

Novel 4-Aminoquinoline Analogs Highly Active against the Blood and Sexual Stages of *Plasmodium* In Vivo and In Vitro

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New drugs to treat malaria must act rapidly and be highly potent against asexual blood stages, well tolerated, and affordable to residents of regions of endemicity. This was the case with chloroquine (CQ), a 4-aminoquinoline drug used for the prevention and treatment of malaria. However, since the 1960s, *Plasmodium falciparum* resistance to this drug has spread globally, and more recently, emerging resistance to CQ by *Plasmodium vivax* threatens the health of 70 to 320 million people annually. Despite the emergence of CQ resistance, synthetic quinoline derivatives remain validated leads for new drug discovery, especially if they are effective against CQ-resistant strains of malaria. In this study, we investigated the activities of two novel 4-aminoquinoline derivatives, TDR 58845, *N*¹-(7-chloro-quinolin-4-yl)-2-methyl-propane-1,2-diamine, and TDR 58846, *N*¹-(7-chloro-quinolin-4-yl)-2,*N*²,*N*²-trimethylpropane-1,2-diamine and found them to be active against *P. falciparum* in vitro and *Plasmodium berghei* in vivo. The *P. falciparum* clones and isolates tested were susceptible to TDR 58845 and TDR 58846 (50% inhibitory concentrations [IC₅₀s] ranging from 5.52 to 89.8 nM), including the CQ-resistant reference clone W2 and two multidrug-resistant parasites recently isolated from Thailand and Cambodia. Moreover, these 4-aminoquinolines were active against early and late *P. falciparum* gametocyte stages and cured BALB/c mice infected with *P. berghei*. TDR 58845 and TDR 58846 at 40 mg/kg were sufficient to cure mice, and total doses of 480 mg/kg of body weight were well tolerated. Our findings suggest these novel 4-aminoquinolines should be considered for development as potent antimalarials that can be used in combination to treat multidrug-resistant *P. falciparum* and *P. vivax*.

Approximately 40% of the world population lives in areas of malarial endemicity. Estimates indicate that the disease causes several hundred million cases and about 1.2 million deaths each year (25, 33). The two most prevalent species of *Plasmodium* that cause malaria in humans are *P. falciparum* and *P. vivax*. Severe disease and resistance to antimalarials have been documented for both species (43), and efforts to control malaria have become more challenging in recent years due to widespread drug resistance (12, 16).

Although *P. falciparum* resistance has been reported for most antimalarials, *P. vivax* resistance is generally limited to chloroquine (CQ), which was first reported in the late 1980s in Papua New Guinea and Indonesia (2, 9, 46). In addition, *P. vivax* has shown innate resistance to sulfadoxine but developed additional resistance when in combination with pyrimethamine in many areas (18). Alternative therapies such as artemisinin combinations for treatment of CQ-resistant malaria parasite infections have been developed. However, these therapies do not meet the simplicity of use and low cost of CQ (55).

For many decades, the least expensive and most effective anti-malarial was CQ, a 4-aminoquinoline drug. CQ has a rapid onset of action and low toxicity and is well tolerated (54). For these reasons, it was widely used worldwide in countries where malaria is endemic. CQ acts by inhibiting hemozoin aggregation in the digestive vacuole of the malaria parasite (14, 37). The heavy use of CQ resulted in the emergence of CQ resistance starting in Southeast Asia, South America, and Oceania and then reaching almost every corner of areas of malaria endemicity (17). Today more than 80% of isolates of *P. falciparum* are resistant to CQ due to mutations in the *P. falciparum* chloroquine-resistant transporter (*pfcr*) (13, 15). Parasite resistance to CQ resulted in a switch to the antifolate combination sulfadoxine-pyrimethamine (SP), which en-

countered resistance within a few years. Later, multidrug resistance (defined as resistance to three or more drugs) appeared in Southeast Asia and South America in response to the switch to either mefloquine (MFQ) or quinine (QUIN) and then in Africa in the late 1970s (6, 8, 53). Recently, the majority of countries have switched to highly efficient artemisinin derivative combinations (ACTs); nevertheless, there is new evidence for clinical resistance to these drugs (11).

Several molecules such as calcium channel blockers (verapamil [VER]) or tricyclic antidepressant (desipramine [DES]) (5, 26), antipsychotic agents (chlorpromazine [CPZ]) (28), and tricyclic histamine (H-1) receptor antagonists (promethazine [PMZ]) (35) have been reported to reverse the CQ resistance in *P. falciparum* in vitro. Of those, CPZ, prochlorperazine, and DES have been shown to have a similar effect in vivo in monkeys and humans (27, 35). The mechanism for resistance reversal is still a matter of debate. The common molecular characteristics of these compounds are two lipophilic aromatic residues and a basic amino alkyl side chain. It has been proposed that the aryl residues interact with a lipophilic pocket in the substrate binding site of the PfCRT, while the protonated amino group restores the positive charge that re-

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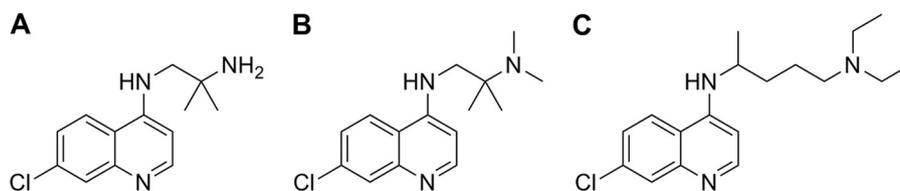


