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Antimalarial Activity of Natural and Synthetic Prodiginines

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Abstract

Prodiginines are a family of linear and cyclic oligopyrrole red-pigmented compounds. Herein we describe the in vitro antimalarial activity of 4 natural (IC₅₀ = 1.7–8.0 nM) and 3 sets of synthetic prodiginines against *Plasmodium falciparum*. Set 1 compounds replaced the terminal non-alkylated pyrrole ring of natural prodiginines and had diminished activity (IC₅₀ >2920 nM). Set 2 and set 3 prodiginines were monosubstituted or disubstituted at either the 3 or 5 position of the right hand terminal pyrrole, respectively. Potent in vitro activity (IC₅₀ = 0.9–16.0 nM) was observed using alkyl or aryl substituents. Metacycloprodiginine and more potent synthetic analogs were evaluated in a *P. yoelii* murine patent infection using oral administration. Each analog reduced parasitemia by more than 90% after 25 mg/kg/day dosing, and in some cases provided a cure. The most favorable profile was 92% parasite reduction at 5 mg/kg/day, and 100% reduction at 25 mg/kg/day without any evident weight losses or clinical overt toxicity.

Introduction

Malaria remains a major global health threat and an enormous economic burden to disease-endemic nations. Despite increased attention and new commitment to malaria eradication, the disease causes more than a million deaths each year, and drug resistance to each new chemotherapeutic approach has developed, most notably in *Plasmodium falciparum*, the parasite causing nearly all malaria-related deaths.¹ On the heels of the global spread of chloroquine-resistant (CQR^a) *P. falciparum*, resistance has also quickly developed to a variety of quinoline analogs, to antifolates, to inhibitors of electron transport, and perhaps most ominously, now to artemisinin.^{2a–b} It is evident that the search for effective novel antimalarial compounds must be comprehensive and must include explorations of chemotypes distinct from the prototypes in clinical use.

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Supporting Information Available. Compounds characterization data and spectra (NMR and HRMS) of all the prodiginine analogs associated with this article are available as supplementary data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

^a**Abbreviations.** CQR, chloroquine-resistant; DNA, deoxyribonucleic acid; SAR, structure-activity relationship; CQ, chloroquine; IC₅₀; half maximal inhibitory concentration; nM, nanomolar; MDR, multidrug-resistant

Prodiginines are a family of linear and cyclic oligopyrrole red-pigmented compounds with antibacterial,³ anticancer,⁴ and immunosuppressive activity,⁵ produced by actinomycetes and other eubacteria. Some prodiginines induce apoptotic effects breaking genomic deoxyribonucleic acid (DNA) strands.⁶ The prodiginines also shown to have potent in vitro activity against *Plasmodium* species, at much lower concentrations than seen with mammalian cells.^{7a-e} Despite potent and selective in vitro activity against *P. falciparum*, the reported efficacy of prodiginines in vivo has either been incomplete, associated with toxicity, or lacking altogether, discouraging their further consideration as antimalarial candidates.

On careful review, however, the previous studies are each limited, either in the identification or variety of compounds tested, by the lack of in vivo correlation studies, or by the route of drug administration in the animals studied. These limitations leave open the question of whether or not there is suitable antimalarial activity among these compounds. Furthermore, previous studies have been limited to naturally occurring prodiginines, leaving open the possibility that some synthetic analogs may have improved in vitro activity or in vivo efficacy or reduced toxicity. We therefore undertook a more comprehensive assessment of the antiplasmodial activity of prodiginines; initially re-assessing the activity of four naturally-occurring prodiginines **1-4** (Figure 1) and subsequently assessing a series of synthetic analogs. Here we report these results, including impressive in vitro potency, SAR, and evidence of in vivo efficacy, including curative efficacy in mice after oral administration.

Results and Discussion

Synthesis

The synthesis of various 2-alkyl pyrrole segments **6a-h** (Scheme 1) was initiated by converting the readily accessible pyrrole to the corresponding 2-acyl pyrroles **5a-h**.⁸ These acyl pyrroles were then smoothly converted to **6a-h** using an excess of NaBH₄, in 2-propanol under reflux.⁹

Synthesis of 3-alkyl pyrrole segments **10a-k** (Scheme 2) used the same pyrrole starting material, which treated with phenylsulfonyl chloride in presence of NaOH provided a *N*-phenylsulfonyl pyrrole (**7**).¹⁰ Use of AlCl₃ permitted the regioselective acylation of **7** at the 3 position with the acyl chlorides to obtain the *N*-phenylsulfonyl-3-acyl pyrroles **8a-k**, which were subsequently hydrolyzed under basic (5N NaOH) conditions to give 3-acyl pyrroles **9a-k**.¹¹ In the final step **9a-k** were reduced to **10a-k** using NaBH under reflux.⁹

Using literature methodologies, the key intermediate involved in the synthesis of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (**13**) and its analogs **14-17**, were prepared via a intermediate **12** from the commercially available 4-methoxy-3-pyrroline-2-one (**11**) (Scheme 3).¹² Acid-catalyzed condensation of an appropriate alkyl pyrrole segments (2,4-dimethyl pyrrole, **6a-h**, and **10a-k**) with **13-17** (Scheme 4)¹³ gave the desired prodiginine analogs **18-25** (Table 1) and **26-43** (Table 2 and 3).

