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Novel Bisquinoline Antimalarials

SYNTHESIS, ANTIMALARIAL ACTIVITY, AND INHIBITION OF HAEM POLYMERISATION

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ABSTRACT. We report the synthesis of two series of novel bisquinoline compounds that inhibit the growth of both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. To study the molecular basis of the action of these novel antimalarial drugs, we examined their ability to inhibit haem polymerisation in the presence and absence of parasite extracts. The level of antimalarial potency was correlated with the level of inhibition of haem polymerisation, suggesting that these bisquinolines exert their antimalarial activity by antagonising the sequestration of toxic haem moieties. BIOCHEM PHARMACOL 52;4:551–559, 1996.

KEY WORDS. malaria; bisquinolines; haem polymerisation; haemozoin; quinoline antimalarials; drug resistance

The intraerythrocytic malaria parasite feeds by ingesting small packets of haemoglobin from the host cytoplasm. These haemoglobin-containing vesicles are transported to the acidic food vacuole where the contents are degraded by a series of proteases [1]. As a by-product of haemoglobin degradation, the toxic haem moiety FPIX§ is released [2, 3]. The haem molecules are detoxified within the food vacuole of the parasite by polymerisation into insoluble granules of haemozoin. Slater and Cerami [2] demonstrated the presence of a polymerisation-promoting activity in parasite preparations which they proposed was due to a parasite-encoded "haem polymerase." Recently, however, Dorn *et al.* [4] suggested that this activity is not enzymic in nature, but is simply an autocatalytic effect of the preformed haemozoin polymers in parasite extracts.

The nature of the haem polymerisation process is of considerable interest as it has been proposed to be the site of action of the quinoline antimalarial drugs. These drugs, which include chloroquine (see Fig. 1), quinine, and mefloquine, are major players in the prophylaxis and treatment of malaria. Inhibition of haem polymerisation by these drugs is thought to lead to a build-up of toxic haem [2, 3], which inhibits a parasite haemoglobinase and disrupts membranes [5, 6].

Unfortunately, resistance to the major quinoline antimalarial drugs is becoming an increasingly serious problem (see Ref. 7 for review). Chloroquine resistance has been studied extensively and is thought to result from a decreased level of accumulation of this drug rather than an alteration in the drug target (reviewed in Ref. 8). This has led to the search for novel quinoline antimalarial drugs that might circumvent the resistance mechanism of the parasite. One series of compounds that show promise in this regard are the bisquinoline antimalarial drugs [9–11]. These drugs show much lower resistance indices than chloroquine, indicating that the bisquinoline structure is less efficiently excluded by drug-resistant parasites.

We previously reported a series of bisquinolines in which the quinoline units were linked by bisamide formation from 6- or 8-amino precursors [11]. These bisquinolines are active against both chloroquine-sensitive and chloroquine-resistant parasites. We have continued this work, and the present paper reports further compounds in which either a chlorine has been incorporated into a slightly modified form of the first series of compounds (see structures **(9)–(11)**, Fig. 1), or the elements of the amide linker have been reversed, with connection through the 6-position to give compounds **(15)–(19)** (Fig. 1).

To examine the mechanism of action of these bisquinolines, we have set up an *in vitro* assay of haem polymerisation under conditions that are designed to resemble the conditions within the food vacuole. We found that the ability of the drugs to inhibit haem polymerisation was directly proportional to their antimalarial potency, suggesting that they exert their activity at the level of haem detoxification.

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§ *Abbreviations:* haem/FPIX, ferriprotoporphyrin IX; haematin, ferriprotoporphyrin IX hydroxide; ESMS, electrospray mass spectrum; and DMF, dimethylformamide.

