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Primaquine synergises the activity of chloroquine against chloroquine-resistant *P. falciparum*

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Abstract

In recent years, resistance to the antimalarial drug, chloroquine, has become widespread. It is, therefore, imperative to find compounds that could replace chloroquine or work synergistically with this drug to overcome chloroquine resistance. We have examined the interaction between chloroquine, a 4-aminoquinoline, and a number of 8-aminoquinolines, including primaquine, a drug that is widely used to treat *Plasmodium vivax* infections. We find that primaquine is a potent synergiser of the activity of chloroquine against chloroquine-resistant *Plasmodium falciparum*. Analysis of matched transfectants expressing mutant and wild-type alleles of the *P. falciparum* chloroquine resistance transporter (PfCRT) indicate that primaquine exerts its activity by blocking PfCRT, and thus enhancing chloroquine accumulation. Our data suggest that a novel formulation of two antimalarial drugs already licensed for use in humans could be used to treat chloroquine-resistant parasites.
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Keywords: Malaria; 8-Aminoquinoline; Primaquine; Chloroquine; Drug resistance; Resistance reversing agent

1. Introduction

Chloroquine (CQ) has been used to treat malaria for nearly 50 years. However, in recent years, resistance to this 4-aminoquinoline has increased to the point where it is virtually useless in many malarious regions. This has contributed to documented increases in malaria-related morbidity and mortality [1] and has led to efforts to understand the molecular basis of CQ action and CQ resistance with the aim of circumventing CQ resistance.

CQ is thought to inhibit the growth of *Plasmodium falciparum* by accumulating in the acidic digestive vacuole of the parasite and interfering with the detoxification of ferriprotoporphyrin IX (FP), a by-product of hemoglobin degradation [2]. Resistant parasites accumulate markedly less CQ than their sensitive counterparts enabling them to survive much higher concentrations of the drug [3–5].

P. falciparum expresses a homologue of the human multidrug resistance (MDR) protein, referred to as P-glycoprotein homolog-1 (Pgh-1) [6]. Pgh-1 does not appear to be the major effector of CQ resistance (see Ref. [7] for review), however, particular alleles of *Pfmdr1* modulate the level of resistance to CQ and the protein is involved in resistance to a related quinoline, mefloquine [8]. Recently, a protein referred to as the *P. falciparum* chloroquine resistance transporter (PfCRT) was identified as the major player in the phenomenon of CQ resistance. Specific polymorphisms in PfCRT have been shown to be tightly correlated with the development of CQ resistance in both field and laboratory isolates [9]. PfCRT is an integral membrane protein with 10 predicted transmembrane domains and is located in the digestive vacuole membrane, i.e. in a suitable location to exert an effect on

Abbreviations: CQ, chloroquine; DMSO, dimethyl sulfoxide; FP, ferriprotoporphyrin IX/haem; PfCRT, *P. falciparum* chloroquine resistance transporter; PQ, primaquine; TQ, tafenoquine

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CQ accumulation. The precise role of this protein has not been delineated, however, it has been suggested that it may function as a transporter that removes CQ from its site of action [10].

PfCRT is predicted to be a member of the drug/metabolite transporter superfamily and may be involved in the export of amino acids and/or peptides from the digestive vacuole [11–13]. The mutant PfCRT allele expressed by CQ resistant parasites has fewer basic residues on the surface predicted to face the digestive vacuole lumen. This may allow entry of positively charged species, such as CQ, thus facilitating exit of the drug down an electrochemical gradient [10].

Interestingly, some weakly basic amphipaths that possess only very weak schizonticidal activity themselves are able to interact synergistically with CQ in inhibiting CQ resistant parasites [14–16]. For example, when CQ is used in combination with the calcium channel blocker, verapamil, or the calmodulin antagonist, chlorpromazine, CQ resistant parasites are killed at CQ concentrations similar to those effective against CQ sensitive parasites [3,14,15,17]. Many of these compounds share certain structural features, such as a hydrophobic heterocyclic ring system and an alkyl side chain, with a terminal amine [18]. Indeed, compounds with resistance reversing activity may be very closely related in structure to compounds with antimalarial activity. We have shown that addition of a tribasic side chain to the resistance reversing agent, chlorpromazine, results in a 100-fold increase in schizonticidal activity but abrogates its ability to interact synergistically with CQ [19]. Moreover, 7-chloro 4-aminoquinoline, which is the CQ nucleus without the basic side chain, shows only very weak schizonticidal activity but is able to act as a resistance reversing agent [20].

