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Examination of the antimalarial potential of experimental aminoquinolines: poor in vitro effect does not preclude in vivo efficacy ☆

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ABSTRACT

Malaria remains a major disease in the developing world and globally is the most important parasitic disease causing significant morbidity and mortality. Because of widespread resistance to conventional antimalarials, including chloroquine (CQ), new drugs are urgently needed. Here we report on the antimalarial efficacy, both in vitro and in vivo, of a series of aminoquinoline derivatives with adamantane or benzothiophene as a carrier. In vitro efficacy was evaluated by a lactate dehydrogenase (LDH) assay in cultures of a CQ-sensitive (3D7) and CQ-resistant (Dd2) strain of *Plasmodium falciparum*. Of a series of 26 screened compounds, 12 that exerted a growth inhibition rate of ≥50% were further examined in vitro to determine the 50% inhibitory concentration (IC_{50}) values. Nine compounds shown in preliminary experiments to be non-toxic in vivo were evaluated in C57BL/6 mice infected with *Plasmodium berghei* ANKA strain using a modified Thompson test. All nine compounds examined in vivo prolonged the survival of treated versus untreated mice, four of which afforded ≥60% survival. Most notably, two of these compounds, both with the adamantane carrier, afforded complete cure (100% survival and parasite clearance). Interestingly, one of these compounds had no in vitro effect against the CQ-resistant *P. falciparum* strain. Better in vivo compared with in vitro results suggest a role for compound metabolites rather than the compounds themselves. The results presented here point to adamantane as a carrier that enhances the antimalarial potential of aminoquinolines.

1. Introduction

Malaria, caused by protozoan parasites of the *Plasmodium* genus, continues to be a major health problem in the developing world and is globally the most important parasitic disease causing significant morbidity and mortality. Human infections are caused by five species of the genus, namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. Infection resulting from the bite of an infected female *Anopheles* mosquito is characterised by blood and liver stages [1].

The World Health Organization (WHO) estimated 214 million cases of malaria and 438 000 deaths in 2015 [2], with most of the deaths caused by *P. falciparum*. One-half of the global human population, residing in tropical and subtropical areas, is estimated to be at a risk of infection, but even the other half is facing an increasing number of imported cases, resulting in deaths and health system burden in non-endemic countries and occasional secondary transmission in areas where malaria has long ago been eradicated [3].

The efficacy of the main conventional antimalarials, including chloroquine (CQ) and artemisinin, is hampered by widespread drug resistance. Coupled with the lack of an effective vaccine, this strongly emphasises the urgent need for novel compounds to treat and prevent malaria [4,5].

The mechanism of action of CQ, like all quinolones, involves activity against the erythrocyte forms of all *Plasmodium* spp. by preventing polymerisation of heme through its selective accumulation in the parasite food vacuole (FV). CQ forms stable complexes with heme and its removal from the FV is prevented by protonation

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Table 1
Investigated compounds grouped according to modifications at the aminoquinoline moiety.