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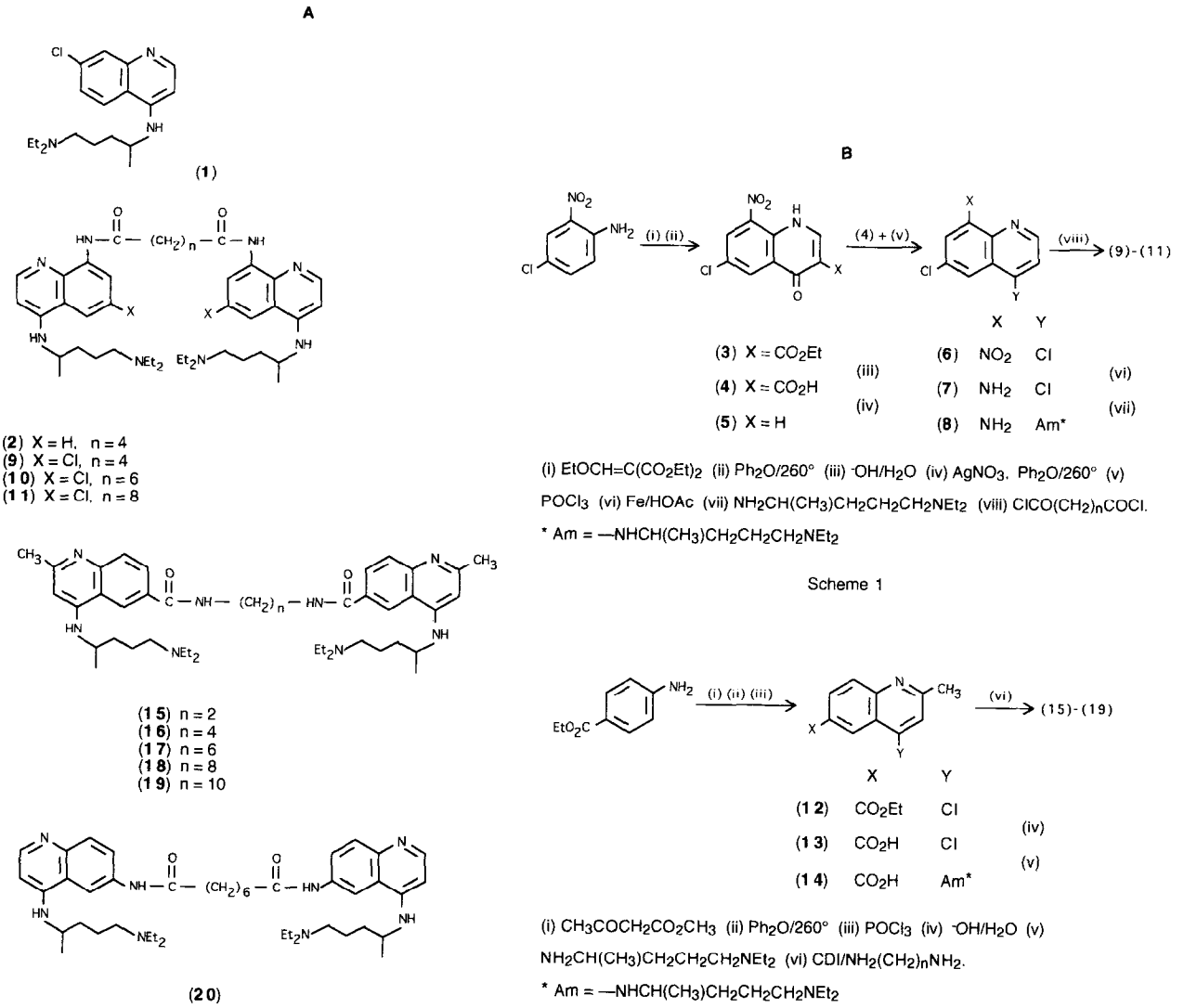

FIG. 1. (A) Structures of the bisquinoline antimalarials prepared in this study: compounds (9)–(11) and (15)–(19). Chloroquine (1), compound (2), and compound (20) are shown for comparison. (B) Schemes for the synthesis of bisquinoline compounds (9)–(11) and (15)–(19).

MATERIALS AND METHODS

Synthesis of Bisquinolines

NMR spectra were recorded on a Bruker AM-300 spectrometer, in $CDCl_3$ unless otherwise stated. Full details are given for the bisamides **(9)** and **(15).** Homologues showed the same patterns, with additional linker CH_2 signals as appropriate. Electrospray mass spectra were obtained on a VG Bio-Q triple quadrupole mass spectrometer using a water:methanol:acetic acid (50:50:1, by vol.) mobile phase.

Ethyl 6-chloro-8-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxylate (3)

A mixture of 4-chloro-2-nitroaniline (12.5 g, 72 mmol) and diethyl ethoxymethylenemalonate (15.5 g, 72 mmol) was heated in an open flask at 110° for 48 hr. The mass solidified on cooling to give the intermediate arylaminomethylenemalonate ester (18.6 g, 75%), m.p. 92–94° (from ethanol), which was dissolved in diphenyl ether (140 mL) and heated to boiling until no more ethanol distilled off. The solution was cooled, and light petroleum (b.p. 60–90°) was added. The solid that separated was filtered off and recrystallised from acetonitrile to give the product as yellow plates (11.1 g, 69%), m.p. 282–284°. ^{1}H NMR (DMSO) δ 1.27, t, *J* 7.0 Hz, CH_2CH_3; 4.22, q, *J* 7.0 Hz, CH_2CH_3; 8.42, d, *J* 2.3 Hz, H-5; 8.52, s, H-2; 8.65, d, *J* 2.3 Hz, H-7; 12.2, br s, NH.

6-Chloro-8-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxylic Acid (4)

A suspension of the ester (8.9 g) in 5% NaOH solution (80 mL) was refluxed for 15 min, during which time the solid disappeared. The solution was then cooled and taken to pH

6–7 with hydrochloric acid. The resulting solid was filtered off to yield the acid (6.8 g, 84%), m.p. 272–273°.

6-Chloro-8-nitroquinolin-4(1H)-one (5)

The acid was decarboxylated by way of the silver salt as reported for 8-nitroquinolin-4(1*H*)-one [12] to give the product, m.p. 230–234° in 55% yield. ^{1}H NMR (DMSO) δ 6.4, d, *J* 7 Hz, H-3; 7.75, br m, H-2; 8.60 + 8.72, d + d, *J* 2.5 Hz, H-5,7; 11.05, br s, NH.

4,6-Dichloro-8-nitroquinoline (6)

A mixture of (5) (2 g) and phosphoryl chloride (5 mL) was refluxed for 40 min. The excess of phosphoryl chloride was removed by reduced pressure distillation. Ice-water was added to the residue, which was then basified with 10% NaOH. The solid that separated was filtered off, dried, and recrystallised from light petroleum (b.p. 90–110°) to give the product (1.6 g, 74%), m.p. 157–158°. ^{1}H NMR δ 7.65, d, *J* 4.8 Hz, H-3; 8.05 + 8.45, d + d, *J* 2.3 Hz, H-5,7; 8.90, d, *J* 4.8 Hz, H-2.

