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Novel phenothiazine antimalarials: synthesis, antimalarial activity, and inhibition of the formation of β-haematin

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

[a]*Department of Chemistry, La Trobe University, Victoria 3086, Australia*
[b]*Department of Biochemistry, La Trobe University, Victoria 3086, Australia*

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Abstract

We report the synthesis of a series of novel phenothiazine compounds that inhibit the growth of both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. We found that the antimalarial activity of these phenothiazines increased with an increase in the number of basic groups in the alkylamino side chain, which may reflect increased uptake into the parasite food vacuole or differences in the toxicities of individual FP–drug complexes. We have examined the ability of the parent phenothiazine, chlorpromazine, and some novel phenothiazines to inhibit the formation of β-haematin. The degree of antimalarial potency was loosely correlated with the efficacy of inhibition of β-haematin formation, suggesting that these phenothiazines exert their antimalarial activities in a manner similar to that of chloroquine, i.e. by antagonizing the sequestration of toxic haem (ferriprotoporphyrin IX) moieties within the malaria parasite. Chlorpromazine is an effective modulator of chloroquine resistance; however, the more potent phenothiazine derivatives were more active against chloroquine-sensitive parasites than against chloroquine-resistant parasites and showed little synergy of action when used in combination with chloroquine. These studies point to structural features that may determine the antimalarial activity and resistance modulating potential of weakly basic amphipaths.

Keywords: Malaria; Phenothiazine; Chlorpromazine; Chloroquine; Haem; Drug resistance

1. Introduction

The antimalarial activity of methylene blue, a phenothiazine compound, was described by Guttmann and Erlich over 100 years ago [1]. Subsequently, analogs of methylene blue with improved antimalarial activity were prepared by replacing one methyl group with a basic side chain. This led to the belief that the basic side chain was beneficial to antimalarial activity, which in turn led German researchers working in the laboratories of IG Farben, at Bayer Corporation, in the 1920s to synthesize the first synthetic antimalarial, pamaquine (see [2] for review). Pamaquine was later found to be toxic, and primaquine was synthesized as a more useful analog. Primaquine is still used to eradicate the refractory hypnozoites (liver reservoirs) of *Plasmodium vivax* and *Plasmodium ovale* (see [3] for review).

Methylene blue, and some of its derivatives, have been shown recently to bind FP and to have antimalarial activities that are partially correlated with their abilities to inhibit β-haematin formation [4]. These data suggest that methylene blue exerts its antimalarial activity by a mechanism that is similar to that of CQ (Fig. 1A). These studies led to the suggestion that the phenathiazine structure should be explored further as a basis for the development of novel antimalarial drugs [4].

CPZ is a phenothiazine that is employed clinically as an antipsychotic agent. It possesses some antimalarial activity, but its potency is too low to be therapeutically useful [5,6]. Of particular interest, however, is the finding that, in common with a number of other weakly basic amphipaths, CPZ can enhance the potency of CQ against CQ-resistant strains of *Plasmodium falciparum*, while having no effect on the activity of CQ against CQ-sensitive parasites [6–9]. Resistance-modulating reagents have been shown to enhance CQ uptake by CQ-resistant parasites, although

* 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; MOG, monooleoyl glycerol; Pgh1, P-glycoprotein homologue-1; CPZ, chlorpromazine; CPT, 2-chlorophenothiazine; PCP, prochlorperazine; CQ, chloroquine.

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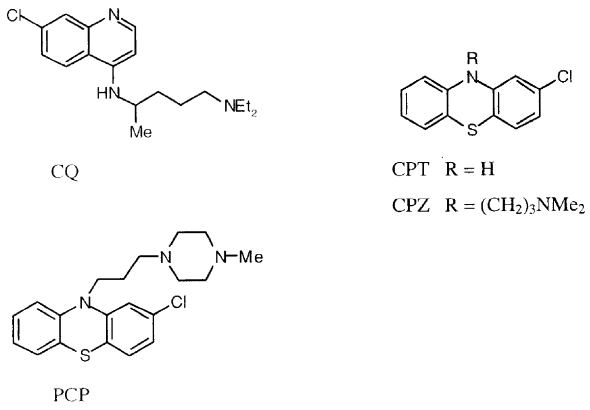
A



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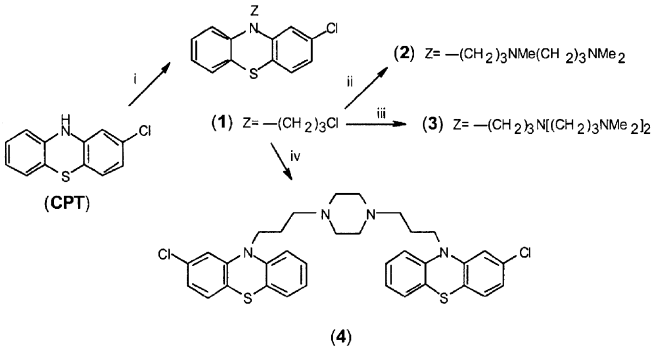
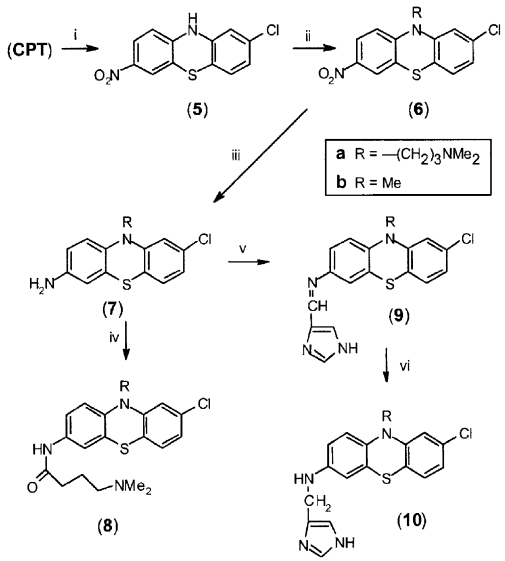


