Journal of Medicinal Chemistry

Brief Article

pubs.acs.org/jmc

# Synthesis of Gallinamide A Analogues as Potent Falcipain Inhibitors and Antimalarials

Trent Conroy,[†] Jin T. Guo,[‡] Nabiha Elias,[†] Katie M. Cergol,[†] Jiri Gut,[∥] Jennifer Legac,[∥] Lubna Khatoon,[‡] Yang Liu,[‡] Sheena McGowan,[§] Philip J. Rosenthal,[∥] Nicholas H. Hunt,[‡] and Richard J. Payne*[,†]

[†]School of Chemistry, The University of Sydney, Building F11, Sydney, New South Wales 2006, Australia

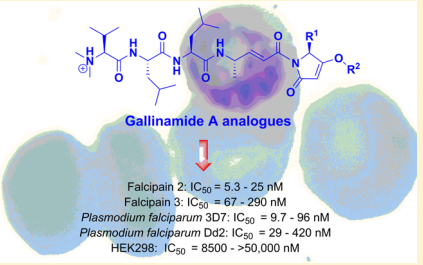
[‡]School of Medical Sciences, The University of Sydney, Sydney, New South Wales 2006, Australia

[§]Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria 3800, Australia

[∥]Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California 94143, United States

Supporting Information

**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.[1] 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.[2] This has resulted in hundreds of millions of *Plasmodium falciparum* infections each year, causing hundreds of thousands of deaths, primarily in children.[2] Nearly all of these deaths are caused by *P. falciparum*, the most virulent human malaria parasite.[2] Unfortunately, the introduction of a highly effective vaccine against malaria has remained elusive[3] and, as a consequence, chemotherapy remains central to control and treatment.[4] Natural products and their derivatives, including quinine, chloroquine (CQ), and artemisinin and its analogues, have led the way as antimalarial drugs used clinically.[5,6] 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.[7] Of growing concern is recently discovered resistance to components of artemisinin-based combination therapies,[8] the cornerstone of treatment of falciparum malaria.[9] 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.[10]

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[11] and stereochemical assignment of the N-terminal isoleucine residue[12] 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 Carribean Coast of Panama[13] and from the *Symploca* genus in Key Largo, Florida[14] (Figure 1). Through our synthetic efforts, we have demonstrated that the natural product exhibits potent activity in vitro against cultured *P. falciparum*, with an $IC_{50}$ of 50 nM. Importantly, gallinamide A did not exhibit hemolytic activity against red blood cells,[11] did not inhibit the proteasome, and displayed weak or no detectable activity against mammalian Vero cells, NCI-H460 lung tumor cells, or neuro-2a mouse neuroblastoma cell lines.[13,14]

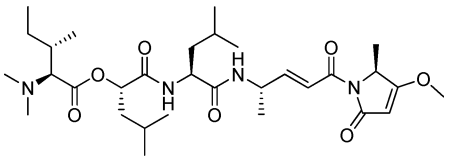



**Figure 1.** Structure of gallinamide A and inhibitory activity against *P. falciparum*[11] and the falcipains (FPs).[15]

Recently, the putative mode of antimalarial action of gallinamide A has been revealed in a study by Stolze et al.[15] Specifically, the natural product has been shown to inhibit a

**Received:** September 18, 2014

   dx.doi.org/10.1021/jm501439w | J. Med. Chem. XXXX, XXX, XXX−XXX

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.[16] 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.[16] Treatment of 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.[15,17−19] Over the past decade, several classes of FP inhibitors have been developed,[17,20−28] some of which have shown efficacy in in vivo models of malaria,[27,29] but no falcipain inhibitors have yet progressed into human clinical trials.[30]

Gallinamide A possesses a number of unique structural features, including a dimethyl-terminated aliphatic depsipeptide backbone, an unusual 4(*S*)-amino-2-(*E*)-pentenoyl moiety, and a C-terminal *N*-acylpyrrolinone unit. On the basis of 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.[31] Given the potent antiplasmodial 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 has 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 amino ester building blocks (as was required for the total synthesis of the natural product[11,12]). The other modification was the incorporation of a dimethylated valine (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 pyrrolinone ring of **1** was reduced, **3**, where the olefinic component of the 4(*S*)-amino-2-(*E*)-pentenoic acid unit was reduced, and analogue **4**, where both olefinic moieties had been reduced. Preparation of the proposed analogues began with the synthesis of N-terminal fragment **5** via Fmoc-strategy SPPS. 2-Cl-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.[26] The compounds were also screened against the chloroquine sensitive 3D7 strain of *P. falciparum* using a [$^3$H]-hypoxanthine incorporation assay (Table 1).[11,12,16] Gallinamide A analogue **1** exhibited potent