8-Amino-4,6-dichloroquinoline (7)

A mixture of nitro compound (6) (1.8 g) and iron powder (0.36 g) in 50% acetic acid (30 mL) was heated at 100° for 0.5 hr. Water (30 mL) was added, and the solid was filtered, dried, and extracted with ether by Soxhlet for 2 hr. Removal of the solvent gave the amine (1.4 g, 89%), m.p. 131–135°. ^{1}H NMR δ 5.10, s, NH_2; 6.81, s, H-7; 7.50, br s, H-3,5; 8.50, d, *J* 4.4 Hz, H-2.

8-Amino-6-chloro-4-(4-diethylamino-1-methylbutyl)aminoquinoline (8)

A mixture of (7) (0.7 g) and 2-amino-5-diethylaminopentane (3.5 g) was heated at 180° for 16 hr in a nitrogen atmosphere. The resulting dark mass was dissolved in chloroform, the solution was washed with 10% sodium hydroxide, the chloroform was removed, and the residue was further evaporated at 1 mm Hg for 2 hr at 100° to leave the product (1.06 g, 96%) as a dark oil that solidified slowly but could not be recrystallised. ^{1}H NMR δ 1.1, t, *J* 7 Hz, CH_2CH_3; 1.35, d, *J* 8 Hz, $CHCH_3$; 1.6–1.8 m, $(CH_2)_2$; 2.5–2.7, m, $CH_2N(CH_2CH_3)_2$; 3.6–3.7, m, $NHCH(CH_3)$; 5.1, s, NH_2; 5.30, d, *J* 8 Hz, NH; 6.4, d, *J* 5 Hz, H-3; 6.85, s, H-7; 7.15, s, H-5; 8.35, d, *J* 5 Hz, H-2. ^{13}C NMR δ 11.3, NCH_2CH_3; 20.1, Cα-CH_3; 23.7, C-γ; 34.5, C-β; 46.8, NCH_2CH_3; 48.1, C-α; 52.5, C-δ; 99.8, C-3; 106.9 + 109.8, C-5,7; 119.6, C-4a; 130.7, C-6; 137.0, C-8a; 145.6 + 148.0 + 148.3, C-2,4,8.

N,N′-Bis[6-chloro-4-(4-diethylamino-1-methylbutyl)aminoquinolin-8-yl]adipamide (9)

Trifluoroacetic acid (0.22 g, 1.9 mmol) was added to a solution of compound (8) (0.77 g, 2.3 mmol) in anhydrous dioxane (10 mL). Then adipoyl chloride (0.2 g, 1.1 mmol) in dry DMF (2 mL) was added dropwise and with stirring. After 2 hr, the dioxane was evaporated, and the residue was dissolved in water, basified with 10% sodium hydroxide, saturated with sodium chloride, and extracted with chloroform (3 × 20 mL). The extracts were dried, the solvent was removed, the residue was extracted with hot light petroleum (b.p. 90–110°), and the solvent was evaporated to leave the amide (0.59 g, 66%) as an oil. ^{1}H NMR ($CDCl_3$) δ 1.1, t, *J* 7 Hz, NCH_2CH_3; 1.35, d, *J* 6 Hz, $NHCH(CH_3)$, 1.6–1.8 (m, 6H, $(CH_2)_2$ + CH_2CH_2CO); 1.85–1.9, br s, CH_2CH_2CO; 2.5–2.7, m, 3 × NCH_2; 3.70, m, $NHCH(CH_3)$; 5.50, d, *J* 8 Hz, NH; 6.43, d, *J* 5 Hz, H-3; 7.35, s, H-5; 8.35, d, *J* 5 Hz, H-2; 8.70, s, H-7; 9.9, s, NHC=O. ^{13}C NMR δ 11.1, NCH_2CH_3; 19.8, Cα-CH_3; 23.5, C-γ; 25.0, $COCH_2CH_2$; 34.4, C-β; 37.6, $COCH_2$; 46.7, NCH_2CH_3; 48.3, C-α; 52.3, C-δ; 100.1, C-3; 112.9 + 116.5, C-5,7; 118.7, C-4a; 130.5, C-6; 135.6, C-8; 137.1, C-8a; 148.6 + 148.6, C-2,4. ESMS: Expected isotope pattern for $C_{42}H_{61}Cl_2N_8O_2$, *m/z* 779–785 (M + 1).

N,N′-Bis[6-chloro-4-(4-diethylamino-1-methylbutyl)aminoquinolin-8-yl]suberamide (10)

ESMS: As expected for $C_{44}H_{65}Cl_2N_8O_2$, *m/z* 807–810 (M + 1).

N,N′-Bis[6-chloro-4-(4-diethylamino-1-methylbutyl)aminoquinolin-8-yl]sebacamide (11)

ESMS: As expected for $C_{46}H_{69}Cl_2N_8O_2$, *m/z* 835–841 (M + 1).

Ethyl 4-chloro-2-methylquinoline-6-carboxylate (12)

This was prepared from ethyl 4-aminobenzoate by a three-step sequence (Scheme 2) to give the product as an orange *solid* (24% overall), m.p. 112–113° (lit. [13], m.p. 113–114°).

4-Chloro-2-methylquinoline-6-carboxylic Acid (13)

The ester (12) was hydrolysed with 10% sodium hydroxide (20 min reflux) as for (4) and gave the *acid* (80%), m.p. 209–210° (from aqueous acetone). ^{1}H NMR (DMSO) δ 2.73, s, CH_3; 7.78, s, H-3; 8.05 + 8.23, d + d, *J* 8 Hz, H-7,8; 8.75, s, H-5.