A new synthetic strategy, outlined in Scheme 5, was used to synthesize 3,5-disubstituted prodiginine analogs **46-55** (Table 2) and **56-75** (Table 3). The 2,4-disubstituted pyrroles **44** were synthesized from either the corresponding 2 or 3-acyl pyrroles (**5** or **9**) with their corresponding acyl chlorides and AlCl_3 .¹⁴ Subsequent reduction with NaBH_4 gave the desired 2,4-disubstituted pyrroles **45**.⁹ Acid-catalyzed condensation of these **45** with **13** provided the desired 3,5-disubstituted prodiginine analogs **46-75** (Table 2 and 3).¹³

Biological Activity

The natural and synthetic prodiginines were assayed for their in vitro antimalarial activity against *P. falciparum* pansensitive D6 with the chloroquine (CQ) as a reference drug. A direct comparative assessment of the antimalarial activity of natural products, prodigiosin (**1**), undecylprodiginine (**2**), metacycloprodiginine (**3**), and streptorubin B (**4**) (Figure 1) revealed they all had remarkably potent activity with very low IC_{50} values (8 nM, 7.7 nM, 1.7 nM, and 7.8 nM, respectively) against *P. falciparum* strain D6, and slightly more active than CQ (11 nM). The natural prodiginine **3** was consistently observed to be the most potent of the 4 natural products, and demonstrated that changes in the alkyl component of prodiginines affected the in vitro antimalarial activity. Based on these observations a series of new analogs of prodiginines with similar core structure were generated.

In the early stages, a set of analogs **18-25** (Table 1), in which an indole, phenyl, thiophen and furan replaced the terminal non alkylated pyrrole ring (left hand side) of core moiety, were generated. In vitro analysis of the activity of these compounds against *P. falciparum* pansensitive D6 demonstrated activity ($\text{IC}_{50} > 2920$ nM) significantly diminished relative to the natural prodiginines **1-4** ($\text{IC}_{50} = 1.7-8.0$ nM). This work suggested that the left hand side pyrrole ring of the prodiginines is important for the potent antimalarial activity. We subsequently tested a hypothesis that the alkyl chain on right hand side pyrrole of the prodiginines might represent an opportunity to make potent and selective antimalarials with the desired “drug-like” properties. Subsequent analogs were synthesized (Scheme 4 and 5) to obtain a SAR for the alkyl groups at the terminal alkylated pyrrole of the prodiginines.

One set of these analogs **26-38** (Table 2), and **39-43** (Table 3) were monosubstituted and contained different alkyl chains or aryl groups at either the 3 or 5 position of the terminal pyrrole ring (right hand side) (Scheme 4). These compounds were screened for their in vitro antimalarial activity *P. falciparum* strain D6 and the results were shown in Table 2 and 3. Of the monoalkylated analogs, compounds **26**, **27**, **31**, **32**, and **38** showed the poorest activity with IC_{50} values above 1700 nM against D6. Compounds **28**, **30**, and **33**, all exhibited slightly better activity with IC_{50} values 375 nM, 300 nM, and 460 nM respectively against D6. Analogs, **29**, **34**, **35**, **36** and **37** were the most potent of the mono alkylated prodiginine analogs with IC_{50} values of 80 nM, 80 nM, 28 nM, 4.6 nM, and 8.0 nM respectively against D6. These observations and the activity of the natural product **2**, demonstrates that $\text{C}_6\text{-C}_{11}$ alkyl chain length at either the 3 or 5 position of right hand side pyrrole ring is required for optimal activity of monoalkylated prodiginines. The presence of either an amine group (see **31**; $\text{IC}_{50} = 1700$ nM (D6) versus **2**; $\text{IC}_{50} = 7.7$ nM (D6)) or methylcarboxylate (see **32**; $\text{IC}_{50} = 4500$ nM (D6) versus **34**; $\text{IC}_{50} = 80$ nM (D6)) at the end of the alkyl chain leads to a decrease in activity. The 3-aryl monosubstituted prodiginine analogs **39-43** (Table 3)

exhibited moderate inhibitory activity with IC₅₀ values of 83 nM, 170 nM, 65 nM, 90 nM, and 56 nM respectively against D6. While the presence of a halogen substituent appeared to lead to increase in activity (see **41**; IC₅₀ = 65 nM (D6) versus **39**; IC₅₀ = 83 nM (D6) and **40**; IC₅₀ = 170 nM (D6)), the activity was poorer than that seen for the more potent monoalkylated prodiginines (such as **2**, and **35-37**).

The final set of prodiginine analogs generated had substituents at both position 3 and 5 of the terminal pyrrole ring (right hand side). The substituents were either two alkyl chains (**46-55**), an alkyl chain and an aryl group (**56-65**), or two aryl groups (**66-75**). These compounds **46-75** (Tables 2 and 3), were screened for in vitro antimalarial activity against *P. falciparum* strain D6. Among the dialkylated analogs **49-51** showed potent in vitro antimalarial activity with IC₅₀ values 1.7 nM, 1.7 nM and 2.1 nM respectively (Table 2) against D6. Other dialkylated prodiginines **47**, **48**, **52**, **53**, and **55** also exhibited excellent activity with IC₅₀ values 4.5 nM, 2.9 nM, 4.9 nM, 6.2 nM, and 5.3 nM respectively against D6, and demonstrated that they are more active than the monoalkylated prodiginines (**2**, and **26-38**). The two exceptions were analogs **46** (IC₅₀ = 8900 nM), and **54** (IC₅₀ = 92 nM) which contained a total alkyl chain length of two and sixteen carbon respectively on right hand side pyrrole ring of the core moiety. From both the mono and dialkylated series the data clearly shown that prodiginines containing a combined C₈-C₁₅ alkyl chain length had the most potent in vitro antimalarial activity (Figure 2).

