Synthesis of Gallinamide A Analogues as Potent Falcipain Inhibitors and Antimalarials

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ABSTRACT: Analogues of the natural product gallinamide A were prepared to elucidate novel inhibitors of the falcipain cysteine proteases. Analogues exhibited potent inhibition of falcipain-2 (FP-2) and falcipain-3 (FP-3) and of the development of Plasmodium falciparum in vitro. Several compounds were equipotent to chloroquine as inhibitors of the 3D7 strain of P. falciparum and maintained potent activity against the chloroquine-resistant Dd2 parasite. These compounds serve as promising leads for the development of novel antimalarial agents.

INTRODUCTION

Malaria, a mosquito-borne disease caused by infection with Plasmodium parasites, is the world's most deadly parasitic infection. Almost half the world's population live in malaria endemic areas, and an estimated 1.2 billion people are at high risk of contracting the disease. This has resulted in hundreds of millions of P. falciparum infections each year, causing hundreds of thousands of deaths, primarily in children. Nearly all of these deaths are caused by Plasmodium falciparum, the most virulent human malaria parasite. Unfortunately, the introduction of a highly effective vaccine against malaria has remained elusive and, as a consequence, chemotherapy remains central to control and treatment. Natural products and their derivatives, including quinine, chloroquine (CQ) and artemisinin and its analogues, have led the way as antimalarial drugs used clinically. However, the control of malaria has been severely compromised in recent years by the widespread resistance of P. falciparum to nearly all frontline therapeutics used for both prophylaxis and treatment. Of growing concern is recently discovered resistance to components of artemisinin-based combination therapies, the cornerstone of treatment of falciparum malaria. Consequently, there is an urgent need for the development of new antimalarials that are structurally distinct from existing drugs and operate through novel mechanisms of action.

We have an interest in utilizing the privileged biological activity of natural products to elucidate new antimalarial drug leads. In this area we have recently reported the efficient total synthesis and stereochemical assignment of the N-terminal isoleucine residue of gallinamide A (also known as symplostatin 4) a depsipeptide natural product that has been isolated independently from a Schizothrix species of cyanobacteria from the Caribbean Coast of Panama and from the Symploca genus in Key Largo, Florida (Figure 1). Through our synthetic efforts we have demonstrated that the natural product exhibits potent activity in vitro against cultured P. falciparum, with an IC50 of 50 nM. Importantly, gallinamide A did not exhibit hemolytic activity against red blood cells, did not inhibit the proteosome and displayed weak or no detectable activity against mammalian Vero cells, NCI-H460 lung tumor cells, or neuro-2a mouse neuroblastoma cell lines.

Figure 1. Structure of gallinamide A and inhibitory activity against P. falciparum and the falcipains (FPs).

Recently, the putative mode of antimalarial action of gallinamide A has been revealed in a study by Tolke et al. Specifically, the natural product has been shown to inhibit a group of cysteine proteases found in the food vacuole of the parasite, known as the falcipains (FPs). P. falciparum is known to possess four falcipains, FP1, FP2, FP2' and FP3, with the last three located in the food vacuole of erythrocytic parasites. All three food vacuole-associated FPs (FP2, FP2' and FP3) were inhibited by gallinamide A at low to mid-nanomolar concentrations. The food vacuole FPs are required for the degradation of hemoglobin and are essential for growth and survival of the organism. Treatment with cultured P. falciparum with gallinamide A leads to swelling of the food vacuole, which fills with undegraded hemoglobin; if not halted, falcipain inhibition leads to parasite death. Over the past decade several classes of FP inhibitors have been developed, some of which have shown efficacy in vivo models of malaria, but no falcipain inhibitors have yet progressed into human clinical trials. Gallinamide A possesses a number of unique structural fea-
structures, including a dimethyl-terminated aliphatic depsipeptide backbone, an unusual 4(α)-amino-2(β)-pentenoyl moiety and a C-terminal N-acylpromeline unit. Based on these features, it is likely that gallinamide A is a covalent, irreversible inhibitor of the FPs via nucleophilic attack by the sulfhydryl side chain of the active site cysteine of the FPs onto one of the two Michael acceptor moieties of the natural product. This hypothesis is supported by a recent study showing that gallinamide A potently inhibits cathepsin L through a covalent, irreversible mechanism. Given the potent anti-plasmodial activity of gallinamide A, coupled with its general lack of toxicity against human cell lines, we envisaged the development of structurally unique gallinamide A analogues as inhibitors of the food vacuole FPs, which may serve as antimalarial drug leads. We proposed that the C-terminal pyrrolinone moiety and N-terminal region of the natural product would be amenable to significant structural change, thus providing scope for dramatic alteration and simplification of the structure to provide the first structure-activity data for this class of natural products.