One approach to prolonging the clinical life of CQ would be to use it in combination with a suitable synergising compound. A number of compounds have been considered for this purpose. For example, the anti-histamine, chlorpheniramine, was shown to reverse chloroquine resistance in African field isolates [21] and a later clinical trial demonstrated enhanced efficacy of a chloroquine/chlorphenariamine combination over chloroquine alone for treating uncomplicated malaria in children [22].

In this work, we show that a number of 8-aminoquinolines, including primaquine (PQ, Fig. 1A), which is frequently used in combination with CQ to treat *Plasmodium vivax*, are able to act synergistically with CQ against CQ resistant parasites. These data suggest that a novel formulation of two antimalarial drugs that are already licensed for use in humans could be used to treat CQ resistant parasites.

2. Materials and methods

2.1. Materials

CQ and PQ were obtained from Sigma Chemical Company (St. Louis, MO, USA). Tafenoquine and carboxyPQ were obtained from Dr. Mike Edstein, Army Malaria Institute, Australia. Fresh human erythrocytes and human serum were obtained from the Red Cross Transfusion Service, Melbourne, Australia.

2.2. Chemical syntheses

2.2.1. 2-Methyl-8-(4-phthalimido-1-methylbutylamino)quinoline (*1a*)

The precursor 4-bromo-1-phthalimidopentane was prepared by a literature procedure [23]. A solution of 8-amino-2-methylquinoline (**1**) (0.25 g, 1.58 mmol) and 4-bromo-1-phthalimidopentane (0.24 g, 0.81 mmol) in *n*-butanol (15 ml) was heated under reflux for 72 h. When cooled, the solvent was removed in vacuo and the residue was dissolved in ethyl acetate (15 ml) and extracted with 15% hydrochloric acid (15 ml). The aqueous phase was basified with sodium hydroxide pellets and extracted with ethyl acetate. The organic extract was dried (magnesium sulfate) and the solvent was removed in vacuo to give the crude (**1a**) as a brown semisolid (0.17 g).

2.2.2. 2-Methyl-8-((4-amino-1-methylbutyl)amino)quinoline (*2*)

A solution of the phthalimido compound (**8a**) (0.10 g, 0.27 mmol) and hydrazine monohydrate (0.02 g 0.4 mmol) in ethanol (10 ml) was heated under reflux for 30 min. During this time a white solid formed. Concentrated hydrochloric acid (3 ml) was added and more solid precipitated. When cooled, the solid was filtered and washed with water. The filtrate was collected and concentrated, then basified with 10% sodium hydroxide and extracted with ether. The extract was dried and the solvent was removed to give (**2**) as a yellow semisolid. A solution of 85% phosphoric acid (0.6 ml) in ethanol (10 ml) was added dropwise to a solution of the free base in ethanol (3 ml) until solid precipitated. This was filtered and recrystallised from ethanol to give the phosphate salt of (**2**) as an orange solid, mp 181–183 °C (0.05 g, 55%).

^{1}H NMR of phosphate salt (d^6-DMSO): δ 1.22 (d, 3H, J = 5.6 Hz, CH_3), 1.65 (m, 4H, $2CH_2$), 2.64 (s, 3H, CH_3), 2.78 (m, 2H, CH_2), 3.68 (m, 1H, CH), 6.00 (m, 1H, NH), 6.67 (d, 1H, J = 7.5 Hz, H-7), 6.99 (d, 1H, J = 7.9 Hz, H-5), 7.28 (t, 1H, J = 8.4 Hz, H-6), 7.36 (d, 1H, J = 8.4 Hz, H-3), 8.07 (d, 1H, J = 8.4 Hz, H-4).
^{13}C NMR of phosphate salt (d^6-DMSO): δ 20.5 (CH_2), 24.2 (2-CH_3), 24.9 (CH_2), 33.2 (α-CH_3), 47.0 (α-CH), 104.8 (C-7), 113.0 (C-5), 122.4 (C-3), 126.5 (C-4a), 126.9 (C-6), 136.3 (C-4), 136.8 (C-8a), 143.0 (C-8), 155.2 (C-2).
ESMS m/z: 244.2 (M + 1).

