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Antimalarial activity of novel pyrrolizidinyl derivatives of 4-aminoquinoline

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ABSTRACT

Two pyrrolizidinylalkyl derivatives of 4-amino-7-chloroquinoline (**MG2** and **MG3**) were prepared and tested in vitro against CQ-sensitive and CQ-resistant strains of *Plasmodium falciparum* and in vivo in a *Plasmodium berghei* mouse model of infection. Both compounds exhibited excellent activity in all tests and low toxicity against mammalian cells. Preliminary studies of the acute toxicity and of the metabolism of the most active compound **MG3** indicate a promising profile as a new antimalarial drug candidate.

Novel, effective, safe and inexpensive antimalarial agents are urgently needed to treat malaria in tropical and subtropical regions, where it still represents a serious health problem, affecting 400–500 million people annually.

Plasmodium falciparum (*Pf*), which is responsible for >1 Mio fatalities, has developed resistance to common antimalarials such as chloroquine (CQ) or antifolates. Nevertheless, several CQ analogs were shown to retain significant activity against CQ-resistant strains of *Pf*, suggesting that resistance could be compound-specific and not related to changes in the structure of the drug target.[1]

Indeed, we have recently demonstrated that analogs of CQ and quinacrine, whose 4-diethylamino-1-methylbutyl chain was replaced by a quinolizidinyl- or a quinolizidinyl-alkyl moiety (Fig. 1) exhibited high antimalarial activity against CQ-S and CQ-R strains of *Pf* in vitro.[2] Some of these compounds also showed efficacy against *P. berghei* and *P. yoelii* when given orally (po) or intraperitoneally (ip) in a murine standard 4-day test.[3]

The presence of a bulky, strongly basic and lipophilic bicyclic moiety (as the quinolizidine ring) appears as an interesting structural feature able to overcome the resistance mechanisms by preventing the metabolic oxidative dealkylation, which affects the usual dialkylaminoalkyl chains of many CQ analogs. This metabolic dealkylation significantly reduces the lipid solubility of the drug and significantly increases cross-resistance up to and beyond that seen with CQ.[1]

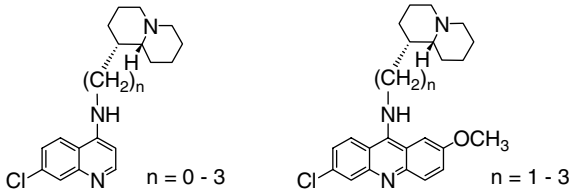

Figure 1. Structures of quinolizidinyl analogs of chloroquine and quinacrine.

In the light of these promising results and pursuing the research of drugs which are cheaper and easier to synthesize, we prepared and studied two novel analogs of CQ (**MG2** and **MG3**), where the amino group of quinoline is linked to a pyrrolizidinylalkyl ((hexahydro-1*H*-pyrrolizin-7a-yl)alkyl) moiety (Fig. 2).

In these new molecules a bulky, basic and lipophilic head is again present, but such a synthetic bicyclic ring is linked in a posi-

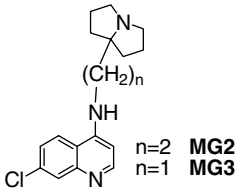

Figure 2. Structures of new pyrrolizidinylalkyl derivatives of 4-amino-7-chloroquinoline.

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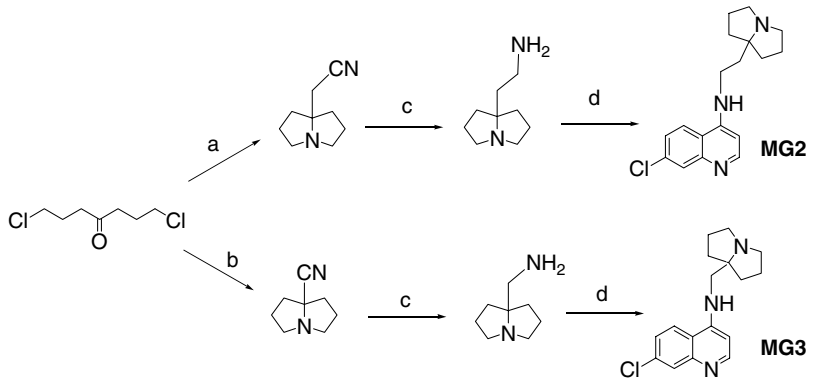

Scheme 1. Reagents and conditions: (a) cyanoacetic acid, NH_4OH, *n*-hexane, 15 °C, 40 h; (b) 2-amino-2-methyl-propanenitrile, NH_3, MeOH, 20 °C, 24 h; (c) $LiAlH_4$, dry Et_2O, rt, 5 h; (d) 4,7-dichloroquinoline, phenol, 180 °C, 4 h.