RESULTS AND DISCUSSION

We were first interested in assessing whether one or both of the olefinic moieties in gallinamide A were crucial for inhibitory activity by serving as Michael acceptors for the active site Cys residue of the FPs. Thus we initially designed and synthesized four analogues of gallinamide A with varied degrees of saturation. This included compound 1 that is structurally identical to gallinamide A, except that the native ester linkage in the depsipeptide natural product had been replaced with an amide bond. We envisioned that this linkage could be formed en bloc from commercially available amino acids, negating the use of synthetically challenging preformed amide building blocks (as was required for the total synthesis of the natural product). The other modification was the incorporation of a dimethylated value (Val) residue, which we had previously shown to be an excellent replacement for the N-terminal dimethylated isoleucine (Ile) moiety in the natural product (see Supporting Information). The three other proposed analogues were 2, where the methoxy-enol moiety in the pyrrolidine ring of 1 was reduced, 3 where the olefinic component of the 4(α)-amino-2(β)-pentenoyl acid unit was reduced, and the completely saturated analogue 4. Preparation of the proposed analogues began with the synthesis of N-terminal fragment 5 via Fmoc-strategy SPPS. 2-Chloro-Trt-Cl resin (6) was first loaded with Fmoc-Leu-OH followed by coupling of Fmoc-Leu-OH and Fmoc-Val-OH. On-resin reductive amination followed by cleavage from the resin using hexafluoroisopropanol (HFIP) provided tripeptide 5 in excellent yield. From here, 5 was coupled to imide fragments 7 and 8, which were prepared using a similar protocol to that adopted for the total synthesis of gallinamide A (see Supporting Information for synthetic details). The coupling was carried out using HATU at low temperature to minimize epimerization and afforded analogues 1 and 2, primarily as single diastereoisomers, in good yields. At this stage 1 and 2 were subjected to hydrogenation to provide 3 and 4 in 85% and 89% yields, respectively, following HPLC purification, which also enabled separation of the diastereoisomers of these compounds.

Having prepared the four target gallinamide A analogues, the compounds were next screened against FP-2 and FP-3 using a fluorescence-based kinetic assay. The compounds were also screened against the chloroquine sensitive 3D7 strain of P. falciparum using a [3H]-hypoxanthine incorporation assay (Table 1). Gallinamide A analogue 1 exhibited potent inhibitory activity against FP-2 (IC50 = 6.78 nM), FP-3 (IC50 = 392 nM) and P. falciparum in vitro (IC50 = 89.3 nM). Interestingly, while 1 was equipotent to gallinamide A against FP-2, the compound exhibited 10-fold weaker inhibition against FP-3 and an almost 2-fold drop in activity against P. falciparum. Reduction of the enol moiety in the acyl-pyrrolinone unit in 2 led to a slight improvement in activity against FP-2 and FP-3, but a two-fold reduction in anti-plasmodial activity (IC50 = 210 nM). In contrast, reduction of the olefin in the α,β-unsaturated imide moiety had a dramatic effect on inhibitory activity. Specifically, analogue 3 exhibited a three orders of magnitude drop in inhibitory potency against FP-2 (IC50 = 3710 nM) and demonstrated no measurable inhibition of FP-3. This compound also showed a marked reduction in activity against P. falciparum. Removal of both olefinic moieties in analogue 4 led to a loss of measurable inhibitory activity against both the FPs and the parasite. In addition, when P. falciparum trophozoites were treated with compounds 1 and 2, both caused swollen food vacuole morphology, a hallmark of FP inhibition (see Supporting Information). In contrast, compounds 3 and 4 (that were inactive against the FPs) did not cause swelling of food vacuoles in the parasite. Taken together, these studies strongly suggest that the olefinic function within the α,β-unsaturated imide moiety is critical for inhibitory activity against both the FPs and against P. falciparum. This indicates the site of diastereoisomeric in 1 and 2.