NMR spectra were recorded on a Bruker Avance-300 spectrometer operating at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C). Various standard techniques were

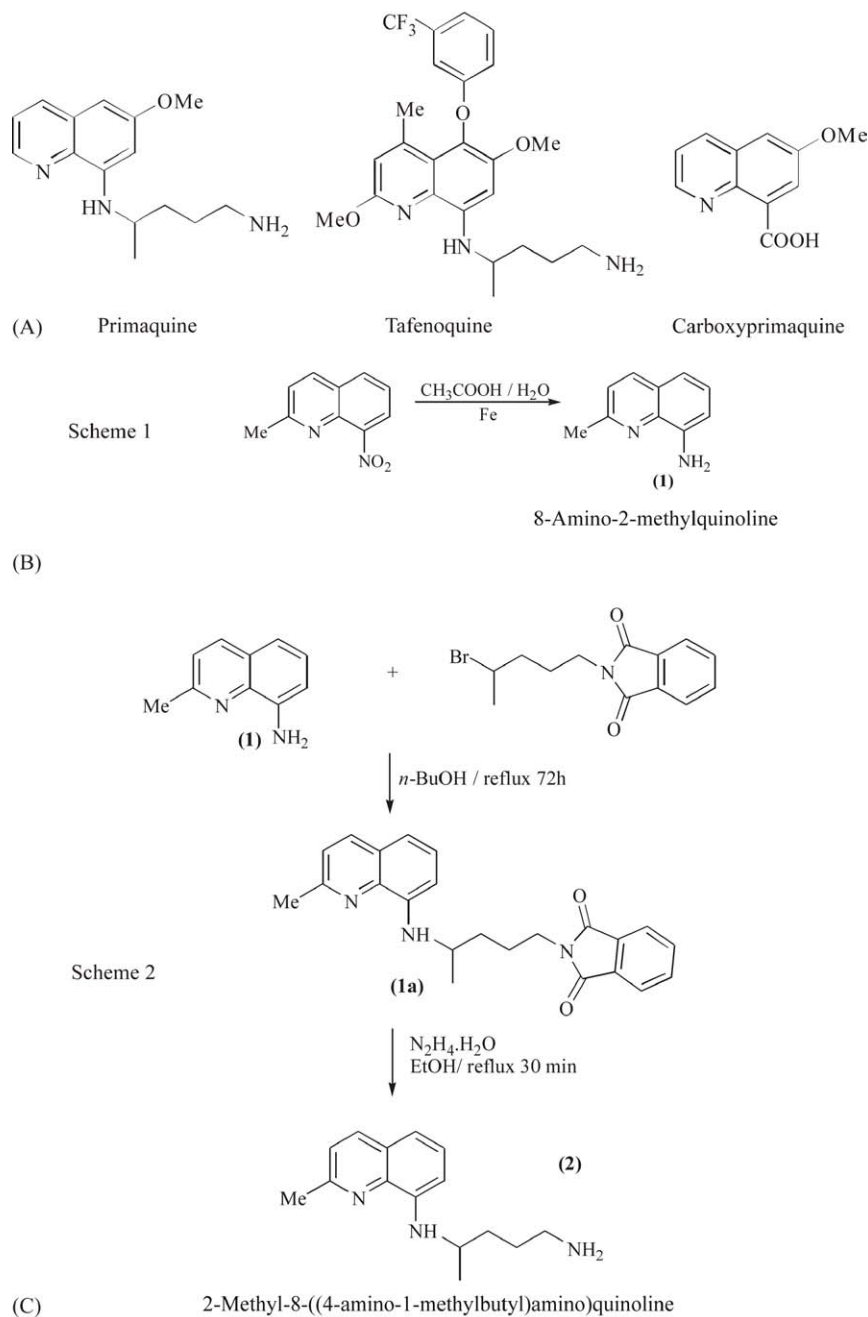

Fig. 1. Structures and synthesis of compounds used in this study. Top panel: pre-existing compounds. Bottom panels: scheme for the synthesis of the new 8-aminoquinoline **(2)**.

used to identify proton-bound carbons in ^{13}C NMR spectra. The electrospray mass spectrum was obtained by Mr. I. Thomas on a VG Bio-Q triple quadrupole mass spectrometer using a water/methanol/acetic acid (50:50:1) mobile phase.

2.3. Drug–hematin interactions

The abilities of compounds to interact with hematin and to inhibit β-haematin formation were determined as described previously [20].

2.4. Parasites

D10 and K1 are CQ sensitive and CQ resistant strains of *P. falciparum* [6]. The $C2^{GC03}$ strain is derived from GC03 (a CQ sensitive progeny line from a genetic cross) [24]. It has been transfected with its own wild-type *PfCRT* allele [25]. $C3^{Dd2}$ is GC03 transfected with the CQ resistant Dd2 *PfCRT* allele [25]. Parasites were maintained in continuous culture under selection with 2.5 nM WR 99210 and 2.5 μM blasticidine S. $D10^{D10}$ is a CQ sensitive parasite line transfected with its own wild-type *PfMDR1* allele,

while D10$^{7G8/3}$ is the same parasite line transfected with the CQ resistant 7G8 *PfMDR1* allele [8].