FIG 1 Structures of the 4-aminoquinoline derivatives used in this study. (A) TDR 58845, N^1 -(7-chloro-quinolin-4-yl)-2-methyl-propane-1,2-diamine; (B) TDR 58846, N^1 -(7-chloro-quinolin-4-yl)-2, N^2 , N^2 -trimethylpropane-1,2-diamine; (C) chloroquine, N^4 -(7-chloro-quinolin-4-yl)- N^1 , N^1 -diethyl-pentane-1,4-diamine.

pels the CQ dication (3). In addition, some authors have proposed that a decrease in the CQ uptake could account for drug resistance, while others propose that the CQ-resistant parasites rapidly expel the intracellular drug via a pump-mediated mechanism (4, 26, 32).

Even though there is widespread resistance of *P. falciparum* and *P. vivax* to CQ, synthetic quinoline derivatives have remained a validated lead class for new drug discovery. Quinoline derivatives such as MFQ and QUIN have strong activity against CQ-resistant strains of *P. falciparum*, and there have been several studies testing the activity of 4-aminoquinoline derivatives. In particular, among several 4-aminoquinoline derivatives, synthesized AQ-13, a three-carbon chain analog, exhibited activity against parasites resistant to CQ and MFQ (10) and was progressed to clinical trials (34). Recently, new classes of 4-aminoquinolines have been recognized and possess strong activity *in vitro* with excellent bioavailability (19, 21, 29, 30, 44). Several of these 4-aminoquinolines have been shown to be more active than CQ in CQ-resistant *P. falciparum* and to have activity in a mouse model *in vivo* (24, 36).

In this study, two novel 4-aminoquinoline derivatives, TDR 58845, N^1 -(7-chloro-quinolin-4-yl)-2-methyl-propane-1,2-diamine and TDR 58846, N^1 -(7-chloro-quinolin-4-yl)-2, N^2 , N^2 -trimethylpropane-1,2-diamine, were evaluated for their ability to eliminate malaria parasites *in vitro* and *in vivo*. These drugs share the quinoline core of CQ and possess a methylpropane and a trimethylpropane chain (Fig. 1). Both compounds, TDR 58845 and TDR 58846, were among a group of 130 synthesized by Hoffmann la Roche (20) and were found to be active against CQ-sensitive and CQ-resistant *P. falciparum*. Also, we have evaluated the potency of TDR 58845 and TDR 58846 against the blood and gametocyte stages of *P. falciparum*. Additionally, we have assessed the curative activities of these two compounds *in vivo* using a rodent malaria model. We show that these two compounds have excellent activity against different *Plasmodium* stages *in vitro* and *in vivo*, suggesting that 4-aminoquinoline analogs can be considered for development in combination with other drugs as antimalarials.

MATERIALS AND METHODS

Parasites. Strains and isolates of *P. falciparum* were cultured in human A+ erythrocytes by standard methods under a low-oxygen atmosphere (5% O₂, 5% CO₂, 90% N₂) according to the method of Trager and Jensen, 1976 (51). The culture medium was RPMI 1640 supplemented with 25 mM HEPES buffer, 10 mM glucose, 2 mM glutamine, and AB+ human plasma. Parasites were maintained in fresh human erythrocytes suspended at a 4% hematocrit in complete medium at 37°C. Stock cultures were subpassaged every 3 to 4 days by transfer of infected red cells to a flask containing complete medium and uninfected erythrocytes.

In this study, the following reference clones of *P. falciparum* were used: NF54 and its clone 3D7 (CQ sensitive, from an airport in the Nether-

lands), W2 (CQ and SP resistant, from Indochina), D6 (MQ resistant, from Sierra Leone), and TM90C2B (CQ, MQ, and atovaquone resistant, from Thailand). Additionally, we included two recent *P. falciparum* isolates, Thai6 and Cam6 (multidrug resistant, from Thailand and Cambodia, provided by François Nosten, Shoklo Malaria Research Unit, Thailand, and Arjen M. Dondorp, Mahidol Oxford Tropical Medicine Research Unit).

Chemicals. The drugs tested in this study were the 4-aminoquinoline derivatives TDR 58845 and TDR 58846 (United Nations' Children's Fund [UNICEF]/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland). Chloroquine diphosphate (MP Biochemicals, Solon, OH) and mefloquine hydrochloride (Sigma, St. Louis, MO) were used as controls in the *in vitro* studies. Each of the CQ resistance-reversing compounds used were obtained from Sigma (St. Louis, MO): verapamil (VER), desipramine (DES), chlorpromazine (CPZ), promethazine (PMZ), and chlorpheniramine (CPR). Dihydroartemisinin (WRAIR Chemical Inventory, Silver Spring, MD) was used as a positive control for gametocyte studies.

