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Adamantane amine-linked chloroquinoline derivatives as chloroquine resistance modulating agents in *Plasmodium falciparum*

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ABSTRACT

Previously we have shown that pentacycloundecylamine-chloroquinoline (PCU-CQ) conjugates possess significant chemosensitizing abilities and can circumvent the resistance associated with chloroquine (CQ) resistant plasmodia. In order to further explore structurally related polycyclic compounds as reversed CQ agents we synthesized a series of eight aza-adamantanol (**1**–**4**) and adamantane-imine (**5**–**8**) CQ conjugates. All conjugates showed limited cytotoxicity against CHO cells (IC_{50} > 37 μM). Compounds **1**, **2** and **5** were highly active (K1 IC_{50} < 100 nM) exhibiting a 3–4-fold increase in antiplasmodial activity against CQ resistant strain K1 compared to CQ. Reduced cross-resistance (resistance index, RI: 2–4.3) relative to CQ (RI = 38) was also observed for these compounds. Compound **1** which showed an 18-fold enhancement at retaining its activity against the K1 strain compared to CQ is a promising candidate to substitute CQ in *P. falciparum* resistant malaria.

Malaria remains a major global health problem with devastating health and socioeconomic outcomes despite decades of research.[1–3] This disease is caused by the *Plasmodium* parasite with *Plasmodium falciparum* as the most common and lethal species implicated in most clinical cases and the cause of most malaria-related deaths.[4,5] Although substantial progress has been made to control and manage the disease in the past decades, it still accounts for millions of malaria-related deaths annually, especially among children below the age of 5 years and in sub-Saharan Africa. In the 212 million clinical malaria cases encountered globally in 2015, 429,000 deaths were registered and sub-Saharan Africa alone contributed 92% to it.[6–9] The burdens of malaria are made worst by the spread of drug resistant *P. falciparum* parasites.[10]

Chloroquine (CQ), a 4-aminoquinoline derivative, was the least expensive and most effective antimalarial agent that was well tolerated with a rapid onset of action and low toxicity profile used to fight malaria.[11,12] These qualities made CQ a 'wonder drug' that was used extensively worldwide as the mainstay drug for the prophylaxis and treatment of blood stage malaria for many decades since its discovery.

However, the spread of CQ resistant *P. falciparum* has limited its current effectiveness and therapeutic use as an antimalarial agent,[13] resulting in high cases of morbidity and mortality.[14,15] *P. falciparum* resistance to CQ arises mainly as a result of mutations in the *P. falciparum* chloroquine resistance transporter (*Pf*CQRT) protein, a putative transporter protein in the parasitic vacuole membrane.[16,17] This transporter promotes CQ efflux out of the parasitic digestive vacuole decreasing CQ accumulation at its site of action rendering it ineffective.[18–20] Furthermore, *P. falciparum* has shown resistance to almost all available antimalarial drugs including the newer artemisinin-based combination therapies.[21] This has limited the number of affordable efficacious drugs available to fight the disease.[22] Therefore, there is an urgent need for continuous research and development of new antimalarial agents effective against *P. falciparum* resistant strains.

One strategy that can be pursued to circumvent the problem of *P. falciparum* CQ resistance is to "reverse" CQ resistance chemically. Several existing compounds, such as the calcium channel blocker verapamil,[23,24] tricyclic antidepressant imipramine[25,26] and antihistaminic drug chlorpheniramine[27,28] have demonstrated promising capability to reverse the CQ resistance in parasite isolates *in vitro*, in animal models, and in human malaria by inhibiting the mechanism(s) of CQ resistance.[29] However, the reversal ability of these compounds is directly proportional to their concentrations[27,38] thus they are optimal as resistance reversers *in vitro* at concentrations higher than 1 μM which may be toxic *in vivo*.[27,30] Moreover, the findings of these studies, though ground breaking, had the limitation of the polypharmacy approach of CQ and

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chemosensitizers, which is costly and constraining. In addition, these individual chemosensitizers are pharmacologically active compounds with multisystem effects that may produce a myriad of unwanted side effects. The search for more effective and safe chemosensitizing agents thus needs to continue.

