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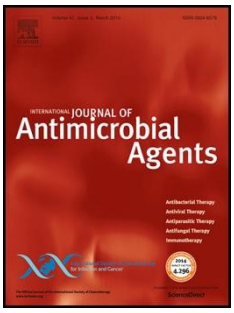

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Examination of the antimalarial potential of experimental aminoquinolines: poor *in vitro* effect does not preclude *in vivo* efficacy

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HIGHLIGHTS

- Antimalarial efficacy of a series of 26 investigational aminoquinolines was examined.
- Two compounds with adamantane as a carrier cured 100% of infected mice.
- Of which one had no *in vitro* effect against a chloroquine resistant *Plasmodium* strain.
- Better *in vivo* than *in vitro* results suggest a role for the compound metabolites.
- Adamantane aminoquinolines warrant further investigation.

ABSTRACT

Malaria remains a major disease of the developing world and globally 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. We here 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 an LDH assay in cultures of a CQ-sensitive (3D7) and a CQ-resistant (Dd2) strain of *Plasmodium falciparum*. Of a series of 26 screened compounds, those 12 that exerted a growth inhibition rate of at least 50% were further examined *in vitro*, to determine the IC_{50} values, and *in vivo*. This way, even the four compounds that exhibited high IC_{50} values, were evaluated *in vivo*, in a modified Thompson test, in C57BL/6 mice infected with the *P. berghei* ANKA strain. However, another three compounds were eventually excluded due to toxicity in mice. All nine compounds examined *in vivo* prolonged survival of treated vs. untreated mice, four of which afforded at least a 60% survival. Most notably, two of these, both with the adamantane carrier, afforded complete cure (100% survival and parasite clearance). One of these, interestingly, had no *in vitro* effect (against the CQR strain). Better *in vivo* than *in vitro* results suggest a role for the compound

metabolites. The presented results point to adamantane as a carrier which enhances the antimalarial potential of aminoquinolines.

Keywords: malaria, aminoquinolines, LDH assay, Thompson test, adamantane

1. Introduction[1]

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

The World Health Organisation estimated 214 million cases of malaria and 438,000 deaths in 2015 [2], with most of the deaths caused by *P. falciparum*. Half of the global human population, residing in the 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].

[1] [1] Chloroquine (CQ); CQ-sensitive (CQS); CQ-resistant (CQR); food vacuole (FV); *P. falciparum* CQ resistance transporter (PfCRT); 4-aminoquinoline (AQ); 7-chloro-4-aminoquinoline (ClAQ); 3-fluoro-4-aminoquinoline (FAQ); 3-fluoro-7-chloro-4-aminoquinoline (FClAQ); 3-fluoro-7-chloro-2-aminoquinoline (FCl2AQ); dimethyl sulfoxide (DMSO); intraperitoneal (i.p.); *per os* (p.o.); lactate dehydrogenase (LDH); 50% inhibitory concentration (IC_{50}); post infection (p.i.); real time PCR (qPCR)

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 emphasizes 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* species by preventing polymerization of heme through its selective accumulation in the parasite food vacuole (FV). CQ forms stable complexes with heme and its removal from FV is prevented by protonation [6, 7]. Mutations in the *P. falciparum* CQ resistance transporter (PfCRT) gene have a central role in CQ resistance. PfCRT 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, 11, 12, 13, 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 ones, which are not recognized 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 against both the liver and blood stages, has been described [19]. We here 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 (CQS) 3D7 and a chloroquine-resistant (CQR) Dd2 strain of *P. falciparum* were maintained in human erythrocytes as described previously [20]. For *in vitro* drug assays, parasites were synchronized with 5% sorbitol, and ring-stage parasites were seeded in 96-well plates to achieve 2% parasitemia and 0.75% hematocrit.

In vivo testing was performed using the *Plasmodium berghei* ANKA strain maintained through serial intraperitoneal (i.p.) passages in C57BL/6 mice.