2.5. Assessment of antiplasmodial activity

For determination of 50% inhibitory concentrations (IC_{50} values) malaria parasites were plated at about 1% parasitemia (2% haematocrit), in 96 well trays and different concentrations of the drugs were added from concentrated stocks in water. Parasites were incubated for 72 h 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 [26] and the concentration of drug required to produce 50% inhibition of growth (IC_{50}) was determined. Isobologram analysis was used to determine interactions between drugs [19,27]. Briefly, three different combinations of CQ with either PQ, TQ or compound (**2**) were diluted at a fixed ratio and examined for their effect on parasite growth. The IC_{50} values were calculated for each drug as though it had been added in isolation. These "apparent" IC_{50} values were divided by the IC_{50} values for the drugs used alone to determine the fractional inhibitory concentration (FIC). The FIC values for the two drugs in the combination were used to construct isobolograms and to determine the sums of the FIC values. A more detailed description of the generation of the isobolograms is available elsewhere [19,28].

2.6. Analysis of CQ accumulation

Prior to use in experiments, ring stage cultures (C2^{GCO3}, C3^{Dd2}, D10^{D10} and D10^{7G8} transfectants) were washed in complete medium without selection agents and cultured for 24 h. [^{3}H]Chloroquine (specific activity, 50.4 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). CQ accumulation was measured as described previously [29] at an extracellular CQ concentration of 3 nM with or without PQ or verapamil. The cellular accumulation ratio (CAR) was calculated as the ratio of chloroquine in the cell pellet to that in the same volume of medium at equilibrium.

3. Results

3.1. Synthesis of 8-amino-2-methylquinolines

The reduction of 2-methyl-8-nitroquinoline to its amino derivative (**1**) was achieved using iron powder and aqueous acetic acid, in 83% yield (Scheme 1, Fig. 1). The amine (**1**) was alkylated with 2-bromo-5-phthalimidopentane to give (**1a**) (Scheme 2, Fig. 1) and the product was confirmed by its ^{1}H NMR spectrum. The doublet for the side chain CH_3 was shifted upfield to 1.28 ppm from 1.66 ppm in 4-bromo-1-phthalimidopentane. Removal of the phthalimide protecting group was performed according to a literature procedure [30] in which (**1a**) was reacted with hydrazine hydrate to give the amine (**2**). Compound (**2**) was isolated by generating the phosphate salt, which was recrystallised from ethanol and obtained as an orange solid. NMR data [^{1}H, PENDANT ^{13}C (five tertiary aromatic carbons and four quaternary carbons) and HMBC] were in accord with this structure.

3.1.1. Schizonticidal activity

The abilities of a number of 8-aminoquinolines to inhibit the growth of the CQ resistant (K1) strain of *P. falciparum* were determined (Table 1) and compared with data for CQ. As previously reported [31], PQ has a weak but measurable schizonticidal activity. Tafenoquine (TQ, Fig. 1A) is a novel 8-aminoquinoline with an enhanced half-life in vivo and reportedly increased activity against blood stage parasites [32,33]. In our hands an increased activity was observed relative to PQ but the increase was modest. The amino side chain appears to be important for blood stage activity as carboxyPQ (Fig. 1A) showed no activity within the range tested (Table 1).

8-Aminoquinolines with 2-alkyl substituents show improved therapeutic activity and decreased methaemoglobin toxicity compared with PQ [34,35]. In this work, we included two such compounds containing 2-methyl substituents. The known compound (**1**), which lacks the alkylamino side chain (Fig. 1B), showed no blood stage activity within the range examined (Table 1). However, the new 8-alkylaminoquinoline, compound (**2**), (Fig. 1C), has measurable though weak blood stage activity. This confirms the importance of the alkylamino side chain for blood stage activity. Some 8-aminoquionolines with substantial blood stage activity have been shown to interact with ferriprotoporphyrin IX and to inhibit the formation of β-hematin [31], however, we found that neither PQ, nor compounds (**1**) or (**2**) were able to interact with FP or inhibit β-hematin formation within the range tested (up to 600 μM, data not shown). These findings for PQ agree with previous reports [36,37]. Nonetheless, the fact that measurable schizontocidal activity was observed only for

