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Aminoquinolines afford resistance to cerebral malaria in susceptible mice☆

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ABSTRACT

Objectives: Malaria treatment is impeded by increasing resistance to conventional antimalarial drugs. Here we explored the activity of ten novel benzothiophene, thiophene and benzene aminoquinolines.
Methods: In vitro testing was performed by the lactate dehydrogenase assay in chloroquine (CQ)-sensitive *Plasmodium falciparum* strain 3D7 and CQ-resistant (CQ^R) *P. falciparum* strain Dd2. In vivo activity was evaluated by a modified Thompson test using C57BL/6 mice infected with *Plasmodium berghei* ANKA strain.
Results: Nine of the ten compounds had a lower 50% inhibitory concentration (IC_{50}) than CQ against the CQ^R strain Dd2. Five of these compounds that were available for in vivo evaluation were shown to be non-toxic. All five compounds administered at a dose of 160 mg/kg/day for 3 days prolonged the survival of treated compared with untreated mice. Untreated control mice died by Day 7 with a mean parasitaemia of 15%. Among treated mice, a dichotomous outcome was observed, with a two-third majority of treated mice dying by Day 17 with a low mean parasitaemia of 5%, whilst one-third survived longer with a mean hyperparasitaemia of 70%; specifically, five of these mice survived a mean of 25 days, whilst two even survived past Day 31.
Conclusions: The significant antimalarial potential of this aminoquinoline series is illustrated by its excellent in vitro activity against the CQ^R *P. falciparum* strain and significant in vivo activity. Interestingly, compounds ClAQ7, ClAQ9 and ClAQ11 were able to confer resistance to cerebral malaria and afford a switch to hyperparasitaemia to mice prone to the neurological syndrome.
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1. Introduction

Malaria is a parasitic infection caused by protozoa of the genus *Plasmodium* that affects millions of people worldwide [1]. The resistance both of parasites and vectors to existing drugs is an ongoing and increasing problem, hampering both prevention and treatment of malaria. Indeed, *Plasmodium* parasites are developing resistance to nearly all conventional antimalarials, and *Anopheles* vectors are becoming resistant to insecticides, with no vaccine existing to date. Additionally, climate changes and migrations may favour further spread and the re-emergence of malaria even in areas considered free of the disease. Given these circumstances, discovering new antimalarial compounds that can overcome parasite resistance has emerged as a global scientific goal [2–7].

Our recent work focused on the antimalarial effect of 26 novel aminoquinolines investigated in both in vitro and in vivo model systems and showed a great potential of adamantane derivatives, with two compounds shown to cure *Plasmodium berghei*-infected mice [8]. The current study examined ten novel benzothiophene, thiophene and benzene derivatives of the same aminoquinoline series, whose synthesis has recently been described [9–11]. Here we report on the activity of these ten aminoquinolines in different in vitro model systems as well as in vivo and note that some

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conferred resistance to cerebral malaria and a switch to hyperparasitaemia of mice prone to the neurological syndrome.

2. Materials and methods

2.1. Parasites

For in vitro drug assays, chloroquine (CQ)-sensitive (CQ^S) *Plasmodium falciparum* strain 3D7 and CQ-resistant (CQ^R) *P. falciparum* strain Dd2 were used. Cell cultures were maintained as described previously [12]. Parasites were synchronised with 5% sorbitol and ring-stage parasites were seeded in 96-well plates to achieve 2% parasitaemia and 0.75% haematocrit.

For in vivo testing, *P. berghei* ANKA strain maintained through serial intraperitoneal (i.p.) passage in C57BL/6 mice was used.

2.2. Mice

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

2.3. Compounds

Ten novel aminoquinoline derivatives with chemical modifications at the aminoquinoline moiety and side chain, synthesised at the Faculty of Chemistry, University of Belgrade [9–11], were examined (Table 1). According to the structure of the aminoquinoline moiety, compounds were classified into 4-aminoquinolines (AQs) (two compounds) and 7-chloro-4-aminoquinolines (ClAQs) (eight compounds).

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

For in vivo experiments, compounds were suspended in 0.5% hydroxyethyl cellulose/0.1% Tween 80 and were administered orally (p.o.).