2.2. *Mice*

Female C57BL/6 mice (Medical Military Academy Animal Research Facility, Belgrade), weighing between 19-21 g, were used. Groups of 4-6 animals were housed in the Institute for Medical Research Animal Facility under a natural photo-period, and 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 synthesized at the Faculty of Chemistry, University of Belgrade, were examined (Table 1).

According to the modifications at the aminoquinoline moiety structure, the compounds belonged to five groups as follows:

1. 4-aminoquinoline - AQ (number of compounds, n=3)
2. 7-chloro-4-aminoquinoline - ClAQ (n=6)

3. 3-fluoro-4-aminoquinoline - FAQ (n=2)

4. 3-fluoro-7-chloro-4-aminoquinoline - FClAQ (n=8)

5. 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% hydroxyethylcellulose - 0.1% Tween 80 and 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 at least 50% were titrated to obtain 50% inhibitory concentration (IC_{50}) values and 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 nontoxic 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 nontoxic were evaluated for antimalarial efficacy at doses of 160 and 80 mg/kg/day. Compound efficacy was evaluated based on parasitemia over time and survival of the treated vs. untreated mice. Cure was defined as survival past day 31 p.i and complete clearance of parasitemia. Survival past day 31 p.i with residual parasitemia indicated survival without cure. If a compound did not afford survival but significantly prolonged time to death of treated vs. untreated mice ($P<0.05$), the effect was defined as prolonged survival. Finally, in case a compound cured mice in a dose of 80

mg/kg/day, efficacy was tested at lower doses, including 40, 20, and 10 mg/kg/day. Parasitemia was determined twice a week, starting from day 3 p.i. (immediately before treatment) and only mice in which parasitemia was detected were submitted to experimental treatment. Parasitemia was evaluated by microscopic examination of Giemsa stained thin blood smears prepared from mouse tail blood on an Axioscope 2+ (Zeiss) optical microscope at 1000X magnification, while parasite clearance was additionally confirmed in treated survivors by qPCR.

2.5. In vitro *examination of compound efficacy*

In vitro testing was performed using a LDH assay. The 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 3 replicates per condition. The assay was performed in flat-bottom 96-well microtiter plates. Briefly, compounds were tested at eight different concentrations, ranging from 256 nM to 2 nM, plated in a volume of 100 µL. Parasites were plated into the wells while in the ring phase at 0.75% hematocrit and 2% parasitemia in a volume of 100 µL. Each well contained the compound and parasite culture in a final volume of 200µL. Following incubation at 37^{o} C for 48 hours in a Heracell 150i incubator (ThermoScientific, Waltham, MA, USA), the parasites were harvested and subjected to three 20-minutes freeze-thaw cycles to resuspend the culture. Cultured erythrocytes without drug were used as the assay blank, while 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 microtiter plate to which a 20 µL sample of each parasite culture was added. Color development of the LDH plate was detected by the Multiscan X (ThermoScientific, Waltham, MA, USA)

microplate reader at 620 nm after an hour incubation in the dark. All reagents used in the assay were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.6. In vivo *examination of compound efficacy*

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

2.7. *PCR*

Residual parasitemia was examined in the surviving mice by the real time PCR (qPCR) method adapted from Rougemont et al., based on the detection of *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, rinsed with Dulbecco's PBS and homogenized. DNA extraction was performed using 100 µl of blood and liver homogenate samples using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each PCR reaction contained 1X MaximaProbe qPCR Mastermix (Thermo Fisher Scientific, Waltham, MA, USA), 200 nM of each primer, 50 nM probe, 1U UNG (Thermo Fisher Scientific Waltham, MA, USA) and 3 µl template gDNA in a final volume of 20 µl. The PCR conditions were as follows: one holding step at 50°C for 2 min, one holding step at 95 °C for 10 min, then 45 cycles of 95 °C

for 15 s, 60 °C for 1 min. Samples with Ct values above 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 compared by the log-rank (two curves) and log-rank test for trends (three or more curves) tests. The level of statistical significance was 0.05. Statistical analysis was performed using GraphPad Prism v. 5.

