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SITE OF ACTION OF THE ANTIMALARIAL HYDROXYNAPHTHOQUINONE, 2-[*trans*-4-(4′-CHLOROPHENYL) CYCLOHEXYL]-3-HYDROXY-1,4-NAPHTHOQUINONE (566C80)

MITCHELL FRY and MARY PUDNEY*†
Biochemical Sciences and †Molecular Sciences, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, U.K.

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Abstract—The site of action of the antimalarial compound 2-[*trans*-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80), would appear to be the mitochondrial respiratory chain. Studies reported herein have demonstrated 566C80 to be a potent and selective mitochondrial inhibitor with mitochondria isolated from *Plasmodium falciparum* and *P. yoelii*. Selective assay of individual respiratory chain complexes has shown the primary site of action of 566C80 to be the cytochrome bc_1 complex (Complex III): supportive evidence from difference spectroscopy indicates the site of inhibition to lie between cytochromes *b* and c_1 of this complex. Using [^{14}C]566C80, evidence is presented which suggests that 566C80 may become irreversibly bound to a polypeptide with an approximate molecular mass of 11,500 Da.

As early as 1946, Wendel [1] observed that certain 2-hydroxy-3-alkyl-naphthoquinones inhibited respiration during a study of the action of these compounds on *Plasmodium*. A year later Ball *et al.* [2] showed certain hydroxynaphthoquinones to completely inhibit beef heart succinate oxidase with the site of action lying between cytochromes *b* and *c* of the respiratory chain. Extensive studies by Fieser *et al.* [3] eventually led to the synthesis of 3-(8-cyclohexyl)-octyl-2-hydroxy-1,4-naphthoquinone (menoctone), a potent inhibitor of NADH- and succinate-cytochrome *c* reductases of yeast submitochondrial particles. The antibacterial and antimalarial properties of hydroxynaphthoquinones have been summarized by Olenick [4] and Hudson [5]. Our own interest in the antiprotozoal properties of the hydroxynaphthoquinones led to the synthesis of novel compounds with high efficacy against *Plasmodium, Eimeria* and *Theileria* species [6]. The compound 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone "Clexon" was developed as an effective antiheilerial agent for the treatment of East Coast fever [7]. Novel hydroxynaphthoquinones were shown to be both potent and selective inhibitors of the mitochondrial electron transport chain in the protozoan *Eimeria tenella* [8]; the primary site of inhibition was localized to the ubiquinol-cytochrome *c* reductase span of the respiratory chain.

The ability of certain hydroxynaphthoquinones to inhibit the mitochondrial respiratory process is well-founded, and in 1974 Porter and Folkers [9] described a new fundamental approach to malaria chemotherapy based on the biochemical rationale of inhibition of the electron transfer mechanisms in the metabolism of *Plasmodia* by antimetabolites of coenzyme Q, a view later reiterated by Gutteridge and Coombs [10]. Our own development of an antimalarial hydroxynaphthoquinone has culminated with the compound 2-[*trans*-4-(4′-chlorophenyl) cyclohexyl] - 3 - hydroxy - 1,4 - naphthoquinone, (566C80‡). This potent antimalarial compound [11] is currently undergoing clinical trials for the treatment of *Plasmodium falciparum* malaria. Following the isolation and partial characterization of the *Plasmodium* mitochondrion [12, 13] it has now become possible to test directly the hypothesis that the antimalarial activity of 566C80 is a conseqence of its inhibition of the parasites' mitochondrial respiratory chain.