To address this problem, Burgess and colleagues in 2006[31] introduced the new attractive strategy of reversing CQ resistance *via* the hybridization of a reversal agent (RA) to the CQ pharmacophore (7-chloro-4-aminoquinoline nucleus) in the development of a single antimalarial molecule. This was termed a 'reversed CQ (RCQ) compound' with the advantage of increasing the accumulation of the compound in the parasite vacuole *in vitro*.[32,33] Many other researchers have demonstrated the feasibility of this strategy by the investigation of different RA design strategies and have produced promising RCQ compounds.[29–35] Additionally, despite resistance to CQ, novel drugs with the CQ-like nucleus and proposed CQ mechanism of action are still an area of intense research interest because the mechanism of action and mechanism of CQ resistance are independent of each other.[36]

We have previously shown that the polycyclic amine NGP1-01 (Fig. 1), a pentacycloundecylamine (PCU) with significant inherent multiple channel blocking activity, acts as a chemosenitizer to CQ. Further investigation of PCU derivatives led to the discovery of aza-derivatives of PCUs represented by PCU-CQ10 (Fig. 1) with better resistance reversal activity when hybridized to a CQ-like nucleus.[34,37]

To further explore the use of polycyclic amines as RAs, this present study focused on the use of adamantane moieties, structurally related to PCUs, as RAs conjugated to the 4-aminoquinoline pharmacophore (CQ-like nucleus). The interest to use the adamantane moiety was fostered by the inherent ability of amantadine (Fig. 1) and its derivatives to block *N*-methyl-d-aspartate (NMDA) channels and voltage dependant calcium channels.[37–40] It is proposed that these channel blocking abilities may enable the adamantane moiety to inhibit the *Pf*CQRT associated with *P. falciparum* CQ resistance. Previous *in vitro* studies have also shown that when amantadine is administered in combination with CQ a synergistic effect is observed in CQ sensitive and CQ resistant strains.[41] Therefore two series of reversed CQ agents with differently tethered adamantane-CQ (AD-CQ) conjugates were developed namely; the aza-adamantanols (**1**–**4**) and the imine adamantanes (**5**–**8**) (Fig. 2). The adamantane moiety will also facilitate the formation of a tertiary amine with the *N*-alkyl amino side chain of the proposed structures which is necessary for antimalarial activity *via* its protonation.[30] Furthermore, the diamondoid and bulky nature of the adamantane moiety could protect the terminal tertiary amine from *N*-dealkylation[42] which could prevent cross resistance and pruritus[43–45] associated with CQ. The main aim of this study was thus to synthesize two series of differently tethered AD-CQ conjugates (**1**–**8**, Fig. 2) as potential improved reversed chloroquine agents to overcome CQ resistance by *Plasmodium falciparum* and to investigate their antimalarial activity on CQ sensitive and resistant strains. We also aimed to investigate the effect of the incorporation of the terminal side chain tertiary amine in the RA structure as well as the presence and absence of the hydroxyl group in the AD-CQ conjugates.

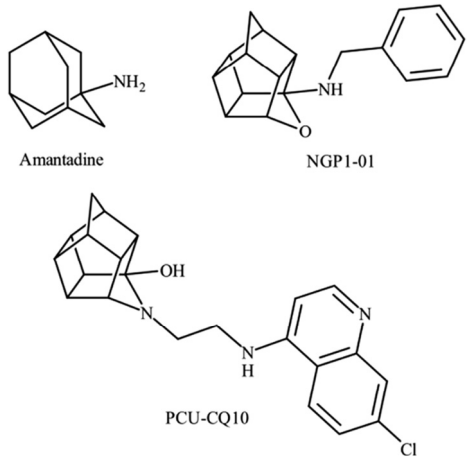


Fig. 1. Polycyclic amines: Amantadine, NGP1-01 and the previously reported reversed chloroquine agent PCU-CQ10.[34]