Table 1
Inhibition of growth of *P. falciparum* in vitro by 8-aminoquinolines

Compound	Inhibition of growth of K1 (IC_{50}, μM)
CQ	0.42 ± 0.10 (3)
TQ	1.95 ± 0.06 (3)
PQ	2.41 ± 0.10 (3)
CarboxyPQ	>100 (2)
(1) 8-Amino-2-methylquinoline	>10.0 (2)
(2) 8-(4-Amino-1-methylbutylamino)-2-methylquinoline	5.0 ± 0.2 (3)

Data for inhibition of parasite growth are average IC_{50} values ± S.D. determined from a series of growth curves prepared on separate days, each of which was performed in triplicate. The numbers in brackets refer to the number of experiments.

compounds with an alkylamino side chain suggests that the active 8-aminoquinolines may exert their activity within the digestive vacuole.

3.2. Effect of 8-aminoquinolines on CQ resistance

It has previously been shown that compounds such as verapamil, chlorpromazine and 7-chloro-4-aminoquinoline can modulate the level of resistance of *P. falciparum* to CQ [15,20,38]. Each of these compounds has weak schizonticidal activity and shows some structural similarity to CQ. We have previously provided evidence suggesting that PQ can also modulate the uptake of CQ by the resistant parasite, K1 [29]. We have, therefore, examined the abilities of our series of 8-aminoquinolines to interact synergistically with CQ against the K1 strain. Initially compounds (**1**) and (**2**) were examined at a single sub-inhibitory concentration (i.e. 2 μM). Compound (**1**) had no effect on CQ activity against the K1 strain, however, compound (**2**) decreased the IC_{50} value for inhibition of the K1 parasite by CQ from 0.39 to 0.04 μM (Fig. 2A). This is equivalent to the value (0.039 ± 0.009 μM) obtained for inhibition of the CQ sensitive parasite, D10.

To examine the interaction of CQ and the 8-aminoquinolines in more detail, different fixed ratios of each 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 isobolograms (Fig. 2B). A concave curve indicates a synergistic interaction, a convex curve, an antagonistic interaction and a straight line, no interaction [28]. Similarly, an SFIC value of less than one gives a numerical indication of a synergistic interaction. The concave curves for CQ with each of PQ, TQ and compound (**2**) and the SFIC values down to 0.3 indicate synergy with CQ. Thus, these 8-aminoquinoline compounds possess substantial resistance reversing activity. CarboxyPQ and compound (**1**) (at a concentration of 10 μM) had no effect on the IC_{50} value for CQ against K1 parasites (data not shown). At sub-inhibitory concentrations (i.e. up to 0.5 μM), neither TQ nor PQ had an effect on the IC_{50} value for CQ against D10 parasites. The IC_{50} values obtained at 0.5 μM TQ and PQ were 0.034 ± 0.010 and 0.045 ± 0.008 μM, respectively, compared with 0.039 ± 0.009 μM for CQ alone.

3.3. Effect of PQ on uptake of CQ in CQ sensitive and CQ resistant parasites

In CQ resistant parasites, a mutant PfCRT is thought to be involved in extrusion of CQ from the digestive vacuole [9]. Resistance reversing agents are postulated to exert a synergistic effect with CQ against CQR parasites by binding to PfCRT and preventing CQ release [10]. In this work, we have examined the effect of PQ on the uptake of [^{3}H]-CQ into matched transfected parasites expressing either a wild-type (CQ sensitive) PfCRT allele (C2^{GC03}) or a mutant (CQ resistant) PfCRT allele (C3^{Dd2}; Ref. [25]). At concentrations above 0.5 μM, PQ significantly enhanced uptake of [^{3}H]-CQ into the parasite strain bearing the CQ resistant allele but not the parasite strain expressing the CQ sensitive allele (Fig. 3A). At a concentration of 5 μM, PQ was slightly more effective than verapamil at enhancing CQ uptake.