MATERIALS AND METHODS

Chemicals. 566C80 and the corresponding *cis*-isomer, 296C85, were prepared by the reaction of 2-chloro-1,4-naphthoquinone with the appropriate cyclohexane carboxylic acid in the presence of ammonium persulphate, followed by hydrolysis to the desired hydroxynaphthoquinone according to procedures described previously [6, 14]. ^{14}C-Labeled 566C80 was prepared with the label at the 1-carbon position (sp. act. 153.72 μCi/mg) via reaction of 4-(4′-chlorophenyl)cyclohexylcarboxylic acid with [1-^{14}C]2-methoxy-1,4-naphthoquinone, followed by alkaline hydrolysis. Hydroxynaphthoquinones were solubilized and used directly from stock solutions in AnalaR dimethyl sulphoxide (DMSO). The structure of 566C80 (and 296C85) and the position of the ^{14}C-label, is shown in Fig. 1. 6-Decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone (DB) was synthesized in-

† Corresponding author.

‡ Abbreviations: 566C80, 2-[*trans*-4-(4′-chlorophenyl)-cyclohexyl]-3-hydroxy-1,4-naphthoquinone; 296C85, the *cis*-isomer of 566C80; DMSO, dimethyl sulphoxide; DM, 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone; UHDBT, undecylhydroxydioxobenzothiazole; DCPIP, dichlorophendindophenol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

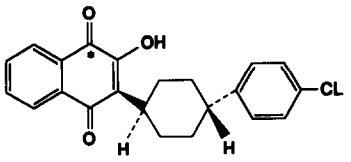

Fig. 1. Structure of 566C80. Asterisk denotes the 1-C position of the ^{14}C-label.

house as described by Wan *et al.* [15]; DB was reduced to DBH by the method of Trumpower and Edwards [16]. UHDBT (undecylhydroxydioxobenzothiazole) was also synthesized in-house according to Friedmann *et al.* [17]. Cytochrome *c* used in these studies was horse heart, Type V1 (Sigma Chemical Co.).

Biological material. Growth, harvesting and the isolation of mitochondrial fractions from *Plasmodium falciparum* and *P. yoelii* were as described previously [12, 18]. Rat liver mitochondria were isolated according to Bustamante *et al.* [19].

Respiratory assays. Cytochrome *c* reductase activity in *Plasmodium* and rat liver mitochondria was measured as described [12]. Assay of individual respiratory chain complexes was made as follows. Isolated mitochondria were sedimented (10,000 *g*, 5 min) and resuspended in 0.05 M potassium phosphate buffer, pH 7.4 containing 0.1% potassium cholate. All assays were made in 1 mL of the phosphate/cholate buffer, at 37°. For assays of ubiquinone reductases, the medium contained 16 mM substrate (or 0.3 mM NADH), 0.1 mM dichlorophenolindophenol (DCPIP), 1 mM azide, 1 μM antimycin A, 1 mM EGTA and 1 μM DB; the reaction was followed by reduction of DCPIP at 600 nm. Cytochrome *c* reductase assays contained 16 mM substrate (or 0.3 mM NADH), 1 mM azide and 100 μM cytochrome *c*; reaction was followed by reduction of cytochrome *c* at 550 nm. Ubiquinol-cytochrome *c* reductase was assayed in a mixture containing 60 μM DBH, 1 mM azide and 100 μM cytochrome *c*, and monitored by reduction of cytochrome *c* at 550 nm. Cytochrome oxidase was assayed using 50 μM reduced cytochrome *c* [20] and followed by the oxidation of reduced cytochrome *c* at 550 nm. Reactions were started by the addition of substrate and corrected for any non-enzymic oxidation–reduction (in the absence of mitochondrial protein) or oxidation–reduction before the addition of substrate. 566C80 was added to the assay immediately following substrate; no preincubation was necessary.