3. Results

A series of 26 aminoquinolines was examined in this work. Of these, 12 compounds inhibited proliferation of either the CQS or the CQR *Plasmodium* strain by at least 50%, while the remaining 14 compounds did not, so they were eliminated from further work.

All 12 compounds were first assayed for toxicity. Four compounds (AQ2, AQ3, ClAQ3, ClAQ6) were shown to cause acute toxicity at a dose of 160 mg/kg/day, which eliminated them from further *in vivo* examination. However, due to chemical similarity with other members of the benzothiophene group, which were nontoxic 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 found to be nontoxic 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, FClAQ2) were subjected to *in vivo* testing. The results showed that, when administered

in doses of 160 and/or 80 mg/kg/day, all nine significantly prolonged survival of treated vs. untreated mice ($P<0.05$; Fig. 1, Fig. 2).

Remarkably, three ClAQ compounds (ClAQ1, ClAQ4, 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 parasitemia in a single animal.

But treatment with 160 and 80 mg/kg/day of the other two compounds, ClAQ1 and FClAQ1, afforded complete cure. All treated infected mice survived beyond d31 (Fig. 3, Fig. 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. We thus 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 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 parasitemia in all mice.

On the other hand, an interesting observation with FClAQ1 was that although all mice treated with 40 mg per kg per day eventually succumbed to the infection, they were able to tolerate very high levels of parasitemia, which amounted up to 62% (ranging from 37.5 to 62.4%). In contrast, the highest level of parasitemia 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 haphazard (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 which afforded survival but not cure, had quite low IC_{50} values and by far the lowest ones against the CQR strain. In contrast, FClAQ1, which cured all infected mice (in two doses), had no *in vitro* effect against the CQR 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 survival time of treated infected mice, even three had much higher IC_{50} values than CQ on both parasite strains (Table 2).

4. Discussion

We here presented the antimalarial efficacy of a series of investigational aminoquinoline compounds. Of the 12 that exhibited at least 50% of growth inhibition in parasite cultures, the efficacy of nine shown to be nontoxic *in vivo* was examined in a mouse infection model. When given in three daily doses of 160 or 80 mg/kg, all nine significantly prolonged survival compared to untreated controls, but most notably, four 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, while compounds ClAQ1 and FClAQ1 afforded cure (with parasite clearance) for 100% mice at doses of both 160 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 in lower doses as well, of which ClAQ1 prolonged time to death (vs. untreated controls) even in a dose as low as 10 mg/kg.

Several important observations arise from these data. To start, we have observed that the best *in vivo* effects did not correlate with *in vitro* efficacy. For instance, AQ1was 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 CQR strain *in vitro*, yet all significantly prolonged survival of infected treated mice, while FClAQ1 even afforded complete cure. Such discordance has been previously reported for some thiophene- and furan-based aminoquinolines synthesized 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 in lower doses did not afford survival, it allowed mice to survive remarkably high parasite burdens (37% to 62%), as opposed to the highest parasitemia of only 14%, seeming 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 presence of the fluorine atom at the C(3) position on the aminoquinoline moiety was attributed to the intrahepatocytic inhibition of parasite growth. The ability of mice treated with this compound to survive massive parasitemia may indicate its impact on the parasite pathogenicity/virulence.

Importantly, the approach we took in this study, to examine all compounds which exerted at least 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 had been missed because of the approach. It is to be hoped that highly advanced techniques including high-throughput technologies will help leave such unfortunate events in the past.

A look at the chemical structures of the four most effective compounds (Table 3) shows that the carrier in ClAQ4 and ClAQ5 is benzothiophene, while it is adamantane in the case of

ClAQ1 and FClAQ1. Since our 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 the compound uptake [25].