**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)**

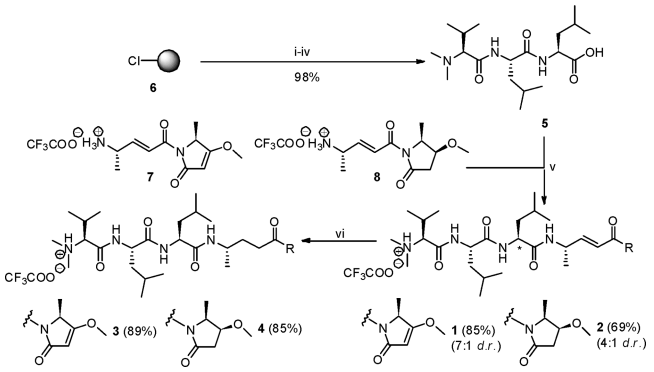
| analogue | $IC_{50}$ FP-2 [nM] | $IC_{50}$ FP-3 [nM] | $IC_{50}$ *P. falciparum* (3D7) [nM] |
|---|---|---|---|
| **1** | 6.78 ± 0.44 | 292 ± 1.3 | 89.3 ± 73 |
| **2** | 2.81 ± 0.39 | 163 ± 21 | 210 ± 80 |
| **3** | 3710 ± 420 | >50000 | 4375 ± 690 |
| **4** | >50000 | >50000 | >50000 |

inhibitory activity against FP-2 ($IC_{50}$ = 6.78 nM), FP-3 ($IC_{50}$ = 292 nM), and *P. falciparum* in vitro ($IC_{50}$ = 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 2-fold reduction in antiplasmodial activity ($IC_{50}$ = 210 nM). In contrast, reduction of the olefin in the $\alpha,\beta$-unsaturated imide moiety had a dramatic effect on inhibitory activity. Specifically, analogue **3** exhibited a 3 orders of magnitude drop in inhibitory potency against FP-2 ($IC_{50}$ = 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).[16,32] In contrast, compounds **3** and **4** (that were inactive against the FPs) did not cause swelling of food vacuoles in the parasite (Scheme 1). Taken together, these studies strongly suggest that the olefinic functionality within the $\alpha,\beta$-unsaturated imide moiety is critical for inhibitory activity against both the FPs and against *P. falciparum*.

Having established the importance of unsaturation in the 4(*S*)-amino-2-(*E*)-pentenoyl unit for FP inhibition and antiparasitic activity, we next explored a small library of gallinamide A analogues (**9**−**16**) as second-generation inhibitors (Scheme 2). The major modification made to these analogues was substitution of the C-terminal *N*-acylpyrrolinone moiety of gallinamide A. Specifically, we proposed that


dx.doi.org/10.1021/jm501439w | J. Med. Chem. XXXX, XXX, XXX−XXX

**Scheme 1. Synthesis of Gallinamide A Analogues 1−4**[a]



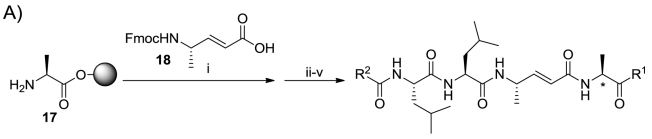

[a]Reagents and conditions: (i) resin loading: Fmoc-Leu-OH, $i$Pr$_2$EtN, DMF/$CH_2Cl_2$; (ii) Fmoc-SPPS (*deprotection*, 10 vol % piperidine/DMF; *coupling*, 4 equiv Fmoc-AA−OH, 4 equiv PyBOP, 8 equiv NMM, DMF; *capping*, 10 vol % $Ac_2O$/pyridine); (iii) HCHO, $NaBH(OAc)_3$, AcOH, DMF; (iv) 30 vol % HFIP/$CH_2Cl_2$; (v) HATU, NMM, 0 °C for 20 min, rt for 2 h; (vi) $H_2$, Pd/C, MeOH. * indicates the site of diastereoisomerism in **1** and **2**.

derivatization of a range of scaffolds at the C-terminus (using simple amides to facilitate more rapid syntheses, $R^1$ 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 be noted that, while Stolze et al. reported that replacement of the C-terminal methylmethoxypyrrolinone moiety in gallinamide A with a C-terminal alanine methyl ester moiety significantly decreased activity against the FPs and *P. falciparum*,[15] we were interested in accessing compounds that possessed greater C-terminal functionalization so as to explore the effect of modifying the acyl-pyrrolinone on FP activity and *P. falciparum* inhibition. We were encouraged by the fact that reduction of the methoxy-enol moiety in analogue **2** did not have a dramatic effect on FP and *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 ($R^2$ in Scheme 2A). Analogues **15** and **16**, extended by one L-Ile residue in the peptide backbone compared to **9**−**14**, were also proposed in order to probe the importance of the length of the analogues for inhibition of the FPs and *P. falciparum* (Scheme 2B).