PAGE and autoradiography. Polyacrylamide gel electrophoresis (PAGE) was performed using precast polyacrylamide gels run on a Micrograd electrophoresis unit (Flowgen; U.K. agents for Gradipore Ltd); 12% homogeneous acrylamide sodium dodecyl sulphate (SDS) gels (72 × 72 × 1 mm) were developed in SDS Tris–Tricine buffer, pH 7.5 (containing 0.1% SDS), and 3–27% concave gradient gels were developed in Tris–borate buffer, pH 8.3, containing 0.05% SDS, at a constant current of 20 mA. Molecular weight standards used included a Coomassie pre-stained molecular mass kit (Sigma), a molecular mass markers kit of range 2512–16,949 Da (Pharmacia/LKB), and ^{14}C-methylated cytochrome *c*, molecular mass 12,384 Da (Sigma). Bromophenol blue was used as tracking dye. Silver staining of proteins was made using the kit and protocol supplied by Sigma. Staining of gels for lipoproteins was made using an acetone/acetic acid solution of Sudan black B [21]. Gels for autoradiography were dried according to Popescu [22]. Autoradiographs of dried gels were developed on Kodak X-OMAT AR film using a Dupont Lightning Plus intensifying screen, at −80°. Mitchondrial samples were prepared for electrophoresis by solubilizing in 1% SDS + 10 mM 2-mercaptoethanol, heating to 90° for 1 hr, centrifuging to remove any particulate matter and finally making 5% (v/v) in glycerol; up to 5 μg of protein were routinely applied to gels.

[^{14}C]566C80 *radiolabelling of mitochondria.* Radiolabelling of *P. falciparum* mitochondria with [^{14}C]566C80 was performed under respiratory assay conditions, i.e. to 50 μg mitochondrial protein, in isoosmotic sucrose/mannitol "H-medium" (see Ref. 12), was added substrate (10 mM α-glycerophosphate) and after 1 min incubation at 37° [^{14}C]-566C80 in DMSO was added to a final concentration of 1.5×10^{-8} M, equivalent to 9177 dpm; the final volume was 1 mL. After a further incubation of 10 min at 37°, the mitochondria were pelleted (10,000 *g*/5 min) and washed twice by resuspension in isoosmotic "H-medium", before being prepared for electrophoresis.

Preparation of mitochondrial low molecular mass fraction. Up to 1 mg of SDS-solubilized *P. falciparum* mitochondrial protein ("cold" or labeled with [^{14}C]-566C80) was applied across the width of a 12% SDS gel and electrophoresed in the normal way. At a point approximately 1 mm above the dye front, a 1 cm deep strip was cut with a scalpel across the width of the gel and placed in an Eppendorf-type centrifuge tube suitable for homogenization purposes (Biomedix; [23]). SDS solution (10%; 0.5 mL) was added and the mixture homogenized sufficiently to break the gel (for about 1 min) and then left to stand at room temperature (22°) for 2 hr. After centrifugation (10,000 *g* 2 min) the supernatant was removed and placed in cellulose dialysis tubing (6 mm diameter; Sigma). The dialysis tubing was overlayed with Aquacide III (flake polyethylene glycol, Calbiochem); sample concentration at room temperature was completed within 20 min. The inside of the dialysis tubing was rinsed with 100 μL of distilled water, the rinse then made 5% (v/v) in glycerol and was used for electrophoresis studies on 3–27% acrylamide gels without further treatment.

Measurement of cytochrome spectra. Cytochrome spectra were studied at room temperature (22°) with mitochondria suspended in sucrose/mannitol medium; rat liver mitochondria were used at 10 mg protein/mL with succinate (5 mM) as substrate, and *P. yoelii* mitochondria at 2 mg/mL with α-glycerophosphate (5 mM) as substrate. Respiration was uncoupled by addition of 0.5 μM carbonyl

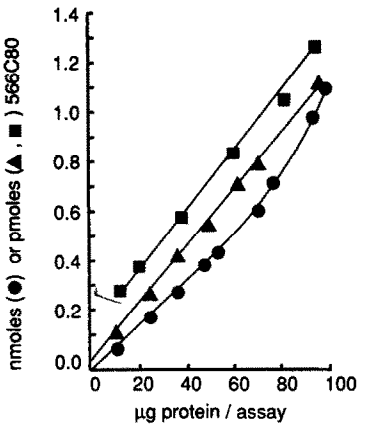

Fig. 2. EC_{50} values for 566C80 determined against different mitochondrial protein concentrations. EC_{50} values for 566C80, i.e. concentrations to give 50% inhibition of cytochrome *c* reductase, were determined for different amounts of mitochondrial protein between 10 and 100 μg protein/assay. Assays were made in a total volume of 1 mL, for succinate-cytochrome *c* reductase (rat liver, ●) or α-glycerophosphate-cytochrome *c* reductase (*Plasmodium falciparum*, ▲ or *P. yoelli*, ■). Note that the 566C80 abscissa is nmoles of drug for inhibition of rat liver, but pmoles of drug for inhibition of *Plasmodium* mitochondria.