Table 1
In vitro antimalarial activities, β-hematin inhibitory activities (BHIA assay) and cellular cytotoxicity of **MG2** and **MG3**

Compound	D-10 (CQ-S) IC_{50}[a] (nM)	W-2 (CQ-R) IC_{50}[a] (nM)	NF-54 (CQ-S) IC_{50}[b] (nM)	K1(CQ-R) IC_{50}[b] (nM)	BHIA Drug:haem molar equivalent	WEHI 13 IC_{50}[a] (nM)	HMEC-1 IC_{50}[a] (nM)	K562 IC_{50}[a] (nM)
MG2	5.5	54.7	6.33	19.6	1.66	61,453	>60,000	17,587
MG3	30.6	28.9	10.9	10.6	1.06	24,717	>60,000	18,069
CQ[c]	16.5	293.5	4.7	48.5	1,69	>40,000	>40,000	31,828

[a] Mean of 3–4 experiments, each performed in triplicate.
[b] Mean of two experiments.
[c] Diphosphate salt.

tion to prevent the chirality present in the previous quinolizidinyl analogs.

It is worth noting that a 7a-substituted pyrrolizidine ring is present in several biologically active substances such as the antiarrhythmic pilsicainide,[4] cerebral function activators,[5] muscarinic M_1 and serotonine 5-HT_4 receptor agonists, useful in improving cognitive functions,[5–7] and as gastro-intestinal prokinetic agents,[8] respectively.

The target compounds were prepared by reacting in the presence of phenol the 4,7-dichloroquinoline with the suitable (hexahydro-1*H*-pyrrolizin-7a-yl)alkylamine.[9] The amino compounds were obtained by $LiAlH_4$ reduction of the corresponding nitriles,[10,11] which in turn were formed by cyclization of 1,7-dichloro-4-heptanone with NH_3 and cyanoacetic acid or 2-amino-2-methylpropanenitrile[6,12,13] (Scheme 1).

Compounds **MG2** and **MG3** were tested in vitro against CQ-S (D-10 and NF-54) and CQ-R (W-2 and K1) strains of *Pf*. The antimalarial activity (IC_{50}) was quantified as inhibition of parasite growth, measured with the production of parasite lactate dehydrogenase (for D-10 and W-2 strains)[14] or the incorporation of [^{3}H]-hypoxanthine (for NF-54 and K1 strains).[15] Moreover, to assess the mechanism of action, the BHIA (β-hematin inhibitory activity) assay was performed as previously described.[16] Cytotoxicity on murine cells WEHI, clone 13, or human cells HMEC-1 and K562, was assayed using the MTT test.[17] Finally, the ability to protect NMRI mice from a lethal CQ-susceptible *P. berghei* GFP-transfected ANKA strain infection[18] was studied, administering the compounds either orally or subcutaneously (sc) as a single dose or repeated doses for 4 consecutive days.[19,20]

The results of these assays are presented in Tables 1–3.

The two compounds **MG2** and **MG3** were active against both CQ-S strains D-10 and NF-54 in a comparable range as CQ (Table 1). Importantly, these compounds exhibited high activity against both CQ-R strains W-2 and K1, being 2.5- to 10-fold more active than the reference drug CQ.

Compound **MG3**, though less active than **MG2** on CQ-S strains, was definitely better than the homolog on CQ-R strains, and was

Table 2
In vivo single dose of **MG2** and **MG3** on *P. berghei* murine model[a]

Compound	Dose (mg/Kg)	Route	Activity (%)	Mouse survival (days) Avg.
MG2	100	po	98	12.3
MG3	100	po	99.5	13.7
CQ[b]	100	po	99.2	14.7
MG2	30	sc	99.1	8.7
MG3	30	sc	99.7	10.3
CQ[b]	30	sc	99.2	9.7
Controls				7.0

[a] Groups of three *P. berghei*-infected NMRI mice were treated 1-day post-infection with compounds formulated in 70/30 Tween 80/ethanol and diluted 10× with water before administration. Antimalarial efficacy was measured by percent reduction in parasitemia on day 3 post-infection and animal survival time was compared to untreated control groups ($n = 5$).
[b] Diphosphate salt.