Synthesis of **9**−**14** began from 2-Cl-Trt Cl resin preloaded with Fmoc-Ala (**17**). Coupling of Fmoc-protected $\alpha,\beta$-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 (including the extended peptide precursor for **15** and **16**) in moderate to good yields over the 10 resin-bound steps following HPLC purification (36−88%, 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

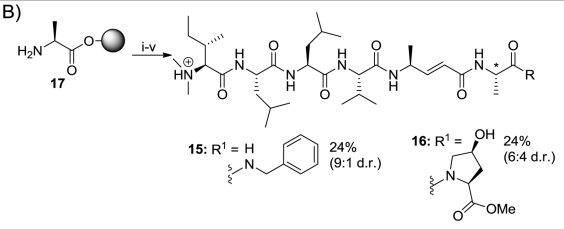
**Scheme 2. (A) Synthesis of Gallinamide A Analogues 9−14 and (B) Synthesis of Extended Analogues 15 and 16 via a Solid-Phase Synthesis Approach (NB: Compounds 9−16 Were Isolated As the Trifluoroacetate Salts)**[a]

A)




| Analogue | $R^2$ | $R^1$ | Yield (based on original resin loading) |
|---|---|---|---|
| **9** | | | 40% (5:1 *d.r.*) |
| **10** | | | 33% (1:1 *d.r.*) |
| **11** | | | 65% (7:1 *d.r.*) |
| **12** | | | 62% (1:1 *d.r.*) |
| **13** | | | 78% (6:1 *d.r.*) |
| **14** | | | 75% (5:1 *d.r.*) |

B)




[a]Reagents and conditions: (i) 4 equiv **18**, 4 equiv PyBOP, 6 equiv NMM; (ii) Fmoc SPPS (*deprotection*, 10 vol % piperidine/DMF; *coupling*, 4 equiv Fmoc-AA-OH, 4 equiv PyBOP, 8 equiv NMM, DMF; *capping*, 10 vol % $Ac_2O$/pyridine); (iii) HCHO, $NaBH(OAc)_3$, AcOH, DMF; (iv) 30 vol % HFIP/DCM; (v) # $R^1$-$NH_2$, PyBOP, DMF, 0 °C. * indicates the site of diastereoisomerism in **9**−**16**. # NMM was added to the coupling reactions for the preparation of inhibitors **10** and **16**.

reverse-phase HPLC, the desired inhibitors **9**, **11**, **13**, **14**, and **15** were isolated in excellent yields based on the original resin loading without significant epimerization. 4-(*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 **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 hydroxyproline methyl ester to the C-terminus of the peptides. Finally, aminothiazole was coupled to the C-terminus of one peptide acid using PyBOP at low temperature, which provided **12** as a 1:1 mixture of diastereoisomers in 62% yield following purification based on the original loading of the resin.

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).


dx.doi.org/10.1021/jm501439w | J. Med. Chem. XXXX, XXX, XXX−XXX

**Table 2. Inhibition of FP-2, FP-3, and the 3D7 Strain of *P. falciparum* by Gallinamide A Analogues 9–16[a]**

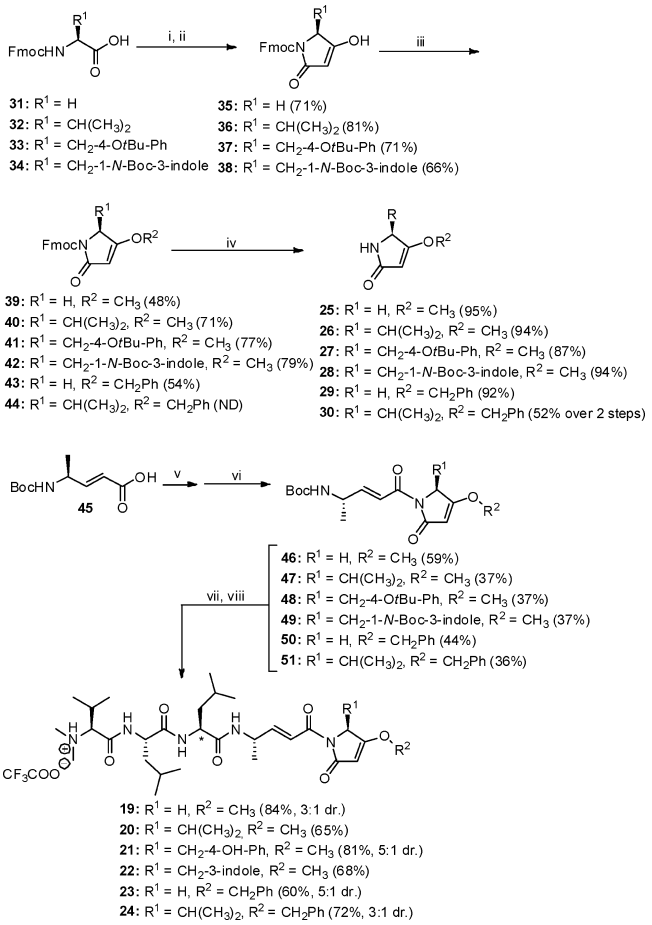
| analogue | $IC_{50}$ FP-2 [nM] | $IC_{50}$ FP-3 [nM] | $IC_{50}$ *P. falciparum* (3D7) [nM] |
|---|---|---|---|
| **9** | 10500 ± 640 | >25000 | 540 ± 210 |
| **10** | 7700 ± 190 | >50000 | 1900 ± 620 |
| **11** | 11500 ± 190 | >25000 | 320 ± 10 |
| **12** | 2500 ± 150 | 34000 ± 460 | 1100 ± 800 |
| **13** | 3400 ± 390 | >25000 | 5400 ± 380 |
| **14** | 6000 ± 160 | 46000 ± 5400 | 6600 ± 3800 |
| **15** | >50000 | >50000 | >50000 |
| **16** | >50000 | >50000 | >50000 |

[a]Errors are standard error of the mean of three experiments.