In summary, the presented results 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 which enhances the antimalarial effect of aminoquinolines.

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Declarations

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Competing Interests: None declared.

Ethical Approval: The study has been carried out in accordance with the ARRIVE guidelines, and was approved by the Veterinary Directorate of the Ministry of Agriculture and Environmental Protection of Serbia (decision no. 323-07-02444/2014-05/1).

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367 **Fig. 1.** Effect of a 3-day treatment with 160 mg/kg/day of the investigational compounds on the
368 survival of mice infected with *P. berghei* ANKA strain. ❒ treatment days
369 **Fig. 2.** Effect of a 3-day treatment with 80 mg/kg/day of the investigational compounds on the
370 survival of mice infected with *P. berghei* ANKA strain. ❒ treatment days
371 **Fig. 3.** Effect of a 3-day treatment with ClAQ in the full dosage regimen on the survival of mice
372 infected with *P. berghei* ANKA strain. ❒ treatment days
373 **Fig. 4.** Effect of a 3-day treatment with FClAQ1 in 3 dosage regimens (40, 80, 160 mg/kg) on the
374 survival of mice infected with *P. berghei* ANKA strain. ❒ treatment days
375

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

GROUP	COMPOUND	ACRONYM	Number in ref. [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**

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

		In vitro (LDH assay)		*In vivo* (Thompson test)		
GROUP	COMPOUND	3D7 (geomeans, nM)	Dd2 (geomeans, nM)	TOXICITY 160 mg/kg/day	TREATMENT DOSE (mg/kg/day)	EFFECT on d31 p.i.
		IC50	IC50			
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** (P=0.0067)
					80	80% survival** (P=0.0031)
	ClAQ5	34.13	16.7	NT	160	60% survival** (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	NS (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

AQ : 4-aminoquinoline ; **ClAQ** : 7-chloro-4-aminoquinoline ; **FAQ** : 3- fluoro-4-aminoquinoline ; **FClAQ** : 3- fluoro-7-chloro-4-aminoquinoline ; **AQ1:** N^1-(1-adamantylmethyl)-N^3-quinolin-4-ylbutane-1,3-diamine; **AQ2:** N^1-[2-(1-adamantyl)ethyl]-N^3-quinolin-4-ylbutane-1,3-diamine; **AQ3:** *N*-(1-adamantylmethyl)-*N*-methyl-*N*'-quinolin-4-ylpropane-1,3-diamine; **ClAQ1:** N^1-(1-adamantylmethyl)-N^3-(7-chloroquinolin-4-yl)butane-1,3-diamine; **ClAQ2:** N^2-(1-adamantylmethyl)-N^1-(7-chloroquinolin-4-yl)propane-1,2-diamine; **ClAQ3:** *N*-(7-chloroquinolin-4-yl)-*N*'-[(5-fluoro-1-benzothiophen-3-yl)methyl]propane-1,3-diamine; **ClAQ4:** *N*-(7-chloroquinolin-4-yl)-*N*'-[(5-fluoro-1-benzothiophen-3-yl)methyl]butane-1,4-diamine; **ClAQ5:** *N*-(7-chloroquinolin-4-yl)-*N*'-[(6-fluoro-1-benzothiophen-3-yl)methyl]propane-1,3-diamine; **ClAQ6:** N^1-[2-(1-adamantyl)ethyl]-N^3-(7-chloroquinolin-4-yl)butane-1,3-diamine; **FAQ1:** N^1-(1-adamantylmethyl)-N^3-(3-fluoroquinolin-4-yl)butane-1,3-diamine; **FClAQ1:** N^1-(1-adamantylmethyl)-N^3-(7-chloro-3-fluoroquinolin-4-yl)butane-1,3-diamine; **FClAQ2:** N4-(7-chloro-3-fluoroquinolin-4-yl)-N1,N1-diethylpentane-