Group	Compound	Acronym	Compound no. in [19]
AQ	N^1-(1-adamantylmethyl)-N^3-quinolin-4-ylbutane-1,3-diamine, $C_{24}H_{33}N_3$	AQ1	**24**
	N^1-[2-(1-adamantyl)ethyl]-N^3-quinolin-4-ylbutane-1,3-diamine	AQ2	**44**
	N-(1-adamantylmethyl)-*N*-methyl-*N*'-quinolin-4-ylpropane-1,3-diamine	AQ3	Not previously published
ClAQ	N^1-(1-adamantylmethyl)-N^3-(7-chloroquinolin-4-yl)butane-1,3-diamine	ClAQ1	**23**
	N^2-(1-adamantylmethyl)-N^1-(7-chloroquinolin-4-yl)propane-1,2-diamine	ClAQ2	**10**
	N-(7-chloroquinolin-4-yl)-*N*'-[(5-fluoro-1-benzothiophen-3-yl)methyl]propane-1,3-diamine	ClAQ3	**58**
	N-(7-chloroquinolin-4-yl)-*N*'-[(5-fluoro-1-benzothiophen-3-yl)methyl]butane-1,4-diamine	ClAQ4	**63**
	N-(7-chloroquinolin-4-yl)-*N*'-[(6-fluoro-1-benzothiophen-3-yl)methyl]propane-1,3-diamine	ClAQ5	**59**
	N^1-[2-(1-adamantyl)ethyl]-N^3-(7-chloroquinolin-4-yl)butane-1,3-diamine	ClAQ6	**36**
FAQ	N^1-(1-adamantylmethyl)-N^3-(3-fluoroquinolin-4-yl)butane-1,3-diamine	FAQ1	**26**
	N^1-[2-(1-adamantyl)ethyl]-N^3-(3-fluoroquinolin-4-yl)butane-1,3-diamine	FAQ2	**39**
FClAQ	N^1-(1-adamantylmethyl)-N^3-(7-chloro-3-fluoroquinolin-4-yl)butane-1,3-diamine	FClAQ1	**25**
	N^4-(7-chloro-3-fluoroquinolin-4-yl)-N^1,N^1-diethylpentane-1,4-diamine	FClAQ2	**74**
	N^1-(1-adamantylmethyl)-N^2-(7-chloro-3-fluoroquinolin-4-yl)propane-1,2-diamine	FClAQ3	**20**
	N^2-(1-adamantylmethyl)-N^1-(7-chloro-3-fluoroquinolin-4-yl)propane-1,2-diamine	FClAQ4	**21**
	N^1-[2-(1-adamantyl)ethyl]-N^3-(7-chloro-3-fluoroquinolin-4-yl)butane-1,3-diamine	FClAQ5	**38**
	N^1-(1-adamantylmethyl)-N^4-(7-chloro-3-fluoroquinolin-4-yl)pentane-1,4-diamine	FClAQ6	**32**
	N^1-[2-(1-adamantyl)ethyl]-N^4-(7-chloro-3-fluoroquinolin-4-yl)pentane-1,4-diamine	FClAQ7	**45**
	N'-(7-chloro-3-fluoroquinolin-4-yl)-*N*,*N*-diethylpropane-1,3-diamine	FClAQ8	**73**
FCl2AQ	N^1-(1-adamantylmethyl)-N^2-(7-chloro-3-fluoroquinolin-2-yl)propane-1,2-diamine	FCl2AQ1	**68**
	N^1-(1-adamantylmethyl)-N^3-(7-chloro-3-fluoroquinolin-2-yl)butane-1,3-diamine	FCl2AQ2	**69**
	N^1-[2-(1-adamantyl)ethyl]-N^3-(7-chloro-3-fluoroquinolin-2-yl)butane-1,3-diamine	FCl2AQ3	**71**
	N^1-(1-adamantylmethyl)-N^4-(7-chloro-3-fluoroquinolin-2-yl)pentane-1,4-diamine	FCl2AQ4	**70**
	N^1-[2-(1-adamantyl)ethyl]-N^4-(7-chloro-3-fluoroquinolin-2-yl)pentane-1,4-diamine	FCl2AQ5	**72**
	N^4-(7-chloro-3-fluoroquinolin-2-yl)-N^1,N^1-diethylpentane-1,4-diamine	FCl2AQ6	**76**
	N'-(7-chloro-3-fluoroquinolin-2-yl)-*N*,*N*-diethylpropane-1,3-diamine	FCl2AQ7	**75**

AQ, 4-aminoquinoline; ClAQ, 7-chloro-4-aminoquinoline; FAQ, 3-fluoro-4-aminoquinoline; FClAQ, 3-fluoro-7-chloro-4-aminoquinoline; FCl2AQ, 3-fluoro-7-chloro-2-aminoquinoline.

[6,7]. Mutations in the *P. falciparum* CQ resistance transporter (*Pf*CRT) gene have a central role in CQ resistance. *Pf*CRT is located in the FV membrane and, when mutated, increases CQ export from the FV and decreases its concentration inside the parasite [6,8,9].