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 ($IC_{50}$ = 3.4–11.5 $\mu$M), no measurable inhibition of FP-3 at 25 $\mu$M, and nanomolar inhibitory activity against *P. falciparum* ($IC_{50}$ = 320–5400 nM). 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 $\mu$M) and FP-3 ($IC_{50}$ = 46 $\mu$M) but exhibited less potent antiparasitic activity ($IC_{50}$ = 6.6 $\mu$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 = 89.3 nM), with the exception that the C-terminal acyl pyrrolinone unit has been replaced by a C-terminal benzylamide. The 3 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 benzylamide-derived compounds against FP-2 and *P. falciparum*. C-terminal functionalization as a thiazole amide in **12** led to moderate inhibitory activity against both FP-2 ($IC_{50}$ = 2.5 $\mu$M) and FP-3 ($IC_{50}$ = 34 $\mu$M) as well as low micromolar antiparasitic activity ($IC_{50}$ = 1.1 $\mu$M). Taken together, these data suggest that the *N*-acyl pyrrolinone moiety is critical for potent FP inhibition and in vitro antimalarial activity. The importance of this functionality may lie in the ability of the aminopentenoyl imide motif to serve as a better Michael acceptor for the active site Cys residue in the FPs (Cys42 in FP-2 and Cys51 in FP-3)[33] when compared to the corresponding amide. Specifically, replacement of the imide moiety with the corresponding amide in analogues **9**–**14** reduces the propensity of the $\alpha,\beta$-unsaturated amide unit to serve as a Michael acceptor, presumably due to the less electron withdrawing nature of the amide compared to the imide in the native acyl-pyrrolinone linkage. Finally, extension of the analogues through the insertion of an additional Val residue in the peptide backbone in **15** and **16** abolished enzyme and parasite inhibitory activity. It is possible that these compounds can no longer be accommodated into the active site of the FPs, as the Leu residue that would be expected to be positioned in the S2 site of the protease[33] has been replaced by a Val or the double bond must be positioned at a suitable distance from the N-terminus to exhibit inhibitory activity.

While the replacement of the C-terminal pyrrolinone unit of gallinamide with simplified functionalities provided a number of compounds with activity against FP-2, FP-3, and *P. falciparum*, these compounds were significantly less potent than **1** and **2** (vide supra). As such, we chose to reinstate the C-terminal acyl pyrrolinone moiety in an additional series of analogues to probe the effect of substitution on the pyrrolinone ring on FP inhibition and antiparasitic activity. In total, six analogues (**19**–**24**) were 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

**Scheme 3. Synthesis of Second-Generation Gallinamide A Analogues 19–24[a]**




[a]Reagents and conditions: (i) (1) Meldrum's acid, EDC, DMAP, $CH_2Cl_2$, 0 °C to rt; (ii) EtOAc, 77 °C; (iii) $R^2$-OH, DIAD, $PPh_3$, $CH_2Cl_2$, 0 °C to rt; (iv) 1:4 v/v piperidine/MeCN, rt, 15 min; (v) $CF_3COOC_6F_5$, pyridine, DMF, 0 °C; (vi) **25**–**30**, *n*-BuLi, THF, −78 °C; (vii) 1:1 v/v TFA/$CH_2Cl_2$; (viii) tripeptide **5**, HATU, NMM, DMF, 0 °C. ND = not determined due to co-contamination with DIAD by-product. * indicates the site of diastereoisomerism in **19**, **21**, **23**, and **24**.

the preparation of the requisite pyrrolinones **25**–**30** from commercially available Fmoc-protected amino acids **31**–**34**. Coupling of Meldrum's acid to amino acids **31**–**34** with EDC in the presence of DMAP followed by reflux of the Meldrum's adduct 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


dx.doi.org/10.1021/jm501439w | J. Med. Chem. XXXX, XXX, XXX–XXX

reacted with methanol under Mitsunobu conditions using DIAD and triphenylphosphine to provide *O*-methylated pyrrolinones **39**–**42** in 48–79% yields. In addition, **35** and **36** were treated with benzyl alcohol under the same conditions to provide **43** and **44**. 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 **25**–**30** in excellent yields. With each of the required pyrrolinone building blocks in hand, attention turned to the modular assembly of the proposed gallinamide A analogues. To this end, Boc-protected amino acid **45**[11] was activated as the corresponding pentafluorophenyl ester by treatment with pentafluorophenyl trifluoroacetate in the presence of pyridine. Separately, pyrrolinones **25**–**30** were deprotonated with *n*-butyllithium at low temperature before addition of the pentafluorophenyl esters to afford imides **46**–**51** in moderate yields (36–59%). Unfortunately, despite efforts to improve the reactions by modifying the temperature and base, these yields could not be improved. From here, acidolysis of the Boc group from **46**–**51** followed by coupling to the *N*-terminal tripeptide **5** using HATU as the coupling reagent and NMM as the base (at low temperature to minimize epimerization), furnished the desired gallinamide A analogues **19**–**24** in good yields following HPLC purification. As with the synthesis of gallinamide A and analogues **1**–**4**, epimerization occurred during the final fragment condensation reaction. The minor epimer could be separated by preparative HPLC for analogues **20** and **22**, while **19**, **21**, **23**, and **24** were isolated as predominantly one diastereoisomer.