2.4. Experimental design

All compounds were screened in vitro and, if appropriate, were titrated to obtain 50% inhibitory concentration (IC_{50}) values and examined for antimalarial efficacy in vivo. Prior to in vivo evaluation of the selected compounds, all were tested for toxicity in mice.

2.5. Assays

In vitro evaluation of the antimalarial activity of the compounds was performed using the colorimetric lactate dehydrogenase assay [13]. Compounds were first screened at a concentration of 500 nM (three replicates per compound for each *P. falciparum* strain) and those that showed a minimum of 50% parasite growth inhibition were further titrated to determine their IC_{50} value. CQ was used as a positive control. Briefly, compounds were titrated using eight two-fold dilutions, starting at a concentration of 256 nM or 1000 nM, depending on the results of the initial screening. Three independent experiments were performed for each compound, with three replicates per compound for each *P. falciparum* strain. More precisely, the IC_{50} value of each compound and CQ was calculated as the geometric mean IC_{50} value of the individual experiment for a particular strain of *P. falciparum*.

In vivo toxicity of the experimental compounds was examined through a series of experiments in which selected compounds were administered at a dose of 160 mg/kg/day p.o. to healthy mice during 3 consecutive days. Mice were monitored daily over a period of 30 days for survival and appearance of symptoms indicating toxicity such as ruffled fur, lethargy, loss of appetite, lacrimation, salivation, diarrhoea, convulsions and weight loss. Compounds not causing signs of gross toxicity were included in further investigations.

The in vivo antimalarial activity of the non-toxic compounds was evaluated by a modified Thompson test [14]. Mice were infected i.p. with 10^6 infected erythrocytes in phosphate-buffered saline (PBS) (250 μL total volume) obtained from the peripheral blood of a *P. berghei.* ANKA-infected donor mouse; this time point was considered Day 0. Mice were treated with investigational compounds once a day for 3 consecutive days on Days 3, 4 and 5 post-infection. All compounds were administered p.o. at a dose of 160 mg/kg/day (200 μL total volume) in accordance with previous experience of both our and other groups [5,15]. Infected mice treated with the same dose of CQ served as a positive control group, whereas untreated infected mice served as a negative control group. Survival and parasitaemia were monitored for 30 days post-infection.

Parasitaemia was monitored by microscopic examination of Giemsa-stained peripheral blood smears using mouse tail blood twice a week, starting immediately before the initiation of treatment (Day 3 post-infection). Up to 1000 erythrocytes were counted and parasitaemia was calculated. The sample obtained immediately before treatment initiation was of particular importance. Blood smears positive for *P. berghei* parasites presented evidence that mice were infected to a degree that would allow microscopic monitoring of treatment effects. In the absence of

Table 1
Compounds grouped according to modifications at the aminoquinoline moiety.

Compound group/compound	Compound	Reference	Compound no.
4-Aminoquinolines			
AQ4	N^1-(4-(5-fluorobenzo[b]thiophen-3-yl)benzyl)-N^3-(quinolin-4-yl)propane-1,3-diamine	[11]	**23**
AQ5	4-(5-(4-((methyl(8-(quinolin-4-ylamino)octyl)amino)methyl)phenyl)thiophen-2-yl)benzonitrile	[11]	**46**
7-Chloro-4-aminoquinolines			
ClAQ7	N^1-(benzo[b]thiophen-3-ylmethyl)-N^2-(7-chloroquinolin-4-yl)ethane-1,2-diamine	[10]	**55**
ClAQ9	N^4-(7-chloroquinolin-4-yl)-N^1-(4-(5-fluorobenzo[b]thiophen-3-yl)benzyl)pentane-1,4-diamine	[11]	**25**
ClAQ10	3-(((3-((7-chloroquinolin-4-yl)amino)propyl)amino)methyl)benzo[b]thiophene-6-carbonitrile	[10]	**61**
ClAQ11	4-(5-(4-(((8-((7-chloroquinolin-4-yl)amino)octyl)amino)methyl)phenyl)thiophen-2-yl)benzonitrile	[11]	**42**
ClAQ12	N^1-benzyl-N^2-(7-chloroquinolin-4-yl)ethane-1,2-diamine	[9]	**6**
ClAQ13	N^1-benzyl-N^3-(7-chloroquinolin-4-yl)propane-1,3-diamine	[9]	**7**
ClAQ14	N^1-(4-bromobenzyl)-N^2-(7-chloroquinolin-4-yl)ethane-1,2-diamine	[9]	**14**
ClAQ15	N^1-(4-bromobenzyl)-N^3-(7-chloroquinolin-4-yl)propane-1,3-diamine	[9]	**15**

microscopic evidence of parasites, the animal was excluded from the experiment.

