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Dual-acting diamine antiplasmodial and chloroquine resistance modulating agents

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ABSTRACT

On the basis of structural features known to be critical for the antimalarial activity, accumulation and uptake of chloroquine (CQ), as well as chemosensitization of CQ resistant *Plasmodium falciparum*, an exploratory novel series of potential dual acting antiplasmodial and chemosensitizing agents was designed and synthesized for biological evaluation. All four compounds contain a common alkyl side chain with two amino groups and differ only in the chemical nature of the hydrophobic aromatic moieties. Among them, *N*′-[4-(biphenyl-2-ylmethoxy)-benzyl]-*N*,*N*-dimethyl-propane-1,3-diamine (P7) displayed the greatest potential as a dual-acting antiplasmodial agent against CQ-resistant strains ($IC_{50}^{K1/RSA11} < 0.6\,\mu M$) and chemosensitizer ($RMI_{K1} = 0.67$; $RMI_{RSA11} = 0.82$) while displaying low in vitro cytotoxicity against a mammalian cell line (CHO). At 1 μM, P7 caused a 8.5 and 4-fold potentiation in CQ accumulation in resistant *P. falciparum* K1 and RSA11 strains, respectively. In a parallel experiment, 1 μM verapamil showed a 6.5 (K1) and 2 (RSA11)-fold increase in CQ accumulation. The preliminary studies point to structural features that may determine antiplasmodial and/or CQ resistance modulating activity in this new series of compounds. An additive effect was observed against both CQ^S (D10) and CQ^R (RSA11) strains when CQ and P7 were used at their corresponding IC_{50} concentrations in isobologram analysis.

1. Introduction

The global picture of malaria is such that 300–500 million people are infected and at least one million die each year, mostly children under the age of 5 [1]. Chloroquine (CQ) has been the most important antimalarial drug for almost half a century. Although this well-tolerated quinoline-based drug appears to have failed as a first-line antimalarial in most parts of the world, it may be resurrected by combination therapy with effective resistance reversal agents [2].

CQ resistance was very slow to develop, suggesting multiple mutations were required to produce the resistance phenotype. Laboratory gene mapping efforts have been carried out by investigators worldwide and a CQ resistance candidate gene was eventually identified on chromosome 7 and thought to be responsible for the difference in CQ accumulation between CQ-sensitive and -resistant isolates [3–5]. Knowledge about CQ resistance and CQ's mode of action has been a subject of debate over the years. A milestone in CQ resistance was established by the discovery of the lysosomal integral protein, PfCRT (*Plasmodium falcparum* CQ resistance transporter) in genetic cross analysis in vitro [6], and its absolute association with CQ resistance.

Work in multi-drug resistance (MDR) in cancer has shown the possibility to restore an anticancer agent's efficacy by using a combination of chemosensitizers at safe therapeutic

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doses to humans [7]. In *Plasmodium*, it had been suggested that a phenomenon similar to the MDR phenotype in cancer cells may be a possible mechanism to explain the CQ resistance since the combination of the calcium channel blocker, verapamil, with CQ was able to sensitize malaria parasites to CQ in vitro [8]. There have been attempts to reverse CQ resistance in *P. falciparum* using chemoreversers like verapamil (VPL), chlorophenaramine, trifluoperazine and desipramine [8–10]. Although the unsuccessful human clinical data was produced with despiramine, the significant finding that despiramine and other chemosensitizers were able to restore CQ's efficacy in vitro is noteworthy [11,12].

Besides the heme binding 7-chloro-4-aminoquinoline nucleus, one other important substructural feature of CQ is the presence of protonatable quinoline and terminal nitrogen atoms, which are believed to be important for the accumulation of CQ in the acidic digestive food vacuole compartment of the parasite [13]. In addition to the importance in accumulation, the presence of basic nitrogens is also important for the uptake. Given the modest binding of CQ to the presumed target ferriprotoporphyrin IX (as suggested by IC_{50} values of 76–100 μM [14,15]), the heightened antimalarial activity of CQ in cell culture may be more to do with uptake and accumulation of this drug. A number of compounds with at least two protonatable nitrogens and a non-quinoline aromatic nucleus such as aminopiperidines show potent antimalarial activity in cell culture presumably due to their enhanced uptake and accumulation in acidic compartments within the parasites [14,16].

On the other hand, a basic chemosensitizing pharmacophore hypothesis has been proposed and exploited in the design of new CQ resistance modulators [17]. According to this hypothesis and previous structure-activity relationship (SAR) studies of chemosensitizers in malaria [18–20], the presence of a hydrophobic moiety and protonatable nitrogens is important for CQ resistance modulation. We have previously identified xanthene-based polyamines showing concentration dependent antimalarial and CQ modulation activities [21,22]. In order to further exploit and validate our initial data, we have designed and synthesized a further new series of diamines for evaluation as concentration-dependent dual-acting antiplasmodial and CQ resistance modulating agents, Fig. 1. The synthesized compounds possess different extended hydrophobic moieties in their respective structures. Compound P4 (Fig. 1) differs from the others with only one aromatic ring in its structure while the others contain at least two aromatic ring systems. The introduction of a methoxy group at position 3 on one of the hydrophobic rings of compound P5 distinguishes compound P6 from P5. In the structure of compound P7, a privileged biphenyl substructure was introduced [23].

2. Materials and methods

2.1. Chemicals

Chloroquine diphosphate, verapamil hydrochloride and daunomycin were purchased from Sigma Chemical Co., USA. 1-Phenylpiperazine and *N*-methylpiperazine were obtained from Lancaster, Scientific, and Sigma–Aldrich South Africa, respectively while 1-benzylpiperazine was synthesized chemically. The remaining reagents and solvents were obtained from Sigma–Aldrich, South Africa.