The synthesis route of compounds **1**–**4** commenced from the commercially available 2-adamantanone. A Baeyer-Villiger oxidation reaction with *m*-chloroperbenzoic acid (*m*-CPBA) was executed to generate the lactone derivative (4-oxatricyclo[4.3.1.1^{3,8}] undecan-5-one) in 97% yield (Scheme 1). The lactone was reduced with lithium aluminium hydride ($LiAlH_4$) to a diol [7-(hydroxymethyl)bicyclo[3.3.1]nonan-3-ol] which was then oxidized with pyridinium dichromate to obtain the adamantane diketone (bicyclo[3.3.1]nonane-3,7-dione), the yields were 54% and 80% respectively.[46,47] The adamantane diketone was subsequently conjugated to the various aminoquinoline (ACQ) intermediates (**1a**–**1d**) by means of an optimized microwave irradiation amination reaction followed by reductive amination and transannular cyclization using sodium triacetoxylborohydride ($NaBH(OAc)_3$) to give the aza-adamantanol derivatives (**1**–**4**) in moderate yields (24–48%, Scheme 1). The ACQ intermediates were synthesized as previously described by our group through the amination of 4,7-dichloroquinoline with different chain length diaminoalkane linkers using a microwave irradiation reaction in good yields (57–83%).[34] The compounds in the adamantane-imine series (**5**–**8**) were synthesized in moderate yields (49–61%) through a direct microwave irradiation amination reaction between 2-adamantanone and the various ACQ intermediates in a 1:1 ratio (Scheme 2).

All compounds (**1**–**8**) were characterized by NMR, MS and IR spectroscopy, providing spectral data in agreement with the postulated structures (Supporting information). In the H^1 NMR, the five aromatic proton peaks of the 4-aminoquinoline moiety were seen down-field (9–6 ppm) in the characteristic multiplicity signals of doublet (d), doublet (d), doublet of singlet (ds), doublet of doublet (dd) and doublet (d) pattern.[48] The protons of the differently tethered linkers appeared around 3.5–2 ppm and the adamantane cage protons were seen up-field at around 2–1 ppm. The C^{13} NMR showed the characteristic C═N signal for compounds **5**–**8** between 170 and 178 ppm, which was absent for compounds **1**–**4**. The molecular mass of each compound was confirmed using MS showing the molecular ion peak and the characteristic M + 2 peak because of the chloride moiety. Characteristic functional moieties such as the hydroxyl group in compounds **1**–**4** and the C═N bond in compounds **5**–**8** as well as C—H, C═C, N—H bonds were identified on the IR spectra. The hydroxyl group was observed at around 3450 cm^{-1} and the imine bond at 1650–1550 cm^{-1}.

The AD-CQ conjugates (**1**–**8**) were tested *in vitro* for their cytotoxicity on a non-parasitic Chinese hamster ovarian (CHO) cell line using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay with emetine as the reference compound. This assay assesses the growth and survival of the CHO cell line used based on viable cells' ability to reduce the yellow MTT into water-insoluble purple-blue formazan mediated by dehydrogenase enzymes of endoplasmic reticulum and mitochondria.[49,50] Results from the assay showed that all the test compounds have high CHO IC_{50} values (IC_{50}: 37–279 μM). The implication of these high values is that the AD-CQ conjugates have very low toxicity towards non-parasitic cells and as such are much