With the small library of pyrrolinone-modified analogues in hand, the compounds were next screened against FP-2, FP-3, and the 3D7 strain of *P. falciparum* (Table 3). Reintroduction of the C-terminal *N*-acyl-pyrrolinone in **19**–**24** led to reinstatement of potent inhibitory activity against the FPs and *P. falciparum*, as observed for gallinamide A and analogue **1**. All compounds also showed swollen food vacuole morphology at a concentration of less than 5 nM, supporting potent inhibition of the FPs as the mode of antiplasmodial action (see Supporting Information). Compound **19**, bearing no substitution on the pyrrolinone moiety, exhibited similar inhibitory activity to **1** against FP-2 ($IC_{50}$ = 24.8 nM), FP-3 ($IC_{50}$ = 225 nM), and *P. falciparum* ($IC_{50}$ = 57.3 nM). Incorporation of a more hydrophobic substituent on the pyrrolinone ring in **20** led to improvement in inhibitory activity against FP-3 ($IC_{50}$ = 131 nM) and *P. falciparum* ($IC_{50}$ = 16.6 nM). Indeed, **20** proved to be equipotent to the antimalarial drug CQ against the CQ-sensitive 3D7 strain of the parasite. Introduction of aromatic side chains onto the pyrrolinone ring in **21** and **22** did not lead to a change in activity against FP-2 but, as with **20**, led to a marked increase in inhibitory activity against FP-3 and *P. falciparum*. Compound **22**, bearing an indole side chain on the pyrrolinone ring, proved to be the most potent inhibitor of FP-3 ($IC_{50}$ = 66.7 nM) and *P. falciparum* ($IC_{50}$ = 9.7 nM) in this series of compounds.

**Table 3. Inhibition of FP-2, FP-3, and the 3D7 Strain of *P. falciparum* by Gallinamide A Analogues 1 and 19–24[a]**

| analogue | $IC_{50}$ FP-2 [nM] | $IC_{50}$ FP-3 [nM] | $IC_{50}$ *P. falciparum* (3D7) [nM] |
|---|---|---|---|
| **1** | 6.78 ± 0.44 | 292 ± 1.30 | 89.3 ± 73 |
| **19** | 24.8 ± 22.9 | 225 ± 26.5 | 57.3 ± 26.0 |
| **20** | 9.52 ± 0.14 | 131 ± 43.6 | 16.6 ± 9.0 |
| **21** | 5.25 ± 2.06 | 81.4 ± 7.73 | 20.0 ± 8.0 |
| **22** | 12.0 ± 3.19 | 66.7 ± 25.4 | 9.7 ± 2.0 |
| **23** | 9.59 ± 0.21 | 196 ± 7.00 | 96.0 ± 74.0 |
| **24** | 6.86 ± 2.53 | 182 ± 15.8 | 62.0 ± 47.0 |
| **CQ** | | | 17.3 ± 3.0 |

[a]Errors are standard error of the mean of three experiments.

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*-acyl-pyrrolinone-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**, and **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, 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 μM (see Supporting Information). In addition, none of the analogues displayed any inhibitory activity against the metalloproteases AP M1, AP M17, or 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[a]**

| analogue | $IC_{50}$ *P. falciparum* (Dd2) [nM] | HEK298 [nM] | AP M1, M17, and M18 [nM] |
|---|---|---|---|
| **1** | 302 ± 126 | 14 200 ± 1050 | >10000 |
| **9** | 421 ± 152 | >50000 | >10000 |
| **11** | 378 ± 20 | >50000 | >10000 |
| **13** | 170 ± 68 | >50000 | >10000 |
| **19** | 419 ± 185 | 16 300 ± 1700 | >10000 |
| **20** | 165 ± 68.0 | 9650 ± 1480 | >10000 |
| **21** | 67.0 ± 30.0 | 18 900 ± 110 | >10000 |
| **22** | 29.0 ± 16.0 | 8500 ± 124 | >10000 |

[a]Errors are standard error of the mean of three experiments.

## 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


dx.doi.org/10.1021/jm501439w | J. Med. Chem. XXXX, XXX, XXX–XXX

functionality found in the natural product. A number of potent inhibitors of FP-2 and FP-3 were elucidated through variation of the side chain on the pyrrolinone 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 Dd2 strain of *P. falciparum* and did not possess noteworthy toxicity to HEK298 cells. These compounds serve as promising leads for assessing in vivo efficacy of this class of inhibitors as well as for crystallographic studies which will aid in the development of second-generation natural-product-based FP inhibitors for the discovery of potential antimalarials. These studies 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.