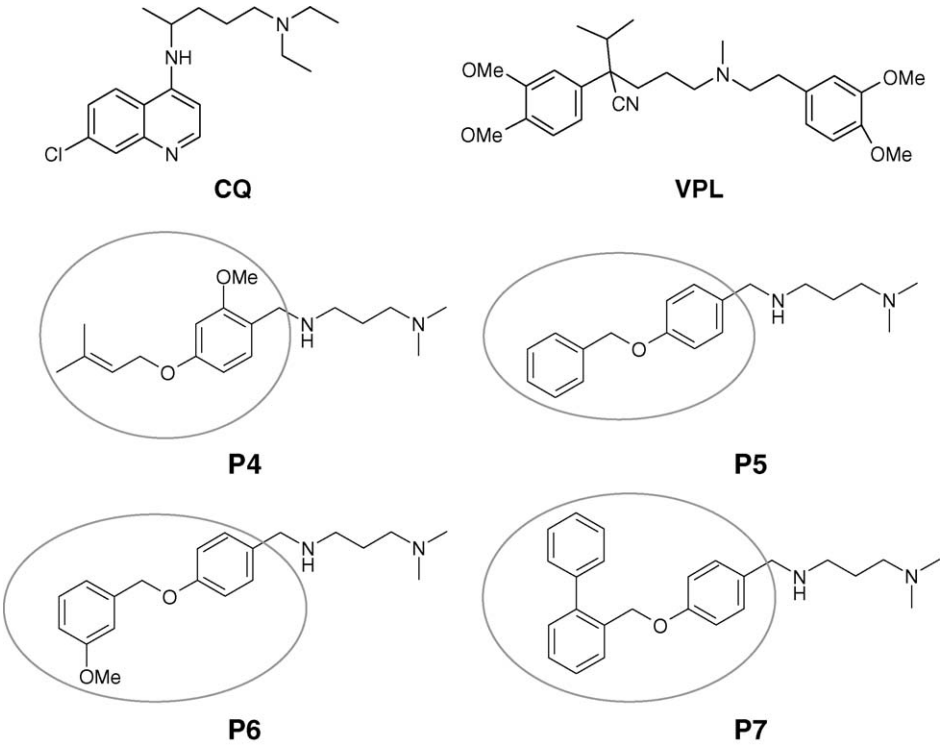


Fig. 1 – Chemical structures of CQ, verapamil and a new class of diamines synthesized and tested.

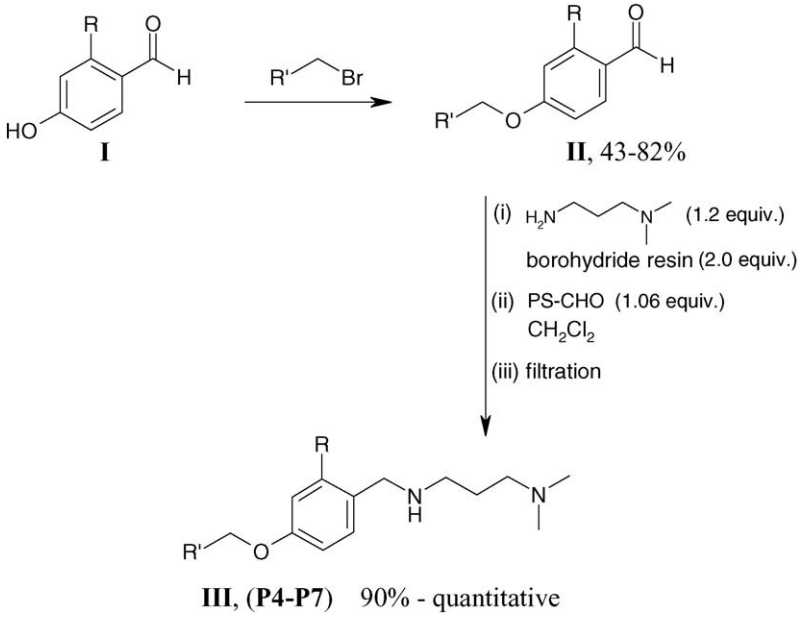


Fig. 2 – Synthetic scheme of potential dual acting antiplasmodial and CQ resistance modulator agents.

Reactions were monitored by thin layer chromatography using aluminium-backed silica gel $60F_{254}$ plates (Merk). Column chromatography was carried out on silica gel (Merk Kieselgel 60:70–230 mesh for gravity). ^{1}H NMR were recorded on a Varian Mercury (300 MHz) or a Varian Unity Spectrophotometer (300 MHz) and were recorded in parts per million (ppm) with respect to tetramethylsilane. ^{13}C NMR were recorded on the same machines but at 75 MHz. The masses were determined by the Department of Pharmacology (University of Cape Town) on an API2000 from Applied Biosystems. Elemental analysis was determined on a Fisons EA 110 CHN elemental analyzer. HPLC system consisted of Waters 1525 Binary HPLC Pump and Waters 996 photodiode array detector with C18 [2] 150 × 4.6 of 3 μm were used. Compounds were further purified using preparative TLC plates where necessary.

2.2. Chemistry

Synthesis of the polyamines P4–P7 (Fig. 1) involved the generation of aldehyde intermediates by alkylation using polymer-supported reagents with appropriate bromides, followed by a reductive amination of the aldehyde intermediates with 3-dimethyaminopropylamine to give the corresponding products. In the first step of the synthesis (Fig. 2), a polymer-assisted O-alkylation of the phenolic benzaldehyde (I) was carried out using a polystyrene-supported base PTBD (1, 5, 7-triazabicyclo[4,4,0]dec-5-ene). This polymer-bound reagent served as a base in the phenol deprotonation step as well as a scavenger to mop up the liberated HBr and the excess unreacted starting phenol. The resulting products (II) were isolated and purified in high yields by simple filtration and evaporation [24].