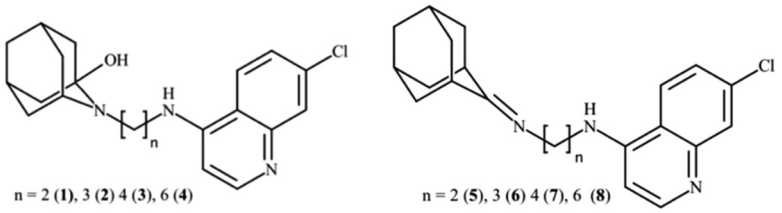
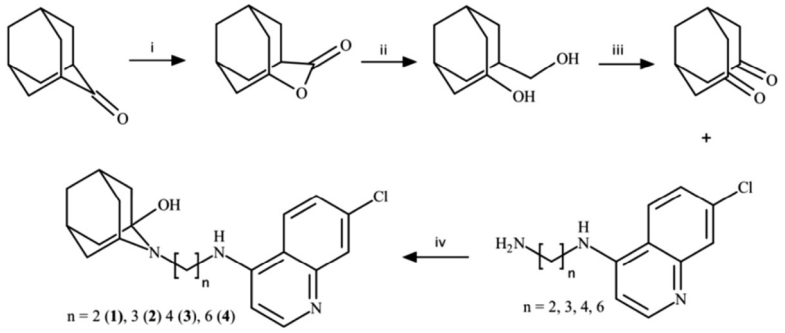
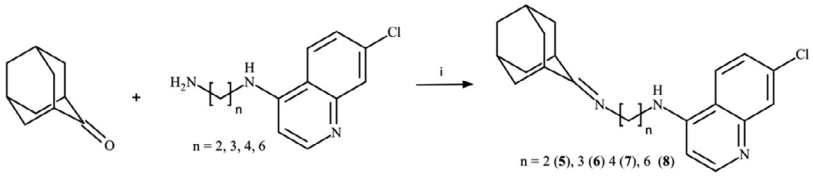


Fig. 2. Aza-adamantanol (**1**–**4**) and imine-adamantane (**5**–**8**) chloroquinoline linked antiplasmodial agents.



Scheme 1. Synthesis route of the aza-adamantanols, **1**–**4**. Reagents and conditions: (i) CH_2Cl_2, m-CPBA, 18 h, rt; (ii) Et_2O, $LiAlH_4$, reflux, 19 h; (iii) CH_2Cl_2, PDC, rt, 66 h; (iv) CH_2Cl_2, AcOH, MW, 85 °C, 100 W, 100 psi for 10 min, then $NaBH(OAc)_3$, rt, until completion, then acetone, 3 M HCl(aq), 6 h.



Scheme 2. Synthetic route of the imine-adamantanes, **5**–**8**. Reagents and conditions: (i) EtOH, HCl, MW, 150 °C. 200 W, 250 psi, 10 h.

safer when compared to the cytotoxic reference drug emetine (IC_{50} = 0.13 μM).

The antimalarial activity of the AD-CQ conjugates (**1**–**8**) was quantitatively determined against both *P. falciparum* CQ sensitive (NF54) and CQ resistant (K1) strains using a modified parasite lactate dehydrogenase (pLDH) assay first described by Makler and Hinrichs,[51] using CQ as the positive control. The resistance index (RI), reversal modification index (RMI) and selectivity index (SI) was subsequently calculated (see Table 1).

Both series of AD-CQ conjugates (**1**–**8**) displayed good antimalarial activity in the nanomolar range (IC_{50} = 5–112.69 nM) when compared to the reference drug CQ (IC_{50} = 7.80 nM) against the CQ sensitive NF54 strain. The results of the compounds on the CQ resistant K1 strain showed that seven of the AD-CQ conjugates (**1**–**7**) were active antimalarials (IC_{50}: 93 nM–784 nM) and compound **8** showed modest activity with an IC_{50} value of greater than 1000 nM (IC_{50} = 1580.28 nM). Three of the compounds (**1**, **2** and **5**) were highly active against K1 (IC_{50}: 98.92, 96.80 and

Table 1
In vitro IC_{50} values of AD-CQ derivatives and reference compounds of cytotoxicity and antiplasmodial activity.