Table 3
In vivo 4-day administrations of **MG2** and **MG3** on *P. berghei* murine model[a]

Compound	Dose (mg/kg): 4×	Route	Activity (%)	Mouse survival (days) Avg.
MG2	30	po	>99.9	30.0 (3/3 mice cured[b])
	10	po	>99.9	14.7
MG3	30	po	>99.9	25 (2/3 mice cured)
	10	po	>99.9	25.7 (2/3 mice cured)
CQ[c]	30	po	>99.9	30 (3/3 mice cured)
	10	po	99.9	22.3
MG2	10	sc	>99.9	15.3
	3	sc	94	9.3
MG3	10	sc	>99.9	29.3 (2/3 mice cured)
	3	sc	96	7.3
CQ[c]	10	sc	>99.9	15.7
	3	sc	99.8	9.3
Controls				7.0

[a] Groups of three *P. berghei*-infected NMRI mice were treated 1 day post-infection with compounds formulated in 70/30 Tween 80/ethanol and diluted 10× with water before administration. Antimalarial efficacy was measured by percent reduction in parasitemia on day 4 post-infection and animal survival time was compared to untreated control groups ($n = 5$).
[b] Mice cured: no parasites present at day 30. Parasitemia was checked by microscopy.
[c] Diphosphate salt.

Table 4
Toxicity and body weight variation after ip administration of **MG3** in mice

Dose (mg/kg)	No. of mice	No. of surviving mice	Mean body weight (g)				
			Initial	After 5 days	% Variation	After 13 days	% Variation
150	4	2[a]	29.5	33.30	+12.9	36.25	+22.9
100	4	4[b]	29.4	32.45	+10.1	36.03	+22.2
50	4	4[c]	29.9	32.38	+8.3	36.12	+20.8
Control	4	4	32.7	34.36	+5.1	38.40	+17.5

[a] Two mice died within 5–10 min; the survivors exhibited shivering and torpor but they recovered in the following 2 h.
[b] Exhibited sluggishness, recovering before 2 h.
[c] Asymptomatic.

not susceptible to the resistance mechanism exhibiting a resistance factor (ratio IC_{50} on CQ-R/ IC_{50} on CQ-S strains) equal 1.

Both compounds displayed a cytotoxicity on murine and human cells that was comparable to that of CQ.

The inhibition of β-hematin formation in the BHIA assay suggests that both compounds interfere with the haem detoxification process of parasites, thus acting with a mechanism similar to that of CQ.

The in vivo studies, in the murine *P. berghei* malaria model, showed that **MG2** and **MG3** behaved similarly to CQ in the single dose treatment.

Repeated treatment (once a day for 4 consecutive days) with **MG2** and **MG3** inhibited parasitemia by >99.9% when given at 10 mg/kg po and sc. Particularly, **MG3** at 10 mg/kg po and sc produced a mean survival of 25.7 and 29.3 days, respectively and 2/3 mice were cured (parasite-free on day 30).

For CQ diphosphate, at the same dose, the mean survival times were 22.3 days (po) and 15.7 (sc), with no mice cured regardless of the route of administration.

The in vivo acute toxicity and the effect on body weight of **MG3** was evaluated in CD1 mice treated with a single dose ip and observed for 13 days (Table 4): the toxic dose (less than 100% survival) was >100 mg/kg for **MG3**.

Finally, preliminary studies of the in vitro metabolism[21] of **MG3** and of its capability to inhibit cytochrome P_{450} isoforms[22] were undertaken.

The in vitro clearance of **MG3** was 1.50 ± 0.58 ml/min/g (human hepatic microsomes), while that of 7-ethoxycoumarine, a common reference standard, was in the range 7–9.9 ml/min/g. Moreover, after 30 min of incubation, only small amounts of two metabolites (with a MH^+at *m/z* 300 and 316; parent compound MH^+at *m/z* 302) were detected at the HPLC-MS, thus supporting the expected stability of the pyrrolizidine ring to phase 1 oxidative metabolism. The interaction of **MG3** (3 μM) with CYP isoforms (1A2, 2C9, 2C19, 2D6, and 3A4) was very low; particularly the inhibition of the last two isoforms (that together are responsible for the metabolism of about 60% of the most clinically important drugs) was only 5.7% and 4.3%, whereas CQ-induced inhibition was 20.4% and 6.8%, respectively. Therefore **MG3** should not interfere extensively with the metabolism of other drugs.

In conclusion, the pyrrolizidinylalkyl derivatives of 4-amino-7-chloroquinoline **MG2** and **MG3** exhibited excellent activity in vitro against CQ-S and CQ-R strains of *Pf* and in vivo against *P. berghei*. Thus the bicyclic pyrrolizidine moiety resulted, as already seen for the quinolizidine, as a structural feature able to overcome the *Pf* resistance mechanism.

