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# Synergistic interaction of a chloroquine metabolite with chloroquine against drug-resistant malaria parasites

Martha Kalkanidis[a], Nectarios Klonis[b], Serena Tschan[b], Leslie W. Deady[a], Leann Tilley[b,*]

[a]*Department of Chemistry, La Trobe University, Melbourne, Vic. 3086, Australia*
[b]*Department of Biochemistry, La Trobe University, Melbourne, Vic. 3086, Australia*

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**Abstract**

We have previously shown that structural modification of chlorpromazine to introduce a basic side chain converts this chloroquine (CQ) resistance-reversing agent into a compound that has activity against *Plasmodium falciparum in vitro* [Biochem. Pharmacol. 63 (2002) 833]. In an effort to further dissect the structural features that determine quinoline antimalarial activity and drug resistance-reversing activity, we have studied a series of aminoquinolines that are structurally related to CQ. We have analysed their haematin-binding activities, their antimalarial activities and their abilities to synergise the effect of CQ against drug-resistant *P. falciparum*. We found that a number of the aminoquinolines were able to interact with haematin but showed no or very weak antiparasitic activity. Interestingly, 4-amino-7-chloroquinoline, which is the CQ nucleus without the basic side chain, was able to act as a resistance-reversing agent. These studies point to structural features that may determine the resistance-modulating potential of weakly basic amphipaths. Interestingly, 4-amino-7-chloroquinoline is a metabolic breakdown product of CQ and may contribute to CQ activity against resistant parasites *in vivo*.


*Keywords:* Malaria; *Plasmodium*; 4-Aminoquinoline; Chloroquine; Haem; Drug resistance

## 1. Introduction

CQ has been used to treat malaria for nearly 50 years, however, in recent years, resistance to CQ has increased to the point where it is virtually useless in many malarial regions (see Refs. [1,2] for reviews). This has led to a critical need to understand the mechanism of action of quinoline antimalarials and the molecular basis of CQ resistance so that novel drugs or drug combinations can be developed to circumvent the development of drug resistance.

The malaria parasite feeds by degrading haemoglobin, producing as a byproduct, free ferriprotoporphyrin IX (FP). FP is highly toxic to the parasite and is neutralised *via* a process of biomineralisation to form innocuous haemozoin crystals. CQ and a number of other quinoline antimalarial drugs are thought to inhibit parasite growth by inhibiting the detoxification of FP [3–6]. CQ appears to trap FP in a μ-oxo dimeric form and prevents the formation of the β-haematin dimers that are required for haemozoin formation [7]. Thus, quinoline antimalarial drugs cause a build-up of toxic FP molecules that eventually destroy the integrity of malaria parasite proteins and membranes [8].

We are now beginning to understand the molecular basis of CQ resistance. It appears to result from a decreased level of CQ uptake [2]. The presence of a mutant form of a parasite protein referred to as the *Plasmodium falciparum* CQ resistance transporter (PfCRT), has been shown to be linked to CQ resistance [9]. The level of CQ resistance is also determined by the expression of mutant forms of the *P. falciparum* P-glycoprotein homologue-1 (Pgh1 [10]). The precise roles of these proteins have not been delineated but they may function as CQ transporters that extrude CQ from its site of action in the food vacuole of CQ-resistant parasites.

Some weakly basic amphipaths that possess only very weak antimalarial activity themselves are able to interact

* Corresponding author. Tel: +61-3-94791375; fax: +61-3-94792467.
*E-mail address:* L.Tilley@latrobe.edu.au (L. Tilley).
*Abbreviations:* FP, haem/ferriprotoporphyrin IX; CQ, chloroquine; Pgh1, P-glycoprotein homologue-1; PfCRT, CQ resistance transporter; SFIC, sums of the fractional inhibitory concentrations.

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synergistically with CQ in inhibiting the growth of CQ-resistant parasites. For example, when CQ is used in combination with the phenothiazine drug, chlorpromazine, it kills CQ-resistant parasites at similar concentrations to those effective against CQ-sensitive parasites [11]. We have recently shown that addition of a tribasic side chain to a phenothiazine nucleus leads to a 100-fold increase in the antimalarial activity of the compound compared with chlorpromazine but abrogates the ability of the compound to interact synergistically with CQ [12]. These data suggest structural similarities in compounds with antimalarial and resistance-reversing activities. One possible explanation for the data is that these resistance-reversing agents might interact with the CQ-binding site of a drug transporter in the food vacuole membrane but not be efficiently extruded, thereby preventing binding of CQ. If the basicity of the compounds is increased, they would be accumulated more efficiently in the food vacuole and therefore would be expected to become more effective antimalarials, however, they may also become suitable substrates for extrusion by the CQ transporter.