The analogs **56-65**, which contains one alkyl and one aryl substituents on right hand pyrrole ring were also tested for in vitro antimalarial activity against *P. falciparum* strain D6. Among these analogs, **62** (IC₅₀ = 0.9 nM) and **63** (IC₅₀ = 1.3 nM) (Table 3) were the most potent and comparable to the most potent dialkylated prodiginines **49-51** and the natural prodiginine **3** (IC₅₀ = 1.7 nM). In this series (**56-65**) all compounds, with the exception of **60** (IC₅₀ = 16.0 nM), were extremely potent (IC₅₀ < 6.3 nM), significantly more than the corresponding analogs **39-43** which have an aryl substituent, but lack the alkyl substituent. The in vitro activity results of chloro (**56-61**), fluoro (**62** and **63**) and bromo (**64** and **65**) substituted analogs of prodiginine indicated that the bromo substituted analogs **64** and **65** were slightly less potent than chloro and fluoro substituted analogs (Table 3).

The final set of analogs **66-75**, contains two aryl substituent groups on right hand side pyrrole ring. In this set, most of the compounds (**66**, **67**, **69-71**, **74**, and **75**) exhibited potent activity (IC₅₀ < 8.3 nM). The exceptions were **68** (IC₅₀ = 14.0 nM), which contains a bromine atom on both aryl substituents, and **72** (IC₅₀ = 12.6 nM) and **73** (IC₅₀ = 14.7 nM) in which each aryl group is dihalogenated (Table 3). A comparison of the most potent compounds in this series, with analogs **47-65** revealed that the in vitro activity for prodiginines bearing two aryl substituents is slightly less than for disubstituted prodiginines in which either one or both of the substituents is an alkyl chain. That said, a general pattern of more potent in vitro activity for disubstituted prodiginines compared to monosubstituted prodiginines was observed.

The majority of the new synthetic prodiginines were also tested alongside CQ against the multidrug-resistant (MDR) Dd2. In all cases there was no significant change in the activity of each analog between the D6 and Dd2 (Table 1-3), indicating no cross-resistance with CQ.

The most potent synthetic prodiginines were active in the 1 nM range against Dd2, compared to 135 nM for CQ.

A number of the synthetic prodiginines were tested against a number of mammalian cell lines and IC₅₀ values in the 500-2000 nM ranges was not observed (data not shown). A general assay with mitogen stimulated murine splenic lymphocytes was used to evaluate all the synthetic prodiginines (Tables 1-3) and to determine if synthetic changes led to reduced toxicity.¹⁵ The majority of the synthetic prodiginines exhibited much greater toxicity (IC₅₀ values < 125 nM) in this particular assay. There were prodiginines which had slightly less toxicity (1000-4000 nM), but these were in all cases also associated with markedly reduced activity against *P. falciparum*, and were still more toxic than CQ (16000 nM). Thus using these particular in vitro assays, the antimalarial activity and toxicity for the current set of synthetic prodiginines exhibit comparable SAR.

The in vivo antimalarial efficacy of the natural prodiginine **3** and a selection of the more potent synthetic analogs were evaluated in a *P. yoelii* murine patent infection. In initial proof-of-principle testing, administration of 25 mg/kg/day **3** by gavage using a caprylic/capric triglyceride vehicle resulted in greater than 99% reduction in parasitemia (0.28% vs 54.1% in controls); at 100 mg/kg/day parasite clearance was complete, and was accompanied by temporary weight loss, and three of four mice were cured. This data provided the first demonstration of oral effectiveness of prodiginines. Equally important, unlike previous reports of parenteral dosing, efficacy after gavage dosing was observed to be without evident toxicity. These findings indicated that antiplasmodial oral efficacy might be achievable and led to similar in vivo testing of some of the more potent synthetic prodiginines. Examples of impressive potency against murine malaria was observed and is briefly summarized here (Table 4). Analogs **48**, **49**, **57**, **58**, and **66** each reduced parasitemia by more than 90%, the exceptions were **41** (38%) and **60** (72%), after 5 mg/kg/day dosing, without any evidence of weight loss or toxicity. **41**, **48**, **49**, **57**, **58**, **60** and **66** each reduced parasitemia by 90%, after 25 mg/kg/day dosing, however, weight loss was observed during the dosing course with a few analogs (Table 4). However, the weight reduction was temporary and for unknown reasons (including dehydration, fasting of tested mice or toxicity at the higher dose). While an exhaustive in vivo analysis of prodiginine analogs was not conducted, the temporary weight loss was primarily associated with analogs with alkyl substituents. The prodiginines **41** and **66** by contrast have no alkyl substituents and have one or two aryl substituents. Of these compounds tested, **66** had the most favorable profile: 92% parasite reduction at 5 mg/kg/day, 100% reduction at 25 mg/kg/day without any evident weight loss or clinical overt toxicity. Although testing consisted only of equidose comparisons between compounds, without full dose ranging or efforts to define curative dose levels, it is worth mentioning that there was one cure each in the **60** and **58** treated groups (25 mg/kg/day).

Conclusions

Although natural prodiginines **3**,^{7d} and heptylprodigiosin (**76**)^{7e,16} have demonstrated remarkable in-vitro potency against growth of *P. falciparum* the mode of action is unknown, and a SAR has not been reported. Herein, we have prepared a set of prodiginine analogs via

simple and efficient pathways from commercially available inexpensive starting materials and reagents. These standardized protocols, can be used to generate a large number of prodiginines in a grams scale from a single batch. This current work has suggested that the non alkylated terminal pyrrole (left hand side) of the natural prodiginines is required for in vitro activity, but that the substituents at the alkylated terminal pyrrole (right hand side) can be varied. Specifically in vitro activity comparable or better ($IC_{50} = 1 \text{ nM}$) than most of the natural prodiginines was observed for analogs with either alkyl or aryl substituents at both the 3 and 5 position of the right hand side pyrrole. In addition these synthetic prodiginines are equally effective against *P. falciparum* pansensitive D6 and MDR Dd2.