cyanide *p*-trifluoromethoxyphenylhydrazone, and oxygenation achieved by addition of 0.5 mM hydrogen peroxide. Additionally, sample cuvettes of rat liver mitochondria contained 1 μM rotenone. Spectra were recorded with a Kontron 930 dual-beam spectrophotometer.

Protein estimation. Protein was estimated using the bicinchoninic acid reagent (Pierce Chemical Co.).

RESULTS

Previous studies on the respiration of isolated *Plasmodium* mitochondria indicated α-glycerophosphate to be a potentially major utilizable substrate; respiration of this substrate was inhibitable by a number of cytochrome bc_1 inhibitors [12]. In initial studies therefore, we assessed the effects of 566C80 on the α-glycerophosphate-cytochrome *c* reductase activity of both *Plasmodium falciparum* and *P. yoelii* mitochondria, and compared this to the effects on succinate-cytochrome *c* reductase activity of rat liver mitochondria. Varying the concentrations of 566C80, EC_{50} values (drug concentration to give 50% inhibition of respiration) were determined in the presence of different amounts of mitochondrial protein. In Fig. 2 are plotted the EC_{50} values vs mitochondrial protein concentration, for rat liver, *P. falciparum* and *P. yoelii*. At the low protein concentrations used in these assays it was apparent that there was a linear relationship between the total *Plasmodium* mitochondrial protein and the EC_{50}; thus, in the range 10–100 μg protein, doubling the protein concentration in the assay approximately doubled the EC_{50}. For rat liver mitochondria a linear relationship was not apparent at higher protein concentrations (above about 50 μg protein/assay) and increasingly greater amounts of 566C80 were required to afford an EC_{50}. The inhibition of *Plasmodium* respiration by 566C80 was similar for *P. falciparum* and *P. yoelli*, but was of the order of a 1000 times less for rat liver mitochondria.

Table 1 provides some EC_{50} values, standardized for 50 μg mitochondrial protein/assay, for 566C80 inhibition of various substrate-cytochrome *c* reductase activities. Potent inhibition of α-glycerophosphate-, succinate- and dihydroorotate-cytochrome *c* reductases were evident in *Plasmodium* mitochondria (α-ketoglutarate and pyruvate + malate do not support respiration in *Plasmodium* mitochondria [12]); NADH-cytochrome *c* reductase was not inhibited by 566C80 (nor by a variety of standard electron transport inhibitors [12]). Again, the far greater efficacy of 566C80 against *Plasmodium* mitochondrial activity was clearly evident (with the exception of NADH respiration). Similarities in the EC_{50} values for 566C80, determined for the various substrate-cytochrome *c* reductases, suggested a site of 566C80 inhibition on the respiratory chain that was common to electron flow from all those substrates, with either rat liver or *Plasmodium* mitochondria.

In order to further delineate the respiratory chain site of action of 566C80, individual respiratory chain complexes were assayed spectrophotometrically in cholate-solubilized preparations of either rat liver or *Plasmodium* mitochondria. The specific activities given in Table 2 are lower for *P. falciparum* compared to rat liver, but were nevertheless readily assayed using approximately 100 μg mitochondrial protein/assay.