If resistance-reversing agents can be rendered antimalarial by increasing the basicity of the side chain, it seemed possible that CQ could be converted to a resistance modulator by removing its basic side chain. To test this hypothesis, we have studied a series of aminoquinolines that lack the CQ side chain and examined their abilities to bind FP and to synergise CQ action.

## 2. Materials and methods

### 2.1. Materials

CQ, BSA (essentially fatty acid free), monooleyl glycerol and FP (porcine haematin) were obtained from Sigma. Fresh human erythrocytes were obtained from the Red Cross Transfusion Service. [$^3$H]Hypoxanthine (50 μCi/0.3 mL stock) was obtained from Amersham. Glass fibre filters were obtained from Nunc. 2-Aminoquinoline (**1**), 3-aminoquinoline (**2**), 4-aminoquinoline (**3**), 4-aminoquinaldine (**7**) and 4,7-dichloroquinoline were from Aldrich.

### 2.2. Absorption spectroscopy

The interaction of the aminoquinolines with FP was examined by monitoring their effect on the absorption profile of FP. Absorption spectra were measured using a Cary 1E spectrophotometer. FP stocks were prepared in 50 mM NaOH on the day of use. For each measurement, the FP was diluted in binding buffer (40% methanol in 10 mM sodium phosphate, pH 6) to a final concentration of 15 μM and absorption spectra were recorded immediately and 2 min after the addition of the compound (from concentrated stocks in ethanol). The absorption spectra of the FP and the FP–drug complexes were stable for at least 30 min under the conditions of the assay. The absorption spectra of FP that are presented have been corrected for contributions from the compound by subtracting spectra recorded in the absence of FP.

### 2.3. β-Haematin formation

Assays of the conversion of FP to β-haematin were performed as described by Kalkanidis *et al*. [12]. A suspension of monooleyl glycerol in 90 mM sodium acetate, pH 5, was prepared by sonication and aliquots (0.5 mL) were mixed with FP from a stock in 50 mM NaOH to final concentrations of 150 μM FP and 0.2 mM monooleyl glycerol. Samples were incubated at 37° for 24 hr with gentle rotation. Following incubation, the β-haematin was pelleted and washed four times in 10 mM sodium phosphate, pH 7.4, containing 2.5% SDS. The remaining pellet was resuspended in 950 μL of 2.5% SDS in phosphate buffer and a 50 μL aliquot of 1 M NaOH was added to dissolve the β-haematin. Drugs were added from stock solutions in ethanol. An equivalent amount of ethanol was added to a control sample. Data are typical results from experiments performed at least twice.

### 2.4. Assessment of antimalarial activity of the 4-aminoquinolines

K1 is a CQ-resistant strain of *P. falciparum* [13]. Malaria parasites were incubated with drugs for 72 hr, with daily replacement of the drug-supplemented medium. Growth curves based on the uptake of [$^3$H]hypoxanthine were obtained in triplicate as described previously [14] and the concentration of drug required to produce 50% inhibition of growth ($IC_{50}$) was determined. The active compounds were tested on at least 3 separate days. The interaction of 4-amino-7-chloroquinoline with CQ was further examined using the method of Berenbaum [15]. Briefly, four different combinations of CQ with 4-amino-7-chloroquinoline (in triplicate) were diluted at a fixed ratio and examined for their effect on parasite growth. The fractional inhibitory concentrations (FIC) were determined and used to construct isobolograms as described previously [15–18].

## 3. Results

### 3.1. Chemistry

2-Aminoquinoline (**1**), 3-aminoquinoline (**2**), 4-aminoquinoline (**3**) and 4-aminoquinaldine (**9**) were Aldrich chemicals. 4-Amino-6-chloroquinoline (**4**) [19], 4-amino-7-chloroquinoline (**5**) [17] and 4-methoxy-7-chloroquinoline (**6**) [20] were prepared by literature procedures.