(i) $NaH/DMSO/THF/I(CH_2)_3Cl/20°C$ (ii) $MeNH(CH_2)_3NMe_2$/butanol/reflux
(iii) $HN[(CH_2)_3NMe_2]_2$/butanol/reflux (iv) piperazine/$NaHCO_3$/butanol/reflux

Scheme 1



(i) $NaNO_2/CHCl_3/HOAc/20°C$ (ii) **a**: $K_2CO_3/NaOH/TBAHS/Me_2N(CH_2)_3Cl$/toluene/reflux;
b: NaH/DMSO/THF/MeI/20°C (iii) H_2/10%Pd-C/EtOH
(iv) $Me_2N(CH_2)_3CO_2H$/CDI/dioxan/reflux (v) 4(5)-imidazolecarboxaldehyde/EtOH/reflux
(vi) $NaBH_4$/MeOH

Scheme 2

Fig. 1. (A) Structures of pre-existing compounds included in the present study. (B) Schemes for the synthesis of new phenothiazine derivatives (**2**)–(**4**), (**8**), and (**10**).

it has been suggested that they may also alter the drug target [10,11]. The mechanisms by which these drugs inhibit parasite growth and reverse CQ resistance are not understood; however, we have made the interesting observation that CPZ and some other resistance-modulating agents bind FP and inhibit the detoxification of FP by H_2O_2-mediated decomposition [12]. This interaction may underlie the weak antimalarial activity of these drugs.

To further explore the usefulness of phenothiazine compounds as potential antimalarials and/or resistance reversing reagents, we have now prepared a series of CPZ analogues and examined their antimalarial activities as well as their activities as inhibitors of the formation of β-haematin.

2. Materials and methods

2.1. Materials

CQ, PCP, CPZ, bovine serum albumin (essentially fatty acid free), 1-monooleoyl-rac-glycerol, and FP (bovine haematin) were obtained from the Sigma Chemical Co. Fresh human erythrocytes were obtained from the Red Cross Transfusion Service, Melbourne, Australia.

2.2. Synthesis of phenothiazine derivatives

NMR spectra were recorded on a Bruker AM-300 spectrometer operating at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C). Various standard techniques were used to identify proton-bound carbons in ^{13}C NMR spectra. The electrospray mass spectra were obtained on a VG Bio-Q triple quadrupole mass spectrometer using a water/methanol/acetic acid (50:50:1) mobile phase. Microanalyses were carried out at the Campbell Microanalytical Laboratory, University of Otago.

2.2.1. 2-Chloro-10-(3-chloropropyl)-10H-phenothiazine (*1*)

This compound was prepared from CPT as reported by Harrold *et al.* [13], and the crude material (85%) was used in further reactions.

2.2.2. 2-Chloro-10-(3-(N-(3-dimethylamino)propyl-N-methyl)amino)propyl-10H-phenothiazine (*2*)

N,N,N′-Trimethyl-1,3-propanediamine (0.33 g, 2.84 mmol) was added to a solution of (**1**) (0.30 g, 0.97 mmol) in *n*-butanol (10 mL), and the reaction was heated under reflux for 18 hr. After being cooled, the solvent was removed at reduced pressure, 10% sodium hydroxide was added, and the mixture was extracted with chloroform. The extract was washed three times with water, dried over magnesium sulfate, and the solvent was removed at reduced pressure to give a dark semisolid (0.31 g, 82%). ^{1}H NMR ($CDCl_3$): δ 1.56 (m, CH_2), 1.89 (m, CH_2), 2.16 (s, NCH_3), 2.18 (s, $N(CH_3)_2$), 2.23 (t, $J = 7.3$ Hz, CH_2), 2.31 (t, $J = 7.3$ Hz, CH_2), 2.42 (t, $J = 6.8$ Hz, CH_2), 3.87 (t, $J = 6.7$ Hz, CH_2), 6.82–7.13 (m, 7H). ESMS *m/z*: 390.1 ^{35}Cl (100%), 392.1 ^{37}Cl (38%) (both $M + 1$).

The bis oxalate salt was a beige powder, m.p. 193–195° (from ethanol). Anal. Calc. for $C_{21}H_{28}Cl_2N_3S{\cdot}2C_2H_2O_4$: C, 52.7; H, 5.7; N, 7.4. Found: C, 52.6; H, 5.8; N, 7.4%.

2.2.3. 2-Chloro-10-[3-(N,N-bis(N,N-(dimethylamino)-propyl)amino)propyl]-10H-phenothiazine (*3*)

A solution of 3,3′-iminobis(*N,N*-dimethylpropylamine) (0.73 g, 3.90 mmol) and (**1**) (0.4 g, 1.29 mmol) in *n*-butanol (20 mL) was heated under reflux for 21 hr. The solvent was then removed at reduced pressure, 10% sodium hydroxide (10 mL) was added, and the mixture was extracted with chloroform. The extract was washed with water and dried over magnesium sulfate, and the solvent was removed at reduced pressure to produce a dark oil. ^{1}H NMR (d^6-DMSO): δ 1.40 (m, $2CH_2$), 1.73 (m, CH_2), 2.04–2.15 (m + s, $2CH_2 + 4N(CH_3)_2$), 2.29 (m, $2CH_2$), 2.41 (m, CH_2), 3.91 (br, t, CH_2), 6.96–7.14 (m, 7H). ESMS *m/z*: 461.3 ^{35}Cl (81%), 463.3 ^{37}Cl (32%) (both $M + 1$); 231.1 ^{35}Cl (100%), 231.9 ^{37}Cl [$(M + 2)/2$].

The tris oxalate salt was a blue solid, m.p. 118–20° (from ethanol). Anal. Calc. for $C_{25}H_{37}ClN_4S{\cdot}3C_2H_2O_4{\cdot}2H_2O$: C, 48.5; H, 6.2; N, 7.3. Found: C, 48.2; H, 5.85; N, 7.4%.