NADH, succinate- and α-glycerophosphate-cytochrome *c* reductases of rat liver mitochondria were inhibited by relatively low concentrations of 566C80, as was the ubiquinol-cytochrome *c* reductase, in contrast to the higher concentrations required to inhibit the corresponding ubiquinone-reductase. There was virtually no effect of 566C80 on cytochrome oxidase activity. A similar pattern of 566C80 inhibition was seen for *P. falciparum* mitochondria; inhibition was particularly marked for α-glycerophosphate- and succinate-cytochrome *c* reductase, and including ubiquinol-cytochrome *c* reductase; NADH-cytochrome *c* reductase was not inhibited by 566C80 (as noted in Table 1), nor was cytochrome oxidase. The results in Table 2 suggest an antagonistic action of 566C80 in those ubiquinone-containing respiratory complexes, but in particular they indicate a primary site of action for 566C80 as being the ubiquinol-cytochrome *c* reductase span of the respiratory chain, i.e. the cytochrome bc_1 complex (Complex III). Table 2 also further emphasizes the high efficacy of 566C80 for the *Plasmodium* respiratory chain and indicates that the 566C80-selectivity for the *Plasmodium* system over the rat liver system resides at the level of the cytochrome bc_1 complex.

The high specificity of the 566C80 interaction with the *Plasmodium* mitochondrion was further emphasized in a comparison with 296C85 (the *cis*-isomer of 566C80). EC_{50} values for these two

Table 1. Inhibition of mitochondrial cytochrome *c* reductase by 566C80

Respiratory substrate	EC_{50} (M) (for 50 μg protein/assay)		
	Rat liver	*P. falciparum*	*P. yoelii*
α-Glycerophosphate	$4.7 \pm 1.8 \times 10^{-7}$ (3)	$8.7 \pm 3.6 \times 10^{-10}$ (7)	$9.7 \pm 2.4 \times 10^{-10}$ (5)
Succinate	$4.8 \pm 1.2 \times 10^{-7}$ (4)	$9.6 \pm 3.8 \times 10^{-10}$ (6)	$1.1 \pm 0.15 \times 10^{-9}$ (3)
Dihydroorotate	$5.1 \pm 1.4 \times 10^{-7}$ (3)	$9.5 \pm 2.7 \times 10^{-10}$ (3)	$9.4 \pm 2.4 \times 10^{-10}$ (3)
Pyruvate + malate	$4.9 \pm 1.7 \times 10^{-7}$ (3)		
α-Ketoglutarate	$4.2 \pm 0.9 \times 10^{-7}$ (3)		
NADH	$5.6 \pm 0.8 \times 10^{-7}$ (3)	$>10^{-5}$	$>10^{-5}$

Substrate cytochrome *c* reductase activity was measured by following the reduction of cytochrome *c* at 550 nm in a total volume of 1 mL of sucrose/mannitol "H-medium". Substrates were added at 5 mM, except for dihydroorotate (0.5 mM) and NADH (0.3 mM), and using 50 μg protein/mL. The temperature was 37°. 566C80 was added immediately after substrate and without preincubation; final concentrations of DMSO in the assay did not exceed 0.5% (v/v). Results are the means of duplicate readings on each number of different mitochondrial preparations (figures in parentheses) ± SD.

Table 2. 566C80 inhibition of respiratory chain complexes in rat liver and *P. falciparum* mitochondria

