

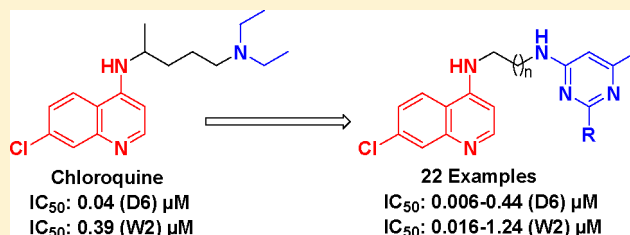
Novel 4-Aminoquinoline-Pyrimidine Based Hybrids with Improved in Vitro and in Vivo Antimalarial Activity

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Supporting Information

ABSTRACT: A class of hybrid molecules consisting of 4-aminoquinoline and pyrimidine were synthesized and tested for antimalarial activity against both chloroquine (CQ)-sensitive (D6) and chloroquine (CQ)-resistant (W2) strains of *Plasmodium falciparum* through an in vitro assay. Eleven hybrids showed better antimalarial activity against both CQ-sensitive and CQ-resistant strains of *P. falciparum* in comparison to standard drug CQ. Four molecules were more potent (7–8-fold) than CQ in D6 strain, and eight molecules were found to be 5–25-fold more active against resistant strain (W2). Several compounds did not show any cytotoxicity up to a high concentration (60 μM), others exhibited mild toxicities, but the selective index for the antimalarial activity was very high for most of these hybrids. Two compounds selected for in vivo evaluation have shown excellent activity (po) in a mouse model of *Plasmodium berghei* without any apparent toxicity. The X-ray crystal structure of one of the compounds was also determined.

KEYWORDS: 4-aminoquinoline, pyrimidine, antimalarial



In terms of human deaths per year, malaria is the third most infectious disease after HIV and tuberculosis.¹ According to the World malaria report 2011, there were 216 million cases of malaria in 106 endemic countries and territories in the world. The World Health Organization (WHO) estimates that over 225 million episodes of malaria occur annually with 0.7–1.0 million deaths a year, and over 85% of deaths are of children below the age of 5 years in sub-Saharan Africa.² *Plasmodium falciparum* is the most virulent species of the malaria parasite and is responsible for most of the malaria-related deaths.^{3,4} The 4-aminoquinoline class of therapeutics remains a frontline drug of choice for combating malaria even after several decades of drug development efforts.⁵ The success of this antimalarial pharmacophore is based on its excellent clinical efficacy, ease of administration, low toxicity, and cheap synthesis.⁵ These features make this pharmacophore so interesting that it is very difficult to abandon. However, a worldwide increase of *P. falciparum*-resistant strains⁶ has severely circumscribed the choice of traditionally used chloroquine (CQ, 1), while amodiaquine was abandoned due to its severe toxic effects (Figure 1). However, despite this global diffusion of resistance at an alarming speed, 4-aminoquinoline derivatives continue to be the most sought out antimalarial agents for chemical modification. To overcome the problem of drug resistance, combination therapy has been used with limited success,⁷ and recently, the concept of hybrid molecules has been introduced in an anticipation that these kind of molecules may overcome drug resistance problems.^{8–10} In hybrid molecules, two or more pharmacophores are linked together covalently, and it is