2.2.4. 1,4-Bis[3-(2-chloro-10H-phenothiazin-10-yl)propyl]piperazine (*4*)

A mixture of piperazine (0.04 g, 0.47 mmol), anhydrous sodium bicarbonate (0.08 g, 0.95 mmol), and (**1**) (0.30 g, 0.97 mmol) in *n*-butanol (15 mL) was heated under reflux for 41 hr. After being cooled, the solvent was removed at reduced pressure, and the residue was taken up into 10% sodium hydroxide and extracted with chloroform. The extract was washed with water and dried over magnesium sulfate, and the solvent was removed at reduced pressure to give the dark product (0.54 g, 86%). This was recrystallized from methanol as a pink solid, m.p. 161–63°, which, from microanalysis, appeared to be a carbonate salt. ^{1}H NMR ($CDCl_3$): δ 1.90 (m, CH_2), 2.40–2.45 (m, $3CH_2$), 3.86 (t, $J = 6.8$ Hz, CH_2), 6.82–7.14 (m, 7 H). Anal. Calc. for $C_{34}H_{34}Cl_2N_4S_2{\cdot}H_2CO_3{\cdot}H_2O$: C, 58.9; H, 5.4; N, 7.8. Found: C, 58.8; H, 5.4; N, 7.9%.

2.2.5. 2-Chloro-7-nitro-10H-phenothiazine (*5*)

CPT (2.50 g, 10.7 mmol) was added portionwise to a solution of sodium nitrite (1.30 g, 18.8 mmol) in chloroform (25 mL and acetic acid (7 mL and stirred at room temperature for 2 hr. The dark mixture was filtered and washed with water to give a dark reddish brown solid (1.87 g, 63%), m.p. 220–225°. ^{1}H NMR (d^6-DMSO): δ 6.67–6.70 (m, H-1,4), 6.87 (dd, $J = 6.1, 2.2$ Hz, H-3), 6.95 (d, $J = 8.3$ Hz, H-9), 7.76 (d, $J = 2.5$ Hz, H-6), 7.86 (dd, $J = 8.9, 2.5$ Hz, H-8), 9.60 (s, NH).

2.2.6. 2-Chloro-7-nitro-10-(3-(dimethylamino)-propyl)-10H-phenothiazine hydrochloride (**6a**)

This compound was prepared from (**5**) by a method reported for CPZ [14]. The reaction mixture was filtered, the solid was washed with toluene, the toluene was removed from the filtrate at reduced pressure, and 15% hydrochloric acid was added. The reddish-brown hydrochloride salt that formed was filtered off (97%), and had m.p. 204–205°. ^{1}H NMR (free base, $CDCl_3$): δ 1.91 (m, $J = 6.9$ Hz, CH_2), 2.21 (s, $N(CH_3)_2$), 2.38 (t, $J = 6.7$ Hz, CH_2), 3.94 (t, $J = 7.1$ Hz, CH_2), 6.88–7.02 (m, 4H), 7.93 (d, $J = 2.4$ Hz, H-6), 8.02 (dd, $J = 9.1$, 2.3 Hz, H-8).

2.2.7. 2-Chloro-10-methyl-7-nitro-10H-phenothiazine (**6b**)

This compound was prepared from (**5**) and methyl iodide, by the same method as for (**1**). The crude product (95%) was an orange solid, m.p. 216–18°. ^{1}H NMR ($CDCl_3$): δ 3.36 (s, CH_3), 6.76–6.81 (m, 2H), 6.96–7.05 (m, 2H), 7.94 (d, $J = 2.5$ Hz, H-6), 8.02 (dd, $J = 8.9$, 2.6 Hz, H-8).

2.2.8. 7-Amino-2-chloro-10-(3-(dimethylamino)propyl)-10H-phenothiazine (**7a**)

A mixture of the free base of (**6a**) (0.83 g, 2.3 mmol) and 10% palladium on carbon (0.40 g) in ethanol was hydrogenated at atmospheric pressure for 5 hr. The catalyst was filtered off through Celite, and the solvent was removed at reduced pressure. The blue residue was taken up in chloroform, washed with water, and dried over magnesium sulfate, and the solvent was removed at reduced pressure to give the red product (0.75 g, 98%). ^{1}H NMR ($CDCl_3$): δ 1.95 (m, CH_2), 2.22 (s, $N(CH_3)_2$), 2.42 (t, $J = 7.0$ Hz, CH_2), 3.80 (t, $J = 6.6$ Hz, CH_2), 6.45–6.50 (m, 2H), 6.66 (d, $J = 8.3$ Hz, H-9), 6.80–6.84 (s + d, 2H), 6.97 (d, $J = 8.0$ Hz, H-4).

2.2.9. 7-Amino-2-chloro-10-methyl-10H-phenothiazine (**7b**)

Hydrogenation of (**6b**) over 10% palladium on carbon, as for the preparation of (**7a**), gave a crude red product (93%) that was used in this state in further reactions. ^{1}H NMR ($CDCl_3$): δ 3.27 (s, CH_3), 6.45–6.51 (m, 2H), 6.60 (d, $J = 8.2$ Hz, H-9), 6.71 (d, $J = 2.2$ Hz, H-1), 6.84 (dd, $J = 8.2$, 2.2 Hz, H-3), 6.97 (d, $J = 8.2$ Hz, H-4). ESMS *m/z*: 263.1 ^{35}Cl (100%), 265.1 ^{37}Cl (43%) (both $M + 1$).

2.2.10. N-[8-Chloro-10-(3-dimethylaminopropyl)-10H-phenothiazin-3-yl]-4-(dimethylamino)butyramide (**8a**)

A solution of 4-(dimethylamino)butyric acid hydrochloride (0.06 g, 0.36 mmol) and 1,1′-carbonyldiimidazole (0.11 g, 0.68 mmol) in dioxan (10 mL) was heated under reflux for 1 hr. The solution was cooled, (**7a**) (0.10 g, 0.27 mmol) was added, and the solution was heated under reflux for 1 hr. After being cooled the solvent was removed at reduced pressure, the residue was taken up in dichloromethane and this was washed with water, 10% sodium carbonate, water, and dried over magnesium sulfate, and the solvent was removed at reduced pressure to give a brown semisolid (40 mg, 34%). ^{1}H NMR ($CDCl_3$): δ 1.80–.93 (m, $2CH_2$), 2.18 (s, $2N(CH_3)_2$), 2.27 (s, $2N(CH_3)_2$), 2.33–2.46 (m, $3CH_2$), 3.82 (t, $J = 6.8$ Hz, CH_2), 6.75–6.85 (m, 3H), 6.96 (d, ^{1}H, $J = 7.7$ Hz), 7.27–7.31 (m, 2H), 9.75 (s, CONH). ESMS *m/z*: 447.1 ^{35}Cl (100%), 449.1 ^{37}Cl (36%) (both $M + 1$); 224.0 ^{35}Cl (98%), 226.0 ^{37}Cl (42%) [both $(M + 2)/2$].