The second step involved a borohydride resin-mediated reductive amination of the aromatic aldehydes. Excess amine relative to the carbonyl intermediate (II) was used and the subsequent imine adduct was formed readily in methanol. Reduction was then performed using polymer-bound borohydride while the polymer-supported aldehyde functioned to selectively separate the desired secondary product and the excess starting primary amine by imine formation [25]. Subsequent filtration and evaporation afforded the secondary amines (P4–P7) in excellent yields and purity (Table 1). The target compounds were fully characterized by spectroscopic (NMR) and analytic techniques. The data obtained in this regard confirmed the integrity and purity of the target compounds.

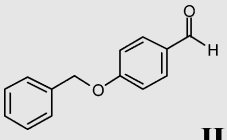
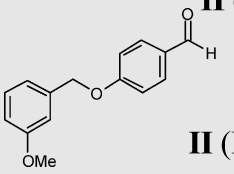
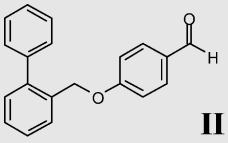
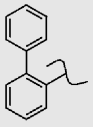
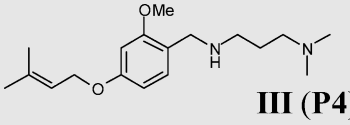
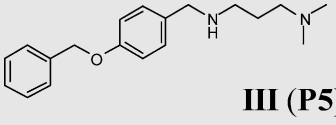
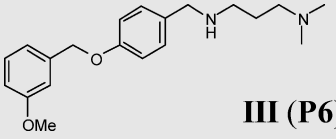
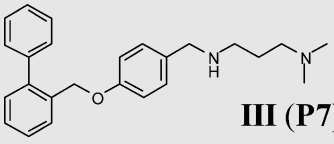
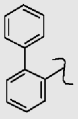
2.3. General procedure A: alkylation using PTBD

4-Hydroxybenzaldehyde (86 mg, 1.2 equiv.) and benzyl bromide (0.07 cm^3, 1.0 equiv.) were added to a 20 cm^3 sealed glass vial containing the PTBD resins (0.10 m equiv.) in 7 cm^3 acetonitrile. The reaction was vortexed vigorously at room temperature under nitrogen atmosphere until the halide was completely consumed. After 18 h, the reaction was filtered free of polymeric material. The filtrate was purified by chromatography on silica gel.

2.4. General procedure B: reductive amination reaction using borohydride resin

3-Dimethylaminopropylamine (0.03 cm^3, 0.27 mmol) and benzaldehydes (0.23 mmol) were dissolved in 1 cm^3 MeOH in a medium screw-capped glass vial. The reaction vial was shaken on an orbital shaker for 4 h to allow imine formation. The resulting reaction mixture was then treated with Amberlite® IRA-400 borohydride resin (2.5 mmol BH_4^-/g resin, 2.0 equiv.) (Aldrich). The slurry was then shaken for an additional 24 h to effect the reduction to the secondary amine. Lastly, the polystyrene-linked benzaldehyde resin (2.5–3.0 mmol/g resin,

Table 1 – Synthesized intermediates I1–I3 and compounds P4–P7 during the initial exploratory phase

Compound	R	R′	% Yield
 II (I1)	H		43
 II (I2)	H		77
 II (I3)	H		82
 III (P4)	OMe		94
 III (P5)	H		90
 III (P6)	H		Quantitative
 III (P7)	H		97

1.06 equiv.) and 1 cm^3 CH_2Cl_2 was added to the reaction. After 18 h, the reaction slurry was filtered through a cotton plug and the residual solids were rinsed with MeOH.

3. *N*′-[2-methoxy-4-(3-methyl-but-2-enyloxy)-benzyl]-*N*,*N*-dimethyl-propan-1,3-diamine (P4)

The conditions employed for the preparation of this compound were those described in Section 2. Simple filtration with MeOH gave the product P4 (76.2 mg, 94%) as a yellowish oil; R_f (MeOH–CH_2Cl_2, 10:90) 0.22; δ_H(300 MHz; $CDCl_3$) 7.12 (1H, d, *J* 8.0), 6.47–6.40 (2H, m), 5.48 (1H, t, *J* 7.5), 4.48 (2H, d, *J* 8.0), 3.79 (3H, s, –OCH_3), 3.70 (2H, s, –Ar–CH_2–N–), 2.63 (2H, t, *J* 7.5), 2.30 (2H, t, *J* 7.5), 2.20 (3H, s, –N–CH_3), 2.15 (3H, s, –N–CH_3), 1.79 (3H, s, CH_3–CH–CH_3), 1.74 (3H, s, CH_3–CH–CH_3) and 1.64 (2H, quintet, *J* 7.5); δ_C(75 MHz; $CDCl_3$)130.6 (2C), 119.6 (2C), 104.6 (2C), 99.3 (2C), 64.8, 58.2, 55.3, 48.5, 47.5, 45.4, 30.8, 27.2, 25.8 and 18.1; EIMS *m*/*z* (50 eV) 306 $[M]^+$, 220 $[C_{13}H_{18}NO_2]^+$, 205 $[C_{13}H_{17}O_2]^+$, 85 $[C_5H_9O]^+$, 30 $[CH_2O]^+$. LC–MS found M^+, 307.2. $C_{18}H_{30}N_2O_2{\cdot}0.04H_2O$ requires *M*, 307.17. (Found C, 64.01; H, 10.09; N, 8.14. $C_{18}H_{30}N_2O_2{\cdot}1.7H_2O$ requires C, 64.14; H, 9.99; N, 8.31%).