Table 1: Inhibition of FP-2, FP-3 and P. falciparum by gallinamide A analogues 1-4. (errors are standard error of the mean of three experiments)

<table>
<thead>
<tr>
<th>Analogue</th>
<th>IC50 FP-2 (nM)</th>
<th>IC50 FP-3 (nM)</th>
<th>IC50 P. falciparum (3D7) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.78 ± 0.44</td>
<td>392 ± 1.3</td>
<td>89.3 ± 73</td>
</tr>
<tr>
<td>2</td>
<td>3.81 ± 0.39</td>
<td>163 ± 21</td>
<td>210 ± 80</td>
</tr>
<tr>
<td>3</td>
<td>3710 ± 420</td>
<td>&gt;50000</td>
<td>4375 ± 690</td>
</tr>
</tbody>
</table>

Reagents and conditions: (i) resin loading: Fmoc-Ile-OH, (Pr3EN), DMF/CHCl3, (ii) Fmoc-SPPS (deprotection: 10vol.% piperidine/DMF; coupling: 4 equiv Fmoc-AA-OH, 4 equiv PyBOP, S equiv NMM, DMF, capping: 10vol.% Ac2O/pyridine), (iii) HCHO, NaBH(OAc)2, AcOH, DMF, (iv) 30vol.% HFIP/CHCl3, (v) HATU, NMM, 0°C for 30 min, rt for 2 h, (vi) H2, Pd/C, MeOH.
Having established the importance of unsaturation in the 4(\text{S})-amino-2-(\text{C})-pentenoyl unit for FP inhibition and antiparasitic activity, we next explored a small library of gallinamide A analogues (\text{9-16}) as second generation inhibitors (Scheme 2). The major modification made to these analogues was substitution of the C-terminal N-acetylpyrrolinone moiety in gallinamide A. Specifically, we proposed that derivatization of this scaffold at the C-terminus (using simple amides to facilitate more rapid syntheses, R1 in Scheme 2A), would enable investigation of structure-activity relationships for this region of the molecule. Introduction of these modifications also enabled the rapid construction of analogues primarily via solid-phase synthesis, without the need for numerous solution-phase fragment condensation and purification steps that were necessary for the synthesis of the natural product (and of 1-4).

It should noted that, whilst Slocombe et al. reported that replacement of the C-terminal methoxy-enol moiety in gallinamide A with a C-terminal alanine methyl ester moiety significantly decreased activity against the FPs and \textit{P. falciparum},\textsuperscript{15} we were interested in accessing compounds that possessed greater C-terminal functionality, so as to explore the effect of modifying the acyl-pyrrolinone on FP activity and \textit{P. falciparum} inhibition. We were encouraged by the fact that reduction of the methoxyl-enol moiety in analogue 2 did not have a dramatic effect on FP and \textit{P. falciparum} inhibitory activity, suggesting that modifications in this region may be tolerated. A number of other changes were also proposed for the N-terminal region, including a range of aliphatic amino acids at the N-terminus (R2 in Scheme 2A). Analogues \text{15 and 16}, extended by one L-Ile residue in the peptide backbone compared to \text{9-14}, were also proposed in order to probe the importance of the length of the analogues for inhibition of the FPs and \textit{P. falciparum} (Scheme 2B).

\textbf{Synthesis of 9-14} began from 2-Cl-Trt Cl resin preloaded with Fmoc-Ala (17). Coupling of Fmoc-protected \textit{\alpha,\beta}-unsaturated amino acid 18 (see Supporting Information for synthesis) followed by elongation via standard Fmoc-strategy SPPS provided the desired resin-bound peptide sequences. Following an en bloc reductive methylation of the N-terminus with formaldehyde and sodium cyanoborohydride, cleavage of the peptides from the resin using HFIP provided the C-terminal peptide acids in moderate to good yields over the ten resin-bound steps following HPLC purification (36-85\%, see Supporting Information for full synthetic details and yields). Having assembled the C-terminal peptide acids, we next installed the C-terminal functionality. Benzylamine was coupled using PyBOP at low temperature and, following purification by reverse-phase HPLC, the desired inhibitors \text{9, 11, 13, 14 and 15} were isolated in excellent yields based on the original resin loading without significant epimerization. 4-(\text{R})-Hydroxy-L-proline methyl ester was also coupled to the C-terminus of two peptides, this time with the addition of NMM as a hindered base, to afford inhibitors \text{10 and 16} in excellent yields following HPLC purification. On this occasion the compounds were isolated as close to 1:1 mixtures of diastereoisomers, reflecting the slower coupling rate of hydroxy-proline methyl ester to the C-terminus of the peptides. Finally, amido-thiazole was coupled to the C-terminus of one peptide acid using PyBOP at low temperature, which provided \text{12} as a 1:1 mixture of diastereoisomers in 62\% yield following purification, based on the original loading of the resin.

