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New 4-aminoquinolines as moderate inhibitors of *P. falciparum* malaria

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Abstract: Synthesis of novel aminoquinoline derivatives has been accomplished and their activity against malaria strains has been examined. The compounds showed moderate *in vitro* antimalarial activity against two *P. falciparum* strains, 3D7 (CQ susceptible clone) and Dd2 (CQ resistant clone). Three aminoquinolines were further examined for antimalarial efficacy in a mouse model using a modified Thompson test. In this model, mice were infected with *P. berghei*-infected red blood cells, and drugs were administered orally. Antimalarial **3** was found toxic at a dose of 320 (mg/kg)/day in 3/6 mice, however, 2/6 mice of the same group survived through day 31, and one of them was cured.

Keywords: quinoline; bromobenzyl derivatives; antimalarials; *β*-hematin inhibitory activity.

INTRODUCTION

Climate change and global warming are closely associated with growing threat from infectious diseases. Increase in global temperatures favors the development and spread of tropical diseases and the associated vector organisms.[1] Warming will contribute to area expansion populated with malaria-transmitting mosquitoes. With 300–500 million clinical cases and nearly one million deaths every year, malaria is a major global public health problem.[2] Among the five known species of the *Plasmodium* genus that cause human malaria (*Plasmodium* species: *P. falciparum*, *P. ovale*, *P. vivax*, *P. malarie* and *P. knowlesi*), *P. falci-*

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parum is th-e major cause of mortality. Malaria parasites use hemoglobin (Hb) as a source of nutrients, and after digestion of host Hb in the acidic food vacuole (FV) of *P. falciparum* amino acids and heme are released. The parasites use Hb amino acids as building blocks for their own protein synthesis. The heme moiety ferriprotoporphyrin-IX is toxic to the host, and the parasite has developed several mechanisms of detoxification: sequestration of heme into insoluble hemozoin,[3] and degradation of heme with hydrogen peroxide[4,5] and glutathione-mediated mechanisms[6] in the FV and cytosol, respectively. The clinical symptoms of malaria appear during the asexual intraerythrocytic stage; consequently, efforts to develop an effective drug have mainly focused on this stage of infection.[7] Malaria has been known for centuries, and there is a lot of information regarding transmission, prevention and treatment of the disease. Despite this, it is still one of the most widespread infectious diseases in the world.[8] One of the main causes is development of widespread drug resistance[9,10] especially to chloroquine (CQ), the most widely used antimalarial (Fig. 1).[11,12] Most of 4-amino-7-chloroquinoline based compounds (ACQs) act as inhibitors of hemozoin formation,[8] but it is also reported that they may act as inhibitors of oxidative[13] and gluthatione--mediated[14] heme degradation. Resistance to ACQs is closely related to reduced accumulation of drugs caused by mutations in drug transporters (PfCRT, Pgh1, and PfMRP).[15,16] The synthesis and development of novel ACQ-based derivatives has been the subject of extensive research. Efforts have focused mostly on three subclasses: bisquinolines, sidechain modified 4-aminoquinolines, and hybrid 4-aminoquinolines.[17,18]

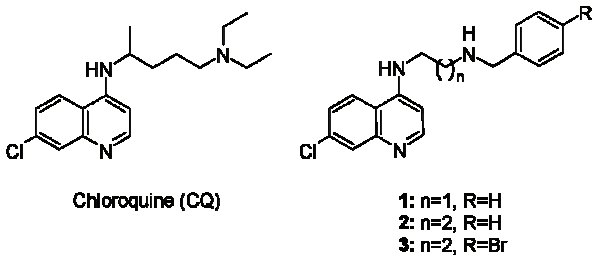
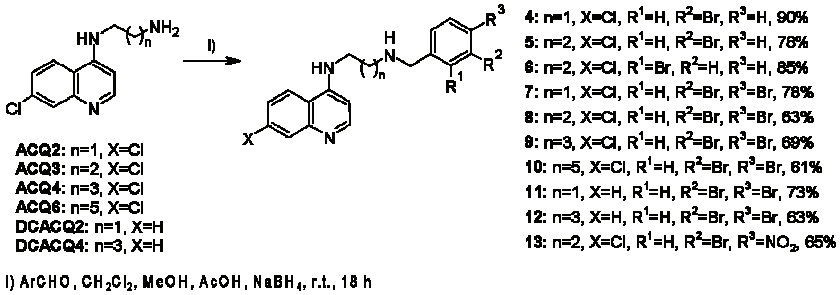


Fig. 1. Structures of CQ and potent antimalarials.