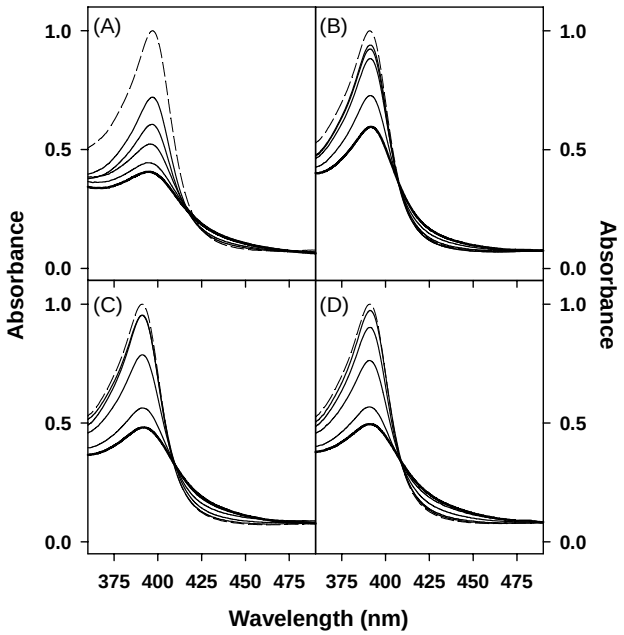



Fig. 1. Spectroscopic analysis of the interaction of some quinolines with FP at pH 6.0. Absorption spectra of FP were recorded in the presence of 0, 2, 4, 16, 64 and 128 μM CQ (A) and in the presence of 0, 4, 16, 64, 250 and 500 μM 4-aminoquinoline (B), 4-amino-6-chloroquinoline (C) and 4-amino-7-chloroquinoline (D). The FP spectra are corrected for the contributions due to the compounds alone and are normalised relative to the spectra recorded in the absence of the compound (dotted line). The spectra recorded at the highest concentration of each compound are represented by the thick line.

### 3.2. Spectroscopic studies of FP–aminoquinoline interactions

Studies of the interaction of FP with drugs under the pH conditions of the food vacuole are complicated by the poor solubility of FP in acidic buffers. However, FP can be maintained in a soluble state in low pH buffers in the presence of organic solvent [12,18]. Under the conditions used to measure the absorption spectrum (40% methanol in 10 mM sodium phosphate, pH 6), FP displays a relatively sharp Soret band centred on 400 nm (Fig. 1A) that represents the presence of monomeric FP species. The addition of CQ results in a decrease in the Soret absorption band (Fig. 1A) which is thought to indicate an association of CQ with an FP μ-oxo dimer to form a π–π donor–acceptor complex [16–18]. The lack of aggregation of FP and its large absorption change upon drug binding under the conditions used facilitates the analysis of the FP–drug interactions [12].

We examined the interactions of each of the aminoquinolines with FP by observing changes in the FP absorption spectrum. 2-Aminoquinoline (**1**) and 4-aminoquinoline (**3**) appear to interact with FP while 3-aminoquinoline (**2**) did not affect the Soret absorbance (Fig. 1B and Table 1). Binding to FP was apparently not saturated even at the highest concentrations of some of the interacting compounds. Therefore, in order to compare the relative affinities of these compounds, we have determined the concentration of compound required to produce a 25% decrease in FP absorption. As shown in Table 1, approximately 150-fold higher concentrations of these compounds compared to CQ were required to produce comparable changes in FP absorption, in agreement with previous studies [18].

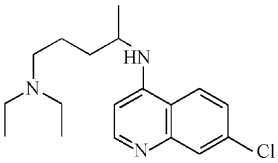
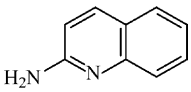
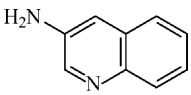
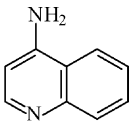
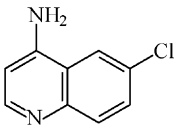
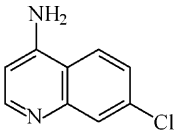
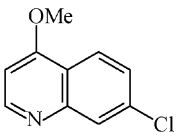
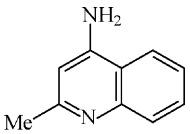
The differential interactions of 2-, 3- and 4-aminoquinoline has been taken to suggest that the amino substituent serves to position the quinoline ring system in an optimal position to allow favourable π–π stacking [17]. However, an examination of the pH dependence of the absorption spectra of the three compounds indicated that 3-aminoquinoline was present in the deprotonated state under the conditions used to measure the binding, whereas the 2- and 4-aminoquinolines were predominantly in the protonated state (data not shown). Interactions of FP and quinolines depend on various factors [18], however, the decreased basicity of 3-aminoquionline may contribute to the lack of interaction with FP at pH 6.