\textbf{Scheme 2.} A) \textit{Synthesis of gallinamide A analogues 9-14} and B) \textit{Synthesis of extended analogues 15 and 16} via a solid-phase synthesis approach. NB: compounds 9-16 were isolated as the trifluoroacetate salts.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Analogue & R2 & R1 & Yield (based on original resin loading) \\
\hline
9 & \text{H} & \text{H} & 40\% (5:1 \text{d.r.}) \\
10 & \text{H} & \text{H} & 33\% (1:1 \text{d.r.}) \\
11 & \text{H} & \text{H} & 65\% (7:1 \text{d.r.}) \\
12 & \text{H} & \text{H} & 62\% (1:1 \text{d.r.}) \\
13 & \text{H} & \text{H} & 78\% (6:1 \text{d.r.}) \\
14 & \text{H} & \text{H} & 75\% (5:1 \text{d.r.}) \\
\hline
\end{tabular}
\end{table}

Reagents and conditions: (i) 4 equiv. 18, 4 equiv. PyBOP, 6 equiv. NMM, DMF; (ii) Fmoc SPPS (deprotection: 10\% piperidine/DMF, coupling: 4 equiv. Fmoc-AA-OH, 4 equiv. PyBOP, 8 equiv. NMM, DMF, capping: 10\% AcO/Oxypyrrolidine); (iii) HCHO, NaBH(OAc)\textsubscript{3}, AcOH, DMF; (iv) 30\% HFIP/DCM, (v) R-\text{NH}\textsubscript{2}, PyBOP, DMF, 0 \textdegree C; NMM was added to the coupling reactions for the preparation of inhibitors 10 and 16.* indicates the site of diastereoseomism in 9-16.

\textbf{Table 2: Inhibition of FP-2, FP-3 and the 3D7 strain of \textit{P. falciparum} by gallinamide A analogues 9-16.} (errors are standard error of the mean of three experiments).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Analogue & \text{IC}\textsubscript{50}FP-2 [\mu M] & \text{IC}\textsubscript{50}FP-3 [\mu M] & \text{IC}\textsubscript{50} \textit{P. falciparum} (3D7) [\mu M] \\
\hline
9 & 10.5 \pm 0.64 & >25 & 0.54 \pm 0.21 \\
10 & 7.66 \pm 0.19 & >50 & 1.90 \pm 0.62 \\
11 & 11.5 \pm 0.19 & >25 & 0.32 \pm 0.01 \\
12 & 2.48 \pm 0.15 & 33.9 \pm 0.46 & 1.10 \pm 0.80 \\
13 & 3.44 \pm 0.39 & >25 & 0.54 \pm 0.38 \\
14 & 6.00 \pm 1.56 & 46.2 \pm 5.35 & 6.60 \pm 3.80 \\
15 & >50 & >50 & >50 \\
16 & >50 & >50 & >50 \\
\hline
\end{tabular}
\end{table}
The gallinamide A analogues 9-16 were next screened for inhibitory activity against FP-2, FP-3 and the 3D7 strain of *P. falciparum* in *vitro* (Table 2). All analogues possessed significant activity, and all led to the swollen food vacuole morphology in trophozoites (see Supporting Information). However, in general the replacement of the N-acyl pyrrolinone moiety in gallinamide A (and analogue 1) with different C-terminal groups was detrimental to activity against both the FPs and the parasite. Analogues 9, 11 and 13, bearing a C-terminal benzylamide moiety, all exhibited similar activity; low micromolar inhibition of FP-2 (IC\(_{50}\) = 3.44-11.5 \(\mu\)M), no measurable inhibition of FP-3 at 25 \(\mu\)M and nanomolar inhibitory activity against *P. falciparum*.