4. *N*′-(4-Benzyloxy-benzyl)-*N*,*N*-dimethyl-propan-1,3-diamine (P5)

The conditions employed for the preparation of this compound were those described in Section 2. Simple filtration with MeOH gave P5 (81.4 mg, 90%) as a yellowish oil; R_f (MeOH–CH_2Cl_2, 10:90) 0.22; δ_H(300 MHz; $CDCl_3$) 7.46–7.21 (7H, m, aromatic), 6.93 (2H, d, *J* 8.7), 5.05 (2H, s), 3.72 (2H, s), 2.65 (2H, t, *J* 7.5), 2.31 (2H, t, *J* 7.5), 2.23 (3H, s, –N–CH_3), 2.21 (3H, s, –N–CH_3)

and 1.72 (2H, quintet, *J* 7.5); δ_C(75 MHz; $CDCl_3$) 157.8, 137.1, 133.0, 129.2 (2C), 128.5, 128.0, 127.9 (2C), 127.4, 114.9, 114.8, 70.1, 58.0, 53.4, 47.8, 45.5 (2C), 28.1; EIMS *m/z* (50 eV) 298 $[M]^+$, 212 $[C_{14}H_{14}NO]^+$, 197 $[C_{14}H_{13}O]^+$, 107 $[C_7H_7O]^+$, 30 $[CH_2O]^+$. LC–MS found M^+, 299.3. $C_{19}H_{26}N_2O{\cdot}0.05H_2O$ requires *M*, 299.33. HPLC found 92.14% (partial decomposition on prolonged storage) at $t_R' = 8.428$ min at λ_{278} using acetonitrile and 0.1% formic acid.

5. *N*′-[4-(3-Methoxy-benzyloxy)-benzyl]-*N*,*N*-dimethyl-propane-1,3-diamine (P6)

The conditions employed for the preparation of this compound were those described in Section 2. Simple filtration with MeOH gave P6 (178 mg, quantitative yield) as a yellowish oil; R_f (MeOH–CH_2Cl_2, 10:90) 0.20; δ_H(300 MHz; $CDCl_3$) 7.32–7.21 (3H, m), 7.03–6.91 (4H, m), 6.95 (1H, dd, *J* 3.0 and 9.0), 5.02 (2H, s), 3.81 (3H, s, $–OCH_3$), 3.76 (2H, s), 2.73 (2H, t, *J* 7.5), 2.37 (2H, t, *J* 7.5), 2.25 (3H, s, $–N–CH_3$), 2.23 (3H, s, $–N–CH_3$) and 1.74 (2H, quintet, *J* 7.5); δ_C(75 MHz; $CDCl_3$) 159.9, 158.0, 138.6, 131.5, 129.6, 129.5, 128.5, 119.6, 114.9 (2C), 113.5, 112.9, 69.9, 58.0, 55.2, 52.7, 47.4, 45.1 (2C), 26.6; EIMS *m/z* (50 eV) 328 $[M]^+$, 242 $[C_{15}H_{16}NO_2]^+$, 227 $[C_{15}H_{15}O_2]^+$, 121 $[C_8H_9O]^+$, 107 $[C_7H_7O]^+$, 30 $[CH_2O]^+$. LC–MS found M^+, 329.2. $C_{20}H_{28}N_2O_2{\cdot}0.04H_2O$ requires *M*, 329.17. HPLC found 80.95% (partial decomposition on prolonged storage) at $t_R' = 8.291$ min at λ_{283} using acetonitrile and 0.1% formic acid.

6. *N*′-[4-(Biphenyl-2-ylmethoxy)-benzyl]-*N*,*N*-dimethyl-propane-1,3-diamine (P7)

The conditions employed for the preparation of this compound were those described in Section 2. Simple filtration with MeOH gave P7 (139 mg, 97%) as a yellowish oil; R_f (MeOH–CH_2Cl_2, 10:90) 0.38; δ_H(300 MHz; $CDCl_3$) 7.61–7.58 (1H, m), 7.39–7.20 (10H, m, aromatic), 6.79 (2H, d, *J* 9.0), 4.89 (2H, s), 3.73 (2H, s), 2.67 (2H, t, *J* 7.5), 2.39 (2H, t, *J* 7.5), 2.26 (3H, s, $–N–CH_3$), 2.25 (3H, s, $–N–CH_3$) and 1.73 (2H, quintet, *J* 7.5); δ_C(75 MHz; $CDCl_3$) 157.8, 141.8, 140.5, 134.1, 130.0, 129.5 (2C), 129.2, 129.1, 128.2, 128.0, 127.6 (2C), 127.3 (3C), 114.8 (2C), 68.1, 57.7, 53.0, 47.3, 45.2 (2C), 27.1; EIMS *m/z* (50 eV) 374 $[M]^+$, 288 $[C_{20}H_{18}NO]^+$, 167 $[C_{13}H_{11}]^+$, 29 $[C_2H_5]^+$. LC–MS found M^+, 375.2. $C_{25}H_{30}N_2O{\cdot}0.04H_2O$ requires *M*, 375.24. (Found C, 70.42; H, 8.12; N, 6.40. $C_{25}H_{30}N_2O{\cdot}2.8H_2O$ requires C, 70.66; H, 8.44; N, 6.59%).

6.1. *In vitro P. falciparum culture*

Three different strains of *P. falciparum* were selected for this study: D10 ($IC_{50}^{CQ} = 23$ nM), a CQ-sensitive strain (provided by the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia), RSA11 ($IC_{50}^{CQ} = 186$ nM) and K1 ($IC_{50}^{CQ} = 190$ nM), CQ-resistant strains (provided by Dr. Janet Freese, Malaria Research Programme, Medical Research Council of South Africa, Durban, South Africa; and isolated at Kanchnaburi, Thailand, respectively).