Compounds (**4**) and (**5**), the 4-aminoquinolines with a chloro substituent in the 6- or 7-position, produced a marked effect on the absorption spectrum of FP (Fig. 1C and D and Table 1), though higher concentrations were needed to achieve an equivalent effect to that of CQ. 4-Amino-7-chloroquinoline has previously been reported to interact with FP [17,18]. The observed interaction of the 4-amino-6-chloroquinoline with FP is, however, surprising given that Vippagunta *et al*. [17] reported that a CQ analogue with the chlorine in the 6-position appeared unable to interact with FP. This apparent discrepancy may reflect the different methods of analysis, isothermal calorimetry vs. optical spectroscopy, or it may reflect the poor solubility of the compounds in aqueous media; the buffer–solvent mixture used in this study may have facilitated the interaction. Alternatively, is possible that the combination of the chlorine at position 6 and the alkylamino side chain on 6-chloroquine destabilises the interaction with FP. Compound (**6**), in which the 4-amino group has been substituted with a methoxy moiety also appeared to interact weakly with FP (Table 1), as did the quinaldine compound (**7**), a 4-aminoquinoline which has a methyl substituent in the 2-position (Table 1).

### 3.3. β-Haematin formation

An important pathway for the detoxification of FP in malaria parasites is the formation of crystals of haemozoin, the characteristic malarial pigment. Bendrat *et al*. [21] proposed that specific lipid components in parasite preparations may contribute to the catalysis of haemozoin formation *in vivo*, and Fitch *et al*. [22] have shown that this catalytic activity can be mimicked *in vitro* using synthetic lipids. CQ and a number of other quinoline drugs with good antimalarial activity inhibit β-haematin formation with an efficacy that is loosely correlated with their antimalarial

Table 1
FP interaction, inhibition of β-haematin formation and inhibition of growth of *Plasmodium falciparum* by some quinoline compounds

| Name | Structure | FP interaction[a] (μM) | Inhibition of β-haematin formation[b] (μM) | Inhibition of growth of K1[c] (μM) | Resistance-reversing activity |
|---|---|---|---|---|---|
| Chloroquine |  | 2.0 | 35 | 0.32 ± 0.03 (8) | N/A |
| 2-Aminoquinoline (**1**) |  | 250 | >500 | >10 | No |
| 3-Aminoquinoline (**2**) |  | >600 | >500 | >10 | No |
| 4-Aminoquinoline (**3**) |  | 300 | >500 | >10 | No |
| 4-Amino-6-chloroquinoline (**4**) |  | 90 | 170 | >10 | No |
| 4-Amino-7-chloroquinoline (**5**) |  | 80 | 200 | 3.0 ± 0.3 (3) | Yes |
| 4-Methoxy-7-chloroquinoline (**6**) |  | ~600 | 200 | >10 | No |
| 4-Aminoquinaldine (**7**) |  | ~600 | >500 | >10 | No |

[a] Concentration of compound producing a 25% decrease in FP absorption at 400 nm under the conditions of the binding assay. Typical results from experiments performed three times.
[b] Data represent typical results from experiments performed at least twice.
[c] Data represent averages $IC_{50}$ values ± SD determined from a series of growth curves performed in triplicate. The number in brackets is the number of experiments.

activity [1]. In this work, we have examined the formation of β-haematin in the presence of monooleyl glycerol as a convenient system for monitoring the ability of the quinolines to inhibit FP sequestration. CQ inhibited the formation of β-haematin with an $IC_{50}$ value of 35 μM, while 2-, 3- and 4-aminoquinolines all failed to inhibit crystal growth (Table 1) in agreement with previous data [12,18]. 4-Aminoquinaldine also had no effect (Table 1). 4-Amino-7-chloroquinoline inhibited β-haematin formation with an $IC_{50}$ value that is several fold higher than that for CQ but is similar to values reported for other weak antimalarials, such as chlorpromazine [12]. Activity of this compound as an inhibitor of parasite extract-catalysed β-haematin formation has been reported previously [17].