Previous results of in vivo testing, have also cast doubt on the suitability of prodiginines as antimalarial candidates. Against *P. berghei* in mice, subcutaneous injection of natural prodiginines **1**,^{7a} **76**,^{7e} **2**, **3**, butylcycloheptylprodiginine (**77**), and cyclononylprodiginine (**78**),^{7b,16} have each been shown to extend survival, however, either without cure or at doses resulting in more toxic deaths than cures. Natural prodiginine **1** was shown to be completely ineffective for antimalarial activity, even at toxic doses, for oral treatment of Rhesus monkeys infected with *P. cynomolgi*.^{7c} In the current study it has been demonstrated for the first time that prodiginines can be administered orally and produce marked parasite clearance, including cures in some cases, without evident weight loss and toxicity. Nonetheless, preliminary in vitro assays continue concerns associated with the toxicity of prodiginines, and the quest for analogs with improved antimalarial activity but reduced toxicity continues.

Experimental Section

Purification of natural prodiginines (1-4)

Natural product **1** was purchased from the developmental therapeutics program NCI/NIH. Using minor modifications to literature procedures, **2**, **4** were isolated from *S. coelicolor* M511,¹⁷ and **3** was isolated from *S. longisporus ruber*.^{13a}

General

¹H NMR spectra were run on Bruker AMX-400 MHz spectrometer in CDCl₃, ¹³C NMR spectra were recorded at 100 MHz in CDCl₃. Chemical shifts are reported as values in ppm relative to CHCl₃ (7.26) in CDCl₃ and TMS was used as internal standard. HRMS (ESI) were recorded on high-resolution (30,000) thermo LTQ-Orbitrap Discovery hybrid mass spectrometry (San Jose, CA). Reagents were from Aldrich and used as supplied, unless otherwise noted reactions which required the use of anhydrous, inert atmosphere techniques were carried out under an atmosphere of Argon/Nitrogen. Chromatography was executed with silica gel (230-400 mesh) and neutral-alumina using mixtures of ethyl acetate and hexane as eluents. Analytical HPLC analyses were performed on a Supelco Discovery HS C18 column (4.6 × 250 mm) with a linear elution gradient ranging from CH₃OH:CH₃CN:H₂O (40%:10%:50%) to CH₃OH (100%) in 0.15% trifluoroacetic acid at a flow rate of 1 mL/min. A purity of 95% has been established for all tested compounds, exception of **25**, **26** and **33** achieved 93%, 81% and 86% respectively.

Representative procedure for the synthesis of 1-(1H-pyrrol-2-yl)-octan-1-one (5e)

A mixture of pyrrole (2.0 g, 29.8 mmol), octanoyl chloride (7.8 g, 44.7 mmol), and zinc powder (3.88 g, 59.7 mmol) in toluene (50 mL) was stirred at room temperature for 4 h. The reaction mixture was quenched with saturated sodium bicarbonate solution (50 mL), and extracted with ethyl acetate (3 × 30 mL). The combined organic layer was washed with water, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The crude product was chromatographed on silica gel to afford the *title compd* **5e** (3.16 g, 55%). ¹H NMR (CDCl₃, 400 MHz) δ 9.71 (br s, 1H), 7.02 (m, 1H), 6.91 (m, 1H), 6.27 (m, 1H), 2.75 (t, *J* = 7.5 Hz, 2H), 1.70 (m, 2H), 1.33 (m, 8H), 0.92 (t, *J* = 7.1 Hz, 3H).

Representative procedure for the synthesis of 2-octyl-1H-pyrrole (6e)

To a stirred solution of **5e** (1.0 g, 5.18 mmol) in 150 mL of 2-propanol (IPA) at 25 °C was added slowly sodium borohydride (NaBH₄) (1.34 g, 36.26 mmol), and the reaction mixture was heated at reflux for 10 h. The hot reaction mixture was poured into 150 mL of ice water, and the solution was acidified with 10% aqueous HCl. The suspension was extracted with dichloromethane (3 × 50 mL), the combined organic extracts were washed with water, brine, and dried over anhydrous Na₂SO₄. The solvent was evaporated under reduced pressure, and the crude product was chromatographed on silica gel to afford the *title compd* **6e** (714 mg, 77%). ¹H NMR (CDCl₃, 400 MHz) δ 7.79 (br s, 1H), 6.56 (m, 1H), 6.04 (m, 1H), 5.83 (m, 1H), 2.50 (t, *J* = 7.6 Hz, 2H), 1.53 (m, 2H), 1.28 (m, 10H), 0.79 (t, *J* = 7.0 Hz, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 132.9, 116.0, 108.2, 104.8, 31.9, 29.7, 29.5, 29.4, 29.3, 27.8, 22.7, 14.1.

Synthesis of compound 7

Pyrrole (10 g, 149 mmol) was added to a well-agitated suspension of NaOH (17.9 g, 447 mmol) in 100 mL of 1,2-dichloroethane. This mixture was then cooled to 0 °C and stirred for 10 min. Following, in which a solution of phenylsulfonyl chloride (31.5 g, 173 mmol) in 20 mL of 1,2-dichloroethane was added drop wise over a period of 20 min. Thirty minutes after the completion of addition, the reaction was allowed to come to room temperature and left stirring overnight. The reaction was quenched by pouring onto 300 mL of distilled water. The organic layer was separated, and the aqueous layer was extracted with dichloromethane (3 × 100 mL). The combined organic extract was washed with distilled water to neutrality and dried over anhydrous Na₂SO₄. Removal of the solvent in vacuo gave **7** as white crystalline solid (21.6 g, 70%). This solid was subsequently washed with hot hexane in greater than 65% overall yield.