The parasites were maintained at 5% haematocrit in continuous culture by a method modified from that of Trager and Jensen [26]. *P. falciparum* parasites were cultivated in type O-positive human erythrocytes (Western Province Blood Transfusion Service, Groote Schuur Hospital, Cape Town, South Africa) with 10% type A-positive human serum. The culture was suspended in RPMI 1640 (Biowhittaeker) culture medium supplemented with 25 mM HEPES buffer (Sigma), 1% sodium bicarbonate and gentamicin (40 mg/ml) and hypoxanthine (44 mg/l). Medium was renewed daily and parasitaemia was determined using Giemsa stained blood smears of the cultures. The cultures were kept continuously at 37 °C under an atmosphere of 3% O_2, 4% CO_2 and 93% N_2. Cultures were synchronized by treatment with 5% D-sorbitol (Sigma) in the ring stage of development [27].

6.2. *Parasite lactate dehydrogenase assay*

The in vitro susceptibility tests for the parasites in the presence and absence of tested compounds were performed using a modified method by Makler et al. [28] The parasites were maintained at 1% haematocrit and 2% parasitaemia for 48h in the presence of the particular drug to be rested to determine the intrinsic antimalarial activity. For combination studies, the parasites were incubated with serially diluted CQ concentrations in the presence of a fixed concentration of each test compound. Using a non-linear regression analysis in GraphPad Prism (GraphPad Software Inc., 5755 Oberlin Drive, #110 San Diego, CA 92121, USA), fractional inhibitory concentrations (IC_{50} = 50% inhibitory concentration) of the synthesized compounds were determined graphically from semi-logarithmic plots.

6.3. *Isobologram analysis*

A fixed-ratio technique was adapted from Chawira and Warhurst, 1987 [29] to investigate the drug interactions of CQ and the hit compound, P7, which showed the most promising results from the primary in vitro screens, against both CQ^S and CQ^R isolates of *P. falciparum* in vitro. Prior to commencing this drug–interaction study, the acquired fractional inhibitory concentration values (IC_{50}) of CQ and P7 in D10 and RSA11 were predetermined from dose response curve experiments. However, it is noteworthy that inhibitory concentration variation between interaction studies is expected to produce different results [30].

The *P. falciparum* were maintained at 1% haematocrit and 2% parasitaemia for 48h. For the IC_{50} drug-interaction study, $2 \times IC_{50}$ concentration of CQ and P7 were prepared and added into the designed plates in the ratio of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90. The Malstat (Flow Inc.) reagent was used as an indicator for the assay and the resulting data were calculated and transformed into isobolograms by Sigmaplot and GraphPad Prism.

6.4. *^{3}H-CQ accumulation*

Synchronous parasitized red blood cells were harvested in the trophozoite growth development (1% haematocrit, 5% parasitaemia) in microcentrifuge tubes. Together with solvent controls, the tubes were pre-incubated in a 37 °C water bath for 15 min in the presence of a fixed concentration of the potential resistance reversers before being exposed to 20 μl of

pre-aliquoted 100 nM ^{3}H-CQ (18.8 Ci/mmol; Amersham) to achieve a final concentration of 1 nM ^{3}H-CQ in each tube. After 1 h incubation, 100 μl of dibutyl phthalate DBP (Sigma) was added into each tube and centrifuged. The supernatant was then aspirated off leaving the parasitized/uninfected erythrocytes behind. The tip of each eppendorff was cut into labeled scintillation vials to which 2 ml of scintillation liquid (Ultra-Gold™, Packard BioScience) was added in advance. It was followed by the addition of 25 μl EDTA into each vial to form a stable chelator complex before being lysed down with 100 μl of Solvable. Lastly, 100 μl of H_2O_2 (30%) was used to quench the colour effect of the red blood cells. The radioactivity within each vial was counted for 10 min in a Packard Tri-Carb 4060 liquid scintillation spectrophotometer.

6.5. *In vitro mammalian cell culture*

Chinese hamster ovarian (CHO) cells were maintained as adherent monolayers in Dulbecos modified eagles medium (DMEM), Ham F-12 (1:1) supplemented with 10% heat-inactivated fetal calf serum (FCS) and gentamycin (0.04 μg/ml). The cells were cultured in 5% CO_2 –95% air humidified atmosphere at 37 °C [31].

6.6. *MTT assay*

Selected synthesized compounds were assessed for cytotoxicity, in terms of mitochondrial impairment, using the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay [31,32].

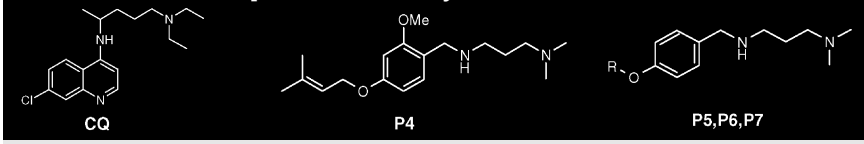
CHO cells were harvested when cells reached confluency and cell density was estimated using the Trypan blue method on a haemocytometer. CHO cells were plated in 96-well flat-bottomed microtitre plates (Costar) at a cell density of 1×10^4 cells per well, in which cells were ensured to be in the exponential growth. Cell dilutions were made accordingly and incubated at 5–95% CO_2, air humidified atmosphere at 37 °C for 24 h. After the incubation, the medium was carefully aspirated off from each well. Appropriate drug concentrations were then added accordingly into the plate. After incubating the plates for 48 h, 25 μl of a MTT stock solution of 5.0 mg/ml was added into each well of the plates, which were allowed to incubate for a further 4h. The plates were then centrifuged at 2050 rpm for 10 min and followed by medium removal from each well before DMSO was used to dissolve the formazan salts formed by viable cells. The plates were shaken cautiously on a microplate shaker for 5 min. Absorbance was monitored at a wavelength of 540 nm with a 7520 Microplate reader from Cambridge Technology Inc.