Compound efficacy was evaluated on the basis of survival of treated versus untreated mice as assessed both by the rate and length of survival (expressed in % and days, respectively) and the presence and level of parasitaemia over time. Cure was defined as survival past Day 31 p.i. and clearance of parasitaemia.

2.6. Statistical analysis

IC_{50} values were obtained using a sigmoidal dose–response model with the variable slope fitted to the results. The rates of survival in particular groups 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. All statistics was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

A series of ten novel aminoquinolines with modifications at the linker and at the quinoline nucleus was examined both in vitro and in vivo for antimalarial efficacy. At a concentration of 500 nM, eight (AQ4, AQ5, ClAQ9–ClAQ13 and ClAQ15) of the ten compounds inhibited the growth both of the CQ^S and CQ^R *P. falciparum* strain by ≥50%, whilst one (ClAQ7) exerted this effect only against the CQ^R strain (Fig. 1). When titrated to determine IC_{50} values, all nine compounds were more active than CQ against the CQ^R strain, whilst none performed better than CQ against the CQ^S strain (Table 2). Although all of these nine compounds qualified for further examination, four (AQ4, AQ5, ClAQ10 and ClAQ12) were unavailable due to technical reasons (not synthesised in sufficient quantity).

The five available compounds (ClAQ7, ClAQ9, ClAQ11, ClAQ13 and ClAQ15) were first assayed for toxicity. No gross toxicity was noted in any mice given any of the compounds at a dose of 160 mg/kg/day, and all five compounds were thus subjected to in vivo testing. Prior to treatment administration, all mice were checked for parasitaemia; the pre-treatment parasitaemia was 0.7 ± 0.4% in mice assigned to the negative control group and 0.6 ± 0.3% in those assigned to treatment ($P > 0.05$). The results showed that compared with untreated control animals that died after 7.3 ± 0.5 days, treatment with all five investigational compounds significantly ($P < 0.05$) prolonged the length of survival (Table 2) to a mean of 17.7 ± 5.3 days for the 23 mice that died during the experiment. However, comparative analysis of the efficacy of the compounds showed different levels of activity (Fig. 2). Compounds ClAQ9 and ClAQ13 afforded survival of one mouse each (out of four and five, respectively) past Day 31, but with high parasitaemia as observed on blood smears, whilst the mice that died survived 17.3 ± 0.6 days and 14.75 ± 1.7 days, respectively. On the other hand, compounds ClAQ7, ClAQ11 and ClAQ15 did not afford survival of any mice but prolonged length of survival of treated animals to 18.6 ± 8.05, 18.6 ± 7.6 and 18.3 ± 3.4 days, respectively (Table 2).

Regular monitoring of parasitaemia in each animal throughout the entire experiment allowed for several interesting observations (Tables 2 and 3). In untreated control mice at the time of death, parasitaemia ranged between 11.8% and 17.3% (mean 14.8 ± 2.3%). In contrast, the mean parasitaemia of the treated animals that died by Day 17 ($n = 16$; mean survival 14.8 ± 2.5 days) was only 4.8 ± 3.5%. Another two animals (both treated with ClAQ15) survived to Day 21 and Day 24, respectively, with a mean parasitaemia of 7.8 ± 3.3%, also significantly ($P < 0.05$) below that of the control mice. On the other hand, all other animals lived longer (five dying between Days 21 and 28, with a mean survival of 25 ± 4 days, and two still alive at Day 31), with a mean parasitaemia of 70 ± 10.5%.

4. Discussion

The results presented here demonstrate a significant antimalarial potential of the examined aminoquinoline series. Nine of the ten compounds were more active than CQ against the CQ^R *P. falciparum* strain Dd2. Moreover, a significant biological activity of five compounds (ClAQ7, ClAQ9, ClAQ11, ClAQ13 and ClAQ15) against *P. berghei* was shown. Whereas all five compounds significantly prolonged the length of survival compared with untreated infected animals, two compounds afforded survival up to Day 31 of 1/4 (ClAQ9) and 1/5 (ClAQ13) treated mice, albeit with residual parasitaemia.