Representative procedure for the synthesis of (1-benzenesulfonyl-1H-pyrrol-3-yl)-(4-chloro-phenyl)-methanone (8i)

To a stirred suspension of anhydrous AlCl₃ (3.85 g, 29 mmol) in 50 mL of 1,2-dichloroethane at 25 °C was added slowly octanoyl chloride (5.0 g, 27 mmol). The resulting solution was stirred at 25 °C for 10 min, a solution of **7** (5.0 g, 24 mmol) in 10 mL of 1,2-dichloroethane was added slowly, and the mixture was stirred at 25 °C for 3 h. The reaction was quenched with ice and water, and the product was extracted into dichloromethane (3 × 100 mL). The combined organic layer was washed with brine, dried over anhydrous

Na₂SO₄, and evaporated under reduced pressure to afford crude product **8i** (8.0 g, 100%) as white solid. The crude product was used for next step without further purification.

Representative procedure for the hydrolysis of **8i**

A solution of **8i** (8.0 g, 24 mmol) in 100 mL of 1,4-dioxane was stirred with 100 mL of 5N NaOH at 25 °C for 20 h. The organic layer was collected, and the aqueous layer was thoroughly extracted with ethyl acetate. The combined extracts were washed with brine, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure to give (4-Chloro-phenyl)-(1H-pyrrol-3-yl)-methanone (**9i**) as a solid material (4.6 g, 100%).

Synthesis of (5-bromo-3-methoxy-pyrrol-2-ylidenemethyl)-diethyl-amine (**12**)

To a mixture of diethylformamide (2.68 g, 26.5 mmol) and chloroform (10 mL) at 0 °C was added drop wise a solution of phosphorus oxybromide (6.32 g, 22.1 mmol) in chloroform (10 mL). The resulting suspension was stirred at 0 °C for 30 min, and the solvent was removed by rotary evaporation to obtain the Vilsmeier complex as a white solid. After drying in vacuo for 20 min, chloroform (10 mL) was added to the solid and cooled to 0 °C. A solution of 4-methoxy-3-pyrrolin-2-one (**11**) (1.0 g, 8.8 mmol) in chloroform (20 mL) was added drop wise and the mixture was warmed to room temperature, and then heated at 60 °C for 5 h. The mixture was poured onto ice water (75 mL), and the pH of the aqueous solution was adjusted to pH 7–8 by treatment with 2N NaOH. Ethyl acetate (40 mL) was added to the resulting precipitate and the mixture was filtered over celite to remove the black solid containing phosphorus salts. The two layers were separated and the aqueous layer was extracted with EtOAc (3 × 100 mL). The organic layers were combined, washed with brine (3 × 200 mL), dried over anhydrous Na₂SO₄, filtered and the solvent was removed by rotary evaporation to furnish the crude bromo enamine **12**. The residue was filtered over a pad of silica gel (50 mL) using a 10% EtOAc/hexanes as eluent to obtain the enamine as an oil, which upon drying in vacuo led to a solid (1.64 g, 72%). ¹H NMR (CDCl₃, 100 MHz) δ 6.90 (s, 1H), 5.50 (s, 1H), 4.05 (q, *J* = 7.1 Hz, 2H), 3.65 (s, 2H), 3.30 (q, *J* = 7.1 Hz, 2H), 1.25 (t, *J* = 7.1 Hz, 3H), 1.20 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 165.3, 138.7, 133.5, 120.8, 96.4, 58.0, 51.2, 44.5, 18.3, 21.2.

Representative procedure for the preparation of compound **13**

To a degassed solution of toluene (5 mL) were added Pd(OAc)₂ (303 mg, 1.3 mmol) and PPh₃ (1.6 g, 6.1 mmol). The mixture immediately turns to bright yellow color and was stirred at 70 °C for 30 min under N₂. A solution of **12** (3.5 g, 13.5 mmol) and *N*-Boc-2-pyrroleboronic acid (3.72 g, 17.6 mmol) in 10% water/dioxane (50 mL) was degassed and purged with N₂. The solution was transferred to the suspension of Pd(PPh₃)₄ in toluene followed by the addition of Na₂CO₃ (4.31 g, 40.6 mmol). The mixture was stirred for 3 h at 100 °C and then poured onto water (100 mL), the pH of the solution was lowered to pH 7 with 2N HCl. The brown precipitate was recovered by filtration over a fritted disc funnel and washed with water then acetone. The yellow solid was washed with 10 mL of CHCl₃ then Et₂O (2 × 10 mL). The desired product **13** is obtained as a yellow solid and used without further purification (2.32 g, 90%). ¹H NMR (DMSO-d₆, 400 MHz) δ 11.40 (br s,

1H), 11.20 (br s, 1H), 9.30 (s, 1H), 6.90 (t, $J = 4.4$ Hz, 1H), 6.75 (s, 1H), 6.25 (d, $J = 4.4$ Hz, 1H), 6.10 (d, $J = 4.4$ Hz, 1H), 3.80 (s, 3H).

Representative procedure for the synthesis of 4'-methoxy-5'-(3-octyl-1H-pyrrol-2-ylmethylene)-1H,5'-H-[2,2']bipyrrolyl (36)