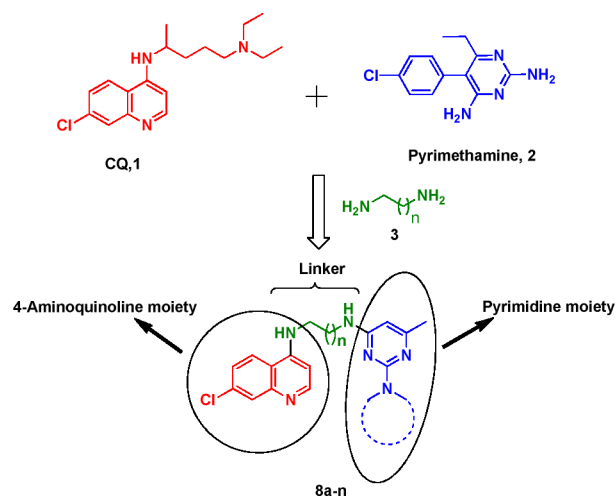


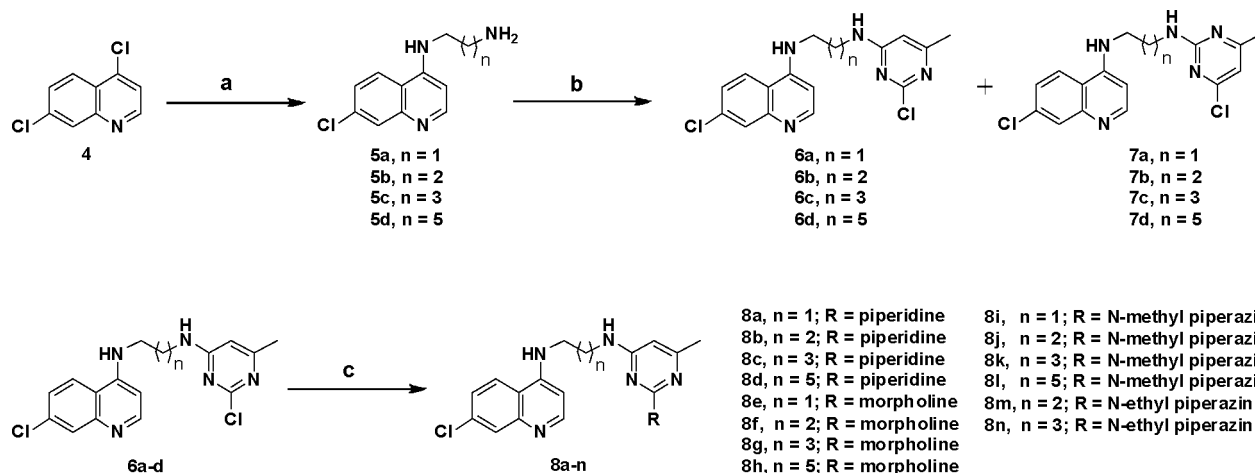
Figure 1. Synthesis of 4-aminoquinoline-pyrimidine hybrids under present investigation.

believed that these compounds act by inhibiting simultaneously two conventional targets. This multiple target strategy led to the discovery of various hybrid molecules including 4-aminoquinoline-trioxane,¹⁰ 4-aminoquinoline-triazine,^{11,12} 4-aminoquinoline-isatin,¹³ 4-aminoquinoline-ferrocene,¹⁴ and,

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Scheme 1^a

^aReagents and conditions: (a) Diaminoalkanes ($n = 1-3, 5$), neat, 120–130 °C, 6–8 h, 80–90%. (b) 2,4-Dichloro-6-methyl-pyrimidine, Et₃N, EtOH, rt, overnight, (6a–d, 70–80%; and 7a–d, 15–25%). (c) R = secondary amines, DMF, 100–120 °C, 10–12 h, 80–85%.

Table 1. In Vitro Antimalarial Activity and Cytotoxicity of 4-Aminoquinoline-pyrimidine Hybrids^a