All five examined compounds that exhibited significant in vivo activity share the same AQ nucleus with a chlorine atom at the C(7) position. It is known that the AQ ring is required for complexation with ferriprotoporphyrin but is not sufficient for the inhibition of haemozoin synthesis [16]. A chlorine atom at position C(7) is introduced into the AQ molecule for this purpose [16–19], and the present results confirm its role. Benzothiophene ClAQ9 is one of the two compounds that afforded survival (if only of one mouse) past Day 31. In our recent investigations of another series of novel aminoquinolines, two benzothiophenes were among the most effective compounds [8], whilst another afforded cure [11]. Taken together, these results illustrate the significance of the benzothiophene structure introduced in the side chain of aminoquinolines. Benzothiophene is suitable for the synthesis of large bioactive molecules owing to its heterocyclic structure, whilst the wide range of pharmacological activities (over 40) of this ring

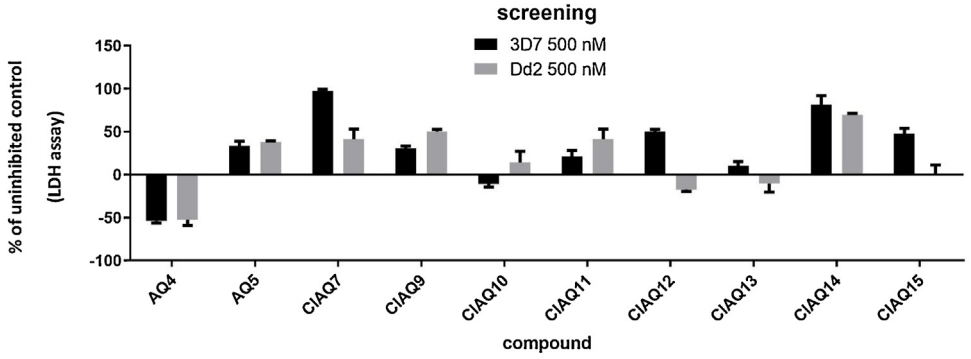

Fig. 1. In vitro activity of ten novel compounds against two *Plasmodium falciparum* strains, chloroquine-sensitive *P. falciparum* strain 3D7 and chloroquine-resistant *P. falciparum* strain Dd2. Three replicates per compound were performed for each *P. falciparum* strain. LDH, lactate dehydrogenase.

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

Compound group/compound	In vitro (LDH assay)		In vivo (Thompson test), treatment with 160 mg/kg/day orally during 3 days (Days 3, 4 and 5 p.i.)		
	GM IC_{50} (nM)				
	3D7[a]	Dd2[b]	No. of mice dead on day (d) p.i.	Mice alive on Day 31/total	Treatment effect
4-Aminoquinolines					
AQ4	18.77	73.35	N/A		
AQ5	119.58	71.52	N/A		
7-Chloro-4-aminoquinolines					
ClAQ7	>500	14.43	2 D12, 1 D15, 1 D24, 1 D30	0/5	Prolonged time to death[c]
ClAQ9	82.35	186.79	2 D17, 1 D18	1/4	Prolonged time to death[c] (3/4); survival[d] (1/4)
ClAQ10	50.23	18.57	N/A		
ClAQ11	97.26	174.12	1 D9, 1 D13, 1 D20, 1 D23, 1 D28	0/5	Prolonged time to death[c]
ClAQ12	32.2	40.82	N/A		
ClAQ13	22.05	37.97	1 D13, 1 D14, 1 D15, 1 D17	1/5	Prolonged time to death[c] (4/5); survival[d] (1/5)
ClAQ15	30.11	18.81	1 D15, 1 D16, 2 D17, 1 D21, 1 D24	0/6	Prolonged time to death[c]
Chloroquine (control)	18.63	270.57	0	5/5	Cure

LDH, lactate dehydrogenase; GM, geometric mean; IC_{50}, 50% inhibitory concentration; p.i., post-infection; d, day; N/A, not available.
[a] Chloroquine-sensitive *Plasmodium falciparum* strain 3D7.
[b] Chloroquine-resistant *P. falciparum* strain Dd2.
[c] Versus infected untreated (control) mice that died 7.33 ± 0.48 days p.i.
[d] With residual parasitaemia.