To a stirred suspension of **13** (220 mg, 1.15 mmol) and compound **10d** (269 mg, 1.5 mmol) in anhydrous methanol (10 mL) was added methanolic 2N HCl (catalytic amount). The resulting bright colored solution was stirred for 5 h at room temperature. The methanol was removed under reduced pressure; the crude solid was dissolved in ethyl acetate (50 mL), and washed with saturated NaHCO₃ solution (2 × 25 mL). The organic layer was dried over anhydrous Na₂SO₄, the solvent was removed under reduced pressure, and the crude product was chromatographed on neutral alumina as stationary phase and hexane-ethyl acetate as mobile phase to afford the final prodiginine analog **36** (256 mg, 63%). ¹H NMR (CDCl₃, 400 MHz) δ 6.92 (s, 1H), 6.77 (m, 1H), 6.72 (dd, $J = 1.2, 2.4$ Hz, 1H), 6.60 (d, $J = 2.4$ Hz, 1H), 6.21 (dd, $J = 0.9, 2.7$ Hz, 1H), 6.06 (s, 1H), 5.97 (d, $J = 2.5$ Hz, 1H), 4.00 (s, 3H), 2.58 (t, $J = 7.6$ Hz, 2H), 1.57 (m, 2H), 1.28 (m, 10H), 0.90 (t, $J = 6.6$ Hz, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 168.5, 159.8, 139.8, 134.6, 128.5, 126.8, 125.3, 122.5, 113.4, 112.6, 110.4, 110.2, 95.1, 58.4, 31.9, 31.4, 29.5 (2C), 29.3, 26.1, 22.7, 14.1; HRMS (ESI) calcd for C₂₂H₂₉N₃O (M+H)⁺: 352.2383, found. 352.2387. Note- Most of these prodiginine analogs were in the form of HCl salt (Addition of ethereal HCl to the pure prodiginine analog).

Representative procedure for the synthesis of 1-(5-octanoyl-1H-pyrrol-3-yl)-octan-1-one (44)

To a stirred suspension of anhydrous AlCl₃ (1.34 g, 10.1 mmol) in 30 mL of 1,2-dichloroethane at 25 °C was added slowly octanoyl chloride (1.34 g, 8.1 mmol). The resulting solution was stirred at 25 °C for 10 min, a solution of **9i** (1.3 g, 6.7 mmol) in 10 mL of 1,2-dichloroethane was added slowly, and the mixture was stirred at 25 °C for 2 h. The reaction was quenched with ice and water, and the product was extracted into dichloromethane (3 × 50 mL). The combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure to afford **44** (2.14 g, 100%) as white solid. The crude product was washed with hot hexane in greater than 96% of overall yield. ¹H NMR (CDCl₃, 400 MHz) δ 10.26 (br s, 1H), 7.60 (dd, $J = 1.5, 1.9$ Hz, 1H), 7.32 (dd, $J = 0.9, 1.5$ Hz, 1H), 2.78 (m, 4H), 1.72 (m, 4H), 1.38-1.25 (m, 16H), 0.89 (m, 6H); ¹³C NMR (CDCl₃, 100 MHz) δ 196.1, 192.2, 132.6, 127.5, 127.2, 115.2, 39.8, 38.1, 31.7 (2C), 29.4, 29.3, 29.1, 29.0, 25.1, 24.7 (2C), 22.6, 14.1 (2C).

Representative procedure for the synthesis of 2,4-dioctyl-1H-pyrrole (45)

To a stirred solution of **44** (1.3 g, 4.0 mmol) in 150 mL of 2-propanol at 25 °C was added slowly sodium borohydride (2.11 g, 57.0 mmol), and the reaction mixture was heated at reflux for 10 h. The hot reaction mixture was poured into 150 mL of ice water, and the solution was acidified with 10% aqueous HCl. The suspension was extracted with dichloromethane (3 × 50 mL). The combined organic extracts were washed with water, brine, and dried over anhydrous Na₂SO₄. The solvent was evaporated under reduced pressure to give **45**, and the crude product was chromatographed on neutral alumina to

afford the *title compd* **45** (865 mg, 73%). ¹H NMR (CDCl₃, 400 MHz) δ 7.60 (br s, 1H), 7.40 (s, 1H), 5.76 (s, 1H), 2.53 (t, *J* = 7.6 Hz, 2H), 2.41 (t, *J* = 7.6 Hz, 2H), 1.57 (m, 4H), 1.33 (m, 18H), 0.87 (m, 6H); ¹³C NMR (CDCl₃, 100 MHz) δ 132.8, 124.9, 112.8, 105.4, 31.9 (2C), 31.2, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 27.9, 27.4, 27.2, 22.7 (2C), 14.1 (2C).

Representative procedure for the synthesis of 4'-methoxy-5'-(5-octyl-3-propyl-1H-pyrrol-2-ylmethylene)-1H,5'H-[2,2']bipyrrolyl, hydrochloride (48**)**

To a stirred suspension of **13** (100 mg, 0.52 mmol) and compound **45** (151 mg, 0.68 mmol) in anhydrous methanol (10 mL) was added methanolic 2N HCl (Catalytic amount). The resulting bright colored solution was stirred for 5 h at room temperature. The methanol was removed under reduced pressure; the crude solid was dissolved in ethyl acetate (50 mL), and washed with saturated NaHCO₃ solution (2 × 25 mL). The organic layer was dried over anhydrous Na₂SO₄, the solvent was removed under reduced pressure, and the crude product was chromatographed on neutral alumina as stationary phase and hexane-ethyl acetate as mobile phase to afford the final prodiginine analog **48** (143 mg, 69%). Note-Most of these prodiginine analogs were in the form of HCl salt (Addition of ethereal HCl to the pure prodiginine analog). ¹H NMR (CDCl₃, 400 MHz) δ 12.79-12.60 (3 br s, 3H), 7.20 (m, 1H), 7.04 (s, 1H), 6.90 (m, 1H), 6.33 (m, 1H), 6.08 (br s, 2H), 4.01 (s, 3H), 2.92 (t, *J* = 7.7 Hz, 2H), 2.62 (t, *J* = 7.5 Hz, 2H), 1.75 (m, 2H), 1.64 (m, 2H), 1.41-1.31 (m, 10H), 0.97 (t, *J* = 7.3 Hz, 3H), 0.87 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 165.6, 153.4, 147.4, 145.5, 126.7, 124.2, 122.3, 120.1, 116.7, 113.2, 112.7, 111.6, 92.7, 58.7, 31.9, 29.4, 29.3, 29.2, 29.1, 28.4, 28.3, 24.2, 22.7, 14.1, 13.9; HRMS (ESI) calcd for C₂₅H₃₆N₃O (M+H)⁺. 394.2853, found. 394.2853.