Interestingly, introduction of an N-methylproline functionality at the N-terminus of the peptide, while retaining the C-terminal benzylamide in 14, led to inhibition of both FP-2 (IC\(_{50}\) = 6.00 \(\mu\)M) and FP-3 (IC\(_{50}\) = 46.2 \(\mu\)M) but exhibited less potent antiparasitic activity (IC\(_{50}\) = 66.0 \(\mu\)M). The loss of activity was particularly striking for 11, which possesses an identical structure to analogue 1 (Table 1, IC\(_{50}\) FP-2 = 6.78 nM, IC\(_{50}\) FP-3 = 292 nM, IC\(_{50}\) 3D7 9.3 nM). Interestingly, the C-terminal acylpyrrolinone functionality at the N-terminus of the peptide, while retaining the C-terminal benzylamide moiety, has been replaced by a C-terminal benzylamide. The three orders of magnitude drop in activity against the FPs and order of magnitude decrease in antiparasitic activity from 1 to 11 suggests that the N-acyl pyrrolinone unit is important for activity. Introduction of a more highly functionalized and flexible hydroxyproline methyl ester to the C-terminus in 10 provided similar inhibitory activity to the C-acyl pyrrolinone unit has been used in the synthesis of Meldrum’s acid to amino acids 31-34 et al.

The synthesis of second generation gallinamide A analogues 19-24 was proposed possessing the identical peptide backbone to 1 and 2 but with variation in the side chain on the pyrrolinone unit and in the substitution of the enol of the pyrrolinone (Scheme 3). The synthesis of 19-24 began with the preparation of the requisite pyrrolinones 25-30 from commercially available Fmoc-protected amino acids 31-34 et al. Coupling of Meldum’s acid to amino acids 31-34 et al. in the presence of DMAP followed by reflux of the Meldum’s acid in ethyl acetate to effect cyclization-condensation provided the corresponding Fmoc-protected pyrrolinones 35-38 in good yield over the two steps. These were each reacted with methanol under Mitsunobu conditions using DIAD and triphenylphosphine to provide O-methylated pyrrolinones 39-42 in 48-79% yield. In addition, 35 and 36 were treated with benzyl alcohol under the same conditions to provide 43 and 44 et al. All that remained for the synthesis of the target pyrrolinones was removal of the Fmoc group, which was effected smoothly by treatment with piperidine in acetonitrile to provide 45-51 et al.

The synthesis of the second generation of gallinamide A analogues 26-31 was achieved by the literature procedures. Reagents and conditions: (i) 1 M Meldum’s acid, EDC, DMAP, **CH**\(_2\)\(_2\)NO, 0 °C to rt; (ii) EtOAc, 77 °C; (iii) 25: R1 = CH(CH\(_3\))2, R2 = CH3 (79%); 26: R1 = CH(CH\(_3\))2, R2 = CH2-1-OH-Ph (77%); 27: R1 = CH2-4-OH-Ph, R2 = CH3 (81%); 28: R1 = H, R2 = CH2-4-OH-Ph (92%); 29: R1 = CH(CH\(_3\))2, R2 = CH2-1-OH-Ph (65%); 30: R1 = CH(CH\(_3\))2, R2 = CH2-1-OH-Ph (52% over 2 steps)