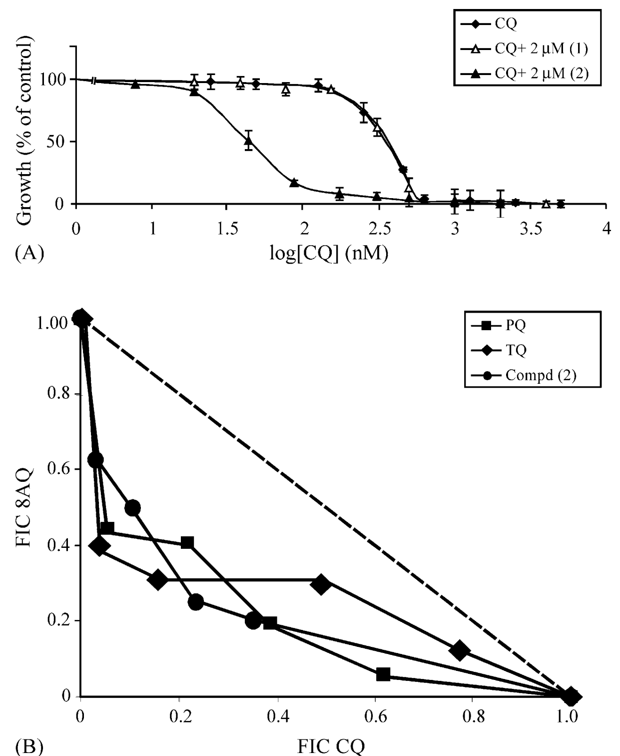

Fig. 2. Effect of 8-aminoquinolines on activity of CQ against the CQ resistant (K1) strain of *P. falciparum*. (A) Dose response curves of CQ alone (◆), or in the presence of 2 μM compound (**1**) (△) or 2 μM compound (**2**) (▲). Results represent the means ± S.D. of triplicate determinations in a typical experiment. (B) Isobolograms 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 PQ and CQ (■) or TQ and CQ (◆), or compound (**2**) and CQ (●) by the IC_{50} values obtained when the drugs were used alone.

Mutations of *PfMDR1* have been shown to modulate the response of parasites to mefloquine and quinine and to a lesser extent chloroquine [8]. In order to test for a contribution of *PfMDR1* mutations we used matched transfected parasites expressing either a wild-type *PfMDR1* allele (D10^{D10}) or a mutant (D10$^{7G8/3}$) allele that has been shown to confer resistance to quinine and increased sensitivity to mefloquine. In contrast to the data for the PfCRT transfectants, there was no significant effect of PQ at any concentration on the uptake of CQ by parasite lines harbouring either *PfMDR1* allele (Fig. 3B). Both the GC03 and the D10 parental lines are fully susceptible to

Table 2
Concentrations of drugs required for 50% inhibition of growth of *P. falciparum* (K1 strain) for combinations of CQ with PQ, TQ or compound (**2**)

Starting concentrations of CQ and PQ	1.2 μM CQ 1.0 μM PQ	0.6 μM CQ 2.0 μM PQ	0.3 μM CQ 4.0 μM PQ	0.15 μM CQ 8.0 μM PQ
Apparent IC_{50} values for individual drugs	0.20 μM CQ 0.16 μM PQ	0.20 μM CQ 0.63 μM PQ	0.09 μM CQ 1.12 μM PQ	0.03 μM CQ 1.78 μM PQ
CQ IC_{50} = 0.45 ± 0.05 μM, PQ IC_{50} = 2.8 ± 0.6 μM				
SFIC	0.50	0.67	0.60	0.70
Starting concentrations of CQ and TQ	1.2 μM CQ 1.0 μM TQ	0.6 μM CQ 2.0 μM TQ	0.3 μM CQ 4.0 μM TQ	0.15 μM CQ 8.0 μM TQ
Apparent IC_{50} values for individual drugs	0.36 μM CQ 0.31 μM TQ	0.22 μM CQ 0.71 μM TQ	0.10 μM CQ 1.00 μM TQ	0.02 μM CQ 1.00 μM TQ
CQ IC_{50} = 0.41 ± 0.06 μM, TQ IC_{50} = 2.05 ± 0.09 μM				
SFIC	0.97	0.74	0.50	0.30
Starting concentrations of CQ and (**2**)	1.2 μM CQ 1.0 μM (2)	0.6 μM CQ 2.0 μM (2)	0.3 μM CQ 4.0 μM (2)	0.15 μM CQ 8.0 μM (2)
Apparent IC_{50} values for individual drugs	0.24 μM CQ 0.21 μM (2)	0.19 μM CQ 0.50 μM (2)	0.09 μM CQ 1.12 μM (2)	0.03 μM CQ 1.78 μM (2)
CQ IC_{50} = 0.39 ± 0.04 μM, (2) IC_{50} = 5.0 ± 1.0 μM				
SFIC	0.66	0.59	0.45	0.44

Two-fold dilutions of drugs combined in a fixed ratio (to a final dilution of 160-fold) were added to parasite cultures and incorporation of [^{3}H]-hypoxanthine was determined over a 72 h incubation period as described in detail elsewhere [19,28]. Data are for an experiment performed in triplicate and are typical of data obtained in experiments performed on at least three different days. The "apparent" IC_{50} value for each drug was estimated as though it had been added in isolation. The actual IC_{50} values ± standard deviations for individual drugs used alone (measured on the same day) are given in the third row of each set. The apparent IC_{50} values for each drug used in the combination were divided by the actual IC_{50} value for the drug to give the fractional inhibitory concentration (FIC). The FIC values for each of the drugs used in the combination were then added to give the sum of the FIC (SFIC).