The bis oxalate salt was a brown solid, m.p. 152–154° (from ethanol). Anal. Calc. for $C_{23}H_{31}ClN_4OS \cdot 2C_2H_2O_4 \cdot 0.5H_2O$: C, 51.0; H, 5.7; N, 8.8. Found: C, 51.0; H, 5.6; N, 8.7%.

2.2.11. N-(8-Chloro-10-methyl-10H-phenothiazin-3-yl)-4-(dimethylamino)butyramide (**8b**)

Amidation of (**7b**) was carried out as for the preparation of (**8a**). The product was a brown solid (52%), m.p. 155–156° after recrystallization from light petroleum (b.p. 90–110°). ^{1}H NMR ($CDCl_3$): δ 1.82 (m, $2CH_2$), 2.28 (s, $N(CH_3)_2$), 2.45 (m, CH_2), 3.29 (s, CH_3), 6.69–6.72 (m, 2H), 6.84 (d, 1H, $J = 8.1$ Hz), 6.97 (d, 1H, $J = 8.3$ Hz), 7.29–7.32 (m, 2H), 9.80 (s, NH). Anal. Calc. for $C_{19}H_{22}ClN_3OS$: C, 60.7; H, 5.9; N, 11.2. Found: C, 60.7; H, 5.9; N, 11.0%.

2.2.12. [8-Chloro-10-(3-(dimethylamino)propyl)-10H-phenothiazin-3-yl]-(3H-imidazol-4-yl-methylene)amine (**9a**)

A solution of (**7a**) (0.73 g, 2.19 mmol) and 4(5)-imidazolecarboxaldehyde (0.23 g, 2.39 mmol) in ethanol (20 mL) was heated under reflux for 20 min. After being cooled, the solvent was removed at reduced pressure and the residue was crystallized from acetonitrile and washed with water to give a yellow solid (0.48 g, 53%), m.p. 258–260°. ^{1}H NMR ($CDCl_3$): δ 1.81 (m, CH_2), 2.20 (s, $N(CH_3)_2$), 2.57 (m, CH_2), 3.64 (t, $J = 6.3$ Hz, CH_2), 6.53–6.71 (m, 6H), 7.15 (s, 1H), 7.31 (s, 1H), 8.01 (s, 1H).

2.2.13. [8-Chloro-10-methyl-10H-phenothiazin-3-yl]-(3H-imidazol-4-yl-methylene)amine (**9b**)

This was prepared from (**7b**) and 4(5)-imidazolecarboxaldehyde, as for (**9a**), as a green solid (77%), m.p. 244–246° (from acetonitrile and washed with water). ^{1}H NMR (d^6-DMSO): δ 3.29 (s, CH_3), 6.71–7.00 (m, 6H), 7.45 (s, 1H), 7.61 (s, 1H), 8.31 (s, 1H).

2.2.14. [8-Chloro-10-(3-(dimethylamino)propyl)-10H-phenothiazin-3-yl]-(3H-imidazol-4-yl-methyl)amine (**10a**)

Sodium borohydride (0.05 g, 1.32 mmol) was added to a solution of (**9a**) (0.44 g, 1.07 mmol) in methanol (15 mL). The reaction mixture was heated under reflux for 15 min. After being cooled, 10% sodium hydroxide (10 mL) was added, and the mixture was extracted with chloroform. The extract was washed with water and dried over magnesium sulfate, and the solvent was removed at reduced pressure to produce a green solid (0.40 g, 90%).

The bis oxalate salt was a light green solid, m.p. 118–120° (from ethanol). ^{1}H NMR (d^6-DMSO): δ 1.97 (m, CH_2), 2.68 (s, $N(CH_3)_2$), 3.07 (m, CH_2), 3.83 (m, CH_2), 4.14 (s, CH_2), 6.51–6.55 (m, 2H), 6.81–7.17 (m, 5H), 7.99 (s, 1H). Anal. Calc. for $C_{21}H_{24}ClN_5S{\cdot}2C_2H_2O_4$: C, 50.5; H, 4.8; N, 11.8. Found: C, 50.3; H, 5.1; N, 11.9%.

2.2.15. [8-Chloro-10-methyl-10H-phenothiazin-3-yl]-(3H-imidazol-4-yl-methyl)amine (**10b**)

This was prepared from (**9b**), as described for (**10a**), and was obtained as a brown solid (87%). ESMS *m/z*: 343.0 ^{35}Cl (100%), 345.0 ^{37}Cl (37%) (both $M + 1$).

The oxalate salt was a beige powder, m.p. 135–137° (from ethanol). ^{1}H NMR (d^6-DMSO): δ 3.19 (s, CH_3), 4.22 (s, CH_2), 6.50–7.31 (m, 7H), 8.54 (s, 1H). Anal. Calc. for $C_{17}H_{15}ClN_4S{\cdot}C_2H_2O_4{\cdot}H_2O$: C, 50.6; H, 4.2. Found: C, 50.5; H, 4.0%.

2.3. Absorption spectroscopy

The interaction of the phenothiazines with FP was examined by monitoring their effect on the absorption profile of FP. Samples were prepared either in 50 mM sodium phosphate, pH 7.4, or in 43% methanol in 10 mM sodium acetate, pH 5.5, and visible absorption spectra were collected using a Cary 1E spectrophotometer. Drugs were added from stocks in water except for CPT, which was added from a stock in DMSO. An equivalent amount of DMSO was added to a control sample.