compd no.	<i>P. falciparum</i> (D6 clone)		<i>P. falciparum</i> (W2 clone)		IC ₅₀ (μM)		
	IC ₅₀ (μM) ^b	SI	IC ₅₀ (μM)	SI	VERO	LLC-PK ₁₁	HepG2
6a	0.16 ± 0.05	>3.70 × 10 ²	0.5 ± 0.2	>1.20 × 10 ²	NC	NC	NC
6b	0.33 ± 0.02	>1.84 × 10 ²	0.70 ± 0.04	>85	NC	NC	NC
6c	0.12 ± 0.03	>5.00 × 10 ²	0.68 ± 0.01	>88	NC	NC	NC
6d	0.44 ± 0.02	74.3	0.54 ± 0.00	60.6	32.7 ± 1.8	15.8 ± 1.0	9.1 ± 0.2
7a	0.21 ± 0.06	2.05 × 10 ²	0.81 ± 0.10	53.1	43.0 ± 0.0	42.3 ± 0.7	43.0 ± 0.0
7b	0.24 ± 0.03	>2.50 × 10 ²	1.17 ± 0.07	>51.3	NC	NC	NC
7c	0.17 ± 0.02	>3.53 × 10 ²	0.64 ± 0.03	>94	NC	NC	46.4 ± 6.6
7d	0.14 ± 0.03	3.53 × 10 ²	0.58 ± 0.01	85.2	49.4 ± 6.2	24.0 ± 0.6	8.1 ± 0.0
8a	ND	ND	ND	ND	ND	ND	ND
8b	0.02 ± 0.001	>3.00 × 10 ³	0.21 ± 0.003	>2.85 × 10 ²	NC	NC	NC
8c	0.02 ± 0.002	>3.00 × 10 ³	0.09 ± 0.01	>6.66 × 10 ²	NC	NC	NC
8d	0.06 ± 0.01	1.46 × 10 ²	0.10 ± 0.02	88	8.8 ± 0.5	6.3 ± 0.4	6.4 ± 0.3
8e	0.02 ± 0.002	>3.00 × 10 ³	0.14 ± 0.02	>4.44 × 10 ²	NC	NC	NC
8f	0.02 ± 0.00	>3.00 × 10 ³	0.05 ± 0.004	>1.20 × 10 ³	NC	35.7 ± 0.6	26.2 ± 4.8
8g	ND	ND	ND	ND	ND	ND	ND
8h	0.03 ± 0.01	5.03 × 10 ²	0.14 ± 0.02	1.07 × 10 ²	15.1 ± 2.0	11.1 ± 1.3	6.4 ± 0.3
8i	0.005 ± 0.001	>1.20 × 10 ⁴	0.03 ± 0.01	>2.00 × 10 ³	NC	NC	NC
8j	0.007 ± 0.00	>8.57 × 10 ³	0.06 ± 0.002	>1.00 × 10 ³	NC	34.6 ± 0.6	26.7 ± 0.9
8k	0.02 ± 0.001	>3.00 × 10 ³	0.02 ± 0.003	>3.00 × 10 ³	NC	34.6 ± 0.6	29.5 ± 2.3
8l	0.007 ± 0.001	1.48 × 10 ³	0.016 ± 0.001	6.50 × 10 ²	10.4 ± 0.1	7.2 ± 0.2	6.3 ± 0.4
8m	0.006 ± 0.00	>1.00 × 10 ⁴	0.06 ± 0.001	>1.00 × 10 ³	NC	NC	NC
8n	0.02 ± 0.00	1.62 × 10 ³	0.02 ± 0.003	1.62 × 10 ³	32.4 ± 0.5	10.7 ± 0.2	15.4 ± 0.6
CQ	0.04 ± 0.004	>1.50 × 10 ³	0.39 ± 0.04	>1.52 × 10 ²	NC	NC	NC
artemisinin	0.01 ± 0.001	>6.00 × 10 ³	0.01 ± 0.00	>6.00 × 10 ³	NC	NC	NC
pyrimethamine	0.01 ± 0.003	1.82 × 10 ³	NA		18.2 ± 0.1	NT	NT
doxorubicin					8.6 ± 0.4	1.05 ± 0.05	1.0 ± 0.0

^aIC₅₀, the concentration that causes 50% growth inhibition; SI, selectivity index (IC₅₀ for cytotoxicity to Vero cells/IC₅₀ for antimalarial activity); and ND, not determined. ^bAverage values of two independent experiments ± standard deviations (SDs). NA, not active up to highest tested concentration (19 μM). NC, no cytotoxicity up to 60 μM; NT, not tested; Vero, monkey kidney fibroblasts; LLC-PK₁₁, pig kidney epithelial cells; and HepG2, human hepatoma cells.

more recently, 4-aminoquinoline-based mannich bases.¹⁵ It is important to mention here that some of these hybrid compounds have also entered into clinical trials.⁹