**$Me_2$-Val-Leu-Leu-Apa-pyVal-OMe·TFA (20).** Imide **47** (20 mg, 57 μmol) was dissolved in 1:1 v/v TFA/$CH_2Cl_2$ (2 mL) and the reaction was stirred for 15 min before it was concentrated in vacuo, and the residue was redissolved in DMF (0.3 mL) and cooled to 0 °C. *N*,*N*-Dimethyltripeptide **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/$H_2O$ (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 mg, 65%). $R_t$ [0−100% MeCN over 30 min] = 20.1 min; $[\alpha]_D$ = 30.2 ($c$ = 0.4, MeOH). IR (thin film) $\nu_{max}$ = 3295, 3073, 2963, 1725, 1670, 1646, 1625, 1550, 1464, 1350 $cm^{-1}$. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 8.95 (d, 1H, $J$ 8.0 Hz, NH), 8.26 (s, 1H, NH), 7.37 (d, 1H, $J$ 15.5 Hz, CH), 7.35 (s, 1H, NH), 6.95 (dd, 1H, $J$ 15.5, 5.0 Hz, CH), 5.09 (s, 1H, CH), 4.83−4.70 (m, 3H, 3 × CH), 4.60 (d, 1H, $J$ 2.5 Hz, CH), 3.87 (d, 1H, $J$ 8.5 Hz, CH), 3.84 (s, 3H, $CH_3$), 3.00 (s, 3H, $NCH_3$), 2.86 (s, 3H, $CH_3$), 2.54 (m, 1H, CH), 2.26 (m, 1H, CH), 1.71−1.53 (m, 6H, 2 × $CH_2$, 2 × CH), 1.26 (d, 3H, $J$ 7.0 Hz, $CH_3$), 1.12−1.07 (m, 6H, 2 × $CH_3$), 0.93−0.82 (m, 15H, 5 × $CH_3$), 0.73 (d, 3H, $J$ 6.5 Hz, $CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 180.0, 172.1, 172.0, 170.7, 166.6, 164.5, 148.5, 122.7, 94.7, 71.7, 64.1, 58.7, 51.7, 51.7, 46.4, 41.7, 41.6, 28.9, 28.1, 25.1, 25.0, 23.2, 23.1, 22.2, 21.7, 19.7, 19.3, 18.9, 18.8, 15.4. MS (ESI): $m/z$ 629 [$(M + Na)^+$, 100%]. HRMS calcd for $C_{32}H_{56}N_5O_6$ M + $H^+$, 606.4230; found M + $H^+$, 606.4225.

**Boc-Apa-pyVal-OMe (47).** To a solution of amino acid **45**[11] (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, 396 mmol), 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 $Et_2O$/EtOAc (20 mL) and washing with 0.2 M aqueous HCl (5 mL), saturated aqueous $NaHCO_3$ solution (5 mL) and brine. The organic phase was then dried ($MgSO_4$) before concentrating in vacuo to afford the pentafluorophenyl ester as a pale-yellow oil which was used immediately in the following reaction. To a solution of pyrrolinone **26** (28.0 mg, 183 μmol) in THF (1.5 mL) at −78 °C was added 2.41 M *n*-butyllithium in hexane (76 μL, 183 μmol), and the reaction was stirred for 10 min. A solution of the freshly prepared pentafluorophenyl ester (99 mg, 235 mmol) in THF (0.5 mL) was subsequently added dropwise over 15 min, and the reaction was allowed to stir for a further hour at −60 °C. The reaction was subsequently quenched with AcOH (50 μL) and concentrated in vacuo, and the residue was purified by column chromatography (eluent: 2:1 v/v hexane/EtOAc) to afford imide **47** as a colorless oil (24 mg, 37%). $R_f$ [1:1 v/v hexane/EtOAc] = 0.65; $[\alpha]_D$ = +69 ($c$ = 1.0, $CHCl_3$). IR (thin film) $\nu_{max}$ = 3343, 3102, 2971, 2934, 2877, 1719, 1673, 1621, 1515, 1455, 1365, 1323 $cm^{-1}$. $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.39 (dd, 1H, $J$ 15.8, 1.6 Hz, CH), 7.02 (dd, 1H, $J$ 15.8, 4.4 Hz, CH), 5.08 (s, 1H, CH), 4.69−4.61 (m, 2H, NH, CH), 4.45 (m, 1H, CH), 3.84 (s, 3H, $OCH_3$), 2.55 (m, 1H, CH), 1.44 (s, 9H, 3 × $CH_3$), 1.29 (d, 3H, $J$ 7.2 Hz, $CH_3$), 1.10 (d, 3H, $J$ 7.2 Hz, $CH_3$), 0.74 (d, 3H, $J$ 7.2 Hz, $CH_3$). $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 179.8, 175.7, 170.7, 164.6, 149.5, 122.1, 94.8, 79.8, 64.1, 58.6, 47.5, 28.5, 20.5, 18.9, 15.5; MS (ESI): $m/z$ 375 [$(M + Na)^+$, 100%]. HRMS: calcd for $C_{18}H_{28}N_2O_5Na$ M + $Na^+$, 375.1896; found M + $Na^+$, 375.1891.

## ASSOCIATED CONTENT

### Supporting Information

Experimental procedures and analytical data for all novel compounds including $^1H$ and $^{13}C$ NMR and analytical HPLC. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION


### Corresponding Author

*Phone: +61 2 9351 5745. Fax: +61 2 9351 3329. E-mail: richard.payne@sydney.edu.au.