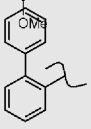
7. Results

7.1. *Intrinsic antimalarial activity*

The IC_{50} values of CQ and the synthesized compounds tested against D10, K1 and RSA11 strains are summarized in Table 2. The synthesized compounds all showed antiplasmodial activity against CQ-sensitive and resistant strains of *P. falciparum*. The intrinsic activity of P7 was comparable to CQ in the CQ resistant strain K1 (CQ: $IC_{50}^{K1} = 0.196\,\mu M$; P7: $IC_{50}^{K1} = 0.148\,\mu M$), even though it was less active than CQ in the RSA11 strain (CQ: $IC_{50}^{RSA11} = 0.181\,\mu M$; P7: $IC_{50}^{RSA11} = 0.600\,\mu M$). In addition to the IC_{50} values, the resistance index ($R_I = IC_{50}^{K1\,or\,RSA11}/IC_{50}^{D10}$) which gives an indication of the relative activity of the compounds in a drug resistant and sensitive

Table 2 – In vitro antiplasmodial activity[a]



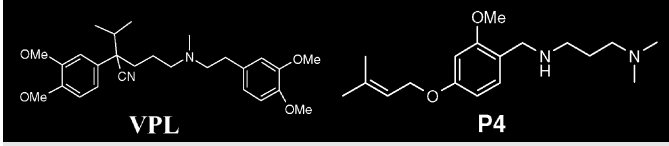
Compoundd	R	*Plasmodium falciparum* (μM)			R_I		CHO (μM)	*c* log P[b]
		D10	K1	RSA11	K1	RSA11		
CQ	–	0.024 ± 0.004	0.196 ± 0.047	0.181 ± 0.045	8.17	7.54	ND	5.06
VPL	–	6.46 ± 0.25	13.51 ± 1.24	11.15 ± 1.39	2.09	1.73	ND	4.47
P4	–	2.45 ± 0.042	2.29 ± 0.039	2.57 ± 0.048	0.93	1.05	>100	3.88
P5		1.10 ± 0.026	1.58 ± 0.055	1.86 ± 0.057	1.43	1.69	>100	3.86
P6		0.977 ± 0.030	0.830 ± 0.028	1.32 ± 0.047	0.85	1.35	>100	3.78
P7		0.174 ± 0.013	0.148 ± 0.032	0.600 ± 0.036	0.85	3.45	125.89 ± 3.15	5.45

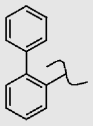
ND: not determined. CHO: Chinese hamster ovarian cells.

[a] Results are expressed as mean IC_{50} (μM) of three independent experiments performed in duplicate.

[b] *c* log *P* values were calculated and/or estimated using ChemDraw Ultra version 6.01. Resistance index, $R_I = IC_{50}^{K1\,or\,RSA11}/IC_{50}^{D10}$.

Table 3 – Resistance reversal effect



Compound	R	IC_{50}^{K1}	RMI_{K1}	IC_{50}^{RSA11}	RMI_{RSA11}
CQ	–	0.196 ± 0.047	–	0.181 ± 0.045	–
CQ + VPL	–	0.126 ± 0.042	0.42	0.109 ± 0.033	0.60
CQ + P4	–	0.144 ± 0.039	0.73	0.335 ± 0.033	1.85
CQ + P5		0.141 ± 0.031	0.72	0.234 ± 0.041	1.29
CQ + P6		0.148 ± 0.043	0.76	0.162 ± 0.019	0.90
CQ + P7		0.132 ± 0.026	0.67	0.148 ± 0.029	0.82

$RMI = IC_{50}^{CQ+drug}/IC_{50}^{CQ\ alone}$.

strain of *P. falciparum*, is reported. For new compounds, this gives an indication of whether or not the compound will be active against CQ^R strains. i.e. the lower the R_I value, the better.

Compounds P4, P5 and P6 were shown to be non-toxic against the CHO cell line with 100% cell viability even at the highest concentration of 100 μg/ml (Table 2). Compound P7 was cytotoxic at a concentration of 125.89 ± 3.15 μM which nevertheless was much higher than its intrinsic antimalarial IC_{50} value ($IC_{50}^{D10} = 0.174$ μM; $IC_{50}^{K1} = 0.148$ μM; $IC_{50}^{RSA11} = 0.600$ μM) among the *P. falciparum* parasites tested, indicating that compound P7 is selectively cytotoxic for *P. falciparum*.

7.2. Resistance reversal effects

The concentration selected for combination studies in CQ^R K1 and RSA11 strains was 0.03 μM for all drugs. This concentration was sub-lethal to the parasite cultures in order to be able to distinguish the resistance reversal from the antimalarial effect (Table 2). Results are shown in Table 3. VPL showed the most potent CQ resistance reversing activity among the drugs tested as its CQ + VPL dose-response experiments revealed 0.126 ± 0.047 and 0.109 ± 0.033 μM in K1 and RSA11, respectively. This was confirmed with the resistance modification indexes which indicate good reversing activity when RMI < 1 (RMI_{K1} = 0.42 and RMI_{RSA11} = 0.60). Amongst the synthesized compounds, the resistance reversal effect of P4, P5 and P6 were shown to be small and similar in K1 (Table 3) and marginal (P6) or antagonistic (P4 and P5) against RSA11. P7 was shown to be the best resistance reverser against both K1 and RSA11. In addition, the marked antiplasmodial activity and moderate resistance reversal effect of compound P7 on both CQ^R strains is noteworthy.