The aminoquinoline structure is very well known as a moiety useful for the design and development of new antimalarial agents [10–14]. Synthetic quinoline derivatives remain the most promising basis for discovery of new drugs [15], especially if they are effective against strains of *Plasmodium* resistant to CQ [16,17], and 4-aminoquinoline derivatives continue to be the most sought after antimalarial agents for chemical modification [18]. Efforts to develop new aminoquinolines include overcoming CQ resistance by adding modifications at the ring or at the side chain, with the main aim of finding new compounds that are not recognised by mutant transporters and thus cannot be pumped out of the parasite FV.

Recently, the synthesis of a series of aminoquinolines and tetraoxanes with demonstrated antiplasmodial activity, including activity both against the liver and blood stages, has been described [19]. Here we report on further examination of the aminoquinoline series in different in vitro model systems and provide further evidence for the complete curative effect observed in vivo by two compounds despite, at least in one case, a poor in vitro effect.

2. Materials and methods

2.1. Parasites

Cultures of a chloroquine-sensitive (CQ^S) 3D7 and a chloroquine-resistant (CQ^R) Dd2 strain of *P. falciparum* were maintained in human erythrocytes as described previously [20]. For in vitro drug assays, parasites were synchronised with 5% sorbitol and ring-stage parasites were then seeded in 96-well plates to achieve 2% parasitaemia and 0.75% haematocrit.

In vivo testing was performed using the *Plasmodium berghei* ANKA strain maintained through serial intraperitoneal passage in C57BL/6 mice.

2.2. Mice

Female C57BL/6 mice (Medical Military Academy Animal Research Facility, Belgrade, Serbia), weighing 19–21 g, were used. Groups of four to six animals were housed in the Institute for Medical Research Animal Facility of the University of Belgrade (Belgrade, Serbia) under a natural photoperiod and were offered drinking water and standard feed ad libitum.

2.3. Compounds

A total of 26 experimental aminoquinoline derivatives with adamantane or benzothiophene as a carrier, synthesised at the Faculty of Chemistry of the University of Belgrade, were examined (Table 1).

According to the modifications at the aminoquinoline moiety structure, the compounds belonged to five groups as follows: (i) 4-aminoquinoline (AQ) ($n = 3$ compounds); (ii) 7-chloro-4-aminoquinoline (ClAQ) ($n = 6$); (iii) 3-fluoro-4-aminoquinoline (FAQ) ($n = 2$); (iv) 3-fluoro-7-chloro-4-aminoquinoline (FClAQ) ($n = 8$); and (v) 3-fluoro-7-chloro-2-aminoquinoline (FCl2AQ) ($n = 7$).

For experimental use in vitro, the compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mM. Compounds were further diluted in complete RPMI 1640 culture medium so that the final DMSO concentration was ≤0.2%.

Compounds further investigated in vivo were suspended in 0.5% hydroxyethyl cellulose/0.1% Tween 80 and were administered per os (p.o.).

2.4. Experimental design

All compounds were screened in vitro by the lactate dehydrogenase (LDH) assay adapted for *Plasmodium* [21], and those that at a defined concentration inhibited proliferation of either *Plasmodium* strain by ≥50% were titrated to obtain 50% inhibitory concentration (IC_{50}) values and were examined for in vivo efficacy.