Compound	NF54: IC_{50} (nM)	K1: IC_{50} (nM)	RI[a]	RMI[b]	CHO: IC_{50} (μM)[c]	SI[d]	pKa[e]
1	46.94	98.92	2.11	18.23	>279	>2820	8.3
2	22.32	96.80	4.34	8.86	45.19	467	8.8
3	33.94	198.2	5.84	6.59	37.86	191	9.1
4	112.5	283.6	3.81	10.09	80.76	285	9.2
5	26.28	93.81	3.56	10.80	98.50	1050	6.3
6	5.00	191.6	16.00	2.40	66.39	346	6.7
7	108.4	783.9	7.23	5.32	57.08	73	7.1
8	112.7	1580	14.02	2.74	>103	104	8.9
PCU-CQ10	9.84	72.60	7.36	5.22	9.54	131	7.5
CQ	7.80	300.0	38.46	1	57.84	193	10.2
Emitine	ND	ND	ND	ND	0.13	ND	ND

[a] Resistance index (RI) = IC_{50}K1/IC_{50}NF54.
[b] Reversal Modification Index (RMI) = the ratio of the RI of the test compounds compared to RI of CQ.
[c] CHO = Chinese Hamster Ovarian.
[d] Selectivity index (SI) = IC_{50} CHO/IC_{50} K1.
[e] The pKa of the tertiary alkyl amine was calculated using the ACE and JChem acidity and basicity calculator, available at http://epoch.uky.edu/ace/public/pka.jsp. ND = not determined.

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93.81 nM, respectively), when compared to CQ (IC_{50} = 300 nM). These results showed that the AD-CQ conjugates exhibit marked antimalarial activity. This was also confirmed by the selectivity index (SI). The high SI values of the compounds (SI = 73–2825) indicates greater selectivity towards the resistant parasite strain K1 compared to the CHO cells and implies that the activity of the conjugates is because of their antiplasmodial activity.[52,53]

When observing the RI, the aza-adamantanol conjugates (**1**–**4**) in general showed better activity with lower resistance factors compared to their imine adamantane (**6**–**8**) counterparts except for **5**. Evaluating the two series structurally in relation to their activity, it was established that the presence of the hydroxyl group may play a role in improved reversed CQ ability of the aza-adamantanols. This may indicate that the hydroxyl group could help or play a part in the binding of the compounds to the *Pf*CQRT and aid in blocking this channel or that the hybrid with the OH is less susceptible towards efflux through this channel.

Furthermore, the calculated pKa values of the tertiary adamantane amine of the aza-adamantanol series (pKa: 8.3–9.2) were closer to that of CQ (pKa = 10.2) compared to the imine-adamantanes (pKa: 6.3–8.9). This increased pKa values of the aza-adamantanols may have influenced their ability to accumulate in the parasitic digestive vacuole and also could explain their improved activity.[54] It is worth mentioning that although the imine-adamantane compounds showed significant activity against the CQ sensitive strain, they had markedly reduced activity against the CQ resistant strain. This loss of activity may be directly linked to the lower pKa values observed or other structural features making the imine-adamantane moiety a weaker RA. Furthermore, imine containing compounds are known to undergo hydrolysis in aqueous media, especially at lower pH values.[55] However, with the imine-adamantane compounds the imine is part of a six-membered ring in a lipophilic adamantine polycyclic system which should provide enhanced stability of the imine function against hydrolysis. Hydrocarbyl imines that are lipophilic are relatively more stable than short chain and hydrophilic hydrocarbyl imines towards hydrolysis.[55] The adamantane moiety and structurally related PCU's have also been shown to provide enhanced chemical stability and metabolic stability of conjugated molecules.[56] In order to explore the stability and if the loss of activity was due to degradation by imine hydrolysis of the imine-adamantanes, **6** was selected as the representative imine-adamantane derivative and evaluated for chemical stability under the same conditions as the pLDH antimalarial assay procedure (see Supplementary material section 1.2.3). Compound **6** was dissolved (1 mg/ml) in HEPES containing aqueous buffer at the same pH (7.4) as the pLDH assay and at a pH within a similar range as the parasites' acidic digestive vacuole pH (4.5)[57] respectively. The solutions were then incubated at 37 °C for 72 h to verify if the observed decreased activity in the antimalarial assays may be due to degradation. Results from this study indicate that **6** remain relatively stable at pH 7.4, however degradation was observed at pH 4.5 after 72 h. This indicates that the imine-adamantanes will remain stable in the assay media (aqueous HEPES-buffer, pH 7.4, 37 °C) and may undergo hydrolysis once inside the parasites' acidic digestive vacuole. It is currently unclear if this observation may have influenced the pLDH assay results, especially in the resistant strain, and further studies are necessary to elaborate on these findings.