7.3. Tritiated CQ accumulation with combinations of compounds

The results presented in combination studies were validated in CQ accumulation experiments. The ability of the potential resistance reversers to stimulate the uptake of CQ was determined at concentrations of 1 and 10 μM of each compound. The results for K1 and RSA11 are presented in Fig. 3 as fold increase versus the CQ combination with different compounds at various concentrations. The parasitized red

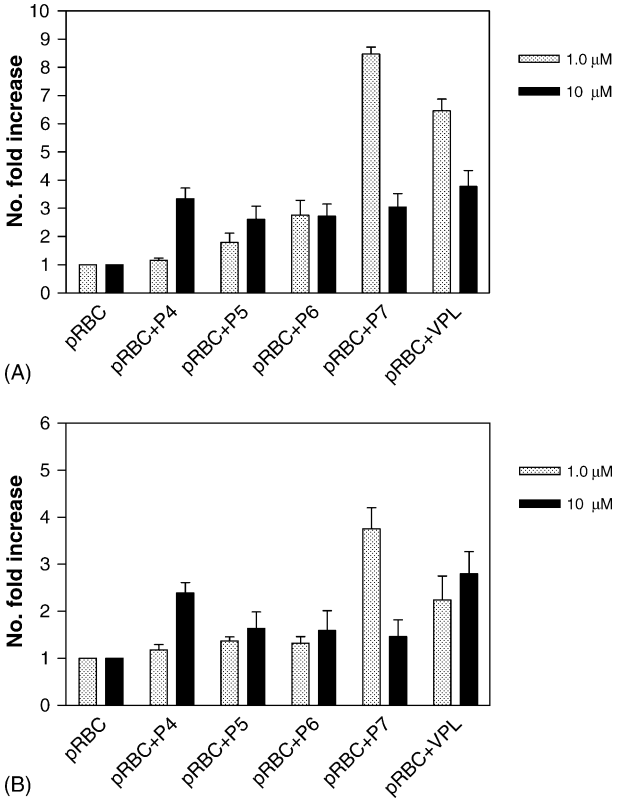


Fig. 3 – (A) Fold increase in ^{3}H-CQ uptake with combinations of drugs at different concentrations used on CQ^R, K1. (B) Fold increase in ^{3}H-CQ uptake with combinations of drugs at different concentrations used on CQ^R, RSA11.

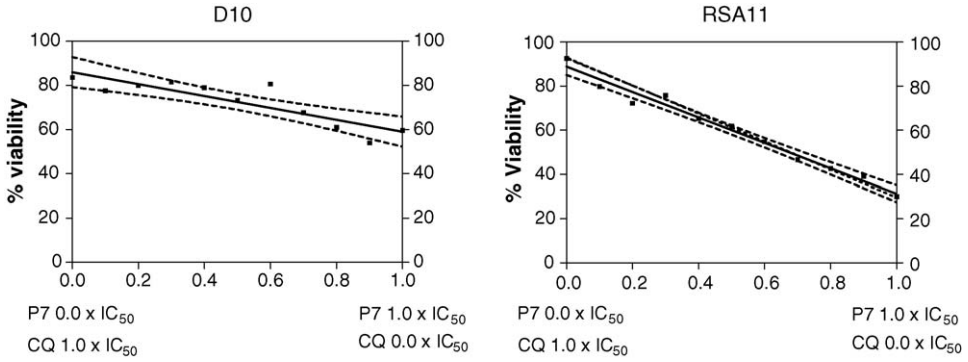


Fig. 4 – Additive effect illustrated by the combinations of CQ and P7 at their predetermined IC_{50} (straight line) against D10 and RSA11 with 95% confidence interval shown in dashed lines.

blood cell served as a control. Fold increase in uptake refers to the ratio of radio-labelled ^{3}H-CQ counts of the combination to the ^{3}H-CQ control. The data for all three strains tested are expressed as three independent experiments performed in triplicate.

Against the CQ^R strain, K1, the compounds potentiated CQ uptake to an extent of three to eight-fold with compound P7 showing an eight-fold increase in CQ uptake and the control VPL exhibiting a six-fold increase at a drug concentration of 1.0 μM in the same assay (Fig. 3). At a concentration of 10 μM both P7 and VPL showed decreased enhancement of CQ accumulation compared to 1 μM most likely due to a cytotoxic effect. A similar pattern of activity was found in RSA11. At the concentration of 1 μM in RSA11, compound P7 revealed a greater activity in potentiating CQ uptake two-fold higher than that of VPL. In contrast to its effect against K1, VPL showed slightly increased CQ potentiation in RSA11 at 10 μM, compared to its activity at 1 μM.

7.4. *Isobologram analysis of compound P7*

The graphic representations using fixed ratios of predetermined concentrations to determine the interactions of two different chemotherapeutic agents are shown in Fig. 4. If the effect is synergistic, the parasite viability values at the fixed ratios for both CQ and P7 are illustrated below the line and antagonism is indicated by values above the line. When the viability values are on the line the effect is additive.

In analyzing whether an additive or synergistic effect was exhibited by compound P7 in combination with CQ, the isobologram method was used. An additive effect was observed against both CQ^S and CQ^R strains when CQ and P7 were used at their corresponding IC_{50} concentrations (Fig. 4).

8. Discussion

Since the synthesized compounds are structurally unrelated to CQ, comparisons were made within this series of compounds produced, in which a lower activity in P4 was observed ($IC_{50} > 2$ μM while the others exhibited $IC_{50} < 2$ μM). This may be explained by the poly-aromatic substructure present in compounds P5, P6 and P7. All the compounds exhibited substantially lower antimalarial activity against the CQ sensitive strain, D10 when compared to CQ.