2.4. β-Haematin formation

Assays of the conversion of FP to β-haematin were performed as described by Fitch *et al.* [15]. A suspension of MOG 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 100 mM FP and 0.2 mM MOG. Samples were incubated at 37° for 24 hr with gentle rotation. Following incubation, the samples were centrifuged at 27,000 *g*, 4° for 15 min. The β-haematin was washed four times by resuspending the pellet in 10 mM sodium phosphate, pH 7.4, containing 2.5% SDS and vortexing for 5 min at 20°, before repelleting. The remaining pellet was resuspended in 950 mL of 2.5% SDS in phosphate buffer, and a 50-mL aliquot of 1 M NaOH was added to dissolve the β-haematin. Drugs were added from stock solutions in water except for CPT, which was added from a stock in DMSO. An equivalent amount of DMSO was added to a control sample.

2.5. Assessment of antimalarial activity of the phenothiazines

D10 and K1 are CQ-sensitive and CQ-resistant strains of *P. falciparum* [16]. 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 hr, with daily replacement of the drug-supplemented medium. Growth curves based on the uptake of [^{3}H]hypoxanthine were determined. Interactions between drugs used in combination were determined by the method of Berenbaum [18]. Briefly, three different combinations of CQ with either CPZ or compound (**3**) 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 used in the combination were added to give the sums of the FIC values (SFIC), which were used to construct isobolograms as described by Adovelande *et al.* [19].

3. Results and discussion

3.1. Chemistry

Two synthetic sequences provided the compounds of Table 1. The known compound (**1**), prepared from CPT [13], was reacted with two secondary amines to produce (**2**) and (**3**), while reaction with one-half mole equivalent of piperazine gave the bis derivative (**4**) (Fig. 1B, Scheme 1).

The second set of compounds all contained a 7-substituent that included an additional basic function, and were accessed through the key nitro compound (**5**) (Fig. 1B, Scheme 2). This compound had been synthesized previously by a Smiles rearrangement [20], but we found that nitration of CPT under conditions used for phenothiazine [21] gave a good yield of **5**, a rather insoluble compound conveniently isolated as the hydrochloride salt. Two variations on ring-N substituent were then introduced; reaction with 3-dimethylaminopropyl chloride by a phase transfer method [14] and with methyl iodide under the conditions used to prepare (**1**) gave (**6a**) and (**6b**), respectively. Nitro group reduction was best carried out by catalytic hydrogenation over palladium/carbon, and the resulting amino functions in (**7a**) and (**7b**) were then derivatized. 1,1′-Carbonyldiimidazole-assisted coupling with 4-dimethylaminobutyric acid gave the amides (**8a**) and (**8b**). Alternatively, ready condensation with 4(5)-imidazolecarboxaldehyde gave the Schiff bases (**9a**) and (**9b**), which were reduced with sodium borohydride in methanol to the final compounds (**10a**) and (**10b**). Compounds (**4**) and (**8b**) were purified by crystallization of the free bases. The remaining compounds of Table 1 were more readily characterized as their oxalate salts.

Table 1
Inhibition of β-haematin formation and IC_{50} values and resistance index values for inhibition of growth of *P. falciparum in vitro* by some novel phenothiazines

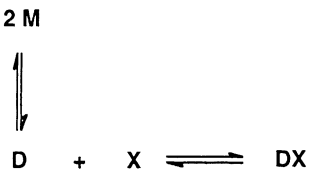
Compound	Inhibition of β-haematin formation (IC_{50}, μM)	Inhibition of growth of D10 (IC_{50}, μM)	Inhibition of growth of K1 (IC_{50}, μM)	Resistance index
CQ	32 ± 4 (14)	0.03 ± 0.01 (10)	0.29 ± 0.05 (8)	10
CPZ	78 ± 9 (3)	10 ± 2 (3)	11 ± 1 (3)	1
CPT	>500 (3)	ND	ND	ND
PCP	71 ± 14 (5)	8 ± 3 (3)	9 ± 3 (3)	1
(**2**)	98 ± 5 (3)	0.5 ± 0.1 (5)	1.0 ± 0.2 (4)	2
(**3**)	57 ± 4 (3)	0.12 ± 0.03 (6)	0.42 ± 0.15 (4)	3.5
(**4**)	>500 (3)	18 ± 1 (3)	12 ± 3 (3)	0.7
(**8a**)	138 ± 9 (3)	0.4 ± 0.1 (3)	2.0 ± 0.3 (3)	5
(**8b**)	135 ± 3 (3)	7 ± 1 (3)	7 ± 1 (3)	1
(**10a**)	124 ± 4 (3)	1.7 ± 0.2 (3)	1.8 ± 0.5 (3)	1
(**10b**)	>500 (3)	7.8 ± 0.7 (3)	12 ± 4 (4)	1.5

Data for inhibition of β-haematin formation are for triplicate measurements in a typical experiment. Data for inhibition of parasite growth are average IC_{50} values ± SD determined from at least three growth curves prepared on separate days, each of which was performed in triplicate. The numbers in parentheses refer to the number of experiments. The Resistance Index was calculated as the IC_{50} value for K1 divided by the IC_{50} value for D10. ND = not determined.

3.2. Spectroscopic studies of FP–phenothiazine interactions

FP is soluble in phosphate buffer at pH 7.4, although it forms self-associated complexes that involve a range of interactions, including ring-stacking, oxo-bridge formation, iron-propionic acid coordination and electrostatic and hydrophobic interactions [22]. The extent of aggregation increases with time. We have examined the spectral characteristics of FP diluted from a stock in 50 mM NaOH in the presence or absence of CQ and a number of phenothiazine compounds. Under these conditions, FP exhibited a Soret absorption band with a peak at 385 nm and a shoulder at 360 nm (Fig. 2). These spectral characteristics reflect the presence of ferric FP as a high-spin pentacoordinate hydroxyl liganded species [23]. The binding of CQ to FP caused a small red-shift and a decrease in the Soret absorption peak (Fig. 2A), as has been reported previously [24]. CPZ also appears to interact with FP, causing a red-shift of the Soret band to a wavelength maximum of 395 nm, and a decrease in the absorbance, particularly at the 360 nm shoulder (Fig. 2B). Mixing of FP with compound (**3**) also caused a red-shift and a decrease in the Soret absorption peak (Fig. 2C). These data suggest an interaction between these phenothiazines and FP that may be similar in nature to that of CQ.