4-(4-Diethylamino-1-methylbutyl)amino-2-methylquinoline-6-carboxylic Acid (14)

A mixture of acid (13) (0.5 g) and 2-amino-5-diethylaminopentane (2.5 g) was heated at 150° for 5.5 hr in a nitrogen atmosphere. The resulting mass was dissolved in water and extracted with chloroform (4 × 20 mL). The aqueous layer was taken to pH 7 with concentrated hydrochloric acid and then evaporated to dryness at reduced pressure. The residue was extracted with hot ethanol, the extract was

filtered, and the ethanol was evaporated from the filtrate to give the product (0.66 g, 86%), m.p. 40–42°. ^{1}H NMR (DMSO) δ 1.1, t + t, 2 × CH_2CH_3; 1.35, d, *J* 6 Hz, $CHCH_3$; 1.6–1.8, m, $(CH_2)_2$; 2.61, s, $ArCH_3$; 2.9–3.1, m, $CH_2N(CH_2CH_3)_2$; 3.9–4.0, m, $NHCH(CH_3)$; 6.73, s, H-5; 7.78, d, *J* 8.7 Hz, H-8; 8.15, d, *J* 8.7 Hz, H-7; 8.28, br s, NH; 9.05, s, H-3.

N,N′-1,2-*Ethanediylbis[4-(4-diethylamino-1-methylbutyl)amino-2-methylquinoline-6-carboxamide]* (15)

Compound **(14)** (0.33 g, 1 mmol) was dissolved in dried DMF (4 mL) at 110°. To this solution was added 1,1′-carbonyldiimidazole (0.32 g, 2 mmol) in anhydrous acetonitrile (5 mL), and the mixture was heated at 110° for 0.5 hr and then left at room temperature for 1 hr. Ethylenediamine (0.03 g, 0.5 mmol), dissolved in acetonitrile (1 mL), was added, and the solution was again heated at 110° for 1 hr. The solvents were removed under reduced pressure, and the residue was dissolved in chloroform. The organic layer was washed with 10% sodium hydroxide, water, and dried ($MgSO_4$), and the solvent was evaporated. The residue was crystallised twice from acetonitrile. On each occasion, the supernatant liquid was decanted from the solid that separated. Finally, the residual acetonitrile was evaporated, and the bisamide became sticky before setting to a glass (0.11 g, 30%), m.p. 129–131°. ^{1}H NMR δ 1.0, t, *J* Hz, CH_2CH_3; 1.35, br s, $CHCH_3$; 1.5–1.7, m, $(CH_2)_2$; 2.35–2.7, m, $CH_2N(CH_2CH_3)_2$; 2.6 s, CH_3; 3.0, br s, H_2O; 3.5–3.7, m, $MHCH(CH_3)$ + $CONHCH_2$; 5.85, br s, NH; 6.28, s, H-3; 7.75 + 7.95, d + d, *J* 7.9 Hz, H-7,8; 8.22, br s, CONH; 8.65, s, H-5. ^{13}C NMR δ 11.0, NCH_2CH_3; 20.0, Cα-CH_3; 23.6, C-γ; 25.2, C2-CH_3; 34.0, C-β; 40.6, $CONHCH_2$; 46.6, NCH_2CH_3; 48.3, C-α; 52.4, C-δ; 99.2, C-3; 116.6, C-4a; 120.8, C-5; 127.5, C-7; 127.6, C-6; 128.4, C-8; 149.0, C-8a; 150.5, C-4; 160.6, C-2; 168.1, CO. ESMS: *m/z* 711 (M + 1). Anal. Calc. for $C_{42}H_{62}N_8O_2 \cdot 2H_2O$: C, 67.5; H, 8.9; N, 15.0. Found: C, 67.2; H, 8.5, N, 14.5.

The following bisamides were prepared in the same way.

N,N′-1,4-*Butanediylbis[4-(4-diethylamino-1-methylbutyl)amino-2-methylquinoline-6-carboxamide]* (16)

23%, m.p. 121–125°. ESMS: *m/z* 739 (M + 1).

N,N′-1,6-*Hexanediylbis[4-(4-diethylamino-1-methylbutyl)amino-2-methylquinoline-6-carboxamide]* (17)

42%, m.p. 119–121°. ESMS: *m/z* 767 (M + 1).

N,N′-1,8-*Octanediylbis[4-(4-diethylamino-1-methylbutyl)amino-2-methylquinoline-6-carboxamide]* (18)

50%, m.p. 102–110° (with shrinking). ESMS: *m/z* 795 (M + 1).

N,N′-1,10-*Decanediylbis[4-(4-diethylamino-1-methylbutyl)amino-2-methylquinoline-6-carboxamide]* (19)

38%, m.p. 88–90°. ESMS: *m/z* 823 (M + 1).

Chloroquine was obtained from Sigma, St. Louis, MO, U.S.A. Mefloquine was donated by Hoffmann-La Roche, Basel, Switzerland. *N,N′*-Bis[4-((4-(diethylamino)-1-methylbutyl)amino)quinolin-8-yl]adipamide (compound 2) was synthesised as described previously [11].

Preparation of Insoluble Trophozoite Material

Plasmodium falciparum was cultured continuously as described by Trager and Jensen [14] using erythrocytes and serum obtained from the Red Cross Transfusion Service, Melbourne. Parasitised erythrocytes (1.8 mL, approx. 5% trophozoite stage parasites, D10 strain) were washed with phosphate-buffered saline, pH 8 (PBS). The cells were lysed in 10 mL of 5% sorbitol in water and the parasites collected by centrifugation at 650 *g* for 5 min. The pellet was mixed with 0.05 vol. of 1% saponin and 0.01 vol. of gentamycin (50 mg/mL) and incubated at room temperature for 10 min. The parasites were pelleted at 1500 *g* for 5 min, resuspended with the aid of sonication (3 min, 20°) in 10 mL of PBS containing 50 μg/mL gentamycin, and repelleted. The washed pellet (approx. 60 μl) was stored at −70° until used (up to 3 weeks). Parasites were thawed by incubation with 1 mL of buffer A (68 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 5 mM glucose, 50 mM sodium phosphate, pH 7.4) for 30 min, 4°, prior to sonication for 10 min, 20°. For stability studies, the trophozoite preparation was subjected to heating to 100° for 30 min.