During our studies a variety of ACQ-based compounds were identified as potential antimalarial agents.[19–22] Recently, we synthesized a series of simplified analogues with the ACQ component attached via an ethylene or propylene linker to different benzyl or pyridyl-groups (Fig. 1).[23] The synthesized compounds showed promising antimalarial activities, and exhibited better potencies against CQ susceptible (CQS) and CQ resistant (CQR) strains as compared to CQ and mefloquine. The structure–activity data indicated that a substitution on the benzene ring had a significant influence on the antimalarial activity.

In order to better understand the possible influence of bromine substituents on the antimalarial activity, we synthesized ten new aminoquinoline derivatives and here we analyze their activity (Scheme 1).



Scheme 1. Synthesis of aminoquinoline derivatives **4**–**13**.

In addition, we have synthesized four hybrid derivatives (Fig. 2) that include previously established antimalarial pharmacophores-aminoquinoline[24] and tetrahydroimidazo[1,2-*a*]pyrazine.[12]

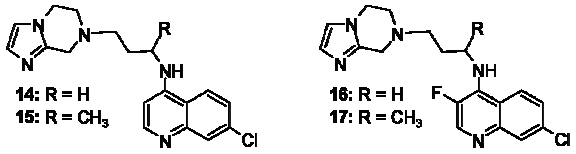


Fig. 2. Structures of hybrid derivatives.

EXPERIMENTAL

For the sake of journal space the full experimental details for the synthesis of tested compounds are given in the Supplementary material to this paper.

In vitro *antimalarial activity*

In vitro cultures of *P. falciparum*, chloroquine-sensitive 3D7 and chloroquine-resistant Dd2 strains were maintained as described previously.[25] For drug assays, parasites were synchronized with 5 % sorbitol and ring-stage parasites were seeded in 96-well plates at a 2 % parasitemia and 0.75 % hematocrit. The compounds were initially dissolved in DMSO at a concentration of 50 mM and further dilutions were made in complete culture medium (final DMSO concentration was ≤0.2 %). After an initial screen at 0.5 μM concentration, compounds that inhibited parasite growth for at least 50 % were further titrated to obtain IC_{50} and IC_{90} values at eight two-fold dilutions (3 independent experiments were performed for each compound, each with 3 replicates per condition). Control experiments using chloroquine and/or artemisinin were performed in parallel with the tested compounds. Parasite inhibition was assayed after 48 h of incubation in the presence of a drug by the colorimetric LDH assay. The test is based on the evaluation of plasmodial lactate dehydrogenase (pLDH) activity and was performed according to the previously described method.[26] IC_{50} and IC_{90} values were obtained using a sigmoidal dose–response model with the variable slope fitted to results using GraphPad Prism.

In vivo *antimalarial activity*

Antimalarial activity of novel compounds was tested in mice infected with *Plasmodium berghei* ANKA strain using a modified version of the Thompson test.[27] Prior to antimalarial efficacy experiments, all compounds were tested for toxicity.

Female C57BL/6 mice aged 12 to 14 weeks and weighing 19–21 g were used. Mice were housed at 5 to 6 animals per cage at the Institute for Medical Research Animal Facility under a natural photo period and were offered drinking water and standard feed *ad libitum*.

Mice were infected intraperitoneally (i.p.) with 250 μL of a PBS suspension containing 10^6 parasitized erythrocytes from the peripheral blood of a donor mouse. Compounds were suspended in 0.5 % hydroxyethylcellulose–0.1 % Tween 80 and administered orally at designated doses in a volume of 200 μL once a day (on day 3, 4 and 5 post-infection (p.i.)).

Mice were monitored daily and any clinical symptoms (*e.g.*, ruffled fur, decreased locomotion, lethargy, loss of appetite, lacrimation, salivation, diarrhoea, convulsions and weight loss) were noted. 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. The first time point served to check the efficacy of experimental infections, whereas all later points served to monitor compound efficacy. Mice were observed for 30 days from the day of infection. Cure was defined as the survival of treated mice with parasite clearance at 31 days p.i.

For toxicity experiments, naive mice were treated according to the described protocol of compound administration. Mice were observed daily during a period of 30 days after the first day of drug administration.

All animal studies were approved by a local (Institute for Medical Research) ethics committee. *In vivo* experiments were approved by the Veterinary Directorate of the Ministry of Agriculture and Environmental Protection of Serbia (decision no. 323-07-02444/2014--05/1).