Prior to in vivo examination, compound toxicity was examined by treating uninfected mice with 160 mg/kg/day (the highest administered dose) of each compound for three consecutive days. A drug was considered non-toxic if mice did not develop any gross clinical symptoms (ruffled fur, lethargy or weight loss) during a 30-day observation period. Compounds determined to be non-toxic were evaluated for antimalarial efficacy at doses of 80 mg/kg/day and 160 mg/kg/day. Compound efficacy was evaluated based on parasitaemia over time and survival of treated versus untreated mice. Cure was defined as survival past Day 31 post-infection (p.i.) and complete clearance of parasitaemia. Survival past Day 31 p.i. with residual parasitaemia indicated survival without cure. If a compound did not afford survival but significantly prolonged time to death of treated versus untreated mice ($P < 0.05$), the effect was defined as prolonged survival. Finally, if a compound cured mice at a dose of 80 mg/kg/day, efficacy was tested at lower doses, including 40, 20 and 10 mg/kg/day. Parasitaemia was determined twice weekly starting from Day 3 p.i. (immediately before treatment) and only mice in which parasitaemia was detected were submitted to experimental treatment. Parasitaemia was evaluated by microscopic examination of Giemsa-stained thin blood smears prepared from mouse tail blood on a Zeiss Axioscope 2+ optical microscope at 1000× magnification, whilst parasite clearance was additionally confirmed in treated survivors by quantitative PCR (qPCR).

2.5. In vitro examination of compound efficacy

In vitro testing was performed using a LDH assay. Compounds were first screened at a concentration of 500 nM and those that showed a minimum of 50% growth inhibition of parasites of either strain (3D7 or Dd2) were further examined to obtain the IC_{50} value. Three independent experiments were performed for each compound, each with three replicates per condition. The assay was performed in flat-bottom 96-well microtitre plates. Briefly, compounds were tested at eight different concentrations ranging from 2 nM to 256 nM, plated in a volume of 100 μL. Parasites were plated into the wells while in the ring phase at 0.75% haematocrit and 2% parasitaemia in a volume of 100 μL. Each well contained the compound and parasite culture in a final volume of 200 μL. Following incubation at 37 °C for 48 h in a Heracell™ 150i incubator (Thermo Scientific, Waltham, MA), parasites were harvested and were subjected to three 20-min freeze–thaw cycles to resuspend the culture. Cultured erythrocytes without drug were used as the assay blank, whilst infected erythrocytes without drug were used as the assay control. CQ was used as the positive control for drug efficacy. To initiate the LDH reaction, 120 μL of the detection reagent mixture (Malstat and NBT/PES) was aliquoted into a new flat-bottom 96-well microtitre plate to which a 20 μL sample of each parasite culture was added. Colour development of the LDH plate was detected using a Multiskan® X microplate reader (Thermo Scientific) at 620 nm following 1 h of incubation in the dark. All reagents used in the assay were purchased from Sigma-Aldrich Inc. (St Louis, MO).

2.6. In vivo examination of compound efficacy

Antimalarial activity in vivo was tested by the modified Thompson test [22]. Infected erythrocytes were obtained from the peripheral blood of a donor mouse infected with *P. berghei*. Mice were inoculated intraperitoneally with 10^6 infected erythrocytes diluted in phosphate-buffered saline (PBS) to a total volume of 250 μL (Day 0). Mice were treated with the investigational compounds once a day for three consecutive days (Days 3, 4 and 5 p.i.). All compounds were administered p.o. at doses ranging from 10 mg/kg/day to 160 mg/kg/day in a total volume of 200 μL. Survival and parasitaemia were monitored for 30 days p.i. Parasitaemia was evaluated by microscopic examination of thin blood smears.

2.7. PCR

Residual parasitaemia was examined in the surviving mice by the real-time qPCR method adapted from Rougemont et al based on detection of the *Plasmodium* species-specific 18S rRNA gene [23]. Briefly, mice alive past day 31 p.i. and with complete parasite clearance were sacrificed and blood (300–500 μL) was sampled from the left ventricle of the heart. The liver was removed, was rinsed with Dulbecco's PBS and was homogenised. DNA extraction was performed using 100 μL of blood and liver homogenate samples using a DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Each PCR reaction contained 1 × Maxima™ Probe qPCR Master Mix (Thermo Fisher Scientific), 200 nM of each primer, 50 nM probe, 1 U of UNG (Thermo Fisher Scientific) and 3 μL of template DNA in a final volume of 20 μL. PCR conditions were as follows: one holding step at 50 °C for 2 min; one holding step at 95 °C for 10 min; followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples with C_t values >40 were considered negative. A positive (*P. berghei* DNA) and a negative (H_2O) control were included in each run.