389 1,4-diamine; **CQ**: chloroquine; **3D7**: *P. falciparum* CQ-sensitive strain; **Dd2**: *P. falciparum* CQ-resistant strain; **IC_{50}**: 50% inhibitory
390 concentration; **T**: toxic; **NT**: nontoxic; * vs. infected untreated (control) mice; **with residual parasitemia; NS: not significant
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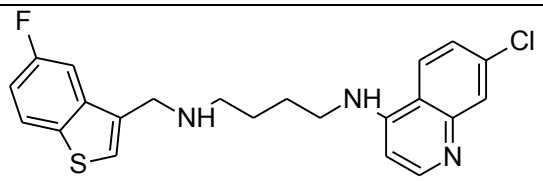
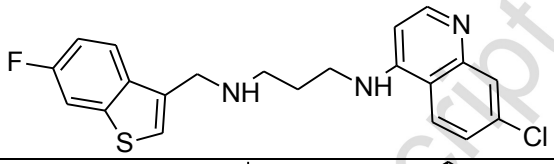
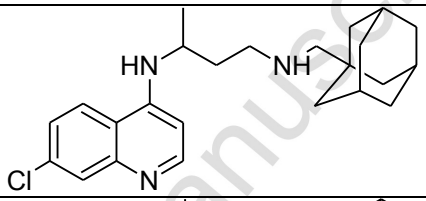
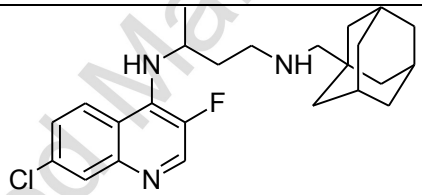
393 Table 3. Chemical structures of the most active investigational compounds

COMPOUND	CHEMICAL STRUCTURE
ClAQ4	
ClAQ5	
ClAQ1	
FClAQ1	

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396 Table 4. Survival and parasitemia of *P. berghei*-infected mice treated with ClAQ1 and FClAQ1 at different doses

COMPOUND mg/kg/day		**No. of mice dead on day**	**No. of mice alive and parasitemia (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) 2 (0.1-0.2)	3 (0) 1 (4)	3 (0)	3 (0)	3 (0)	3/5 (60)
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	2	1	-					0/4

			(0.3-2.3)	(3.8-4)	(10)						(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)

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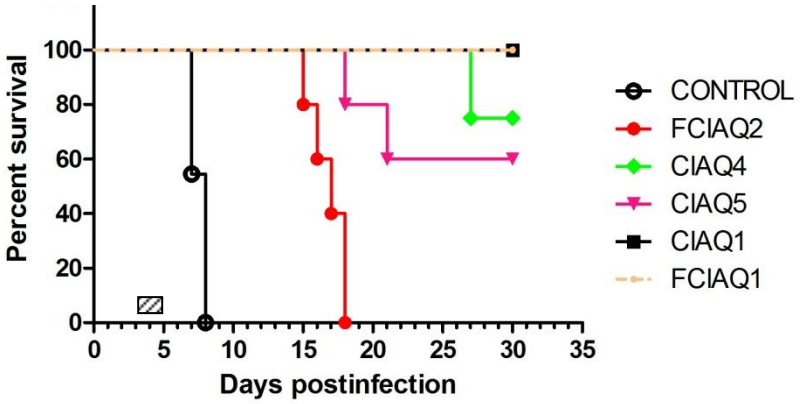
400 Figure 1