### Author Contributions

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

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS


We thank Dr. Ian Luck, School of Chemistry, University of Sydney, for assistance with NMR, the John Lamberton Research Scholarship and the Australian Postgraduate Award Programs (T.C. and N.E.), and a National Health and Medical Research Council of Australia (NHMRC) Postgraduate Scholarship (J.G.) for Ph.D. support. R.J.P. and S.M. are supported by Australian Research Council Future Fellowships (R.J.P., FT130100150; S.M., FT100100690). This research was funded by a NHMRC project grant 1062216.


## ABBREVIATIONS USED

CQ, chloroquine; FP, falcipain; NMM, *N*-methylmorpholine; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; DIAD, diisopropyl azodicarboxylate; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphon-ium hexafluorophosphate; DMAP, 4-dimethylaminopyridine; HATU, 1-[bis-(dimethylamino) methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate

## REFERENCES


(1) Persidis, A. Malaria. *Nature Biotechnol.* **2000**, *18*, 111−112.

(2) *Malaria Report*; World Health Organisation: Geneva, 2012; http://www.who.int/malaria/publications/world_malaria_report_2012/.

(3) Birkett, A. J.; Moorthy, V. S.; Loucq, C.; Chitnis, C. E.; Kaslow, D. C. Malaria vaccine R&D in the decade of vaccines: Breakthroughs, challenges and opportunities. *Vaccine* **2013**, *31*, B233−243.

(4) Kokwaro, G. Ongoing challenges in the management of malaria. *Malar. J.* **2009**, *8*, S2.

(5) Newman, D. J.; Cragg, G. M.; Snader, K. M. The influence of natural products upon drug discovery. *Nat. Prod. Rep.* **2000**, *17*, 215−234.

(6) Rosenthal, P. J. Antimalarial drug discovery: old and new approaches. *J. Exp. Biol.* **2003**, *206*, 3735−3744.