2.8. Statistical analysis

IC_{50} values were obtained using a sigmoidal dose–response model with the variable slope fitted to the results. Survival rates in each particular group were estimated by the Kaplan–Meier product limit method and were compared by the log-rank (two curves) and log-rank for trends (three or more curves) tests. The level of statistical significance was 0.05. Statistical analysis was performed using GraphPad Prism v.5 (GraphPad Software Inc., La Jolla, CA).

3. Results

A series of 26 aminoquinolines was examined in this work. Of these, 12 compounds inhibited proliferation of either the CQ^S or CQ^R *Plasmodium* strain by ≥50%; the remaining 14 compounds did not and thus were eliminated from further study.

All 12 compounds were first assayed for toxicity. Four compounds (AQ2, AQ3, ClAQ3 and ClAQ6) were shown to cause acute toxicity at a dose of 160 mg/kg/day, three of which were eliminated from further in vivo examination. However, due to chemical similarity with other members of the benzothiophene group, which were non-toxic even at the highest applied dose, one of the latter compounds (ClAQ3), although toxic at 160 mg/kg/day, was further tested for toxicity at 80 mg/kg/day and was found to be non-toxic at this dose. ClAQ3 was thus included in the in vivo examination (Table 2).

A total of nine compounds (AQ1, ClAQ1, ClAQ2, ClAQ3, ClAQ4, ClAQ5, FAQ1, FClAQ1 and FClAQ2) were subjected to in vivo testing. When administered at doses of 80 mg/kg/day and/or 160 mg/kg/day, all nine compounds significantly prolonged survival of treated versus untreated mice ($P < 0.05$; Figs 1 and 2).

Remarkably, three ClAQ compounds (ClAQ1, ClAQ4 and ClAQ5) and one FClAQ compound (FClAQ1) (chemical structures presented in Table 3) afforded survival of 60–100% of treated mice past Day 31. Of these, ClAQ4 and ClAQ5 afforded a 60–80% survival rate of infected mice, although not even the highest dose of either compound eradicated parasitaemia in a single animal.

However, treatment with 80 mg/kg/day and 160 mg/kg/day of the other two compounds (ClAQ1 and FClAQ1) afforded complete cure. All treated infected mice survived beyond Day 31 (Figs 3 and 4) and, moreover, survival was associated with parasite clearance as determined by microscopic examination and by qPCR of murine blood and liver tissues after Day 31. Thus, we next examined their effect at lower doses, which revealed a strong dose-dependent effect ($P = 0.0141$ and $P = 0.0362$ for ClAQ1 and FClAQ1, respectively), but

Table 2
Antimalarial effect of experimental aminoquinolines examined in vitro and in vivo.

Group	Compound	In vitro LDH assay [GM IC_{50} value (nM)]		In vivo Thompson test		
		Strain 3D7 [a]	Strain Dd2 [b]	Toxicity at 160 mg/kg/day	Treatment dose (mg/kg/day)	Effect on Day 31 p.i.
AQ	AQ1	14.08	118.2	NT	80	Prolonged time to death * ($P = 0.0031$)
	AQ2	99.86	195.3	T		
	AQ3	67.33	223.0	T		
ClAQ	ClAQ1	34.75	58.4	NT	160, 80	100% cure
					40, 20, 10	Prolonged time to death * ($P = 0.0031$, 0.0067, 0.0031)
	ClAQ2	142.70	>500	NT	80	Prolonged time to death * ($P = 0.0031$)
	ClAQ3	43.48	34.8	T	80	Prolonged time to death * ($P = 0.0067$)
	ClAQ4	32.53	13.7	NT	160	75% survival [c] ($P = 0.0067$)
					80	80% survival [c] ($P = 0.0031$)
	ClAQ5	34.13	16.7	NT	160	60% survival [c] ($P = 0.002$)
	ClAQ6	67.07	35.9	T		
FAQ	FAQ1	185.38	>500	NT	80	Prolonged time to death * ($P = 0.0290$)
FClAQ	FClAQ1	41.13	>500	NT	160, 80	100% cure
					40	Prolonged time to death * ($P = 0.0031$)
					20, 10	N/S ($P > 0.05$)
	FClAQ2	145.36	>500	NT	160	Prolonged time to death * ($P = 0.0020$)
Control	CQ	18.74	249.1	NT	160	100% cure