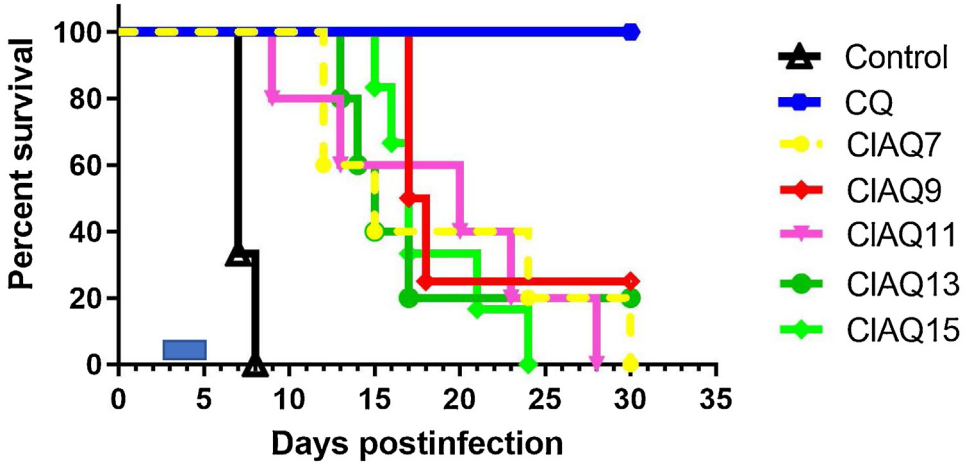

Fig. 2. Effect of a 3-day oral treatment with 160 mg/kg/day of chloroquine (CQ) and five investigational compounds (ClAQ7, ClAQ9, ClAQ11, ClAQ13 and ClAQ15) on the survival of mice ($n = 5$ per compound, except for ClAQ9 and ClAQ15 where there were 4 and 6 mice, respectively) infected with *Plasmodium berghei* ANKA strain. The blue box indicates treatment days.

has been associated with the presence of a sulfur atom [20]. Among the many benzothiophene activities [21], in vitro antimalarial activity has recently been shown for benzothiophene-2-carboxamide [22] and bromobenzothiophene carboxamide derivatives [23], which were also active in vivo.

The observation of the different levels of parasitaemia associated with early or late death (and occasional survival with parasitaemia in the range from 70–90% defined as hyperparasitaemia [24]), as the end result of treatment with compounds ClAQ7, ClAQ11 and ClAQ9, respectively (Table 3), deserves consideration. The C57BL/6 mouse strain is highly susceptible to the neurological syndrome with an incidence of cerebral malaria ranging from 60–100%, and mice die with low parasitaemia (on average < 15%) within 6–10 days after being challenged with 10^6 *P. berghei* ANKA blood-stage parasites [24]. On the other hand, in cerebral malaria-resistant mouse strains,

Table 3
Parasitaemia in individual *Plasmodium berghei*-infected mice treated orally with five experimental compounds (160 mg/kg/day) and in positive (CQ) and negative (untreated infected) control groups at various sampling points.