***In vitro* drug sensitivity assay.** The sensitivity of the parasites *in vitro* to the different drugs was tested using a SYBR green I fluorescence-based method and confirmed by radioisotopic method as described previously (28, 49). The experiments were set up in 96-well plates with 2-fold dilutions of each drug across the plate in a total volume of 150 μ l and at a final red blood cell concentration of 1.5% (vol/vol). Stock solutions of each drug were prepared in dimethyl sulfoxide (DMSO) at 1 mg/ml. All assay procedures were performed by automated pipetting and dilution with the aid of a programmable Biomek 3000 robotic station (Beckman Coulter, Brea, CA). The experiment was started at an initial parasitemia of 0.5% (>80% rings) synchronous parasite-infected red blood cells (PRBC). The plates were incubated for 72 h at 37°C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. The SYBR green I dye-lysis mixture (100 μ l) was added to 100 μ l of the parasites in black plates that were incubated at room temperature for an hour in the dark. The plates were then read using a fluorescence plate reader (Spectramax Gemini-EM; Molecular Diagnostics) at excitation and emission wavelengths of 480 and 535 nm, respectively. The readings, in relative fluorescence units (RFUs), were plotted against the logarithm of the drug concentration and analyzed by nonlinear regression analysis (DataAspects Corporation, CA) to determine the drug concentration that produced 50% of the observed decline from the maximum readings in the drug-free control wells (50% inhibitory concentrations [IC₅₀s]). In addition, a regression coefficient (R^2) of >0.9 was considered significant. All the experiments were done in duplicate and repeated twice.

***In vitro* analysis of 4-aminoquinolines in combination with potentiating compounds.** Dose-response assays were carried out to obtain the IC₅₀ of TDR 58845 and TDR 58846 alone and in combination with CQ resistance-potentiating compounds (VER, DES, CPZ, PMZ, and CPR). A fixed concentration of 0.9 μ M potentiating compound was used, while the concentration of TDR 58845, TDR 58846, CQ, or MQ was varied. The IC₅₀s were determined by nonlinear regression analysis of the fluorescence readings against the logarithm of the drug concentration. In order to determine the degree of action of the CQ resistance-potentiating compounds, we used a response modification index (RMI), that is, the ratio of

TABLE 1 IC₅₀ and IC₉₀ of *P. falciparum* laboratory lines and field isolates exposed to 4-aminoquinoline derivatives, chloroquine, and mefloquine^a

Strain	TDR58845		TDR58846		Chloroquine		Mefloquine	
	IC ₅₀	IC ₉₀						
3D7 ^b	7.75 (4.81)	11.2 (3.73)	10.3 (11.4)	15.3 (9.50)	8.36 (6.16)	10.2 (5.88)	19.3 (2.15)	44.1 (22.2)
W2 ^c	89.8 (45.6)	124 (60.5)	23.1 (10.6)	32.9 (13.2)	271 (81.2)	332 (82.8)	10.0 (3.01)	20.1 (7.46)
D6 ^b	9.22 (3.63)	11.3 (3.12)	11.8 (9.56)	14.9 (8.97)	9.78 (4.77)	11.1 (4.93)	26.3 (22.3)	41.1 (25.3)
C2B ^c	35.5 (4.51)	57.9 (9.69)	16.3 (12.1)	21.0 (9.84)	112 (50.1)	277 (78.9)	17.4 (13.7)	51.9 (3.62)
Cam6 ^c	22.4 (11.8)	126 (149)	10.3 (8.08)	17.1 (7.33)	160 (95.9)	287 (268)	39.5 (22.5)	229 (39.8)
Thai6 ^c	7.65 (0.50)	13.4 (2.39)	5.52 (2.71)	10.4 (3.41)	26.0 (3.56)	49.6 (9.92)	58.1 (34.7)	135 (94.4)

^a Values are in nM (± standard deviation).

^b CQ sensitive.

^c CQ resistant.

the IC₅₀ of CQ to the IC₅₀ of the potentiating compound in combination with CQ or with one of the 4-aminoquinolines tested (28). An RMI of 1.0 represents no change in the IC₅₀. RMI values of <1.0 represent the degree of potentiation or synergism. All the experiments were done in duplicate and repeated twice.

In vitro *P. falciparum* gametocyte production and gametocyte drug sensitivity assays. Asexual and mature gametocytes of NF54 parasites were cultivated in duplicate as previously described (22, 47) in six-well plates. The CQ-sensitive NF54 clone was used because of its ability to form male and female viable gametocytes and to infect mosquitoes. In brief, 0.1% parasitemia cultures were inoculated in 2.5 ml of culture medium and 7.0% hematocrit (day 0) and were grown for 3 days. On day 4 post-inoculation (PI), the volume of media was doubled. The plates were kept in 5% O₂ and 5% CO₂.

On days 7, 8, and 9 PI, when the gametocytes were at stages I, II and III, respectively, the TDR drugs, CQ, or DHA was added to the cultures to reach a final concentration of 0.1 μM or 1 μM. This procedure was used to determine the effect of the drug on early stages of gametocytemia. Similarly, on days 11, 12, and 13 PI, when the gametocytes are usually at stages III, IV, and V, respectively, drug was added in the cultures at the same concentration range to determine the effect of the drug on late stages of gametocytes. Each plate had two untreated controls and four drug-treated wells. Blood smears were made each day starting on day 7 to document the progression of asexual- as well as sexual-stage parasitemia. A minimum of 1,000 cells were counted. Finally, on day 15 PI, the gametocytes were checked for exflagellation as previously described (41). The experiments were repeated twice, and duplicates were used in each case. The *t* test was used to calculate significance.