β-*Hematin inhibitory activity assay*. The inhibition of *β*-hematin formation is expressed as the molar equivalent of compound, relative to hemin, that inhibits *β*-hematin formation by 50 % and determined by slightly modified BHIA assay introduced by Parapini *et al.*[28] Briefly, as described previously,[29] 50.0 μL of 16 mM solution of hemin in DMSO was distributed to 1.5 mL centrifuge tubes; compound dissolved in DMSO (C_{comp}≈80 mM) was added to hemin in doses ranging from 0.125 to 5 mole equivalents (pure DMSO was added to control samples). 100.0 μL of ultrapure water was added to each tube, and β-hematin formation initiated by the addition of 200.0 μL of 8 M acetate buffer (pH 5.2). The final concentration of DMSO per tube was kept constant at 25 %. Tubes were incubated at 37.0±0.1 °C for 18 h and then centrifuged. The remaining pellet was resuspended in 0.500 mL DMSO to remove unreacted hematin. Tubes were then centrifuged again, DMSO-soluble fraction removed and the pellet, consisting of a pure precipitate of *β*-hematin, dissolved in 1.000 mL 0.1 M NaOH. 10.0 μL aliquots were transferred to 96-well microplate and diluted with 190.0 μl 0.1 M NaOH (200.0 μl of 0.1 M NaOH was used as a blank). The absorbance was measured at 405 nm, with correction at 670 nm (LKB 5060-006 Micro Plate Reader, Vienna, Austria). Experiments were performed in duplicate. A calibration curve of hemin dissolved in 0.1 M NaOH was made in the c_{HE} range of 0.4×10^{-5}–4.0×10^{-5} M, samples were diluted, if needed, to fit the calibration curve range.

RESULTS AND DISCUSSION

Synthesis

All compounds were synthesized in high yield by coupling commercially available aryl aldehydes with aminoquinolines via reductive amination using sodium borohydride and acetic acid (Scheme 1). Each of the final compounds was then purified by dry-flash chromatography, and tested as > 95 % pure based on m.a. and/or HPLC analysis.

Antimalarial activity

The *in vitro* antimalarial activity of the synthesized compounds was evaluated against the CQS 3D7 and the CQR Dd2 *P. falciparum* strains, using CQ and artemisinin (ART) as positive controls (Table I). Eight compounds (**1**–**3**, **5**, **6**, **8**, **11** and **12**) were quite potent, with IC_{50} values within 19–48 nM range and appeared more active against the CQR Dd2 strain than CQ. Among them, derivatives **3** and **6** were 5-fold more active than CQ.[30] In addition, **2** and **6** were as active as CQ and ART against the CQS 3D7 strain (**2**: IC_{50} = 22.05 nM; **6**: IC_{50} = 14.02 nM). The results indicate that the length of the methylene chain affects the antiplasmodial activity. The potency of our compounds against the CQS 3D7 strain was increased up to C4 spacer, and further chain lengthening resulted in a decrease of the activity (**7**–**9** *vs.* **10**). On the other hand, the substitution of chlorine atom with hydrogen at the 7-position of the quinoline ring did not significantly affect the *in vitro* antimalarial activity against the CQS strain (**9**: IC_{50} = 30.04 nM; **12**: IC_{50} = 25.45 nM).

TABLE I. *In vitro* antimalarial activity, IC_{50} / nM (geometric mean)

Compound	Strain	
	3D7[a]	Dd2[b]
1	32.20[c]	40.82[c]
2	22.05[c]	37.97[c]
3	30.11[c]	18.81[c]
4	N.D. (>500)[d]	N.D.[d]
5	43.50	34.04
6	14.02	26.07
7	41.08	N.D.[e]
8	45.75	35.22
9	30.04	N.D.[d]
10	61.66	N.D.[d]
11	291.10	48
12	25.45	30.72
13	76.22	216.71
CQ	16.17(5)[e]	129.18(5)[e]
ART	13.09(3)[e]	15.93(2)[e]

[a]*P. falciparum* CQ susceptible clone; [b]*P. falciparum* CQ resistant clone; [c]taken from reference[25]; [d]N.D. – not determined; [e]number of replicates

In order to get an initial insight into the compound's antimalarial mechanism of action, antimalarials **2**, **3** and **8** were submitted to BHIA.[28] The results showed that **3** and **8** interfered with hematin polymerization with the same potency as CQ (**3**: IC_{50} = 1.40; **8**: IC_{50} = 0.81 *vs*. CQ: IC_{50} = 1.23), indicating the same mechanism of action. On the other hand, compound **2** (without bromine attached to benzene ring) showed significantly lower BHIA (**2**: IC_{50} = 3.46). This difference in blocking the hemozoin formation requires further research, and we will pay full attention to this issue in the near future.