The pyrimidine-based compounds are well-known for their wide range of biological activity such as fungicidal,¹⁶ herbicidal,¹⁷ analgesic,¹⁸ anti-inflammatory,¹⁹ antitumor,²⁰ and antimalarial²¹ activities apart from their role in the nucleic acid

synthesis.²² The pyrimethamine (2), a pyrimidine analogue, has been used for the treatment of malaria with limited success.²³

On the basis of these observations and in continuation of our efforts to develop new structurally diverse molecular scaffolds for the treatment of malaria,^{24–26} we proposed to link 4-aminoquinoline and pyrimidine entities together via flexible linear-chained diaminoalkane (3) linkers (Figure 1), so that the

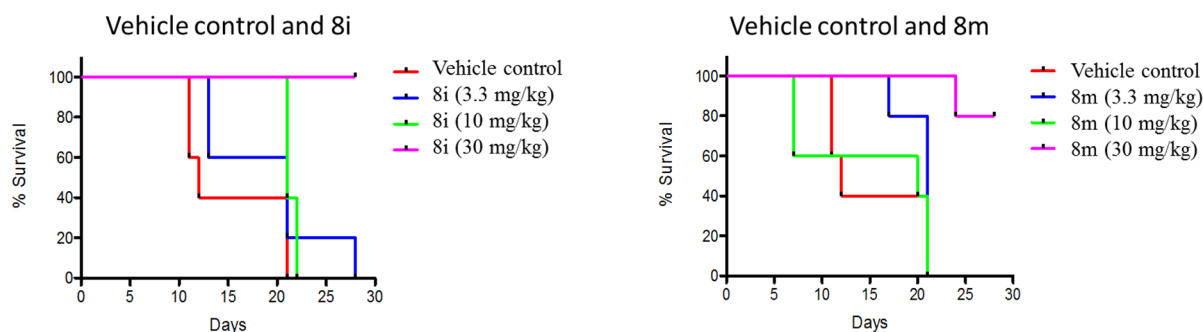


Figure 2. Dose regime of compounds **8i** and **8m**.

molecule has enough flexibility to fit in the binding site of the target, and as a result, this kind of hybrid molecules may show better antimalarial activity. It is important to mention here that Chauhan et al. reported the synthesis of 4-aminoquinoline-pyrimidine conjugates in which 4-aminoquinoline and pyrimidine moieties were linked through an aromatic ring,²⁷ and some of these compounds have shown moderate activity.

The 4-aminoquinoline-pyrimidine conjugates were synthesized using a three-step procedure as outlined in Scheme 1.²⁸ The commercially available starting material 4,7-dichloroquinoline (**4**) was reacted with an excess of aliphatic linear chain diaminoalkanes via a S_NAr type of reaction in neat conditions as reported in the literature to afford substituted 4-aminoquinolines (**5a–d**) with free terminal amino groups in good to excellent yield.²⁹ These intermediates (**5a–d**) on reaction with commercially available 2,4-dichloro-6-methyl-pyrimidine yielded two regioisomers viz. **6a–d** in major and **7a–d** in minor yield. The major regioisomers **6a–d** were subjected to nucleophilic substitution with different cyclic secondary amines at an elevated temperature in DMF as the solvent to yield 4-aminoquinoline-pyrimidine conjugates (**8a–n**) in excellent yield. The crystal structure of one of the conjugate **8f** was also determined.