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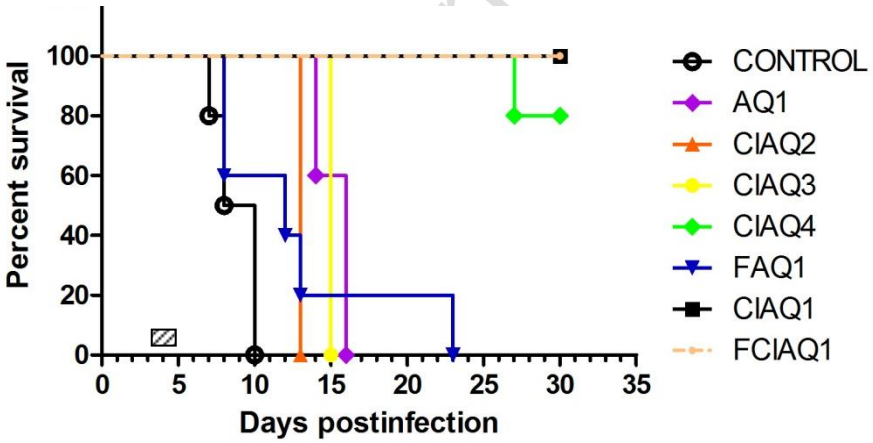
404 Figure 2



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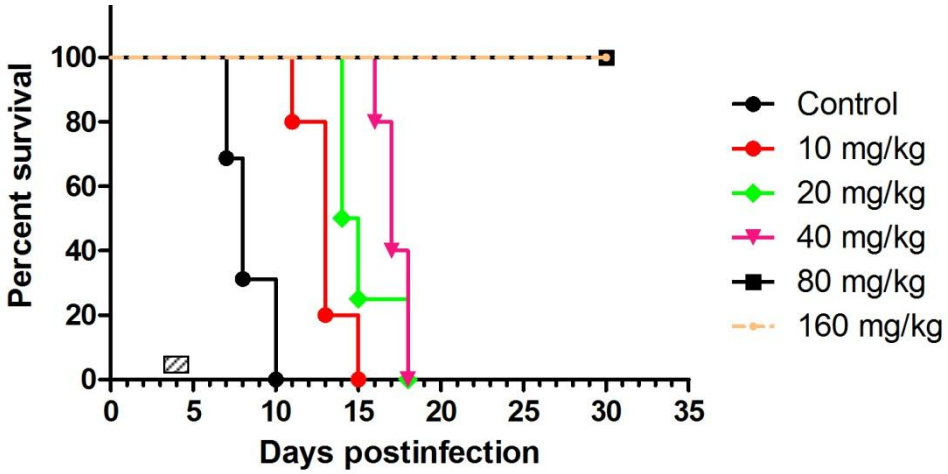
408 Figure 3



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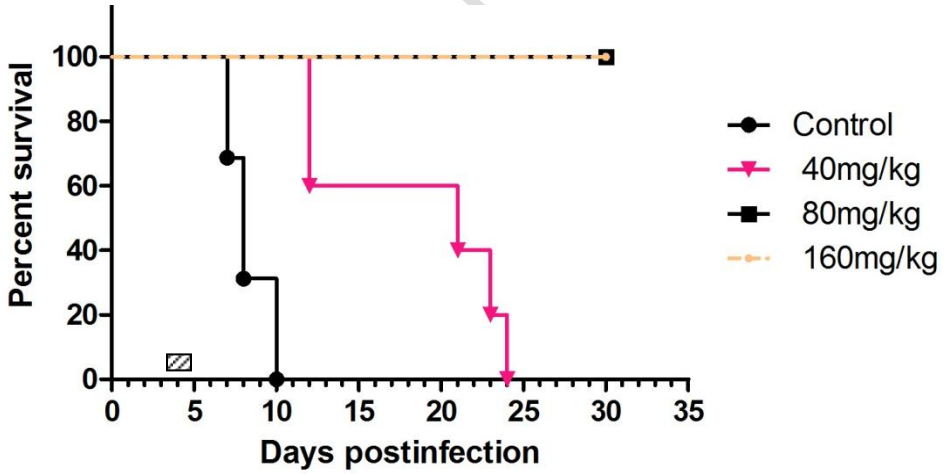
413 Figure 4



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