In vivo efficacy study tests using the murine model *P. berghei* NK65 (modified Thompson test). To test the TDR compounds for their *in vivo* activity, *P. berghei* NK65-infected BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in a modified Thompson test (50). Endpoints for efficacy included survivability of mice to 30 days and suppression of parasitemia on day 6 postinfection following administration of the drug. In brief, 5 mice were randomly assigned to each dosage group, and 1 × 10⁶ *P. berghei*-infected erythrocytes (NK65 strain) were inoculated into the intraperitoneal cavity of female mice weighing 15 to 20 g. On day 3 postinfection, parasitemia was around 1%. Each drug was suspended in 0.5% hydroxyethylcellulose (HEC)–0.1% Tween 80 and was administered orally (p.o.) at 40, 80, and 160 mg/kg of body weight once daily for 3 days on days 3 to 5 postinfection. Blood films were made from tail vein puncture on days 3, 6, 9, 13, 20, 27, and 30 postinfection, and a minimum of a 1,000 cells were counted to monitor and determine parasitemia progression. Mice without a parasitemia on day 30 postinfection were considered cured. To evaluate the toxicity of the drugs, we looked for signs of morbidity or death compared to the untreated controls. The study was approved by the Institutional Animal Care and Use Committee for the University of South Florida.

RESULTS

4-Aminoquinoline derivatives are potent against asexual erythrocytic stages of *P. falciparum*. The efficacy of TDR 58845 and TDR 58846 was assessed against a series of lab lines and field isolates of *P. falciparum* that possess a range of susceptibilities and resistance to antimalarial drugs (Table 1). Both 4-aminoquinolines tested had low-nM IC₅₀s against the CQ-sensitive lines 3D7 and D6. In these parasites, the activities of TDR 58845 and TDR 58846 were equivalent to the activities of CQ for a CQ-sensitive parasite (IC₅₀s < 12 nM). TDR 58845 was more active than CQ against the Southeast Asian CQ-resistant laboratory lines W2 and C2B (89.8 and 35.5 nM, respectively). The IC₅₀s of TDR 58845 for Thailand-Cambodia multidrug-resistant isolates were considerably lower than CQ IC₅₀s but higher than for CQ-sensitive lines (<25 nM). Conversely, TDR 58846 was equipotent against all laboratory lines and field isolates tested (<25 nM) and was significantly more active than CQ. A trend for cross-resistance with CQ was evident for TDR 58845 but was not observed with TDR 58846.

TDR 58845 is synergistic with verapamil, desipramine, and chlorpromazine. Previous studies have established the potential of VER and other compounds to reverse CQ resistance *in vitro*. Response modification indexes (RMI) were used to quantify the synergism of this resistance reversal. RMIs decrease in CQ-resistant parasites when treated with reversal compounds (28, 31, 35). Our data show that in combination with 0.9 μM CQ resistance-reversing compounds, there was an increased sensitivity of TDR 58845 in CQ-resistant isolates, indicating synergism of TDR 58845 with VER, DES, and CPZ (Tables 2 to 4). In addition, the RMIs decreased in combinations of TDR 58845 with VER, DES, or CPZ. The IC₅₀ and RMI values of CQ and TDR 58845 were reduced 2- to 3-fold in W2, C2B, and the multidrug-resistant isolates Thai6 and Cam6 (Tables 2 to 4). In addition, TDR 58845 was synergistic with the CQ resistance reversal drugs chlorpheniramine (CPR) and PMZ (data not shown). Conversely, these compounds were not synergistic with mefloquine. Interestingly, the activity of TDR 58846 is not enhanced by VER, DES, CPZ, CPR, or PMZ; rather, it is reduced slightly by the resistance reversal compounds (Tables 2 to 4).

4-Aminoquinoline derivatives are active against gametocyte stages of *P. falciparum*. To compare the effect of 4-aminoquinoline derivatives against the sexual stages of *P. falciparum*, we treated stage I to III or stage III to V gametocytes for 3 days with 0.1 μM or 1 μM TDR 58845, TDR 58846, CQ, or DHA. DHA eliminates early-stage gametocytes; therefore, it was used as a control in

TABLE 2 Susceptibility of *P. falciparum* to chloroquine, TDR 58845, TDR 58846, and mefloquine in the absence and presence of verapamil

Parasite isolate	VER ^a (0.9 μM)	Chloroquine		TDR 58845		TDR 58846		Mefloquine	
		IC ₅₀ ^b (SD)	RMI ^c	IC ₅₀ (SD)	RMI	IC ₅₀ (SD)	RMI	IC ₅₀ (SD)	RMI
3D7	–	8.36 (6.16)		7.75 (4.81)		10.3 (11.4)		19.3 (2.15)	
	+	9.44 (8.12)	1.13	10.2 (4.30)	1.32	16.1 (9.41)	1.55	13.5 (0.28)	0.70
W2	–	271 (81.2)		89.8 (45.6)		23.1 (10.6)		10.0 (3.01)	
	+	47.7 (20.8)	0.18	22.9 (2.72)	0.25	22.2 (3.65)	0.96	4.70 (0.43)	0.47
D6	–	9.78 (4.77)		9.22 (3.63)		11.8 (9.56)		26.3 (22.3)	
	+	11.8 (5.09)	1.20	11.4 (2.33)	1.24	15.0 (11.5)	1.27	34.5 (29.8)	1.31
C2B	–	112 (50.1)		35.5 (4.51)		16.3 (12.1)		17.4 (13.7)	
	+	35.4 (7.64)	0.31	15.1 (3.63)	0.42	12.3 (8.91)	0.75	8.65 (10.8)	0.59
Cam6	–	160 (95.8)		22.4 (11.8)		10.3 (8.08)		39.5 (22.5)	
	+	59.1 (24.7)	0.37	18.6 (0.24)	0.83	13.5 (6.40)	1.32	30.5 (12.8)	0.77
Thai6	–	26.0 (3.56)		7.65 (0.50)		5.52 (2.71)		58.1 (34.7)	
	+	13.1 (10.1)	0.51	4.57 (0.33)	0.60	6.71 (0.70)	1.22	28.8 (0.19)	0.50