Assay	Specific activity (nmoles substrate oxidized/min/mg protein)		566C80 EC_{50} (M)	
	Rat liver	*P. falciparum*	Rat liver	*P. falciparum*
NADH-UR	502 ± 52 (2)	86 ± 15 (2)	$5.7 \pm 1.3 \times 10^{-5}$ (2)	$>10^{-4}$
NADH-cR	870 ± 61 (3)	392 ± 32 (3)	$1.2 \pm 0.8 \times 10^{-6}$ (3)	$>10^{-4}$
Succinate UR	508 ± 42 (2)	95 ± 12 (2)	$6.1 \pm 2.2 \times 10^{-5}$ (2)	$5.4 \pm 2.3 \times 10^{-5}$ (2)
Succinate-cR	851 ± 28 (5)	126 ± 21 (3)	$9.8 \pm 2.6 \times 10^{-7}$ (3)	$4.6 \pm 2.5 \times 10^{-9}$ (3)
αGP-UR	462 ± 38 (2)	149 ± 31 (4)	$8.2 \pm 3.1 \times 10^{-5}$ (2)	$4.8 \pm 1.1 \times 10^{-5}$ (2)
α-GP-cR	601 ± 89 (3)	196 ± 38 (4)	$1.5 \pm 1.1 \times 10^{-6}$ (3)	$1.2 \pm 0.8 \times 10^{-9}$ (4)
Ub-cR	565 ± 88 (3)	112 ± 18 (3)	$3.2 \pm 1.8 \times 10^{-6}$ (3)	$1.7 \pm 1.3 \times 10^{-9}$ (3)
Cytochrome oxidase	1026 ± 119 (2)	302 ± 42 (2)	$>10^{-4}$	$>10^{-4}$

Activities were assayed using 100 μg mitochondrial protein/mL and specific activities expressed per mg of protein. Results are the means of duplicate determinations (for rat liver) or single determinations (for *P. falciparum*) on a number of different mitochondrial preparations (figures in parentheses) ± SD.

Abbreviations: UR, ubiquinone reductase; cR, cytochrome *c* reductase; αGP, α-glucerophosphate; Ub, ubiquinone.

Table 3. Comparison of 566C80 and 296C85 inhibition of cytochrome *c* reductase

Compound	EC_{50} (M)		
	Rat liver	*P. falciparum*	*P. yoelii*
566C80 *trans*	$4.7 \pm 1.8 \times 10^{-7}$ (3)	$8.7 \pm 3.6 \times 10^{-10}$ (7)	$7.7 \pm 2.4 \times 10^{-10}$ (5)
296C85 *cis*	$7.2 \pm 3.2 \times 10^{-7}$ (2)	$3.4 \pm 1.8 \times 10^{-8}$ (2)	$3.5 \pm 2.7 \times 10^{-8}$ (2)

566C80 or 296C85 were added from DMSO immediately following the addition of substrate (succinate for rat liver or α-glycerophosphate for *Plasmodium* mitochondria). Protein concentration was 50 μg/mL. Results are the means of duplicate readings on each of a number of different mitochondrial preparations (figures in parentheses) ± SD.

compounds are compared in Table 3 for the inhibition of succinate-cytochrome *c* reductase (rat liver) or α-glycerophosphate cytochrome *c* reductase (*Plasmodium*). For rat liver mitochondria, inhibition of succinate-cytochome *c* reductase by 296C85 was only marginally less than with 566C80. In contrast, 296C85 was almost 50 times less effective than 566C80 in inhibiting the α-glycerophosphate-cytochrome *c*