Haem Polymerisation Assay

For routine measurements of spontaneous haem polymerisation, haematin (Sigma, bovine) was dissolved in 0.1 M NaOH and warmed to 37°. An aliquot (300 μL) of this stock was mixed with warmed aliquots of 1 M HCl (30 μL) and sodium acetate, pH 5 (to a final volume of 1 mL). The final concentration of acetate was either 60 mM or 10.3 M, while the final concentration of haem was either 0.3 or 1.6 mM. In some experiments, a final concentration of 10 mM haem was achieved by mixing 300 μL of a 33 mM stock of haem in 1 M NaOH with 30 μL of 10 M HCl. It should be noted, however, that upon acidification, partial precipitation of the haem was observed at the higher haem concentrations, so the concentration of haem in solution may have been somewhat lower than estimated. Samples were incubated at 37° for 4 hr with gentle rotation. To assess the effect of the parasite extract on haem polymerisation, a 50-μL aliquot of the trophozoite preparation (equivalent to approx. 4×10^7 parasites) was added to the haem/acetate mixture. To assess the effect of the antimalarial drugs on haem polymerisation, the compounds were dissolved in methanol/sodium acetate, pH 5 (50:50, v/v), and a 30-μL aliquot was added to the warmed acetate solution prior to mixing with the other components.

Determination of Haemozoin Content

Haemozoin content was determined using the procedure of Chou and Fitch [3]. Following incubation, the samples were centrifuged at 27,000 g, 15 min, 4°. The pellet was resuspended in buffer A and repelleted. The pellet was resuspended in SDS (2.5% in buffer A) and sonicated for 10 min, 20°. The polymerised haem, which is insoluble in SDS, was collected by centrifugation at 27,000 g, 30 min, 20°. The pellet was washed repeatedly until the supernatant was clear (usually 4 times, over a period of 18 hr). After the final wash, the supernatant was removed and the remaining pellet was resuspended in 900 μL of 2.5% SDS in buffer A. A 100-μL aliquot of 1 M NaOH was added to dissolve the polymerised haem. Following incubation for 1 hr at room temperature, the concentration of haemozoin was determined by measuring the absorbance at 404 nm, assuming a molar extinction coefficient of $9.08 \times 10^4\ cm^{-1} \cdot M^{-1}$ [15]. For incubations performed in the presence of trophozoite material, the amount of haemozoin formed during the incubation period was corrected for endogenous haemozoin. The concentration of drug required to produce 50% inhibition of polymerisation (IC_{50}) was determined.

Assessment of Antimalarial Activity of the Bisquinolines

Two strains of *P. falciparum* were used. D10 is a chloroquine-sensitive strain [16]; FAC8 is a chloroquine-resistant line cloned from ITG2F [16]. Malaria parasites were plated at about 1% parasitemia (2% hematocrit), in 96-well trays and different concentrations of the quinoline drugs were added from concentrated stocks in DMSO. Parasites were incubated for 72 hr, with daily replacements of the drug-supplemented medium. Growth curves were obtained in duplicate as described by Barnes *et al.* [17], and the concentration of drug required to produce 50% inhibition of growth (IC_{50}) was determined.

RESULTS AND DISCUSSION

Chemistry

We previously prepared a series of bisquinoline compounds that showed antimalarial activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* [11]. In an effort to increase the potency of these reagents, we have now synthesised a series of bisquinolines, compounds **(9)–(11),** which have a chlorine substituent on the quinoline ring. . We were unable to obtain amide formation when a 7-chloro substituent was also present in the 8-amino precursor; therefore, the chlorine was placed at the 6-position, and 4-chloro-2-nitroaniline (Fig. 1B, Scheme 1) was converted to **(8)** by the same sequence of reactions used previously to prepare the 6-amino precursor [11]. Reaction with the appropriate diacid chloride under the conditions devised previously [11] gave the amides **(9)–(11).** These were not obtained as crystallisable solids, but NMR and mass spectral data were in accord with the proposed bis structures. We also examined the effect of reversing the elements of the amide linker. Compounds **(15)–(19)** (Fig. 1B, Scheme 2) were prepared by converting ethyl 4-aminobenzoate to the known compound **(12)** in a reaction sequence slightly modified from the literature procedure [13]. Hydrolysis of the ester was achieved without affecting the chloro group, and the amine side-chain was attached to give the key precursor **(14).** Formation of the various diamides was achieved by firstly, reacting **(14)** with 1,1′-carbonyldiimidazole and then with the appropriate diamine. The amides were glassy, hydrated solids. Satisfactory microanalysis figures were obtained for **(14),** and mass spectra were again consistent with all the proposed structures.