**Scheme 3.** Synthesis of second generation gallinamide A analogues 26-31. Reagents and conditions: (i) 1 M Meldum’s acid, EDC, DMAP, **CH**\(_2\)\(_2\)NO, 0 °C to rt; (ii) EtOAc, 77 °C; (iii) 25: R1 = CH(CH\(_3\))2, R2 = CH3 (79%); 26: R1 = CH(CH\(_3\))2, R2 = CH2-1-OH-Ph (77%); 27: R1 = CH2-4-OH-Ph, R2 = CH3 (81%); 28: R1 = H, R2 = CH2-4-OH-Ph (92%); 29: R1 = CH(CH\(_3\))2, R2 = CH2-1-OH-Ph (65%); 30: R1 = CH(CH\(_3\))2, R2 = CH2-1-OH-Ph (52% over 2 steps)
Having elucidated a number of gallinamide A analogues as potent FP and *P. falciparum* inhibitors, we were next interested in investigating whether selected compounds were capable of maintaining activity against a CQ-resistant (Dd2) strain of *P. falciparum* and whether the compounds exhibited selective killing of parasites over human cells by screening against a HEK298 cell line (Table 4). Finally, we were interested in investigating the selectivity of the compounds in inhibiting the FPs over other parasitic proteases. To this end, the compounds were screened against three aminopeptidase (AP) enzymes from *P. falciparum*, namely, AP M1, AP M17 and AP M18. The compounds selected included the potent N-acetylpyrrolinones-containing analogues 1 and 19-22 as well as the C-terminal amide derivatives, 9, 11 and 13, which exhibited IC\(_{50}\) values < 600 nM against the 3D7 strain of *P. falciparum* (see Table 2). All of the tested compounds (1, 9, 11, 13, 19-22) exhibited potent inhibition of the CQ-resistant Dd2 strain of *P. falciparum* (IC\(_{50}\) = 29.0-421 nM). Despite exhibiting potent inhibitory activity against human cathepsin D, the compounds were selective inhibitors of *P. falciparum* over HEK298 cells, with 9, 11 and 13, showing no measurable inhibition of this cell line at a concentration of 50 nM (see Supporting Information). In addition, none of the analogues displayed any inhibitory activity against the metalloproteases AP M1, AP M17 and AP M18 from *P. falciparum* (see Supporting Information). Taken together, these data suggest that structural analogues of gallinamide A are promising leads for the pursuit of potent food vacuole FP inhibitors as antimalarial compounds.

**Table 4: Inhibition of the CQ-resistant Dd2 strain of *P. falciparum*, HEK298 cells and *P. falciparum* aminopeptidase M1, M17 and M18 (AP M1, M17 and M18) by gallinamide A analogues.** *(errors are standard error of the mean of three experiments)*

<table>
<thead>
<tr>
<th>Analogue</th>
<th>IC(_{50}) <em>P. falciparum</em> (Dd2) [nM]</th>
<th>IC(_{50}) HEK298 [nM]</th>
<th>IC(_{50}) AP M1, M17 and M18 [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.76 ± 0.30</td>
<td>14.00 ± 0.30</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>9</td>
<td>421 ± 152</td>
<td>&gt;50 000</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>11</td>
<td>378 ± 20</td>
<td>&gt;50 000</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>13</td>
<td>170 ± 68</td>
<td>&gt;50 000</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>19</td>
<td>419 ± 185</td>
<td>16.30 ± 170</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>20</td>
<td>165 ± 68</td>
<td>96.50 ± 1480</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>21</td>
<td>67.0 ± 30</td>
<td>18.90 ± 110</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>22</td>
<td>39.0 ± 16.0</td>
<td>8300 ± 54</td>
<td>&gt;10 000</td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

In summary, a number of potent new inhibitors of the food vacuole FPs, FP-2 and FP-3, were discovered based on the structure of the cyanobacterium-derived natural product gallinamide A. The importance of the α,β-unsaturated imide moiety of the natural product for inhibitory activity was initially demonstrated through the synthesis of selectively reduced analogues and through the synthesis and evaluation of several derivatives bearing a C-
terminal amide in place of the imide functionality found in the natural product. A number of potent inhibitors of FP-2 and FP-3 were also identified through variation of the side chain on the pyrroli- dinone ring. Several of these compounds also demonstrated potent inhibition of the CQ-sensitive 3D7 strain of P. falciparum, with a number of these proving similarly potent to CQ. Gratifyingly, these analogues maintained potent activity against the CQ-resistant D2 strain of P. falciparum and did not possess noteworthy toxicity to HEK293 cells. These compounds serve as promising leads for the development of second generation natural-product-based FP inhibitors for the discovery of potential antimalarials, as will be the focus of ongoing research in our laboratories.

EXPERIMENTAL SECTION

Final inhibitors 19–24 were synthesized as detailed in the representative example for compound 20 below. The purity of all final compounds was determined to be ≥95% by NMR and HPLC-MS analysis. General methods, full experimental details and original NMR spectra for all analogues can be found in the Supporting Information.