LDH, lactate dehydrogenase; GM, geometric mean; IC_{50}, 50% inhibitory concentration; p.i., post-infection; AQ, 4-aminoquinoline; ClAQ, 7-chloro-4-aminoquinoline; FAQ, 3-fluoro-4-aminoquinoline; FClAQ, 3-fluoro-7-chloro-4-aminoquinoline; CQ, chloroquine; NT, non-toxic; T, toxic; N/S, not significant.
* Versus infected untreated (control) mice.
[a] CQ-sensitive *Plasmodium falciparum* strain 3D7.
[b] CQ-resistant *P. falciparum* strain Dd2.
[c] With residual parasitaemia.

did not afford survival. ClAQ1 is particularly interesting in this respect as treatment with even the lowest dose (10 mg/kg) prolonged survival ($P = 0.0031$). However, dose reduction resulted in persistence of parasitaemia in all mice.

On the other hand, an interesting observation with FClAQ1 was that although all mice treated with 40 mg/kg/day eventually succumbed to the infection, they were able to tolerate very high levels of parasitaemia, which amounted up to 62% (range 37.5–62.4%). In contrast, the highest level of parasitaemia observed with any other treatment regimen ranged from as low as 0.1% to not more than 13.9% (Table 4).

Interestingly, the correlation between the in vivo and in vitro results appeared indiscriminate (Table 2). The four compounds with the highest in vivo efficacy did not show the best in vitro results, i.e. the lowest IC_{50} values for both strains. For instance, ClAQ4 and ClAQ5, the two compounds that afforded survival but not cure, had quite low IC_{50} values and by far the lowest ones against the CQ^R strain. In contrast, FClAQ1, which cured all infected mice (in two doses), had no in vitro effect against the CQ^R strain (>500 nM). On the other hand, AQ1, the single compound that had lower IC_{50} values than CQ against both strains, did not have remarkable in vivo efficacy. Of the remaining four compounds, which all significantly prolonged the

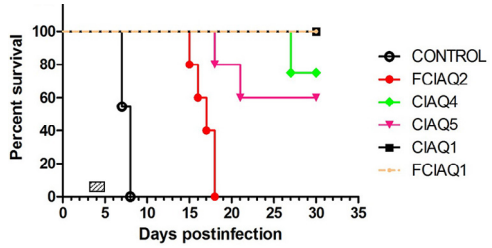


Fig. 1. Effect of a 3-day treatment with 160 mg/kg/day of the investigational compounds on the survival of mice infected with *Plasmodium berghei* ANKA strain. ▨, treatment days.

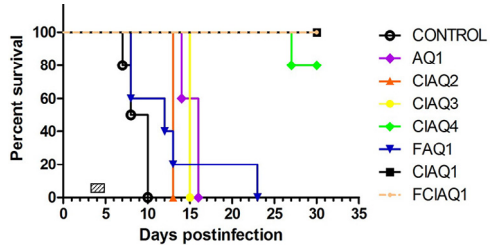
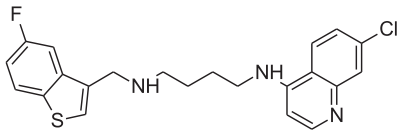
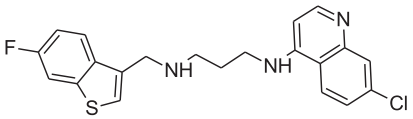
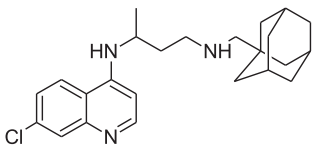
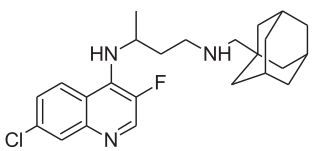


Fig. 2. Effect of a 3-day treatment with 80 mg/kg/day of the investigational compounds on the survival of mice infected with *Plasmodium berghei* ANKA strain. ▨, treatment days.