^a +, with VER; –, without VER.^b IC₅₀ values are in nM.^c RMI is the ratio of IC₅₀ with VER to IC₅₀ without VER.

this experiment. Our findings show that CQ (1 μM) kills early-stage gametocytes, as demonstrated in the reduction of stage V gametocytes at day 14 compared to the control (Fig. 2). Both 4-aminoquinoline derivatives TDR 58845 and TDR 58846 are effective at eliminating the early gametocyte stages at 1 μM but were not effective at significantly decreasing the number of stage V gametocytes at 0.1 μM ($P = 0.13$ and $P = 0.16$) (Fig. 2). As expected, DHA (0.1 μM) completely inhibited the development of early-stage gametocytes to stage V. The 4-aminoquinoline derivatives tested in this study did not completely eliminate *P. falciparum* gametocytes but significantly decreased the stage V gametocytemia when gametocyte cultures in stages III to V were treated with 1 μM concentrations ($P = 0.02$ and $P = 0.01$). Only 1 μM

DHA completely blocked development to stage V gametocytes when treated in stages III to V (Fig. 3).

4-Aminoquinoline drugs (TDR 58845 and TDR 58846) cure *P. berghei* infection in mice. The activity of the 4-aminoquinoline derivatives was tested *in vivo* using a modified Thompson test (3 consecutive days of dosing) with *P. berghei*-infected mice. A summary of the *in vivo* results is presented in Table 5. Both 4-aminoquinolines (TDR 58845 and TDR 58846) showed potent activity *in vivo* against *P. berghei*. In fact, 100% suppression of parasitemia was observed on day 6 postinfection, and both compounds produced cures at all doses tested (40, 80, and 160 mg/kg). In particular, 40 mg/kg of TDR 58845 cured 4 of 5 mice over the course of 30 days, and only one mouse had delayed parasitemia on day 30

TABLE 3 Susceptibility of *P. falciparum* to chloroquine, TDR 58845, TDR 58846, and mefloquine in the absence and presence of desipramine

Parasite isolate	DES ^a (0.9 μM)	Chloroquine		TDR 58845		TDR 58846		Mefloquine	
		IC ₅₀ ^b (SD)	RMI ^c	IC ₅₀ (SD)	RMI	IC ₅₀ (SD)	RMI	IC ₅₀ (SD)	RMI
3D7	–	8.36 (6.16)		7.75 (4.81)		10.3 (11.4)		19.3 (2.15)	
	+	10.5 (6.69)	1.26	11.9 (2.05)	1.53	14.5 (12.3)	1.40	18.6 (1.45)	0.96
W2	–	271 (81.2)		89.8 (45.6)		23.1 (10.6)		10.0 (3.01)	
	+	18.9 (1.28)	0.07	10.4 (5.71)	0.12	15.3 (6.41)	0.66	2.44 (1.04)	0.24
D6	–	9.78 (4.77)		9.22 (3.63)		11.8 (9.56)		26.3 (22.3)	
	+	11.2 (5.65)	1.14	11.3 (2.60)	1.23	11.0 (5.14)	0.94	34.8 (25.4)	1.32
C2B	–	112 (50.1)		35.5 (4.51)		16.3 (12.1)		17.4 (13.7)	
	+	21.5 (17.3)	0.19	9.84 (6.21)	0.28	11.7 (8.82)	0.72	10.6 (4.38)	0.72
Cam6	–	160 (95.9)		22.4 (11.8)		10.3 (8.08)		39.5 (22.5)	
	+	31.6 (3.93)	0.20	13.0 (0.88)	0.58	12.2 (4.53)	1.19	26.9 (8.73)	0.68
Thai6	–	26.0 (3.56)		7.65 (0.5)		5.52 (2.71)		58.1 (34.7)	
	+	6.51 (1.58)	0.25	5.03 (2.50)	0.66	5.36 (0.06)	0.97	26.8 (3.61)	0.46

^a +, with DES; –, without DES.^b IC₅₀ values are in nM.^c RMI is the ratio of IC₅₀ with DES to IC₅₀ without DES.

TABLE 4 Susceptibility of *P. falciparum* to chloroquine, TDR 58845, TDR 58846, and mefloquine in the absence and presence of chlorpromazine