CQ. This enables the testing of the contribution of drug resistant alleles of PfCRT and PfMDR1 to the PQ effect against a drug sensitive background. Unfortunately, the only available allelically exchanged PfMDR1 lines with a CQ resistant background harbour a different PfCRT allele (7G8) to the one employed here (Dd2). In the absence of a full allelic analysis, the most obvious interpretation of our results is that PQ is interacting with mutant PfCRT. However, since the allelic substitution of PfMDR1 did not alter CQ susceptibility of the D10 line [8] we cannot rule out an additional interaction of PQ with PfMDR1 in other parasite lines that are resistant to CQ.

4. Discussion

German research workers in the Bayer laboratories of IG Farben in the 1920s synthesized the 8-aminoquinoline, pamaquine, as the first synthetic antimalarial (see Ref. [39], for review). Pamaquine was found to have unacceptable toxicity in vivo and primaquine was synthesized as a more useful analogue. Primaquine is still widely used to treat *P. vivax* infections (see Refs. [40,41], for reviews). Surprisingly, despite several decades of use, the mechanism of action of primaquine is not well understood. It is believed that it is converted in the liver to an active quinone metabolite and probably exerts its activity by interfering with parasite mitochondrial function [42]. Redox cycling of the quinone metabolite also exerts a substantial oxidative stress on the erythrocyte. This can lead to problems in patients with glucose 6-phosphate dehydrogenase (G6PD) deficiency [43].

The 4-aminoquinoline, CQ, was developed in the 1940s, as a more effective treatment for *P. falciparum* [44]. Despite ever-increasing levels of CQ resistance, this drug continues to be employed as a treatment for both falciparum and vivax malaria due mainly to the fact that alternative therapies that are more effective are considered too expensive. PQ and CQ are commonly used in sequential combination for the radical cure of vivax malaria [40,41]. CQ/PQ combinations are not normally used to treat *P. falciparum*, as until now, no benefit of this treatment was evident.

Our data indicate that PQ acts as a synergiser of the action of CQ against CQ resistant *P. falciparum* in a manner that appears to be PfCRT-dependent. As PQ is rapidly metabolised in vivo to carboxyPQ [41] it was important to determine whether his metabolite possessed similar activity. We found that carboxyPQ had no schizontocidal activity in the range examined and did not synergise the action of CQ. This indicates that the resistance reversing activity of these compounds is dependent on the presence of a basic side-chain. It also suggests that CQ and PQ would need to be administered concurrently to achieve maximal effect in patients. Levels of PQ and carboxyPQ peak a few hours after treatment at levels that have been reported to range from 0.4–2 to 2–13 μM, respectively [45,46]. Thus, we anticipate that the levels