Table 3
Chemical structures of the most active investigational compounds in vivo.

Compound	Chemical structure
ClAQ4	
ClAQ5	
ClAQ1	
FClAQ1	

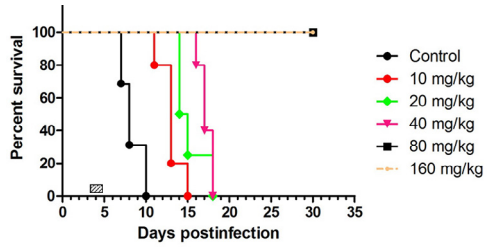


Fig. 3. Effect of a 3-day treatment with ClAQ1 in the full dosage regimen on the survival of mice infected with *Plasmodium berghei* ANKA strain. ▨, treatment days.

survival time of treated infected mice, three had much higher IC_{50} values than CQ against both parasite strains (Table 2).

4. Discussion

Here we present the antimalarial efficacy of a series of investigational aminoquinoline compounds. Of the 12 compounds that exhibited ≥50% growth inhibition in parasite cultures, the efficacy of nine compounds shown to be non-toxic in vivo was examined in a mouse infection model. When given in three daily doses of 80 mg/kg or 160 mg/kg, all nine compounds significantly prolonged survival compared with untreated controls, but most notably, four compounds afforded survival of mice past Day 31. Of these, compounds ClAQ4 and ClAQ5 afforded a high protection rate although with residual infection in mice that survived the observation period, whilst compounds ClAQ1 and FClAQ1 afforded cure (with parasite clearance) for 100% of mice at doses both of 160 mg/kg/day and 80 mg/kg/day. At the latter dose, the survival rate afforded by ClAQ1 and FClAQ1 was even superior to that of CQ. Furthermore, these two compounds showed significant activity at lower doses as well, of which ClAQ1 prolonged time to death (versus untreated controls) even at a dose as low as 10 mg/kg.

Several important observations arise from these data. First, we have observed that the best in vivo effects did not correlate with in vitro efficacy. For instance, AQ1 was the single compound that had lower IC_{50} values than CQ against both strains, but its in vivo efficacy did not go beyond prolonging survival of infected treated mice. On the other hand, none of the three examined compounds with fluorine on the aminoquinoline moiety had any effect of against the CQ^R strain in vitro, yet all significantly prolonged survival of infected treated mice, whilst FClAQ1 even afforded complete cure. Such discordance has been previously reported for some thiophene- and furan-based aminoquinolines synthesised by the same group [24]. The discrepancy between in vitro and in vivo effects suggests that the antimalarial efficacy of such compounds is due to their metabolites rather than the compounds themselves.

The second interesting observation was that although FClAQ1 at lower doses did not afford survival, it allowed mice to survive

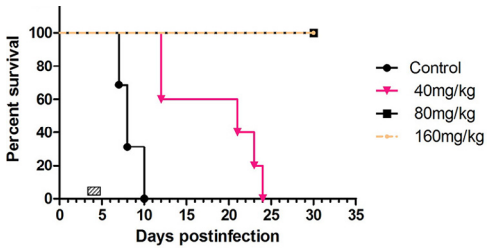


Fig. 4. Effect of a 3-day treatment with FClAQ1 in three dosage regimens (40, 80 and 160 mg/kg) on the survival of mice infected with *Plasmodium berghei* ANKA strain. ▨, treatment days.

Table 4
Survival and parasitaemia of *Plasmodium berghei*-infected mice treated with ClAQ1 and FClAQ1 at different doses.