Interestingly, the inhibitory activity is maintained when the 4-amino moiety is replaced with a methoxy substituent (Table 1; compound (**6**)) indicating that the amino group is not essential for activity. We also observed inhibitory activity for 4-amino-6-chloroquinoline (Table 1). This is again contrary to previous reports of the inability of 6-chloroquine to inhibit β-haematin formation [17] but is in keeping with our finding that it does form a complex with FP as monitored by our spectrophotometric assay. The data are in general agreement with the previous report of Egan [18] that the chlorine substituent is essential for inhibition

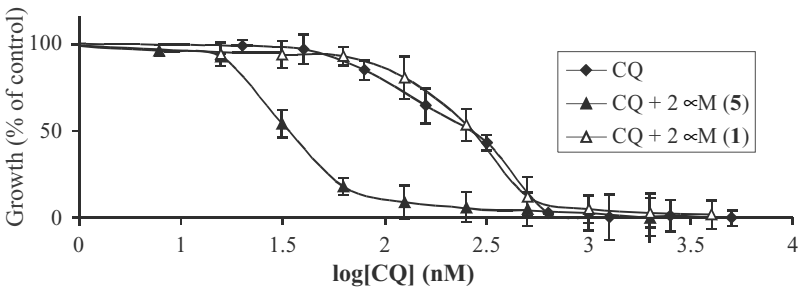



Fig. 2. Dose–response curves for CQ against the CQ-resistant strain of *Plasmodium falciparum* strain K1 in the presence and absence of aminoquinolines. These curves were generated by using a serial dilution of CQ alone (◆), or in presence of 2 μM (**5**) (▲) or 2 μM (**1**) (△). Results represent the means ± SD for a typical experiment performed in triplicate.

of β-haematin formation even though it has relatively little effect on FP-binding affinity.

### 3.4. Antimalarial activity

The abilities of the aminoquinolines to inhibit the growth of a CQ-resistant strain (K1) of *P. falciparum* were determined (Table 1) and compared with the data for CQ. CQ inhibits growth of this strain with an $IC_{50}$ value of 0.32 μM in agreement with previous reports [3,12]. By contrast, CQ inhibits the CQ sensitive D10 strain of *P. falciparum* with an $IC_{50}$ value of 0.03 ± 0.01 μM [12]. Of the various quinolines examined, only the 4-amino-7-chloroquinoline showed any antimalarial activity and this was at 10 times the $IC_{50}$ for CQ against the K1 strain. Similar values for antimalarial activity of this compound have been reported previously [17,23]. The much weaker antimalarial activity of 4-amino-7-chloroquinoline compared with CQ is likely to be due to poor accumulation in the parasite food vacuole.

### 3.5. Effect of the aminoquinolines on CQ resistance

To investigate the relative abilities of the aminoquinolines to reverse CQ resistance, we examined the antimalarial activities of combinations of these compounds with CQ. Initially, all of the compounds were examined at a single sub-inhibitory concentration (i.e. 2 μM). Compounds **1**–**4**, **6** and **7** had no effect on CQ activity against the K1 strain (Table 1), however, 4-amino-7-chloroquinoline (**5**) decreased the $IC_{50}$ value for inhibition of the K1 parasite by CQ from 0.32 to 0.03 μM (Fig. 2). This is similar to the values we routinely obtain for the CQ-sensitive parasite, D10 ([12] and data not shown). To examine the interaction of CQ and 4-amino-7-chloroquinoline in more detail, different fixed ratios of the test compounds were diluted over a 300-fold range (Table 2). The apparent $IC_{50}$ values for inhibition of the growth of the K1 strain were determined for each combination of compounds, and the data were used to estimate the sums of the fractional inhibitory concentration (SFIC) values (Table 2) and to prepare an isobologram (Fig. 3). A concave curve indicates a synergistic interaction, a convex curve, an antagonistic interaction and a straight line, no interaction [24]. Similarly, an SFIC value of less than one gives a numerical indication of a synergistic interaction. The concave curve for CQ and compound (**5**) and the SFIC values down to 0.42 indicate synergy with CQ. Thus, the 4-amino-7-chloroquinoline compound is only

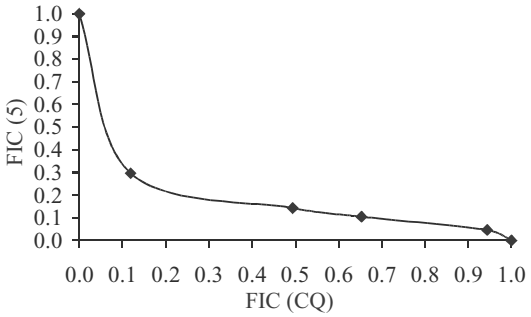



Fig. 3. Isobologram constructed from $IC_{50}$ values in Table 2. For each drug combination, the fractional inhibitory concentrations (FIC) were calculated by dividing the measured "apparent" $IC_{50}$ values for individual drugs in the different combinations of compound (**5**) and CQ by the $IC_{50}$ values obtained when these drugs were used alone.