Moreover, the novel compounds exhibited low toxicity against mammalian cells, whereas in preliminary in vivo studies the most active **MG3** was also well tolerated up to a dose of 100 mg/kg.

These data combined with the absence of chiral centers and the low cost of the intermediates required for its synthesis, make **MG3** an interesting compound deserving further study as a promising antimalarial agent.

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9. A mixture of 2-(hexahydro-1*H*-pyrrolizin-7a-yl)ethanamine[10] or (hexahydro-1*H*-pyrrolizin-7a-yl)methanamine[11] (1.94 mmol), 4,7-dichloroquinoline (384 mg, 1.94 mmol) and phenol (1.21 g, 13.58 mmol) was heated for 4 h at 180 °C, stirring under nitrogen. After cooling, the mixture was basified with 2 N NaOH and the product extracted thoroughly with ether. The organic extracts were washed with 2 N NaOH, then with water and finally with 5% acetic acid. The acetic solution was alkalized with concd NH_3 and extracted with ether. After evaporation of the solvent, **MG2** or **MG3** as white solid were obtained. *Compound* **MG2**: Yield: 66%; mp (Büchi): 123.5–125.5 °C (Et_2O). ^1H NMR (Varian Mercury 300VX, $CDCl_3$): δ = 9.10 (br s, 1H, collapses with D_2O); 8.45 (d, *J* = 5.5 Hz, 1H); 7.90 (d, *J* = 1.9 Hz, 1H); 7.65 (d, *J* = 9.0 Hz, 1H); 7.35 (dd, *J* = 1.9, 8.8 Hz, 1H); 6.25 (d, *J* = 5.5, 1H); 3.40 to 3.30 (m, 2H); 3.20 to 3.00 (m, 2H); 2.80 to 2.60 (m, 2H); 2.00 to 1.60 (m, 10H). Anal. (Carlo Erba-EA-1110 CHNS-O instrument) Calcd for $C_{18}H_{22}N_3Cl$: C, 68.45; H, 7.02; N, 13.30, found: C, 68.62; H, 7.14; N, 13.32. *Compound* **MG3**: Yield: 59.4%; mp: 112–113 °C (Et_2O). ^1H NMR ($CDCl_3$): δ = 8.50 (d, *J* = 5.5 Hz, 1H); 7.95 (d, *J* = 1.9 Hz, 1H); 7.70 (d, *J* = 9.0 Hz, 1H); 7.35 (dd, *J* = 1.9, 8.8 Hz, 1H); 6.40 (d, *J* = 5.5 1H); 6.00 (br s, 1H, collapses with D_2O); 3.15 (m, 4H); 2.80 to 2.60 (m, 2H); 2.00 to 1.60 (m, 10H). HRMS (APEX II ICR-FTMS Bruker Daltonics, ESI) *m/z* calcd for $C_{17}H_{21}N_3Cl$ $[M+H]^+$ 302.14185, found 302.14187.
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21. Test compound at the final concentration of 1 μM was dissolved in DMSO and pre-incubated for 10 min at 37 °C in potassium phosphate buffer pH 7.4, with human microsomes (Xenotech) at the final concentration of 0.5 mg/ml. After the pre-incubation period, the reaction was started by adding the cofactor mixture (NADP, G6P, G6P-DH, $MgCl_2$ 3 mM and UDPGA 1 mM); samples were taken at time 0 and 30 min and added to acetonitrile to stop reaction, centrifuged and supernatant analyzed by LC–MS/MS (PremiereXE, Waters, in full scan ESI pos mode). Acquired data were processed by Metabolynx software. A control sample without cofactor was always added in order to check the chemical stability of test compound. 7-Ethoxycoumarin was used as reference standard.
22. The inhibition of the P450 isoforms was measured using specific substrates that become fluorescent upon CYP metabolism. Compounds, dissolved in DMSO, are tested at least in triplicate (n = 3 or 4) at the single concentration of 3 μM in a 96-well plate containing incubation/NADPH regenerating buffer. Specific isoenzymes and substrates are added and incubated at 37 °C. Reaction was terminated at different times, depending on the assays, and plates are read on a Fluoroskan Ascent at the appropriate emission/excitation wavelengths. Concentration–response curves (seven concentrations) performed in duplicate for known inhibitors for each isoenzyme are tested in every assay as positive control. For each compound the percentage inhibition vs. control without inhibitor is calculated. An inhibition <5% is considered no effect. For standards the IC_{50} (concentration at 50% inhibition) is then determined by using Grafit v. 6.0.