The in vitro antimalarial activity of all of the conjugates was evaluated against both CQ-sensitive (D6 clone) and CQ-resistant (W2 clone) strains of *P. falciparum*, while mammalian cell cytotoxicity was determined against Vero, LLC-PK₁₁, and HepG2 cells (Table 1) using the procedure as described earlier.³⁰ Most of the compounds showed very promising antimalarial activity in the in vitro screening. Out of these, 11 compounds (**8b,c**, **8e,f**, and **8h–n**) have displayed better antimalarial activity ($IC_{50} = 0.005–0.03 \mu M$) against the CQ-sensitive strain, while 12 compounds (**8b–f** and **8h–n**) have displayed better antimalarial activity ($IC_{50} = 0.01–0.21 \mu M$) against the CQ-resistant strains of *P. falciparum*. Four hybrids (**8i**, **8j**, **8l**, and **8m**) showed 1.6–2.0-fold higher activity in comparison to pyrimethamine against both drug-sensitive and drug-resistant strains (D6 and W2) of *P. falciparum*, in contrast to pyrimethamine, which was inactive up to 19 μM concentration against W2 strain. Pyrimidine-based compounds N1-[6-methyl-2-(4-methyl-piperazin-1-yl)-pyrimidin-4-yl]-ethane-1,2-diamine and N-(7-chloro-quinolin-4-yl)-N'-[2-(4-ethyl-piperazin-1-yl)-6-methyl-pyrimidin-4-yl]-propane-1,3-diamine, an integral part of the most active compounds **8i** and **8m** showed appreciable activity against D6 strain ($IC_{50} = 0.12$ and 0.18 μM , respectively), while these compounds showed poor antimalarial activity ($IC_{50} = 10$ and 16 μM , respectively) against the drug-resistant (W2) strain. As evident from Table 1, these

hybrids showed efficacy in the nanomolar range against both of the strains (D6 and W2), and it indicates an improved activity of the two molecules in the conjugate form as compared to the individual form.

The selectivity index of antimalarial activity (calculated as a ratio of IC_{50} for cytotoxicity to Vero cells and IC_{50} for antimalarial activity) was very high for most of these compounds as compared to standard drug CQ. The activity of compounds **8i**, **8j**, **8l**, and **8m** was 6–8-fold higher than CQ and 2-fold higher than artemisinin in CQ-sensitive strain, and the activity of compounds **8c**, **8f**, and **8i–n** was 6–26-fold higher than CQ-resistant strains, while compounds **8l** and **8n** showed comparable activity as artemisinin in the CQ-resistant strain, revealing their strong potency. The comparison of antimalarial activity of **6a–d** with **8a–n** clearly showed that substitution of Cl from compounds **6a–d** ($IC_{50} = 0.12–0.44 \mu M$ for CQ-sensitive and 0.50–0.70 μM for CQ-resistant) with secondary amines (**8b–f** and **8h–n**, $IC_{50} = 0.005–0.06 \mu M$ for CQ-sensitive and 0.02–0.21 μM for CQ-resistant) improves the antimalarial activity of these compounds. The comparison of antimalarial activity of two groups of regioisomers **6a–d** ($IC_{50} = 0.12–0.44 \mu M$ for CQ-sensitive and 0.50–0.70 μM for CQ-resistant) and **7a–d** ($IC_{50} = 0.14–0.24 \mu M$ for CQ-sensitive and 0.58–1.17 μM for CQ-resistant) clearly indicates that both the regioisomers displayed more or less similar potency against both of the strains of *P. falciparum*. These results indicate that the point of attachment of the spacer to the pyrimidine nucleus may not have a great impact on activity profile. For a particular amino-substituted 4-aminoquinoline-pyrimidine hybrids (**8a–d** or **8e–h** or **8i–l**), a structure–activity relationship (SAR) study demonstrated no obvious trend of activity with increasing or decreasing carbon spacers from C2 to C6, but changing the amino groups for a particular C2 (**8e** and **8i**), C3 (**8b**, **8f**, **8j**, and **8m**), C4 (**8c**, **8k**, and **8n**), or C6 (**8d**, **8h**, and **8l**) spacer changes the activity significantly in a decreasing order of 4-ethyl piperazine > 4-methyl piperazine > morpholine > piperidine.

Among the most active compounds (**8b–f** and **8h–n**), **8d**, **8h**, and **8l** showed toxicity to all of the three cell lines (VERO, LLC-PK₁₁, and HepG2), but their selectivity index of antimalarial activity was considerably high (Table 1). In general, the cytotoxicity of the most of the conjugates appeared at much higher concentrations than the concentrations responsible for their antimalarial activity. Some of the hybrids were not cytotoxic at all up to the highest tested concentration of 60 μM , indicating their high selectivity index of antimalarial activity versus cytotoxicity to mammalian cells.