Antimalarial Activity

The ability of the bisquinolines **(9)–(11)** and **(15)–(19)** to inhibit the growth of chloroquine-sensitive (D10) and chloroquine-resistant (FAC8) strains of *P. falciparum* was determined (Table 1) and compared with data for chloroquine **(1),** and the previously described bisquinoline compounds **(2)** and **(20).** All the bisquinoline compounds prepared in this study showed some antimalarial activity, although members of the 8-series had significantly greater activities than members of the 6-series. This is in agreement with our previous data for 8- and 6-amino bisquinolines (Table 1 and [11]). Compounds **(9)** and **(10)** were the most effective inhibitors of parasite growth. This is consistent with our previous findings for the 8-aminoquinoline series in which a linker length of 6 carbons was associated with the most potent antimalarial activity [11]. Interestingly, the addition of the chlorine substituent in compounds **(9)–(11)** did not increase substantially the antimalarial activity of these bisquinolines. Compound **(9)** showed a similar activity to compound **(2),** which lacks the chlorine substituent. This result was somewhat surprising given that previous studies have shown that the 7-H derivative of chloroquine has a 14-fold lower antimalarial activity than chloroquine itself [18]. A comparison of the results for compound **(17)** and compound **(20)** reveals that reversal of the atoms of the amide link so that linkage to the quinoline ring is through the carboxyl group rather than the amino group was associated with a decrease in the IC_{50} value of the quinoline compound. We have not made the missing combination, i.e. the amide link of **(17)** through the 8-position, as it is reported that the standard Conrad-Limpach quinoline synthesis does not work with derivatives of *o*-aminobenzoic acid [19].

The marked resistance of the FAC8 strain of *P. falciparum* to chloroquine is indicated by the resistance index value of 9 (Table 1). By contrast, the resistance indices for members of the two quinoline series prepared in this study were much lower, ranging from 0.5 to 1.8. Thus, compound **(9)** is a substantially more potent inhibitor of the growth of the chloroquine-resistant strain, FAC8, than chloroquine. The molecular basis for this apparent circumvention of the chloroquine-resistance mechanism is not known. Chloro-

TABLE 1. IC_{50} Values and resistance index values for inhibition of the growth of *P. falciparum in vitro* by bisquinolines

Compound	Series*	n†	IC_{50} (μM) D10	IC_{50} (μM) FAC8	Resistance Index
1			0.03 ± 0.02 (3)‡	0.27 + 0.02 (2)	9
2§	8	4	0.13 ± 0.06 (3)	0.12 ± 0.07 (3)	0.9
9	8	4	0.08 ± 0.05 (3)	0.13 ± 0.07 (3)	1.6
10	8	6	0.12 ± 0.06 (3)	0.14 ± 0.03 (3)	1.2
11	8	8	0.25 ± 0.12 (3)	0.35 ± 0.06 (3)	1.4
15	6	2	2.1 ± 0.4 (3)	3.7 ± 0.3 (2)	1.8
16	6	4	3.0 ± 0.2 (3)	3.5 ± 0.3 (2)	1.2
17	6	6	2.7 ± 0.3 (2)	1.5 ± 0.2 (2)	0.6
18	6	8	2.5 + 0.1 (2)	1.6 ± 0.4 (2)	0.6
19	6	10	1.1 ± 0.1 (3)	0.6 ± 0.2 (2)	0.5
20§	6	6	5.0 ± 0.63 (3)	5.2 ± 1.2 (2)	1.0

* Position of linker attachment.

† Number of CH_2 groups in linker.

‡ The numbers in parentheses refer to the number of growth inhibition experiments, each of which was performed in duplicate. Values are means ± SD.

§ Data were taken from Raynes *et al.* [11].

quine resistance has been proposed to derive from the action of a drug efflux protein, though more recent work has suggested an alternative explanation, namely, that resistant parasites operate at a higher food vacuole pH, which decreases the level of chloroquine accumulation (see Ref. 7 for review). If a drug effluxer is involved, it is possible that the bulky bisquinoline structure may not be a good substrate for this enzyme. Alternatively, the higher charge on the bisquinolines may facilitate their accumulation in the food vacuole in the face of a decreased pH gradient. Further studies are required to distinguish between these two possibilities.

Studies of Haem Polymerisation

Quinoline antimalarial drugs have been proposed to exert their anti-parasitic activity at the level of haem polymerisation [2, 3]. In this study, we have made a detailed examination of the polymerisation of haem *in vitro*, in the presence and absence of extracts of parasite material, in an attempt to determine the molecular basis of the antimalarial activity of the bisquinolines prepared in this study. We followed the conversion of free haem (FPIX) to polymerised haem by monitoring the appearance of an SDS-insoluble precipitate [3]. The absorption spectrum of this precipitate was identical to that of a suspension of haemozoin in SDS (data not shown), indicating that the polymers formed *in vitro* have a structure similar to that of the endogenous product.

Under experimental conditions that were designed to mimic the environment within the food vacuole, spontaneous polymerisation of haem occurred only at a low rate (Fig. 2A). The rate of haem polymerisation increased with increasing acetate concentration (Figs. 2B and 3A), probably due to an increased solubility of the haem molecules at the higher acetate concentrations. The rate of polymerisation was also increased with increasing haem concentration (Fig. 3B), but was still rather slow at a concentration of 1 mM, which probably represents an upper limit for the concentration of free haem within the food vacuole (Raynes K