It is more difficult to study the interaction of the drugs with FP under the pH conditions of the food vacuole due to the poor solubility of FP at low pH. However, FP can be maintained in a monomeric state in low pH buffers in the presence of organic solvents [25]. Under the conditions used (43% methanol, 10 mM sodium acetate, pH 5.5), FP displayed a sharp Soret band centered on 400 nm (Fig. 3A, top curve in the inset). The addition of CQ resulted in a concentration-dependent decrease in the Soret absorption band (Fig. 3A, inset), which may indicate an association of CQ with an FP μ-oxo dimer to form a π–π donor–acceptor complex [26,27] according to the following model:



where M is the FP monomer, D is the FP μ-oxo dimer, and X is the interacting drug. The lack of aggregation of FP and

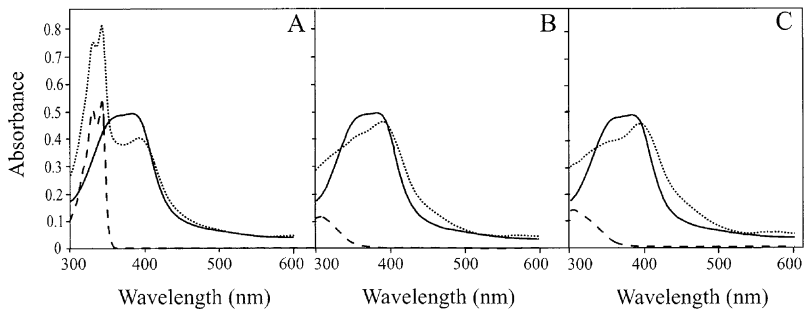


Fig. 2. Optical spectroscopic analysis of the interaction of some phenothiazines with FP at pH 7.4. Samples of (A) 15 μM FP (—), 30 μM CQ (– – –), and a mixture 15 μM FP and 30 μM CQ (···) or (B) 15 μM FP (—), 30 μM CPZ (– – –), and a mixture 15 μM FP and 30 μM CPZ (···) or (C) 15 μM FP (—), 30 μM compound (**3**) (– – –), and a mixture 15 μM FP and 30 μM compound (**3**) (···) were prepared in phosphate buffer at pH 7.4, and visible absorption spectra were collected using a Cary 1E spectrophotometer.

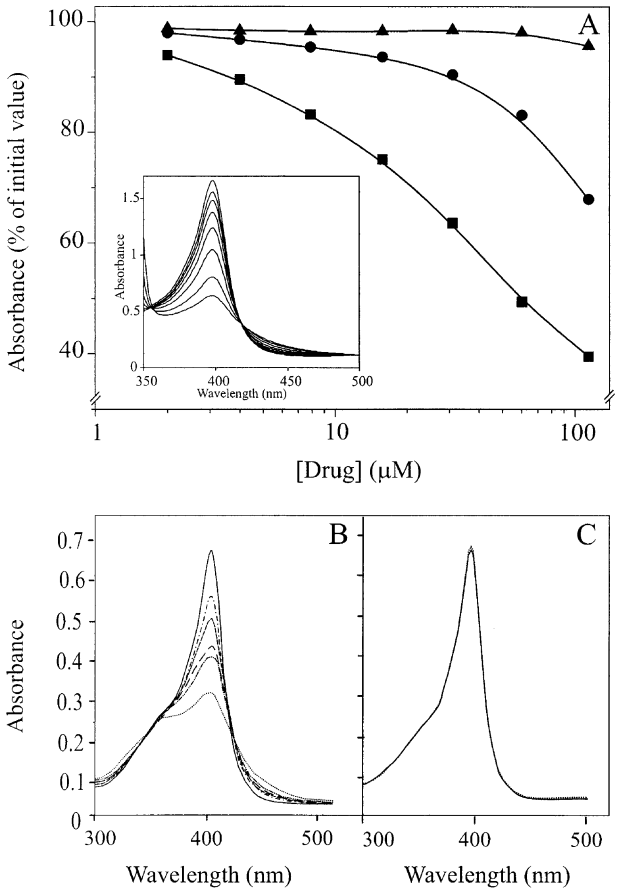


Fig. 3. Optical spectroscopic analysis of the interaction of some phenothiazines with FP at pH 5.5. Samples were prepared in 43% methanol in 10 mM sodium acetate, pH 5.5, and visible absorption spectra were collected using a Cary 1E spectrophotometer. (A) Titration of FP (15 μM) with CQ (■), CPZ (●) and CPT (▲). The interaction of the drugs with FP was monitored by the decrease in the Soret absorption at 400 nm. Inset: Spectral analysis of FP in the presence of 0, 2, 4, 8, 16, 31, 60, and 114 μM CQ. All data have been corrected for dilution. (B) Absorption spectra of 13 μM FP alone (—), or in the presence of 130 mM CPZ (—·—·—), PCP (— — —), compound (**2**) (- - -), compound (**3**) (—··—··—), and CQ (···) (curves listed in order of decreasing absorbance at 400 nm). (C) Absorption spectra of 13 mM FP alone (—) or in the presence of 130 mM CPT (···). Drugs were added from stock solutions in water except for CPT, which was added from a stock in DMSO. An equivalent amount of DMSO was added to the control sample. The absorbance due to the drugs has been subtracted from the curves.

its large absorption change upon drug binding under these conditions facilitate the analysis of the drug–FP interactions. Moreover, shifts in titration curves can be directly related to relative changes in the affinity of the drug for the μ-oxo dimer. Thus, Fig. 3A shows that CPZ binds FP with an approximately 10-fold lower affinity than CQ, while no significant binding occurs between CPT and FP under the conditions of the assay.

Interactions of the various phenothiazines with FP were compared by observing changes in the FP absorption spectra at a single drug concentration. All the alkylamino phenothiazines caused a decrease in the FP Soret absorbance (Fig. 3B), suggesting that they associate with FP in a manner similar to CQ, i.e. by binding to the μ-oxo dimer form. The order of the decrease in absorbance is CPZ < PCP < compound (**2**) < compound (**3**), indicating that the strength of the interaction increases with an increase in the number of basic groups in the side chain. This suggests that the side chain plays an important role in the interaction of the drugs with FP, possibly by positioning the quinoline ring system in an optimal position to allow favorable π–π stacking [27].