Reported results of *in vitro* antimalarial activity for **2** and **3** showed that these compounds are good candidates for *in vivo* testing. Additionally, low toxicities to human liver carcinoma cell line HepG2 and high metabolic stability in mouse and human liver microsomes of antimalarial **2** further supported antimalarial efficacy studies in a mouse model.[23] The bromo derivatives **3** and **8** were examined for antimalarial efficacy. A modified Thompson test model of malaria was used to determine the blood schizonticidal efficacy of the test compounds. Mice were infected with *P. berghei*-infected red blood cells. The test compounds were administered orally at doses of 80, 160 and 320 (mg/kg)/day on days 3, 4, and 5 after parasite inoculation; infection was confirmed by positive blood smear results for all the mice on study day 3 (prior to drug administration).

At a dose of 320 (mg/kg)/day, compound **3** appeared active, since it afforded survival of 2/6 mice, with parasite clearance in one and parasite recrudescence in the other mouse. However, these results should be taken with caution since 3 experimental animals died on day 7 (D7), indicating no activity. Of the two mice that survived to D31, one had a positive blood smear and was therefore not cured, but the other one (1/6 tested) was cured based on a negative blood smear as well as absence of detection of *P. berghei* DNA by PCR (performed as previously described[31]) of blood, liver and spleen. At concentrations of 160 and 80 (mg/kg)/ /day only suppression activity was observed. No acute toxicity of compound **3** was detected, the mice that died or were euthanized prior to day 31 showed typical gross lesions such as gray swollen liver, dark spleen, and pale emaciated carcass (like the control mice group), which indicated fatal malaria infection. Antimalarial **8** was tested at doses of 160 and 80 (mg/kg)/day. At 160 mg/kg/day it was found toxic, and at the lower concentration only suppression activity was observed, Table II.

The hybrid derivatives **14**–**17** did not meet our expectations as they exhibited extremely poor *in vitro* activity, of IC_{50} > 1000 nM, against the CQR strain. However, their BHIA ($IC_{50} \approx 1$) indicated that they may interfere with hematin polymerization to the same extent as CQ (CQ: IC_{50} = 1.23; **14**: IC_{50} = 0.95; **15**: IC_{50} = 1.19; **16**: IC_{50} = 0.72; **17**: IC_{50} = 0.96). The inconsistency between *in vitro* activity and BHIA could indicate the inability of hybrid derivatives to accumulate in the food vacuole of the parasite.

TABLE II. *In vivo* antimalarial activity; groups of four, five, or six, *P. berghei* (ANKA strain) infected mice were treated p.o. once per day on days 3–5 post infection with aminoquinolines suspended in 0.5 % hydroxyethylcellulose-0.1 % Tween 80. Mice alive on day 31 with no parasites as detected by PCR are considered cured

Compd.	Dose, (mg/kg)/day	Mice dead/day died	Mice alive on day 31/total	Mean survival time, day
2[b]	160c	1/13, 1/14, 1/15, 1/17	1/5	18
	80	1/12, 2/13, 1/14, 1/28	0/5	16
3	320	3/7, 1/22	2/6	18
	160c	1/15, 1/16, 2/17, 1/21, 1/24	0/6	18
	80	1/12, 1/13, 2/14	0/4	13
8	80	2/14, 2/15, 1/19, 1/26	0/6	17
Infected controls	0	All mice died on day 6–8		

[a]Taken from reference[22]

CONCLUSIONS

In this manuscript we reported our findings on the antimalarial activity of simple aminoquinoline tethered benzyl derivatives with introduced bromines in positions C3 and C4. Although the synthesized compounds showed acceptably low *in vitro* activities, the antimalarial **3** was found toxic *in vivo* at a dose of 320 (mg/kg)/day in 3/6 mice, however, 2/6 mice of the same group survived through day 31, and one of them was cured. The detected toxicity of compound **3** indicates that the basic structure of presented aminoquinolines has yet to be improved in the near future.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website:.https://www.shd-pub.org.rs/index.php/JSCS/index, or from the corresponding author on request.

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ИЗВОД

НОВИ ДЕРИВАТИ 4-АМИНОХИНОЛИНА КАО УМЕРЕНИ ИНХИБИТОРИ ПАРАЗИТА *Plasmodium falciparum*

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Синтетисани су нови деривати аминохинолина и испитана је антималаријска активност једињења на два соја *P. falciparum*, хлорокин осетљивом клону 3D7 и хлорокин резистентом клону Dd2. У наставку истраживања испитана је *in vivo* активност деривата који су у *in vitro* условима испољили највећу активност. Применом аминохинолина **3** у дози 320 (mg/kg)/дан преживела су два од шест мишева, при чему је један миш излечен.

Међутим, при истој дози у 3/6 тестиране животиње исказана је токсичност аминохинолина **3**, што указује да је потребно даље истраживање на побољшању структуре овог типа једињења.

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