g. (40,41)] (presumably via non-pantetheinase related mechanisms; e.g. [42]), such a strategy may prove problematic.

The observed attenuating effect of pantotenone on the antimalarial activity of pantothamides in aged medium is consistent with the growth inhibition resulting from inhibition of pantotenone and/or CoA utilization, whether it be via inhibition of (i) the uptake of pantotenone; (ii) the biosynthetic conversion of pantotenone to CoA; or (iii) CoA-utilizing enzymes (by pantothamid-derived CoA analogues). Notably, this is the first report of compounds that inhibit growth of P. falciparum at sub-micromolar concentrations by such a mechanism and, as such, this study provides important further validation of pantotenone and CoA utilization as potential antimalarial drug targets. Addition of 100 μM pantotenone was observed to alleviate growth inhibition in the presence of pantothamides to a much lesser extent than in aged medium (Figure 4). To reconcile this finding, it is important to consider that one of the products of pantothamide hydrolase is panK. For this reason, where pantothamide hydrolase has occurred, the pantotenone concentration will have increased from the initial 1 μM present in the medium. This will not only attenuate the antimalarial effect of the pantothamides, but will mean that addition of 100 μM pantotenone will increase the pantotenone concentration less than 100-fold. We did investigate the possibility that the antimalarial effect observed in fresh medium in the presence of pantothamides was due to the primary amines generated upon pantothamide hydrolase, however, at least for the amines tested (those that should result from hydrolisis of compounds 12, 17 and 19), little-to-no inhibition was observed at a concentration of 200 μM (data not shown).

We showed that all 22 pantothamides tested inhibit P. falciparum PanK-catalysed pantotenone phosphorylation, consistent with the pantothamides either inhibiting or serving as alternate substrates of P. falciparum PanK. From the data presented it is not, however, possible to discriminate between these two possibilities. It has been shown previously that P. falciparum PanK accepts pantothenol as a substrate for phosphorylation [43]. If pantothamides also serve as P. falciparum PanK substrates, it should be investigated whether the resultant 4'-phosphopantethamides are metabolized by P. falciparum CoA biosynthesis enzymes downstream of PanK, as they are by bacterial CoA levels downstream of PanK, as they are by bacterial CoA biosynthesis enzymes [17], and whether this has an effect on CoA levels in the parasite. Inhibition of fatty acid biosynthesis (as a result of inactivation of acyl carrier protein with pantothamide derived CoA analogues) has been implicated as a primary cause of pantothamide toxicity in E. coli [19]. As fatty acid biosynthesis is not required during the pantothamide-susceptible erythrocytic stage of P. falciparum development [44,43] it is unlikely that the key target of pantothamides in P. falciparum is fatty acid synthesis.

For the SAR generated in this study (Figure 2) to inform future pantothamide design, it will be important to determine the extent to which they reflect (i) relative efficacy against the target, (ii) relative cell permeabilities (and/or susceptibility to efflux), (iii) relative rates of pantetheinase-mediated hydrolysis, and (iv) inactivation by other mechanisms including serum binding. In light of the demonstration in this study that pantothamides are hydrolyzed by serum pantetheinase in vitro, it is likely that they will also be subject to pantetheinase-mediated hydrolysis in vivo, and thereby rendered ineffective as antimalarial agents in vivo. Consistent with this, compound 12 had little-to-no effect on parasite growth at concentrations up to 200 μM in the presence of human serum (Figure 6A). Therefore to exploit the antimalarial potency of pantothamides it will be important to consider strategies for circumventing pantetheinase-mediated hydrolysis in vivo. This is also crucial for the future development of pantothamides as antibacterial agents, as serum stability will be required for all but topical applications. One strategy is to develop antimalarial (or antibacterial) pantothamide analogues that are resistant to degradation by pantetheinases by, for example, using a biospecific replacement strategy [46,47] to replace the key hydrolyzable amide bond. Another strategy for circumventing pantetheinase-mediated pantothamide hydrolysis in vivo is to simultaneously inhibit host pantetheinase. Recently, however, genetic studies in mice have provided evidence that a reduction in pantetheinase activity increases susceptibility to malaria, perhaps as a result of modulation of the inflammatory response [48]. The design of pantetheinase-resistant pantothamides may therefore be a preferable strategy for circumventing pantetheinase-mediated degradation.

In conclusion, in this study we present, for the first time, analogues of pantotenone that inhibit growth of P. falciparum at sub-micromolar concentrations through inhibition of pantotenone and/or CoA utilization, and propose the identification of pantetheinase-resistant pantothamide analogues as a viable strategy for the discovery of antimalarial agents.

Acknowledgments

We are grateful to the Canberra Branch of the Australian Red Cross Blood Service for the provision of red blood cells and serum. We thank Leanne Barnard for assistance in the preparation of additional supplies of compound 12.

Author Contributions

Interpreted data and discussed experimental design: CS CM ZLG REL ES KJS. Conceived and designed the experiments: CS CM ES KJS. Performed the experiments: CS CM ZL KJS. Analyzed the data: CS CM ZL KJS. Contributed reagents/materials/analysis tools: KGV REL ES KJS. Interpreted data and discussed experimental design: CS CM ES KJS. Contributed reagents/materials/analysis tools: KGV REL ES KJS. Analyzed the data: CS CM ZL KJS. Wrote the paper: CS ES KJS.

References