In vitro antimalarial activity: *P. falciparum* growth inhibition

P. falciparum pansensitive D6 and MDR Dd2 (parasites MRA-285, MRA-156, ATCC, Manassas, Virginia) were cultivated under standard conditions, as previously described,¹⁸ and in vitro susceptibility to test compounds was determined by measuring growth after 72 hours in the presence of varying concentrations of test compound or CQ (positive control), or no drug; each condition in quadruplicate. Growth inhibition was determined using the SYBR Green I-based fluorescence assay¹⁹ with minor modifications as previously described,²⁰ and expressed as the compound concentration inhibiting growth by 50% (IC₅₀).

In vivo antimalarial efficacy: *P. yoelii* murine patent infection

Female outbred CF-1 mice (Charles River, Wilmington, MA) were injected intravenously via tail vein with 5 × 10⁵ erythrocytes infected with *P. yoelii*-Kenya (parasite MRA-428, ATCC, Manassas, VA), and groups of five mice were then treated either with test compound or vehicle only, once each day for three days beginning 24 hours after infection. All compounds were administered by gavage in caprylic/capric triglyceride vehicle (Miglyol 812, Sasol, Hamburg, Germany). One day after the final dose, thin blood smears were made from all mice, stained using a modified Giemsa method, and parasitemia ((infected erythrocytes/total erythrocytes) × 100) was determined using microscopy and compared to untreated controls. Mice without detectable parasitemia were observed and monitored by

serial examination of blood smears. Infected mice were euthanized; those remaining parasite-free for 28 days were considered cured.

Alamar Blue assay for mammalian cell viability

The general cytotoxic effects of prodiginine analogs on host cells were assessed by a functional assay as described previously¹⁵ using murine splenic lymphocytes induced to proliferate and differentiate by concanavalin A. Splenic lymphocytes isolated from C57BL/6J mice were washed twice in RPMI 1640 medium, and resuspended in complete RPMI containing 10% fetal bovine serum (FBS), 50 µg/mL penicillin/streptomycin, 50 µM β-mercaptoethanol, and 1 µg/mL concanavalin A. Cells (100 µL/well) then were seeded into 96-well flat-bottom tissue culture plates containing drug solutions (100 µL) serially diluted with complete culture medium to a final cell density of 2×10^5 per well. The plates were then incubated for 72 h in a humidified atmosphere at 37 °C and 5% CO₂. An aliquot of a stock solution of resazurin (Alamar Blue, prepared in 1 × PBS) was then added at 20 µL per well (final concentration, 10 µM), and the plates were returned to the incubator for another 24 h. After this period, the fluorescence in each well was measured in a Gemini EM plate reader with an excitation wavelength at 560 nm and an emission wavelength at 590 nm. IC₅₀ values were determined by non-linear regression analysis of logistic concentration-fluorescence intensity curves (GraphPad Prism software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Naturally occurring alkyl prodiginines (**1-4**)

Figure 2.
SAR of combined alkyl chain length for mono and dialkylated prodiginines and in vitro antimalarial activity against D6 strain of *P. falciparum*

Scheme 1.
Synthesis of 2-alkylated pyrroles (**6a-h**)

Scheme 2.
Synthesis of 3-alkylated pyrroles (**10a-k**)

Scheme 3.

Synthesis of 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (**13**) and its analogs (**14-17**).

R_1 = Phenyl, 2-heteroaryl groups; R_2, R_3 = CH_3 or R_2 = $n\text{-C}_{11}\text{H}_{23}$, R_3 = H (**18-25**)

R_1 = Alkyl groups, R_2 = H (**26-31**); R_1 = H; R_2 = Alkyl/aryl groups (**32-43**)

Scheme 4.
Synthesis of various prodiginine analogs (**18-43**)

Scheme 5.

Synthesis of 2,4-disubstituted pyrroles (**45**), and the corresponding prodiginine analogs (**46-75**).

In vitro antimalarial activity of prodiginine analogs where the non alkylated terminal pyrrole (left hand side) has been substituted

Table 1

18-25

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)			Cytotoxicity (nM)
				D6	Dd2	135	
CQ	-	-	-	11	135	16000

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
18		CH ₃	CH ₃	4250	4950	< 125

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
19		n-C ₁₁ H ₂₃	H	4060	6150	3410

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
20		n-C ₁₁ H ₂₃	H	10470	15750	3580

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
21		CH ₃	CH ₃	19410	13640		7950

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
22		n-C ₁₁ H ₂₃	H	2920	3810		980

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
23		CH ₃	CH ₃	> 25000	> 25000		3530

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
24		n-C ₁₁ H ₂₃	H	5940	7770		2120

Compd	R ₁	R ₂	R ₃	IC ₅₀ (nM)		Cytotoxicity (nM)	
				D6	Dd2		
25		CH ₃	CH ₃	> 25000	> 25000		3890

Table 2

In vitro antimalarial activity of prodiginines containing alkyl substituents at the 3 and 5 positions of the terminal pyrrole ring

26-38 and 46-55

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
CQ	-	-	11	135	16057	
26	n-C ₃ H ₇	H	2300	N.D.	N.D.	
27	n-C ₄ H ₉	H	1780	1590	< 125	
28	n-C ₆ H ₁₃	H	375	450	< 125	
29	n-C ₈ H ₁₇	H	80	130	< 125	
30	n-C ₁₆ H ₃₃	H	300	400	< 125