Table 2
Concentrations of drugs required for 50% inhibition of growth of *Plasmodium falciparum* for combination of CQ with compound (**5**)

| | | | | |
|---|---|---|---|---|
| Starting concentrations of CQ and (**5**) | 1.2 μM CQ<br>0.5 μM (**5**) | 0.6 μM CQ<br>1.0 μM (**5**) | 0.3 μM CQ<br>2.0 μM (**5**) | 0.15 μM CQ<br>4.0 μM (**5**) |
| $IC_{50}$ values for individual drugs | 0.28 μM CQ<br>0.14 μM (**5**) | 0.21 μM CQ<br>0.32 μM (**5**) | 0.16 μM CQ<br>0.43 μM (**5**) | 0.04 μM CQ<br>0.89 μM (**5**) |
| SFIC | 0.92 | 0.76 | 0.64 | 0.42 |

2-fold dilutions of drugs combined in a fixed ratio were added to parasite culture and incorporation of [$^3$H]hypoxanthine was determined over a 72-hr incubation period. The "apparent" $IC_{50}$ values for each of the drugs were estimated as though each drug had been added in isolation and divided by the $IC_{50}$ for the drug alone to give the fractional inhibitory concentrations (FIC). The sums of the individual FIC (SFIC) were also calculated, indicating a synergistic effect if less than 1. Results represent the mean values for a typical experiment performed in triplicate.

weakly antimalarial but possesses substantial resistance-reversing activity.

## 4. Discussion

The data presented here allow us to analyse some of the structural features that are associated with resistance-reversing activity. A number of the aminoquinolines were able to interact with FP and some (those with a chlorine substituent) were able to inhibit β-haematin formation. This was not, however, a good predictor of antimalarial activity. Indeed, it has previously been shown that accumulation at the site of action is also critical for antimalarial activity [18]. Ability to interact with FP was also not a good predictor of resistance-reversing activity. This indicates that binding to FP is not directly responsible for resistance-reversing activity. Indeed, the only active compound, 4-amino-7-chloroquinoline, was distinguished from the other compounds in the group by the fact that it had weak antimalarial activity. Similarly, chlorpromazine has been shown to bind FP, to inhibit β-haematin formation and to display weak intrinsic antimalarial activity [12].

One possible explanation for the observed correlation is that resistance-reversing compounds may function by mimicking the molecular structure of the CQ ring system in a way that allows a specific interaction with components of the resistance apparatus. For example, if PfCRT or Pgh1 function by transporting CQ out of the food vacuole, they may bind tightly to the closely related 4-amino-7-chloroquinoline. However, because this compound is more hydrophobic than CQ, it may be less efficiently extruded from the food vacuole. In this context, it is interesting to note that a linear correlation has been observed between hydrophobicity and activity against resistant parasites for a series of quinoline antimalarials [25]. Alternatively, the resistance reversers could bind to PfCRT or Pgh1 and indirectly affect CQ accumulation by altering the proton gradient across the food vacuole membrane [7,26]. Whatever the mechanism, it appears likely to rely on a very specific interaction of 4-amino-7-chloroquinoline with a proteinaceous receptor as the closely related 4-amino-6-chloroquinoline was not active. Clearly, it would be interesting to examine other antimalarial quinolines and related compounds, such as mefloquine, quinacrine and pyronaridine, to determine whether removal of their amino side chain also switched their activities to that of resistance reversers. In this context, it is interesting that CQ analogues with shortened side chains show activity against CQ-resistant parasites [27,28].

In summary, we have identified 4-amino-7-chloroquinoline as a novel resistance-reversing agent that could be used in combination with CQ to extend its clinical life. It is important to point out that 4-amino-7-chloroquinoline is a metabolic breakdown product of CQ. There is some discrepancy in the literature about the level of this metabolite [29–31]. The bulk of the literature suggests that it is a relatively minor metabolite but one report suggests that it can reach levels even higher than that of the parent drug [31]. If 4-amino-7-chloroquinoline does reach significant levels *in vivo*, it may contribute to activity against resistance strains and may help explain why CQ is sometimes clinically active against parasites that are CQ-resistant when assayed *in vitro*. If physiological levels of 4-amino-7-chloroquinoline are low under most treatment conditions supplying it in combination with CQ may provide a means of circumventing CQ resistance. The compound can be readily prepared from inexpensive precursors and the fact that it is formed *in vivo* during CQ breakdown suggests that it is not toxic to humans. Clearly, further investigations of this drug combination are indicated.

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