Compound (mg/kg/day)		No. of mice dead/Day	No. of mice alive and parasitaemia [range in %] at time point:							Mice alive on Day 31/total (% survival)
			Before treatment	Day 7	Day 10	Day 14	Day 17	Day 21	Day 24	Day 28	
CQ	160	–	5 [0.4–0.9]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5/5 (100)
	80	1/17, 1/18	5 [0.5–0.9]	5 [0]	5 [0]	3 [0]	3 [0]	3 [0]	3 [0]	3 [0]	3/5 (60)
						2 [0.1–0.2]	1 [4]				
ClAQ1	160	–	5 [0.7–1.2]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5/5 (100)
	80	–	5 [0.4–0.5]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5 [0]	5/5 (100)
	40	1/16, 2/17, 2/18	5 [0.3–2.4]	5 [0]	5 [0]	5 [0.2–1.2]	2 [2.1–4.6]	–			0/5 (0)
	20	2/14, 1/15, 1/18	4 [0.5–3.5]	4 [0]	4 [0.2–0.4]	2 [1–3.9]	1 [3.5]	–			0/4 (0)
	10	1/11, 3/13, 1/15	5 [0.4–1.6]	5 [0.18–0.5]	5 [1.6–8.9]	1 [4.3]	–				0/5 (0)
FClAQ1	160	–	4 [0.3–0.5]	4 [0]	4 [0]	4 [0]	4 [0]	4 [0]	4 [0]	4 [0]	4/4 (100)
	80	–	6 [0.3–1]	6 [0]	6 [0]	6 [0]	6 [0]	6 [0]	6 [0]	6 [0]	6/6 (100)
	40	2/12, 1/21, 1/23,1/24	5 [0.5–3]	5 [1–4.7]	5 [3.1– 16.3]	3 [5.6–23]	3 [30–52.4]	2 [37.5–62.4]	–		0/5 (0)
	20	2/7, 1/8, 1/14	4 [0.3–2.3]	2 [3.8–4]	1 [10]	–					0/4 (0)
	10	2/7, 1/8, 1/11, 1/12	5 [0.6–2.3]	3 [2.3–5]	2 [3.2–13.9]	–					0/5 (0)

CQ, chloroquine.

remarkably high parasite burdens (37–62%), as opposed to the highest parasitaemia of only 14% appearing to be the survival limit by any other treatment. Importantly, this compound (designated compound **25** in [19]) has been shown to have significant activity in the plasmodial liver stage infection [19], where the intrahepatocytic inhibition of parasite growth was attributed to the presence of the fluorine atom at the C(3) position on the aminoquinoline moiety. The ability of mice treated with this compound to survive massive parasitaemia may indicate its impact on parasite pathogenicity/virulence.

Importantly, the approach taken in this study, i.e. to examine all compounds that exerted ≥50% parasite growth inhibition in vitro in parallel with their effects in an in vivo infection model, allowed us to observe a therapeutic potential that would have gone unnoticed had we chosen the usual approach to examine in vivo only those compounds with an IC_{50} lower than that of the control drug. This observation also suggests that there may have been drug candidates in the past that have been missed because of the approach. It is to be hoped that highly advanced techniques including high-throughput technologies will help avoid such unfortunate events in the future.

A look at the chemical structures of the four most effective compounds (Table 3) shows that the carrier in ClAQ4 and ClAQ5 is benzothiophene, whilst in the case of ClAQ1 and FClAQ1 it is adamantane. Since the results showed that both compounds with adamantane afforded cure of mice, it appears that the higher in vivo activity may be attributed to its use as a carrier. Among its many biological properties, adamantane has been shown to substantially increase drug solubility in lipophilic membranes and may thus increase compound uptake [25].

In summary, the results presented here illustrate the enormous potential of aminoquinoline derivatives bearing an adamantane group as antimalarials whose metabolites and mechanisms of action warrant further investigation and put adamantane into the spotlight as a carrier that enhances the antimalarial effect of aminoquinolines.

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