Two of the potent molecules **8i** and **8m** were selected for further in vivo evaluation. In vivo antimalarial activity was determined through oral route of administration in *P. berghei*–mouse malaria model. The compounds were administered to the *P. berghei*-infected mice, through oral gavage, once daily on days 0, 1, and 2 days postinfection and monitored for apparent signs of toxicity, parasitemia, and survival until day 28 postinfection (Table 2). Both compounds exhibited excellent

Table 2. In Vivo Antimalarial Activity of Selected 4-Aminoquinoline-pyrimidine Hybrids in the *P. berghei*–Mouse Malaria Model

treatment (po)	dose (mg/kg × no. of days postinfection)	% suppression in parasitemia ^a				
		day 5	day 7	survival ^b	MST ^c	cure ^d
vehicle	NA × 4			0/5	15.2	0/5
CQ	100 × 3	100.0	100.0	5/5	28	0/5
8i	3.3 × 3	23.5	35.2	1/5	19.2	0/5
8i	10 × 3	97.0	2.0	0/5	21.4	0/5
8i	30 × 3	100.0	100.0	5/5	28	4/5
8m	3.3 × 3	20.1	19.1	0/5	20.2	0/5
8m	10 × 3	73.9	27.1	0/5	15.2	0/5
8m	30 × 3	100.0	100.0	4/5	27.2	1/5

^aThe % suppression in parasitemia is calculated by considering the mean parasitemia in the vehicle control as 100%. Parasitemia suppression <80% is considered as nonsignificant. ^bNumber of animals that survived on day 28/total animals in the group (the day of the death postinfection). ^cMST, mean survival time (days). ^dNumber of mice without parasitemia (cured) until day 28 postinfection.

in vivo antimalarial activity without any toxicity. The compound **8i** was better active as compared to **8m** and CQ, the control used in this study. Treatment with compound **8i**, at three doses of 30 mg/kg, produced almost complete suppression of parasitemia and cured 80% of the treated mice, as compared to only 20% cured by **8m** and no cure by CQ (Figure 2). Although CQ is a highly tolerated drug and produces cure in mice at higher doses, in the present in vivo study, the dose was selected to get the comparative results with the test compounds. The mechanistic studies involving inhibition of hemozoin formation (a potential target of CQ) and dihydrofolate reductase (a target for the pyrimethamine) and structural modification of the lead molecules are in progress, and results will be published in due course of time.

In summary, a series of 4-aminoquinoline-pyrimidine hybrids were prepared in an attempt to search for more potent molecule that can be effective against both drug-sensitive and drug-resistant strains of *P. falciparum*. The in vitro antimalarial activity profile of these compounds indicates that in comparison to CQ, 11 hybrids (**8b**, **8c**, **8e**, **8f**, **8h–n**) showed better antimalarial activity, while in comparison to pyrimethamine, four hybrids (**8i**, **8j**, **8l**, and **8m**) showed better activity against both drug-sensitive and drug-resistant (D6 and W2) of *P. falciparum*. Out of these hybrids, **8i**, **8j**, **8l**, and **8m** were 6–8 times more potent than CQ and 1.6–2.0 times more potent than pyrimethamine and artemisinin against the D6 strain. In the W2 strain, they were 6–26 times more potent than CQ and equally potent as artemisinin. In contrast, pyrimethamine was inactive against the drug-resistant strain. The pyrimethamine- and pyrimidine-based compounds are either not active or

weakly active against W2 strain, while these hybrids showed nanomolar range activity against both the strains, which confirms and indicates the synergistic effect of covalent binding of pyrimidine to aminoquinolines.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic method and characterization of compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

D.S.R. conceived and designed the work; S.M. and U.C.R. performed the synthesis and characterization of the compounds; and S.I.K. and B.L.T. are responsible for evaluation of in vitro and in vivo antimalarial activity.

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Notes

The authors declare no competing financial interest.

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