3.3. Inhibition of 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. It has been revealed recently that the structure of haemozoin (and its synthetic equivalent, β-haematin) is not a polymer, as had been proposed previously [28], but a repeating array of coordinated dimers, with the ferric iron of each FP moiety chelated onto the carboxyl side chain of its partner, held together in a crystalline matrix by hydrogen bonding interactions [29]. β-Haematin can be distinguished from FP aggregates by its insolubility in SDS at neutral pH [30]. Bendrat *et al.* [31] proposed that specific lipid components in parasite preparations may contribute to the catalysis of haemozoin formation *in vivo*, and Fitch *et al.* [15] 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 [17,32,33]. By contrast, epiquinine, a quinoline compound with very low antimalarial activity, has little inhibitory effect [32]. In the present work, we examined the formation of β-haematin in the presence of MOG as a convenient system for monitoring the ability of the novel phenothiazines to inhibit FP sequestration. In the presence of a 0.5 mM suspension of MOG, a yield of about 15% of β-haematin was formed during the 24-hr incubation. In the absence of the lipid catalyst, only a small amount of β-haematin (about 1%) was formed in this *in vitro* reaction (data not shown). CQ inhibited the formation of β-haematin with an IC_{50} value of 32 mM (Table 1), which is similar to the IC_{50} values found using other methods of promoting FP sequestration [17,34]. CPZ inhibited the sequestration of FP into the β-haematin crystal with an IC_{50} value that is about twice that of CQ (Table 1). The novel phenothiazines were also examined for activity as inhibitors of β-haematin formation. Each phenothiazine that possesses an alkylamino side chain linked to the ring nitrogen showed some inhibitory activity, the most potent of the novel drugs being compound (**3**), which has a branched tribasic side chain (Table 1). Compound (**8b**), which has an amide-linked basic side chain in the 4-position on the phenothiazine ring, also showed some activity in this assay. By contrast, CPT, which has no attached side chain, and compound (**10b**), which has an imidazole side chain, showed no activity (Table 1). The bis-linked phenothiazine, compound (**4**),

also showed no activity as an inhibitor of β-haematin formation (Table 1).

3.4. Antimalarial activity

The abilities of the novel phenothiazines to inhibit the growth of CQ-sensitive (D10) and CQ-resistant (K1) strains of *P. falciparum* were determined (Table 1) and compared with data for CQ, CPZ, and PCP. As previously reported [5,6], CPZ had a weak but measurable antimalarial activity (IC_{50} value of about 10 μM). A number of other phenothiazines, including PCP, the bisphenothiazine (**4**), one of the compounds with an imidazole side chain [compound (**10b**)], and compound (**8b**), which has an amide-linked side chain but no 10-alkylamino side chain, showed no improvement in antimalarial activity relative to CPZ. By contrast, compounds (**2**) and (**3**) showed appreciable antimalarial activity. Indeed, the activities of compound (**2**) and compound (**3**), as inhibitors of the growth of D10 parasites, were 20 and nearly 100 times better than that of CPZ, respectively. The only difference in structure between these compounds is the substitution of the monobasic side chain of CPZ for a dibasic [compound (**2**)] or tribasic [compound (**3**)] side chain. Compounds (**8a**) and (**10a**), which have a CPZ-type side chain in the 10-position and an amide or an imidazole side chain in the 7-position, also showed significantly better activity than either CPZ or the equivalent compounds lacking the 10-position side chain. These data suggest that an increase in the basicity of the side chain(s) is strongly linked to an increase in antimalarial activity, perhaps due to an increased uptake of these compounds into the parasite food vacuole or increased toxicities of the drug–FP complexes.

The marked resistance of the K1 strain of *P. falciparum* to CQ is indicated by the resistance index value of 10 (Table 1). The resistance indices for the three most potent compounds (**2**), (**3**), and (**8a**), were reduced; however, these compounds were still up to 5-fold more active against the CQ-sensitive strain than the CQ-resistant strain. Thus, while the addition of a more basic side chain has improved the antimalarial activities of the phenothiazine derivatives relative to CPZ, it also appears to have increased recognition of the drugs by the CQ resistance mechanism.

An analysis of the structural features of the more active phenothiazines may point to chemical characteristics that are important determinants of the antimalarial activities of these compounds against different strains of the malaria parasite. Several of the phenothiazines examined in this study were good inhibitors of β-haematin formation; however, only a sub-set showed potent antimalarial activity. The most active of the novel phenothiazines [compound (**3**)] inhibited β-haematin formation with an efficiency similar to that for CQ, which is consistent with the suggestion that inhibition of FP detoxification does indeed underlie the mechanism of action of the novel phenothiazines; however, the ability of individual phenothiazines to inhibit β-haematin formation was not a good predictor of the relative potencies of these compounds as inhibitors of parasite growth. It is likely that different levels of uptake of the drugs also contribute to their antimalarial activities. The 100-fold improved activity of compound (**3**) compared with CPZ is associated with the presence of the tribasic arm on the phenothiazine ring structure, which may enhance uptake via the weak base effect [35]. There is currently some debate as to the relative roles of vacuolar pH and binding to FP in determining the level of uptake of quinoline compounds into the parasite food vacuole (see [36,37] for reviews). The spectroscopic data presented here suggest that compound (**3**) may bind to FP somewhat more tightly than CPZ, and it also is a somewhat more effective inhibitor of β-haematin formation. There is, however, a dramatic difference in antimalarial activity with the more basic amphipath, compound (**3**), being a much more potent inhibitor of parasite growth. This enhanced activity of compound (**3**) may be due to enhanced uptake of this drug into the parasite food vacuole due to a combination of ion trapping and FP binding. Alternatively, the enhanced activity may be due to differences in the relative toxicities of individual drug–FP complexes.