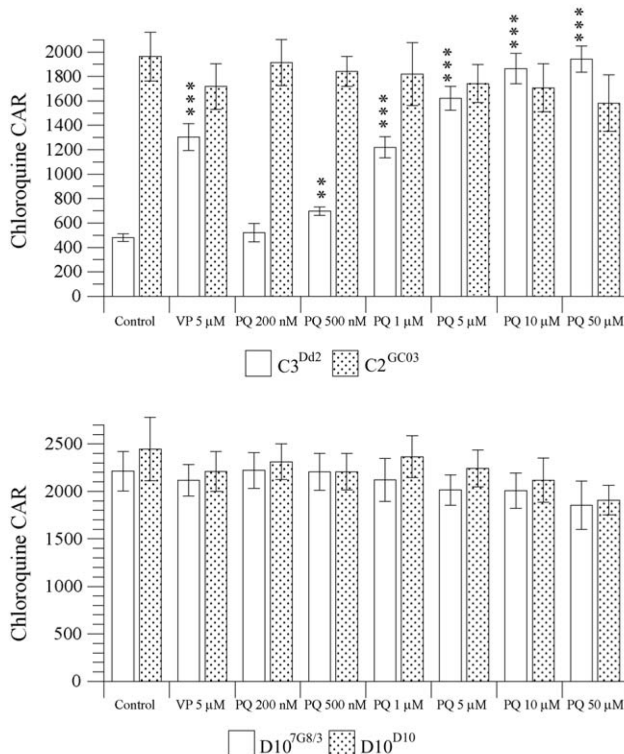

Fig. 3. (A) Effect of PQ and verapamil on uptake of CQ into CQ sensitive and CQ resistant parasites. $C2^{GC03}$ (a CQ sensitive parasite line transfected with its own wild-type PfCRT allele) and $C3^{Dd2}$ (the same parasite line transfected with the CQ resistant Dd2 PfCRT allele) were sampled at mid-trophozoite stage (2% haematocrit, ~5% parasitaemia in RPMI without bicarbonate). The parasitised erythrocytes were incubated with 3 nM $[^3H]$-CQ in the presence or absence of PQ or verapamil (VP) at the indicated concentrations for 1 h at 37 °C. (B) The same experiment performed using $D10^{D10}$ (a CQ sensitive parasite line transfected with its own wild-type PfMDR1 allele) and $D10^{7G8/3}$ (the same parasite line transfected with the CQ resistant 7G8 PfMDR1 allele). The bar graphs show means plus or minus standard deviations for four independent experiments each performed in triplicate. $^{**}p < 0.01$; $^{***}p < 0.001$; Mann–Whitney *U*-test comparing PQ treated with control. Steady state CQ uptake is expressed as the cellular accumulation ratio (CAR).

of PQ that are achieved in vivo may be sufficient to act synergistically with CQ.

TQ is a novel 8-aminoquinoline with a much longer half-life in patients than PQ [47]. We found that TQ also interacts synergistically with CQ. Our data suggest that a combination of CQ and TQ might be even more effective for treating *P. falciparum* infections.

In an effort to understand the molecular features of the 8-aminoquinolines that are needed for resistance reversing activity we synthesized two 8-amino-2-methylquinolines lacking the methoxy substituent. These compounds are substituted with a primary amino group or with a 4-amino-1-methylbutylamino substituent equivalent to the side chain of PQ and TQ. Compound (**2**) interacts synergistically with CQ indicating that the basic side chain is essential for activity but that the methoxy substituent is not required. A number of 8-aminoquinolines with substituents on the 2-position have been prepared previously; some of these exhibit improved therapeutic activity and reduced methemoglobin toxicity [34,48]. Given the similarity of these compounds to compound (**2**), it is likely that they will also interact synergistically with CQ against CQ resistant parasites.

CQ resistance is thought to result from amino acids changes in PfCRT that allow the extrusion of positively charged CQ down an electrochemical gradient [10]. Resistance reversing compounds may function by mimicking the molecular structure of the CQ ring system. Resistance modulators are similar to CQ in that they are usually heterocyclic compounds with basic amphipathic characteristics. However, while CQ is dibasic at physiological pH values, resistance modulators are monobasic and more hydrophobic. Thus, the 8-aminoquinolines may enter the pore of the mutated PfCRT because of their structural similarity to CQ, however, because of their relatively lipophilic character bind to sites within the pore, and thereby block the passage of CQ [10,49].

There is one report of an interaction of PQ and CQ in the field. CQ combined sequentially with PQ was tested against uncomplicated *P. falciparum* malaria in northeastern Papua, Indonesia. This treatment regimen led to a significant decrease in treatment failure rates at day 14, though not at longer times [50]. However, it is important to note that this regimen was not optimised to promote CQ/PQ synergism, i.e. the drugs were not co-administered. A synergistic interaction between PQ and pyronaridine (which may have a similar mechanism of action to CQ) in inhibiting growth of *P. falciparum* has also been observed [51].

There are concerns about possible side-effects and the long duration of PQ therapy, however, shorter and better tolerated PQ regimens are now being tested which will remove some of these objections [40]. We suggest that the main advantage of CQ/PQ combinations compared with other possible combinations is the low cost. Treatment with CQ costs US$ ~0.10 and PQ costs US$ ~0.15, while artemisinin costs US$ ~1–2 and should also be used in combination [52]. A novel synthetic endoperoxide currently being trialed [53] also has a target price of US$ 1 per treatment.

In conclusion our data indicate that PQ or TQ can interact synergistically with CQ. Our findings suggest that a novel formulation of two antimalarial drugs that are already licensed for use in humans could be used to treat CQ resistant parasites, thereby providing a cheap and effective means of extending the clinical life of CQ. Clearly trials of optimised combinations of these drugs in humans are needed to determine whether any beneficial effect can be observed in field situations.

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