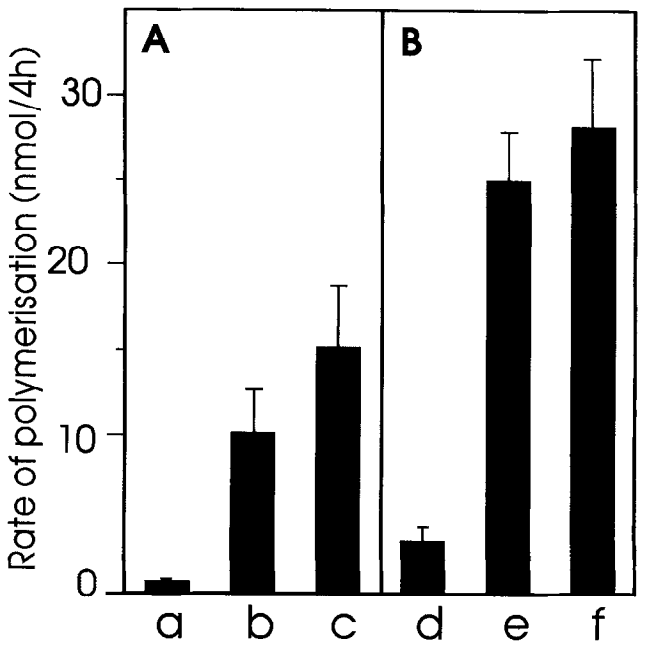

FIG. 2. Haem polymerisation in the presence and absence of malaria parasite extract. Haem (1.6 mM) was incubated for 4 hr at 37° in sodium acetate, pH 5 (A, 60 mM, B, 10.3 M), either alone (a and d) or in the presence of a preparation of malaria parasites (D10 strain, approx. 4 × 10^7 parasites; b, c, e, and f). In some experiments (c and f), the parasite preparation was boiled for 30 min prior to addition to the haem polymerisation assay. Polymerised haem was determined by SDS insolubility as described in Materials and Methods. Data represent means ± SEM from three separate experiments, each performed in triplicate.

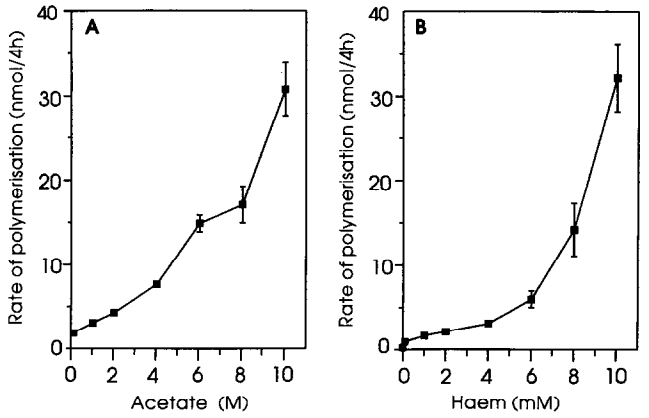

FIG. 3. Effects of acetate and haem concentrations on the rate of spontaneous haem polymerisation. (A) Haem (10 mM) was incubated for 4 hr at 37° in the presence of increasing concentrations of sodium acetate, pH 5. (B) Increasing concentrations of haem were incubated in 10.3 M acetate, pH 5, for 4 hr at 37°. Data represent the means ± SEM for two separate experiments, each performed in triplicate.

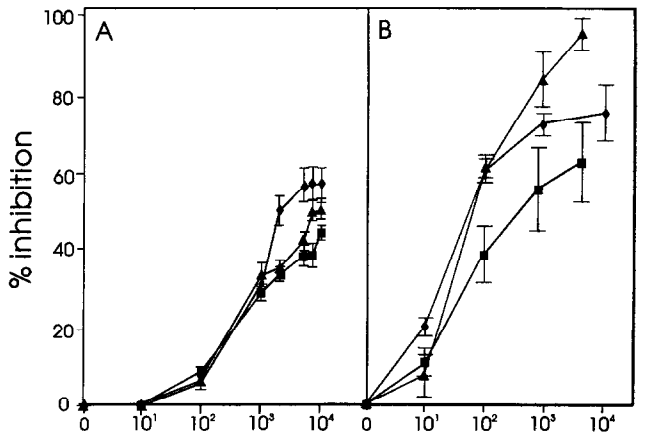

FIG. 4. Effect of quinoline-containing antimalarial drugs on spontaneous and parasite extract-catalysed haem polymerisation. (A) Haem (1.6 mM) was incubated for 4 hr at 37° in 10.3 M sodium acetate, pH 5, in the presence of increasing concentrations of chloroquine (♦), 8AQ6 (▲), or mefloquine (■). The rate of haem polymerisation in the absence of any drug was 1.8 ± 0.2 nmol/4 hr. (B) Haem (0.3 mM) was incubated for 4 hr at 37° in 60 mM sodium acetate, pH 5, in the presence of a preparation of approx. 4 × 10^7 parasites and increasing concentrations of chloroquine (♦), 8AQ6 (▲), or mefloquine (■). The level of haem polymerisation during incubation incubation was corrected for the preformed haemozoin in the parasite extract. The rate of haem polymerisation in the absence of any drug was 3.4 ± 0.2 nmol/4 hr. Data represent the means ± SEM for a typical experiment, which was performed in triplicate.

and Tilley L, unpublished data). Assuming that the conditions of our assay mimic the conditions of the food vacuole, we estimate that spontaneous polymerisation is, at best, sufficient to allow polymerisation of about 2% of the haem molecules over a 24-hr period. This cannot account for the known efficiency of haem polymerisation *in vivo* and suggests that polymerisation-enhancing factors must facilitate haem sequestration in the food vacuole.