In terms of the relative activity of the compounds in different strains of *P. falciparum*, compound P4 was equally active against CQ^S and CQ^R strains ($R_I^{K1} = 0.93$; $R_I^{RSA11} = 1.05$). Compound P5 was slightly less active in the CQ resistant strains while P6 ($R_I^{K1} = 0.85; R_I^{RSA11} = 1.35$) and P7 ($R_I^{K1} = 0.85; R_I^{RSA11} = 3.45$) showed better activity against K1 than RSA11. This suggests that the hydrophobic structural feature incorporating substituents (methoxy group or another lipophilic aromatic ring) on the aromatic ring may improve their antiplasmodial activity against the K1 strain of malaria parasites as the more lipophilic compound, P7, (high *c* log *P* value) may be able to readily cross the parasite membrane and accumulate via pH trapping within the acidic parasite food vacuole. It is also noteworthy that none of the results obtained for a particular strain were significantly different from the others.

Like CQ, the compounds P4–P7 contain a hydrophobic aromatic nucleus and basic protonatable nitrogen atoms, which may be important for binding to the heme as well as uptake and concentration of these agents in the acidic compartment(s) of the parasites. Non-aminoquiline agents, the 4-aminopiperidines, in which basic nitrogens are arrayed in a similar fashion to CQ have been reported to be potent antimalarials [14,16]. Although evidence suggests that the 4-aminopiperidines act by inhibiting heme polymerization, the basic nitrogens are critical to the antimalarial potency of these agents.

Within the context of CQ modification, it was recently found that the antiplasmodial activity of phenothiazine derivatives of chloropromazine, increased with an increase in the number of basic groups in the alkylamino side chain, which among other things, may reflect increased uptake into the acidic parasitic digestive food vacuole [33].

All synthesized compounds showed moderate resistance modulating properties in the K1 strain whereas compound P7 demonstrated the best resistance reversal effect in both CQ^R strains ($RMI_{K1} = 0.67$ and $RMI_{RSA11} = 0.82$). Compound P4 was shown to be the least potent resistance reverser although it exhibited the least intrinsic toxicity. When the alkyl chain was substituted with an aromatic ring (P4 versus P5), the intrinsic antiplasmodial activity as well as the resistance reversal effect

were improved, suggesting the importance of an aromatic hydrophobic moiety. Furthermore, when an additional methoxy group was attached to the substituted aromatic ring (compound P5 versus P6), further enhancement in resistance reversing properties was observed in addition to increased antiplasmodial activity. Lastly, the effect of the biphenyl substructure in compound P7 was evident since compound P7, the analogue of P4, showed significant chemosensitization as well as antimalarial effects. The poly-aromatic structural element may be the pharmacophore that is responsible for interacting with protein(s) involved in the resistance mechanism. The moderate chemosensitizing activities observed with the drugs investigated in this study was most likely due to the fact that the chosen sub-lethal concentration of 0.03 μM was minimal. It is expected that the higher concentrations of these drugs would produce greater chemosensitization and antiplasmodial activity against CQ resistant *P. falciparum*. However, it would not be possible under experimental conditions to distinguish the antiplasmodial activity from resistance reversal effects at higher concentrations of the compounds due to the dual acting properties of the compounds.

Substantial literature on the accumulation of CQ by falciparum infected erythrocytes has shown that the cytotoxic effect of CQ is due to the increased uptake of CQ within malaria parasites. In CQ^R parasites, an effective chemosensitizer leads to a potentiation of CQ uptake within the malaria-infected red blood cells. ^{3}H-CQ was used in this accumulation assay and CQ^S strain D10 served as a control in this experiment (data not shown). Typical resistance reversal agents are characterized by increased CQ accumulation in addition to the shift in the CQ curve.

When the compounds are compared within CQ^R strains, clearly compound P7 is the most probable candidate as a resistance reversal agent among all the synthesized compounds. This compound not only exhibited moderate intrinsic antimalarial activity against K1 (Table 2), but also enhanced CQ action in a resistance reversal experiment (Table 3), a result confirmed in CQ accumulation experiments (Fig. 3). Additionally, it is noteworthy that compound P7 was shown to possess the highest antimalarial activity of all the compounds tested, with comparable activity to CQ against CQ^R K1 strain as well as the most cytotoxic.

Comparing the antiplasmodial activities of compound P7 and CQ (Table 2), the general structural similarities observed are the basic nitrogens and the aromatic structural features, which may suggest a similar mechanism of action. This may also suggest that the highest antiplasmodial activity P7 possesses among the compounds tested may be due to the tricyclic hydrophobic chemical feature. The importance of the 4-amino-7-chloroquinoline moiety in binding to the heme may account for the superior antiplasmodial activity of CQ and relative to compound P7. The tricyclic moiety of P7 may be involved in binding to heme, albeit presumably weakly, which may account for the superior antiplasmodial activity of P7 relative to the other synthesized compounds. Possible binding to heme of compound P7 may be enhanced by further structure–activity relationship studies focusing on the nature and relative position (relative to the side chain) of the tricyclic (or even bicyclic) moiety.

This study demonstrated that novel exploratory compounds synthesized all possess intrinsic (albeit weak to modest) antiplasmodial activity against CQ-sensitive and -resistant *P. falciparum* parasites. Further, in vitro studies of resistance reversal in *P. falciparum* and cytotoxicity on mammalian cells showed that compound P7 exhibited the greatest potential as a dual-acting antimalarial agent and CQ resistance reverser. From a therapeutic point of view, it is in fact an advantage to develop dual-acting antimalarial agents as drugs showing both antimalarial and CQ potentiating activity would be active against CQ^R parasites strains [21]. In addition, compound P7 showed selective cytotoxicity and high resistance reversal effects that were comparable to VPL. If SAR trends could be clearly delineated, the biphenyl nucleus could be a useful scaffold on which to base antiplasmodial and/or CQ resistance modulators drug discovery efforts.

Acknowledgements

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