Parasite isolate	CPZ ^a (0.9 μM)	Chloroquine		TDR 58845		TDR 58846		Mefloquine	
		IC ₅₀ ^b (SD)	RMI ^c	IC ₅₀ (SD)	RMI	IC ₅₀ (SD)	RMI	IC ₅₀ (SD)	RMI
3D7	–	8.36 (6.16)		7.75 (4.81)		10.3 (11.4)		19.3 (2.15)	
	+	12.2 (4.02)	1.46	13.5 (0.22)	1.73	13.7 (13.3)	1.33	19.1 (2.32)	0.99
W2	–	271 (81.2)		89.8 (45.6)		23.1 (10.6)		10.0 (3.01)	
	+	31.8 (9.86)	0.12	18.8 (7.87)	0.21	23.8 (8.37)	1.03	3.44 (0.34)	0.34
D6	–	9.78 (4.77)		9.22 (3.63)		11.8 (9.56)		26.3 (22.3)	
	+	11.7 (4.89)	1.19	11.7 (2.02)	1.27	16.1 (10.2)	1.37	35.3 (26.3)	1.34
C2B	–	112 (50.1)		35.5 (4.51)		16.3 (12.1)		17.4 (13.7)	
	+	24.7 (16.4)	0.22	11.6 (6.37)	0.33	14.2 (14.6)	0.87	9.91 (11.43)	0.67
Cam6	–	160 (95.9)		22.4 (11.8)		10.3 (8.08)		39.5 (22.5)	
	+	35.3 (1.16)	0.22	14.2 (0.28)	0.63	13.0 (7.27)	1.27	28.6 (23.0)	0.72
Thai6	–	26.0 (3.56)		7.65 (0.5)		5.52 (2.71)		58.1 (34.7)	
	+	7.76 (0.80)	0.30	6.58 (3.71)	0.86	9.17 (4.84)	1.66	38.9 (17.0)	0.67

^a +, with CPZ; –, without CPZ.

^b IC₅₀ values are in nM.

^c RMI is the ratio of IC₅₀ with CPZ to IC₅₀ without CPZ.

postinfection (Fig. 4A). An 80-mg/kg dose of TDR 58846 was completely curative in all the experimental mice, and recrudescence was observed in only one mouse at 40 mg/kg dose of TDR 58846. No toxicity was observed at 160 mg/kg of TDR 58846 (total dose, 480 mg/kg; Fig. 4B). In summary, our results demonstrate remarkable efficacy and tolerability of 4-aminoquinoline derivatives in the *P. berghei* model *in vivo*.

DISCUSSION

In this study, we assessed the activity of two novel 4-aminoquinoline derivatives (TDR 58845 and TDR 58846) against the erythrocytic and gametocyte stages of *P. falciparum* *in vitro* and *in vivo*. Our results indicate that the 4-aminoquinoline derivatives show

excellent potential as a treatment for erythrocytic stages of CQ-sensitive and -resistant *P. falciparum*, as well as gametocyte stages of a CQ-sensitive line. In addition, both 4-aminoquinolines cured *P. berghei*-infected mice at concentrations as low as 40 mg/kg per day and were well tolerated at a total dose of 480 mg/kg over 3 days.

One of the most important findings of this study is that both novel 4-aminoquinolines are more potent than CQ against CQ-resistant *P. falciparum*. In particular, TDR 58846 produced consistently low IC₅₀s when tested against CQ-resistant parasites. The highest IC₅₀ for TDR 58846 was 23 nM for the CQ-resistant W2, while none of the IC₅₀s in the multidrug-resistant isolates exceeded 11 nM. These values are much lower than reported in previous studies (45) of four different 4-aminoquinoline analogs (related to the compounds in this study) against the K1 CQ-resistant line (IC₅₀s ranged between 46 and 61 nM). Interestingly, overcoming CQ cross-resistance was not complete for TDR 58845. The IC₅₀s of TDR 58845 against CQ-resistant laboratory lines and field isolates have a positive correlation with the IC₅₀s of CQ in these parasites, suggesting that TDR 58845 shares cross-resistance with CQ. Our TDR 58845 data are in accordance with the results of previous studies by Ridley et al. (45) that show cross-resistance of the 4-aminoquinolines Ro 47-0543, Ro 41-3118, Ro 47-9396, and Ro 48-0346 with CQ. In particular, in our study the highest IC₅₀ for TDR 58845 was 90 nM in the CQ-resistant W2, yet IC₅₀s were lower than 23 nM for the recent multidrug-resistant field isolates. These results indicate a potential role of the novel 4-aminoquinolines in this study to treat CQ-resistant malaria and demonstrate the potential of these drugs to treat multidrug-resistant malaria.

Here, we report potency of two novel 4-aminoquinolines that is similar to activities reported for AQ-13 in the CQ-resistant lines Dd2, TM91-C235, and TM92-C815 (10). In comparison to CQ, both TDR compounds and AQ-13 have shorter alkyl side chains that are likely to affect cross-resistance with CQ (10). Interestingly, the two compounds containing a tertiary amine at the end of

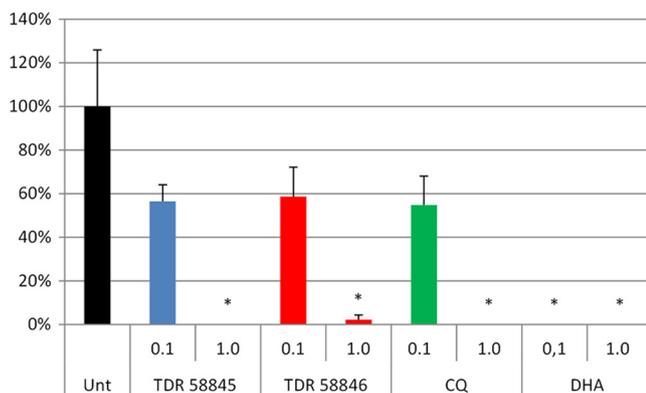


FIG 2 *P. falciparum* stage V gametocytemia (as a percentage of the control) that develops after treatment of stage I to III gametocytes with TDR 58845, TDR 58846, CQ, or DHA. *P. falciparum* gametocytes were treated on days 7, 8, and 9 postinoculation with 0.1 μM or 1 μM concentration of the compound, and the percentage of stage V gametocytes was calculated on day 14. CQ, TDR 58845, and TDR 58846 treatments showed significantly fewer stage V gametocytes than the control (Unt) at 1 μM ($P = 0.0015$). DHA (0.1 μM) was more potent and completely prevented formation of stage V gametocytes. *, statistically significant compared to the untreated control (t test).