Addition of a preparation of insoluble material from approximately 4 × 10^7 malaria parasites substantially enhanced the polymerisation rate (Fig. 2 b, c, e, and f), indicating the presence of a haem-polymerising "activity" in preparations of trophozoite-stage parasites. The haem-polymerising "activity" of trophozoite pellets was found to be markedly heat-stable (Fig. 2, c and f) in agreement with the recent report of Dorn *et al.* [4]. These authors proposed that preformed haemozoin polymers in parasite extracts catalyse polymerisation by providing nucleation sites for the formation of haem polymers. Bendrat *et al.* [20] recently proposed that lipidic components in parasite preparations may also contribute to the catalysis of haem polymerisation. Very recent work suggests that histidine-rich protein may also contribute to the catalysis in the early stages of parasite growth [21].

Inhibition of Haem Polymerisation

In our attempts to find a set of reaction conditions suitable for assays of the inhibitory activity of the novel bisquinolines, we initially examined the effect, on the polymerisation process, of three quinoline antimalarials: chloroquine, mefloquine and the previously described bisquinoline compound (2) (Fig. 4). Although spontaneous polymerisation occurred only rather slowly under physiological conditions, it was none the less possible to examine the effect of the quinoline antimalarials on this reaction. Spontaneous haem polymerisation (using 1.6 mM haem) was inhibited by each of the quinoline antimalarials examined, but only at rather high concentrations (Fig. 4A). A 10 mM concentration of chloroquine, mefloquine, and bisquinoline compound (2) inhibited haem polymerisation by 59 ± 5, 47 ± 2, and 53 ± 2%, respectively. The high concentrations of drugs required to inhibit polymerisation made this reaction format unsuitable for routine purposes. Parasite extract-catalysed polymerisation (using 300 μM haem) was inhibited by significantly lower concentrations of the drugs. Chloroquine, mefloquine, and compound (2) inhibited the extract-catalysed polymerisation process by 50% at concentrations of 50, 320, and 60 μM, respectively (Fig. 4B). These data for chloroquine and mefloquine are in general agreement with previous reports [2, 4, 8]. Concentrations of the quinoline antimalarial drugs in this range are achieved easily, and therefore this reaction scheme was used in further assays. The lower concentration of drugs required for inhibition of catalysed polymerisation probably reflects the lower haem concentration used in this assay. This supports the proposal that inhibition occurs via a direct interaction of the quinoline drugs with free haem molecules [22].

The potency of the novel bisquinoline compounds as inhibitors of haem polymerisation was very closely correlated with their activity as inhibitors of growth of *P. falciparum in vitro* (Fig. 5). Compound (2) and members of the 8-aminoquinoline series (compounds 9–11) inhibited

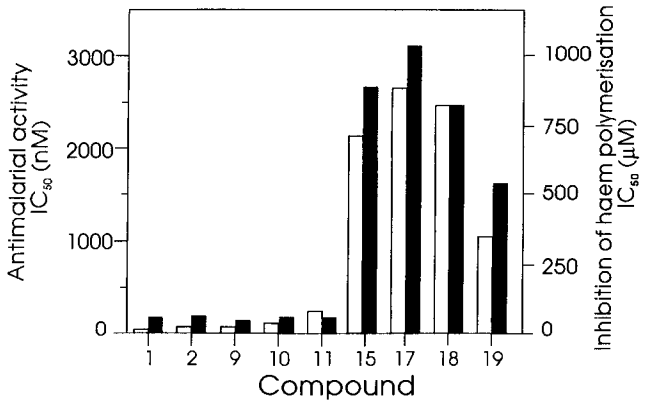

FIG. 5. Correlation of the antimalarial activity of a range of drugs with the ability of the drugs to inhibit haem polymerisation. Antimalarial activity against the D10 strain of *P. falciparum* (open bars) was assessed as described in Materials and Methods. Haem polymerisation inhibitory activity (filled bars) was assessed by incubating haem (0.3 mM) for 4 hr at 37° in 60 mM sodium acetate, pH 5, in the presence of a preparation of approx. 4 × 10^7 parasites and increasing concentrations of the different drugs. The level of haem polymerisation during incubation was corrected for the preformed haemozoin in the parasite extract, and the drug concentration that inhibited growth by 50% was determined. Data represent the results for a typical experiment.

haem polymerisation with an efficiency similar to that of chloroquine. These compounds were also the most potent inhibitors of parasite growth, indicating that these quinolines probably exert their antimalarial effect by inhibiting haem detoxification. The 6-series of compounds were much less effective inhibitors of haem polymerisation. In fact, the least active antimalarial (compound **16**) inhibited the reaction by only 47% at the highest concentration used (5 mM) and, thus is not included in Fig. 5.

It is important to note that the concentrations of the quinoline drugs that were associated with 50% inhibition of haem polymerisation *in vitro* were several 100-fold higher than the concentrations required to inhibit parasite growth in cellular assays. Haem polymerisation is, none the less, likely to be the site of action of these drugs as the quinoline antimalarials will be concentrated inside the acidic food vacuole of the parasite. The dibasic drug, chloroquine, is predicted to be concentrated by 1000- to 5000-fold [23, 24]. The bisquinoline drugs used in this study would bear an overall charge of +4 at the pH of the food vacuole and would be efficiently trapped in this acidic environment. Indeed, their efficient accumulation may be very important for the potency of these compounds against chloroquine-resistant *P. falciparum*.

To summarise, some of the bisquinolines synthesised in this study have potent activity against chloroquine-resistant strains of *P. falciparum*. It appears that the optimal chain length for the linker group is 6 carbons, that linking through the 8-position is better than through the 6-position, that a chlorine substituent is not necessary, and that an amide of the type in **(17)** may be better than one of the type in **(20)**. The low resistance indices for these bisquinolines indicates that they may be useful in circumventing the increasingly serious problem of chloroquine resistance.

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