26-38 and 46-55

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
31	n-C ₁₁ H ₂₂ NH ₂	H	1700	N.D.	N.D.	
32	H	(CH ₂) ₃ COOCH ₃	4500	N.D.	N.D.	
33	H	CH ₂ CH(CH ₃) ₂	460	230	< 125	
34	H	n-C ₄ H ₉	80	18	< 125	
35	H	n-C ₆ H ₁₃	28	7	< 125	
36	H	n-C ₈ H ₁₇	4.6	1.8	< 125	
37	H	n-C ₁₀ H ₂₁	8.0	10	< 125	
38	H	n-C ₁₆ H ₃₃	> 25000	> 25000	3628

26-38 and 46-55

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
46	CH ₃	CH ₃	8900	8130	< 125	< 125
47	n-C ₆ H ₁₃	n-C ₃ H ₇	4.5	4.0	< 125	< 125
48	n-C ₈ H ₁₇	n-C ₃ H ₇	2.9	2.7	< 125	< 125
49	n-C ₃ H ₇		1.7	1.3	< 125	< 125
50	n-C ₆ H ₁₃	n-C ₆ H ₁₃	1.7	1.1	< 125	< 125
51	n-C ₇ H ₁₅	n-C ₆ H ₁₃	2.1	1.2	< 125	< 125

26-38 and 46-55

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)
			D6	Dd2	
52	n-C ₆ H ₁₃	n-C ₈ H ₁₇	4.9	2.0	< 125
53	n-C ₇ H ₁₅	n-C ₈ H ₁₇	6.2	2.9	3906
54	n-C ₈ H ₁₇	n-C ₈ H ₁₇	92	129	31250
55			5.3	3.5	< 125

N.D.: not determined

Table 3

In vitro antimalarial activity of prodiginines containing aryl substituents at either the 3, or the 3 and 5 positions, of the terminal pyrrole ring

39-43 and 56-75

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
CQ	-	-	11	135		16057
39	H	C ₆ H ₅ CH ₂	83	86		< 125
40	H	4-OCH ₃ C ₆ H ₄ CH ₂	170	156		< 125
41	H	4-ClC ₆ H ₄ CH ₂	65	81		< 125
42	H	4-BrC ₆ H ₄ CH ₂	90	108		< 125
43	H	2-NaphthylCH ₂	56	N.D.		N.D.
56	C ₂ H ₅	4-ClC ₆ H ₄ CH ₂	6.3	6.2		< 125

39-43 and 56-75

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
57	n-C ₃ H ₇	4-ClC ₆ H ₄ CH ₂	3.0	2.6	< 125	
58	n-C ₆ H ₁₃	4-ClC ₆ H ₄ CH ₂	2.0	1.8	< 125	
59	n-C ₇ H ₁₅	4-ClC ₆ H ₄ CH ₂	2.8	2.2	< 125	
60	n-C ₈ H ₁₇	4-ClC ₆ H ₄ CH ₂	16.0	12.0	1105	
61	4-ClC ₆ H ₄ CH ₂		3.9	2.9	1007	
62	n-C ₆ H ₁₃	4-FC ₆ H ₄ CH ₂	0.9	0.9	1462	
63	n-C ₈ H ₁₇	4-FC ₆ H ₄ CH ₂	1.3	1.2	< 125

39-43 and 56-75

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
64	n-C ₆ H ₁₃	4-BrC ₆ H ₄ CH ₂	2.9	2.8	< 125	
65	n-C ₈ H ₁₇	4-BrC ₆ H ₄ CH ₂	4.0	2.9	< 125	
66	4-ClC ₆ H ₄ CH ₂	4-ClC ₆ H ₄ CH ₂	6.1	4.8	< 125	
67	4-FC ₆ H ₄ CH ₂	4-FC ₆ H ₄ CH ₂	5.6	5.7	< 125	
68	4-BrC ₆ H ₄ CH ₂	4-BrC ₆ H ₄ CH ₂	14.0	11.0	< 125	
69	4-FC ₆ H ₄ CH ₂	4-ClC ₆ H ₄ CH ₂	6.1	6.1	< 125	
70	4-BrC ₆ H ₄ CH ₂	4-ClC ₆ H ₄ CH ₂	8.3	7.7	< 125	
71	4-BrC ₆ H ₄ CH ₂	4-FC ₆ H ₄ CH ₂	5.7	5.1	< 125	
72	2,4-Cl ₂ -C ₆ H ₃ CH ₂	2,4-Cl ₂ -C ₆ H ₃ CH ₂	12.6	11.0	< 125

39-43 and 56-75

Compd	R ₁	R ₂	IC ₅₀ (nM)		Cytotoxicity (nM)	
			D6	Dd2		
73	2,6-F ₂ C ₆ H ₃ CH ₂	2,6-F ₂ C ₆ H ₃ CH ₂	14.7	18.3	< 125	
74	3-FC ₆ H ₄ CH ₂	3-FC ₆ H ₄ CH ₂	5.1	6.7	< 125	
75	2-ClC ₆ H ₄ CH ₂	2-ClC ₆ H ₄ CH ₂	3.6	4.9	< 125	

N.D.: not determined

Table 4In vivo antimalarial efficacy of prodiginine analogs in *P. yoelii* murine infection

Compd	Dose mg/kg/day	No. of treated mice	% Group mean weight; Day3 vs Day0	% Parasitemia reduction vs controls; Day3
Controls	-	5	100.8	0
41	5	5	101.2	38
41	25	5	105.0	90
48	5	5	100.1	90
48	25	4	92.4	100
49	5	5	101.5	97
49	25	4	84.5	100
57	5	5	102.9	94
57	25	5	87.6	100
58	5	5	100.3	99
58	25	4	96.0	100
60	5	5	100.2	72
60	25	3	93.4	100
66	5	5	107.5	92
66	25	5	102.5	100