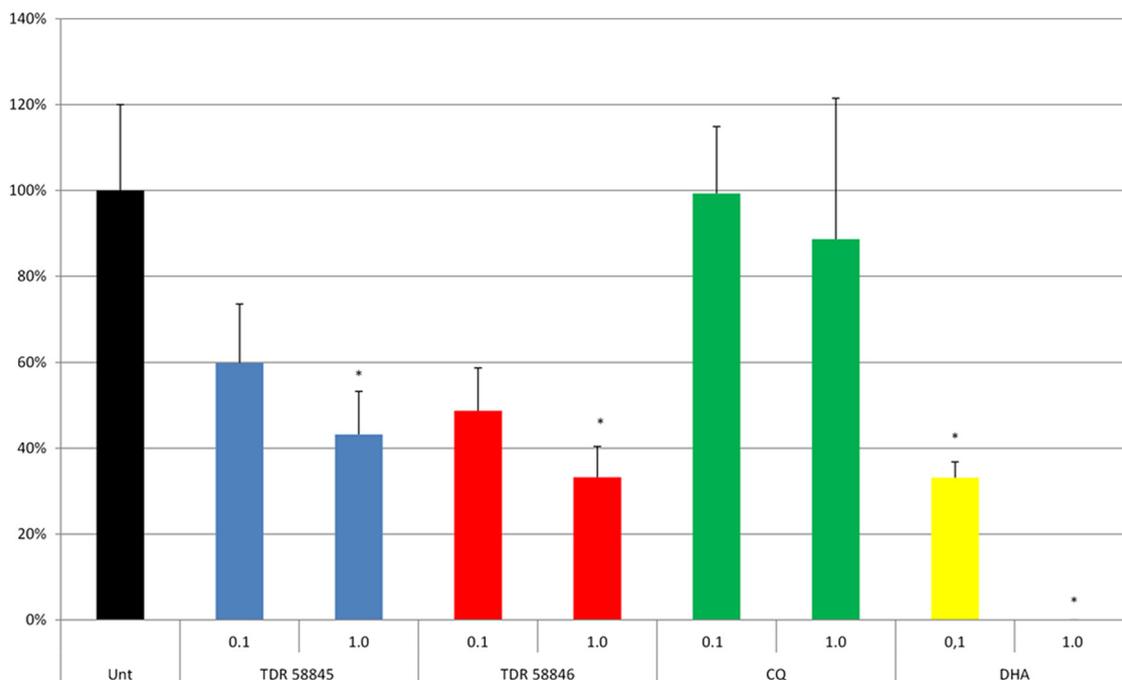


FIG 3 *P. falciparum* stage V gametocytemia (as a percentage of the control) that developed after stage III to V gametocytes were treated with TDR 58845, TDR 58846, CQ, or DHA. *P. falciparum* (NF54) gametocytes were treated on days 11, 12, and 13 postinoculation with a 0.1 μ M or 1 μ M concentration of the compound, and the percentage of stage V gametocytes was calculated on day 14 postinoculation. No decrease in gametocytemia was observed in CQ-treated parasites. TDR 58845- and TDR 58846-treated gametocytes showed a decrease in stage V gametocytemia that was significant at 1.0 μ M ($P = 0.02$ and $P = 0.01$). No stage V gametocytes were found in 1 μ M DHA-treated cultures. *, statistically significant compared to the untreated control (Unt) by the *t* test.

their side chains (AQ-13 and TDR 58846) are the most potent and possess no cross-resistance with CQ *in vitro*. In contrast, TDR 58845, with its side chain terminated by a primary amine, is potent, yet cross-resistance with CQ was evident. Similarly, the activities of AQ-13 and TDR58846 are not potentiated by resistance reversal drugs VER, CPR, and DES (reference 10 and this study). The position of the N₂ in the alkyl chain should affect the pK of the compounds, with TDR 58846 expected to be more basic by approximately 1.5 pK units.

Since the initial observation on the CQ-reversing potential of VER and DES (5, 26), more than 40 compounds that enhance the activity of CQ have been identified (42, 52). We examined the classical series of CQ reversal drugs in combination with TDR 58845 and TDR 58846. We found that the combination of TDR 58845 with VER, DES, CPZ, PMZ, and CPR increases the sensitivity *in vitro* to this drug with the CQ-resistant lines (W2 and

C2B) and the field isolates Cam6. The activity of TDR 58845 in Thai6 was slightly improved by the CQ reversal drugs. Ridley found the activity of four 4-aminoquinolines with shortened side chains was not potentiated by DES (45). The fact that TDR 58845 activity is potentiated by CQ reversal compounds is in agreement with the observation of TDR 58845 showing cross-resistance with CQ and suggests a similar mechanism of action for these two drugs. More importantly, we found that activity of TDR 58846 was not improved by any of the CQ resistance-reversing compounds, nor was there evidence of cross-resistance with CQ. This observation is in accordance to what has been reported for AQ-13 and other aminoquinoline derivatives when combined with verapamil (10). In this work, we included CQ-resistant parasites with multiple unique *pfcr* alleles, and TDR 58846 was equally effective against them all. These data suggest TDR 58846 may not interact with mutant *pfcr*. This may be due to structural differences