Compound	Parasitaemia (%)								Mouse
	Before treatment	Day 7	Day 10	Day 14	Day 17	Day 21	Day 24	Day 28	
ClAQ7	0.4	0.2	5.3	Death d12					1
	0.4	0.3	2.9	4	4.6	57	Death d24		2
	0.2	0.2	0.3	6.9	Death d15				3
	1	0.2	2.7	5	9.3	55.2	82	86	4
	0.7	0.2	7	Death d12					5
ClAQ9	0.4	0.4	0.5	0.7	Death d17				1
	0.6	0.2	0.3	0.5	1.5	2.7	52	72	2
	0.5	0.3	0.4	0.5	Death d17				3
	0.5	0.4	0.6	0.8	3.7	Death d18			4
ClAQ11	0.4	6.6	13.5	47	60	Death d20			1
	0.3	0.2	9.2	Death d13					2
	0.2	9	Death d9						3
	0.6	10	15	23.4	53.2	70	Death d23		4
	0.4	8.9	12	12.8	50	64	75	Death d28	5
ClAQ13	0.3	0	0.1	n.s.	5.1	17.1	n.s.	n.s.	1
	1.1	0	1.1	n.s.	Death d15				2
	n.s.	n.s.	n.s.	n.s.	Death d17				3
	n.s.	n.s.	n.s.	Death d13					4
	n.s.	n.s.	n.s.	Death d14					5
ClAQ15	1.1	0	0.1	n.s.	0.5	10.1	Death d24		1
	1.6	0	1.2	n.s.	5.5	Death d21			2
	n.s.	n.s.	n.s.	n.s.	Death d15				3
	n.s.	n.s.	n.s.	n.s.	Death d16				4
	n.s.	n.s.	n.s.	n.s.	Death d17				5
	n.s.	n.s.	n.s.	n.s.	Death d17				6
CQ	0.6	0	0	0	0	0	0	0	1
	0.8	0	0	0	0	0	0	0	2
	0.4	0	0	0	0	0	0	0	3
	0.9	0	0	0	0	0	0	0	4
	0.7	0	0	0	0	0	0	0	5
Untreated infected mice	1	17.3, Death d7							1
	0.5	Death d7							2
	0.9	Death d7							3
	0.4	15.1	Death d8						4
	0.3	15.2	Death d8						5
	0.2	Death d7							6
	1.2	Death d7							7
	1.2	11.8	Death d8						8
	0.9	Death d7							9

d, day; n.s., not sampled.

death generally occurs during the third or fourth week post-infection due to anaemia caused by hyperparasitaemia [24]. However, delayed death associated with hyperparasitaemia in *P. berghei*-infected mice owing to pharmacological treatment or genetic background has been observed [25,26]. In our study, untreated infected animals died on average after 7.3 days, with a parasitaemia of 14.8%, exactly in line with the study by Bagot et al. [24]. In contrast, the investigational compounds all significantly prolonged survival, with a mean survival of mice that died during the experiment of 17.7 days. Among the mice treated with all five compounds, despite high variability within particular treatment groups, the animals clearly segregated into roughly a two-third majority that died within the first 17 days (mean 14.8 days) with a low parasitaemia of 5%, and one-third that survived a mean of 25 days with a mean parasitaemia of 70% (Fig. 3). It stands to reason that the first developed cerebral malaria as expected in *P. berghei*-infected C57BL/6 mice, but with a delay, whereas those that died at later times succumbed to anaemia caused by hyperparasitaemia. Hence, the results presented herein indicate that the compounds afforded prevention of cerebral malaria or a delay

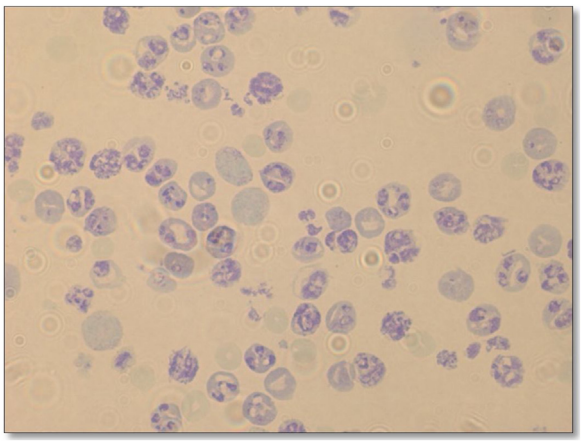

Fig. 3. Example of peripheral blood smear at Day 24 post-infection showing hyperparasitaemia in *Plasmodium berghei*-infected C57BL/6 mouse treated with ClAQ11 (Giemsa staining, light microscope, magnification 1000×).

in its development in a mouse strain susceptible to cerebral malaria.

One limitation of this study is that we have not studied the residual parasite populations in mice that survived to the end of the experiment with a hyperparasitaemia of up to 86%, which could have shed more light on their virulence and pathogenicity. However, this was not possible since microscopic examination of the blood smears was not performed immediately following the sampling because of the large number of blood smears, and the residual parasites were not preserved.

In conclusion, this series of aminoquinolines exhibited significant antimalarial activity. Excellent in vitro activity against the CQ^R strain of even nine of the ten compounds is highly relevant, since the primary goal of new treatment options is to overcome parasite resistance. Interestingly, several compounds conferred resistance to cerebral malaria to mice prone to the neurological syndrome, an observation that merits further investigation.

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Competing interests

None declared.

Ethical approval

This study was 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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