(7) Fidock, D. A.; Rosenthal, P. J.; Croft, S. L.; Brun, R.; Nwaka, S. Antimalarial drug discovery: efficacy models for compound screening. *Nature Rev. Drug Discovery* **2004**, *3*, 509−520.
(8) Dondorp, A. M.; Nosten, F.; Yi, P.; Das, D.; Phyo, A. P.; Tarning, J.; Lwin, K. M.; Ariey, F.; Hanpithakpong, W.; Lee, S. J.; Ringwald, P.; Silamut, K.; Imwong, M.; Chotivanich, K.; Lim, P.; Herdman, T.; An, S. S.; Yeung, S.; Singhasivanon, P.; Day, N. P.; Lindegardh, N.; Socheat, D.; White, N. J. Artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* **2009**, *361*, 455−467.
(9) Nosten, F.; White, N. J. Artemisinin-based combination treatment of falciparum malaria. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 181−192.
(10) Guiguemde, W. A.; Shelat, A. A.; Garcia-Bustos, J. F.; Diagana, T. T.; Gamo, F. J.; Guy, R. K. Global phenotypic screening for antimalarials. *Chem. Biol.* **2012**, *19*, 116−129.
(11) Conroy, T.; Guo, J. T.; Hunt, N. H.; Payne, R. J. Total synthesis and antimalarial activity of symplostatin 4. *Org. Lett.* **2010**, *12*, 5576−5579.
(12) Conroy, T.; Guo, J. T.; Linington, R. G.; Hunt, N. H.; Payne, R. J. Total synthesis, stereochemical assignment, and antimalarial activity of gallinamide A. *Chem.—Eur. J.* **2011**, *17*, 13544−13552.
(13) Linington, R. G.; Clark, B. R.; Trimble, E. E.; Almanza, A.; Ureña, L.-D.; Kyle, D. E.; Gerwick, W. H. Antimalarial peptides from marine cyanobacteria: isolation and structural elucidation of gallinamide A. *J. Nat. Prod.* **2009**, *72*, 14−17.
(14) Taori, K.; Liu, Y.; Paul, V. J.; Luesch, H. Combinatorial strategies by marine cyanobacteria: symplostatin 4, an antimitotic natural dolastatin 10/15 hybrid that synergizes with the coproduced HDAC inhibitor largazole. *ChemBioChem* **2009**, *10*, 1634−1639.
(15) Stolze, S. C.; Deu, E.; Kaschani, F.; Li, N.; Florea, B. I.; Richau, K. H.; Colby, T.; van der Hoorn, R. A. L.; Overkleeft, H. S.; Bogyo, M.; Kaiser, M. The antimalarial natural product symplostatin 4 is a nanomolar inhibitor of the food vacuole falcipains. *Chem. Biol.* **2012**, *19*, 1546−1555.
(16) Rosenthal, P. J. In *Cysteine Proteases of Pathogenic Organisms*; Robinson, M. W., Dalton, J. P., Eds.; Springer-Verlag: Berlin, 2011; Vol. *712*, p 30.
(17) Rosenthal, P. J.; Sijwali, P. S.; Singh, A.; Shenai, B. R. Cysteine proteases of malaria parasites: targets for chemotherapy. *Curr. Pharm. Des.* **2002**, *8*, 1659−1672.
(18) Sijwali, P. S.; Rosenthal, P. J. Gene disruption confirms a critical role for the cysteine protease falcipain-2 in hemoglobin hydrolysis by *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 4384−4389.
(19) Sijwali, P. S.; Koo, J.; Singh, N.; Rosenthal, P. J. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **2006**, *150*, 96−106.
(20) Verissimo, E.; Berry, N.; Gibbons, P.; Cristiano, M. L. S.; Rosenthal, P. J.; Gut, J.; Ward, S. A.; O'Neill, P. M. Design and synthesis of novel 2-pyridone peptidomimetic falcipain 2/3 inhibitors. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4210−4214.
(21) Kerr, I. D.; Lee, J. H.; Farady, C. J.; Marion, R.; Rickert, M.; Sajid, M.; Pandey, K. C.; Caffrey, C. R.; Legac, J.; Hansell, E.; McKerrow, J. H.; Craik, C. S.; Rosenthal, P. J.; Brinen, L. S. Vinyl sulfones as antiparasitic agents and a structural basis for drug design. *J. Biol. Chem.* **2009**, *284*, 25697−25703.
(22) Coterón, J. M.; Catterick, D.; Castro, J.; Chaparro, M. A. J.; Díaz, B.; Fernández, E.; Ferrer, S.; Gamo, F. J.; Gordo, M.; Gut, J.; de las Heras, L.; Legac, J.; Marco, M.; Miguel, J.; Muñoz, V.; Porras, E.; de la Rosa, J. C.; Ruiz, J. R.; Sandoval, E.; Ventosa, P.; Rosenthal, P. J.; Fiandor, J. M. Falcipain inhibitors: Optimization studies of the 2-pyrimidinecarbonitrile lead series. *J. Med. Chem.* **2010**, *53*, 6129−6152.
(23) Breuning, A.; Degel, B.; Schulz, F.; Büchold, C.; Stempka, M.; Machon, U.; Heppner, S.; Gelhaus, C.; Leippe, M.; Leyh, M.; Kisker, C.; Rath, J.; Stich, A.; Gut, J.; Rosenthal, P. J.; Schmuck, C.; Schirmeister, T. Michael acceptor based antiplasmodial and anti-trypanosomal cysteine protease inhibitors with unusual amino acids. *J. Med. Chem.* **2010**, *53*, 1951−1963.
(24) Shah, F.; Mukherjee, P.; Gut, J.; Legac, J.; Rosenthal, P. J.; Tekwani, B. L.; Avery, M. A. Identification of novel malarial cysteine protease inhibitors using structure-based virtual screening of a focused cysteine protease inhibitor library. *J. Chem. Inf. Model.* **2011**, *51*, 852−864.
(25) Shah, F.; Gut, J.; Legac, J.; Shivakumar, D.; Sherman, W.; Rosenthal, P. J.; Avery, M. A. Computer-aided drug design of falcipain inhibitors: virtual screening, structure−activity relationships, hydration site thermodynamics, and reactivity analysis. *J. Chem. Inf. Model.* **2012**, *52*, 696−710.
(26) Rosenthal, P. J.; Olson, J. E.; Lee, G. K.; Palmer, J. T.; Klaus, J. L.; Rasnick, D. Antimalarial effects of vinyl sulfone cysteine proteinase inhibitors. *Antimicrob. Agents Chemother.* **1996**, *40*, 1600−1603.
(27) Olson, J. E.; Lee, G. K.; Semenov, A.; Rosenthal, P. J. Antimalarial effects in mice of orally administered peptidyl cysteine protease inhibitors. *Bioorg. Med. Chem.* **1999**, *7*, 633−638.
(28) Shenai, B. R.; Lee, B. J.; Alvarez-Hernandez, A.; Chong, P. Y.; Emal, C. D.; Neitz, R. J.; Roush, W. R.; Rosenthal, P. J. Structure−activity relationships for inhibition of cysteine protease activity and development of *Plasmodium falciparum* by peptidyl vinyl sulfones. *Antimicrob. Agents Chemother.* **2003**, *47*, 154−160.
(29) Rosenthal, P. J.; Lee, G. K.; Smith, R. E. Inhibition of a *Plasmodium vinckei* cysteine proteinase cures murine malaria. *J. Clin. Invest.* **1993**, *91*, 1052−1056.
(30) Ettari, R.; Bova, F.; Zappalà, M.; Grasso, S.; Micale, N. Falcipain-2 inhibitors. *Med. Res. Rev.* **2010**, *30*, 136−167.
(31) Miller, B.; Friedman, A. J.; Choi, H.; Hogan, J.; McCammon, J. A.; Hook, V.; Gerwick, W. H. The marine cyanobacterial metabolite gallinamide A is a potent and selective inhibitor of human cathepsin L. *J. Nat. Prod.* **2014**, *77*, 92−99.
(32) Rosenthal, P. J.; McKerrow, J. H.; Aikawa, M.; Nagasawa, H.; Leech, J. H. A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum*. *J. Clin. Invest.* **1988**, *82*, 1560−1566.
(33) Kerr, I. D.; Lee, J. H.; Pandey, K. C.; Harrison, A.; Sajid, M.; Rosenthal, P. J.; Brinen, L. S. Structures of falcipain-2 and falcipain-3 bound to small molecule inhibitors: implications for substrate specificity. *J. Med. Chem.* **2009**, *52*, 852−857.