TABLE 5 Summary of activities of TDR 58845 and TDR 58846 against blood stage infections with *P. berghei* *in vivo*

Drug	Dose (mg/kg/day for 3 days)	Vehicle	Route	Outcome or no. of days to death	Activity
None	None	None	None	7–8	NA ^a
TDR 58845	40	0.5% HEC–0.1% Tween 80	p.o.	30	Curative
	80			All mice survived	Curative
	160			4/5 mice survived	Curative
TDR 58846	40	0.5% HEC–0.1% Tween 80	p.o.	27 (4/5 survived)	Curative
	80			All mice survived	Curative
	160			All mice survived	Curative

^a NA, not applicable.

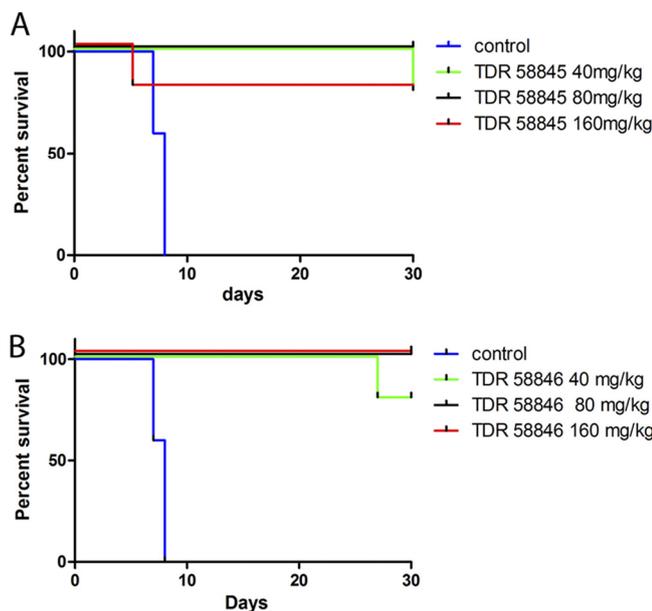


FIG 4 Survival curves of mice untreated or treated with the 4-aminoquinoline analogs TDR 58845 and TDR 58846. (A) TDR 58845 treatment. All mice at doses of 40 and 80 mg/kg survived until the end of the experiment (day 30), although one mouse had parasitemia at day 30 and one mouse died for unknown reasons in the 160-mg/kg group on day 5. (B) TDR 58846 treatment. Recrudescence was observed only in the lower-dose group. All the mice in groups receiving 80 and 160 mg/kg survived and showed no sign of parasitemia.

caused by the variation in the diamine groups and in the number of methyl groups between the two tested compounds. We hypothesize that TDR 58846 is unable to interact with the CQ transporter receptor, which would imply that this compound has great promise as a CQ replacement drug.

CQ has been reported to kill early-stage gametocytes *in vitro* at concentrations greater than 1 nM (48). To determine if TDR 58845 and TDR 58846 have gametocytocidal activity, we tested two different concentrations of the drugs in an *in vitro* gametocyte assay. We found that in comparison to DHA, which eliminates gametocytes at 0.1 μ M, both TDR 58845 and TDR 58846 completely eliminate early-stage gametocytes of *P. falciparum* NF54 at 1 μ M. Other studies have observed an increase in the number of gametocytes when subcurative doses of CQ were used. Interestingly, these studies did not show a significant difference between CQ-sensitive and CQ-resistant laboratory strains in gametocyte production (7). In our studies, we did not observe an increase in the number of gametocytes when treated in early stages with 0.1 μ M CQ, TDR 58845, or TDR 58846. The observation that CQ, TDR 58845, and TDR 58846 eliminate gametocytes at 1 μ M suggests a mode of action similar to that of CQ against early-stage gametocytes. In addition, we found that 1 μ M TDR 58845 or TDR 58846 significantly decreases the number of mature gametocytes when added to late-stage gametocytes (stages III to V). The fact that these 4-aminoquinoline derivatives affect both early and late gametocyte stages as well as erythrocytic stages makes them attractive candidates for further development.

Our experiments *in vitro* indicate that the 4-aminoquinoline derivatives are efficacious against several multidrug-resistant *P. falciparum* strains. In addition, both TDR 58845 and 58846 cured

100% of *P. berghei*-infected mice at doses of 80 mg/kg/day and cured 80% of mice dosed at 40 mg/kg/day. As a result, both drugs are more efficacious than CQ in this model, where daily doses of 160 mg/kg/day clear parasitemia by day 6 but do not cure infections (data not shown) (23, 38).

Our findings illustrate the enormous potential of these 4-aminoquinoline derivatives as antimalarials. In fact, TDR 58845 can overcome parasite resistance through the use of CQ resistance-reversing compounds *in vitro*. More importantly, TDR 58846, to our knowledge, is one of the first 4-aminoquinoline derivatives that are potent *in vitro* and *in vivo* and do not have cross-resistance with CQ. Given the fact that CQ has not been used for many years in areas of the world, these novel 4-aminoquinolines could be part of a combination therapy to efficiently treat malaria. Our results suggest that these novel compounds, particularly TDR 58846, should be considered as partner drugs in antimalarial combinations to treat multiple-drug-resistant *P. falciparum* and *P. vivax* malaria.

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