The IC_{50} values of the AD-CQ derivatives increased as the chain length of the alkyl linker increased. This trend is significant in the resistant strain K1 and concurs with literature in that chain length changes has little influence on activity against CQ sensitive strains but has a profound influence in CQ resistant strains.[36,58] This can be explained in that as the chain length increases the degree of flexibility of the compounds in the *Pf*CQRT protein increases and may adopt an unfavourable conformation whereas the short chain conjugates may adopt a more favourable conformation and thus improves the efficacy of the compounds. From both series, the conjugates with alkyl linkers of 2–3 carbons showed optimum activity for both NF54 and K1.

All the compounds tested showed reduced cross-resistance, with a resistance index (RI) factor in the range 2.11–16 compared to 38 for CQ. The aza-adamantanols generally had improved RI factors when compared to their imine-adamantane counterparts, which indicates that the aza-adamantanol conjugates are better RCQ compounds. The observed RI of the three most active compounds (**1**, **2** and **5**) was between 2.11 and 4.34. Furthermore, the reversal effect of the adamantane moiety as a CQ resistance RA compared to the structurally related PCU moiety used as a RA in our previous study[34] was compared. This was done by comparing the RI of the AD-CQ conjugates with that of the PCU-CQ derivatives, represented by PCU-CQ10 (see Fig. 1 and Table 1) identified as the most promising RCQ agent in our previous work.[34] To easily and clearly compare the reversal effect, the reversal modification index (RMI) was calculated (see Table 1). It revealed that PCU-CQ10 (with a 2C linker) was 5.22 times better at retaining activity than CQ whereas compound **1** (also with a 2C linker), was 18 times better at retaining activity than CQ. In addition, six out of the eight AD-CQ derivatives (**1**–**5**, **7**; RMI: 5.3–18) showed improved RMIs compared to PCU-CQ10 and the adamantane moieties, as used in the AD-CQ conjugates, are thus more potent reversal agents *in vitro* than the PCU moiety.

In conclusion, resistance to antimalarial drugs especially CQ is a major setback in the use of chemotherapy to control malaria. This created the need to identify and develop new improved antimalarial agents. A total of eight non-toxic AD-CQ reversed CQ derivatives were synthesized and compounds **1**–**6** exhibited potent antimalarial activity *in vitro* superior to CQ against the CQ resistant strain K1 and overcame *P. falciparum* resistance to CQ. Compound **1** stood out as a potent conjugate (K1 IC_{50} = 98.92 nM) with the lowest resistance index (RI = 2.11) and was identified as the most promising AD-CQ conjugate. Its ability to retain activity in the CQ resistant strain was 18-fold better than that of CQ. The adamantane moiety, especially in the aza-adamantanol series, was shown to be a significant *P. falciparum* CQ resistance reversal agent compared to the previously used structurally related PCU moiety. Hence, the hybridization of a CQ-like nucleus to adamantane moieties results in reversed CQ molecules with improved antimalarial activity that could overcome *P. falciparum* CQ resistance. The next step of this study will be to carry out further *in vitro* and *in vivo* biological and mechanistic studies to elaborate on the molecular mechanism(s) involved in parasite-killing and reversal of the *Pf*CQRT efflux effect. Also, the role of the hydroxyl group on the activity of the aza-adamantanol conjugates should be investigated further to build on the structure activity relationships of the compounds.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.03.026.

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