**Mc-Val-Leu-Leu-Apa-pyVal-OMe.TFA (20)** In vitro (40 mg, 57 µmol), was dissolved in 1:1 v/v TFA/DCM (2 mL) and cooled to 0 °C. N,N-dimethylaminopropyli (5 (46.5 mg, 95 µmol) and HATU (36.1 mg, 95 µmol) were added, followed by NMM (21 µL, 190 µmol), and the reaction was stirred for 20 min at 0 °C, then 2 h at room temperature. The reaction was subsequently quenched with TFA (20 µL), diluted with 1:1 v/v MeCN/H2O (4 mL) and purified by preparative reverse phase HPLC (gradient: 0-60% MeCN over 40 min) to afford 20 as a white amorphous solid and a single diastereomer (27% yield). R: [D-100% MeCN over 30 min] = 20.1 mm; [i,µ] = 30.2 (c = 0.4, MeOH); IR (thin film) νmax = 3295, 3073, 2963, 1725, 1670, 1646, 1625, 1550, 1464, 1350 cm−1; 1H NMR (500 MHz, CDCl3) δ 8.95 (d, 1H, J = 8.5 Hz, CH), 8.37 (d, 1H, J = 8.5 Hz, CH), 3.84 (3H, CH3), 3.00 (3H, NCH3), 2.86 (3H, CH3), 2.54 (1H, CH), 2.26 (1H, CH), 1.71-1.53 (6H, 2 x CH2, 2 x CH), 1.26 (3H, J = 7.0 Hz, CH2), 1.12-1.07 (6H, 2 x CH2, 2 x CH), 0.93-0.82 (15H, 5 x CH3), 0.73 (3H, J = 6.5 Hz, CH3). C29H39N5O6S2 (549.746) [M+Na]+, 100%; HRMS Calcd for C29H39N5O6S2Na 571.8109; Found 571.8102.

**Boc-Apa-pyVal-Ome.TFA** (47) To a solution of amino acid 46 (101 mg, 396 µmol) in DMF (2 mL) at 0 °C was added pentafluorophenyl trifluoroacetate (89 µL, 0.51 mmol), followed by pyridine (32 µL, 366 µmol) and the reaction was allowed to warm to room temperature. The reaction was subsequently stirred for 1 h before diluting with 1:1 v/v EtO/EtOAc (20 mL) and washing with 0.2 M aqueous HCl (5 mL), saturated aqueous NaHCO3 solution (5 mL) and brine. The organic phase was then dried (MgSO4), before concentrating in vacuo to afford the pentafluorophenyl ester as a white amorphous solid (24 mg, 37%). R: [D-100% MeCN over 30 min] = 20.1 mm; [i,µ] = 30.2 (c = 0.4, MeOH); IR (thin film) νmax = 3343, 3102, 2971, 2934, 2877, 1719, 1673, 1621, 1515, 1455, 1363 cm−1; 1H NMR (500 MHz, CDCl3) δ 7.39 (dd, 1H, J = 15.5, 1.6 Hz, CH), 7.02 (dd, 1H, J = 15.4, 8.4 Hz, CH), 5.08 (1H, CH), 4.69-4.61 (2H, 2H, NCH2), 4.45 (1H, CH), 3.84 (3H, CH3), 2.55 (1H, CH), 1.44 (3H, 3 x CH3), 1.29 (1H, J = 7.2 Hz, CH). C13N5O4S2F5Na (555.994) [M+Na]+, 100%; HRMS Calcd for C13N5O4S2F5NaNa 577.8552; Found 577.8542.

ASSOCIATED CONTENT

**Supporting Information.** Experimental procedures and analytical data for all novel compounds including 1H and 13C NMR and analytical HPLC.

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All authors have given approval to the final version of the manuscript.

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**ABBREVIATIONS**

CQ, chloroquine; FP, falcipain; NMM, N-methylmorphone; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DIAD, diisopropyl azodicarboxylate; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DMAP, 4-dimethylaminoypyridine; HATU, 1-[bis(dimethylamino) methyl]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate.

**REFERENCES**

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Gallinamide A analogues

Falcipain 2: IC_{50} = 5.3 - 25 nM
Falcipain 3: IC_{50} = 67 - 250 nM
Plasmodium falciparum 3D7: IC_{50} = 9.7 - 96 nM
Plasmodium falciparum Dd2: IC_{50} = 29 - 420 nM
HEK293: IC_{50} = 8500 - >50,000 nM