3.5. Effect of phenothiazines on CQ resistance

It has been shown previously that CPZ and PCP can modulate the level of resistance of *P. falciparum* to CQ [8,9]. To further investigate the relative abilities of CPZ and the most potent of the novel phenothiazines, compound (**3**), to reverse CQ resistance, we examined the antimalarial activities of combinations of CPZ and compound (**3**) with CQ. The drugs were diluted over a 300-fold range starting with different fixed ratios of the test compounds (Table 2). The antimalarial activity of compound (**3**) against the CQ-resistant strain, K1, was about 30 times higher than that of CPZ. Therefore, the concentrations of this drug used in the mixtures were correspondingly lower. The IC_{50} values for inhibition of the growth of the CQ-resistant strain, K1, were determined for each combination of drugs (Table 2), and the data were used to estimate the Sums of the Fractional Inhibitory Concentration (SFIC) values (Table 2) and to prepare isobolograms (Fig. 4). A concave curve indicates a synergistic interaction; a convex curve, an antagonistic interaction; and a straight line, no interaction [18]. Similarly, an SFIC value of less than one gives a numerical indication of a synergistic interaction. The concave curve for CQ and CPZ and the SFIC values of 0.5–0.7 confirm the previously reported synergy that is achieved with this drug combination [6,8]. By contrast, the combination of CQ and compound (**3**) showed, at best, a weak synergistic interaction (Table 2, Fig. 4).

Thus, compound (**3**) appears to be less effective than CPZ as a modulator of the activity of CQ against CQ-resistant parasites. This suggests that the molecular functions of inhibition of FP detoxification and interaction with

Table 2
Concentrations of drugs required for 50% inhibition of growth of *P. falciparum* (K1 strain) for combinations of CQ with CPZ or compound (**3**)

Starting concentrations of CQ and CPZ	0.6 μM CQ 10 μM CPZ	0.3 μM CQ 20 μM CPZ	0.15 μM CQ 40 μM CPZ
Apparent IC_{50} values for individual drugs	0.10 μM CQ 1.59 μM CPZ	0.05 μM CQ 3.55 μM CPZ	0.03 μM CQ 7.08 μM CPZ
SFIC	0.489	0.495	0.747
*Starting concentrations of CQ and (**3**)*	0.6 μM CQ 0.3 μM (**3**)	0.3 μM CQ 0.6 μM (**3**)	0.15 μM CQ 1.2 μM (**3**)
Apparent IC_{50} values individual drugs	0.20 μM CQ 0.9 μM (**3**)	0.10 μM CQ 0.20 μM (**3**)	0.04 μM CQ 0.28 μM (**3**)
SFIC	0.928	0.821	0.805

Two-fold dilutions of drugs combined in a fixed ratio were added to parasite cultures, and the incorporation of [^{3}H]hypoxanthine was determined over a 72-hr incubation period. Experiments were performed in triplicate. Data are for a typical experiment. The "apparent" IC_{50} value for each drug was estimated as though it had been added in isolation. The IC_{50} value for each drug used in the combination was divided by the IC_{50} value for the drug used alone (data from Table 1) to give the Fractional Inhibitory Concentration (FIC). The FIC values for each of the drugs used in the combination were added to give the sum of the FIC (SFIC).

the CQ resistance mechanism require different structural characteristics. The molecular basis of CQ resistance is only poorly understood; however, it seems to result from a decreased level of CQ uptake [38–40]. Transfection experiments have been used recently to confirm that CQ resistance arises, at least in part, from the expression of a mutant form of the *P. falciparum* Pgh1 [41]. In addition, the presence of a mutant form of another parasite protein, referred to as the *P. falciparum* CQ resistance transporter (PfCRT), also has been shown to be linked to CQ resistance [42]. The roles of these proteins have not been established, but they may affect either the pH of the food vacuole or the physical state of FP within the food vacuole, which in turn may affect the level of uptake of CQ and other quinoline antimalarials [42,43] or the extent of interaction of FP with the antimalarial drugs [44]. The phenomenon of CQ resistance has parallels with tumor multi-drug resistance (see [45] for review), and CPZ has been shown to bind to human P-glycoprotein and modulate the MDR of tumor cells [46]. Indeed, it now seems increasingly likely that CPZ may exert its resistance-reversing activity by interacting with Pgh1 [41]. It is possible that the addition of a tribasic side arm may inhibit the interaction of compound (**3**) with Pgh1.

In conclusion, we have prepared a series of novel phenothiazine-based compounds and examined their potential as antimalarial drugs. The phenothiazine nucleus shows some promise as a scaffold for the assembly of novel antimalarial drugs. In addition, studies of these drugs may provide some insight into the mode of action of CQ and other quinoline antimalarials. If structure–function relationships could be delineated clearly, and the molecular basis of resistance could be defined, it might be possible to develop novel drugs that target the FP detoxification processes, but that somehow evade the resistance mechanism(s).

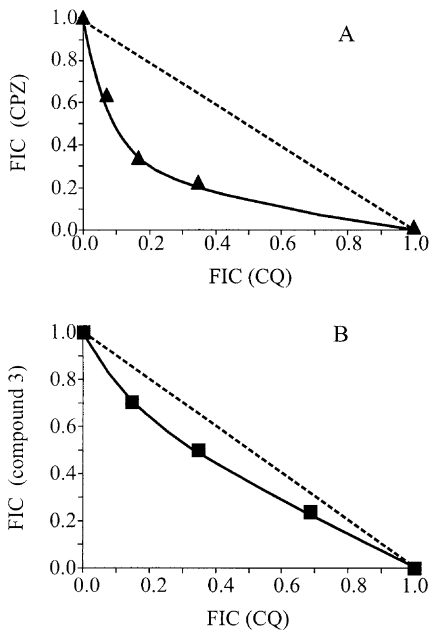


Fig. 4. Isobolograms constructed from IC_{50} values in Tables 1 and 2. For each drug combination, the Fractional Inhibitory Concentrations (FIC) were calculated by dividing the measured "apparent" IC_{50} values for individual drugs in the different combinations of (A) CPZ and CQ or (B) compound (3) and CQ by the IC_{50} values obtained when the drugs were used alone.

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