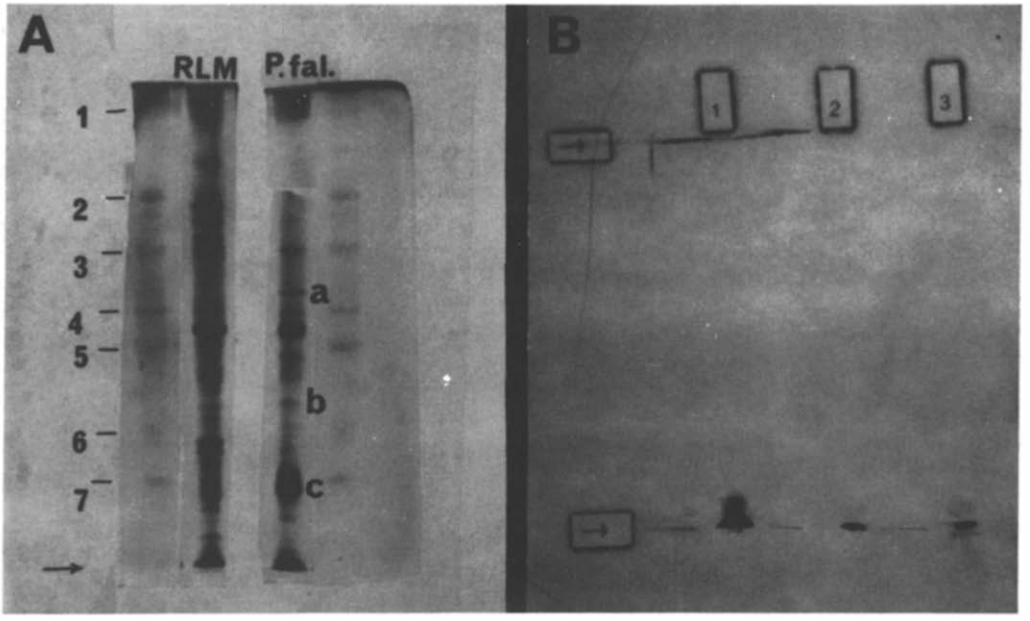

Fig. 3. (A) Acrylamide gel (12%) profile, silver stained for rat liver mitochondria (RLM) and *P. falciparum* mitochondria (P. fal.). Five micrograms of each mitochondrial preparation were applied to the gel. Numbers 1–7 denote the positions of Coomassie blue pre-stained markers [(1,α_2-macroglobin (molecular mass 180,000 Da); 2,β-galactosidase (116,000); (3, fructose-6-phosphate kinase (84,000); 4, pyruvate kinase (58,000); 5, fumarase (48,500); 6, lactic dehydrogenase (36,500) and 7, triosephosphate isomerase (26,600)]. a, b and c denote apparently unique *P. falciparum* mitochondrial components. Arrow marks tracking dye front. (B) Autoradiograph of 12% acrylamide gel run with [^{14}C]566C80-labelled *P. falciparum* mitochondria. Gel was loaded with 50 μg of protein. Column 1, mitochondria labelled with [^{14}C]566C80 according to Materials and Methods; column 2, mitochondria incubated 1 min following substrate addition with "cold" 566C80 (10^{-7} M) before addition of [^{14}C]566C80; column 3, mitochondria treated for 1 hr at 60° prior to incubation with [^{14}C]566C80. Top arrow shows gel origin, bottom arrow position of tracking dye. Fifty micrograms of protein applied to column 1 contained ~1213 dpm. Autoradiograph was developed over 14 days at −80°.

reductase, of either *P. falciparum* or *P. yoelii*. This would indicate a high degree of stereospecificity for the 566C80 interaction with *Plasmodium* mitochondria.

Gel electrophoresis profiles of *P. falciparum* mitochondria, visualized by silver staining, are compared and contrasted with rat liver mitochondria in Fig. 3A. There are numerous bands on the rat liver mitochondrial profile that are absent or barely detectable in the *Plasmodium* profile. Furthermore, there are some bands on the *Plasmodium* gel profile that appear to have no counterparts in the rat liver profile, e.g. bands a, b and c (Fig. 3A). For both mitochondrial preparations there was a significant amount of lower molecular weight material running close to the tracking dye front. When [^{14}C]566C80-labelled mitochondria (see Materials and Methods) were run on 12% gels and subjected to autoradiography, results indicated (Fig. 3B) the presence of radiolabelled low molecular mass material running behind and at the dye front (column 1). However, prior incubation of mitochondria with unlabelled 566C80, before addition of the [^{14}C]566C80, generated only a small amount of radiolabelled material running at the dye front (column 2), as did heat-inactivation of mitochondria prior to addition of [^{14}C]566C80 (column 3). Sudan black B staining of 12% gels, such as that shown in Fig. 3A, indicated the presence of appreciable amounts of lipoprotein material running at the dye front (results not shown). Therefore, in addition to those results shown in columns 2 and 3 of Fig. 3B, further controls were attempted in which [^{14}C]566C80 was applied directly to gels, either in SDS, 5% glycerol or as a lipid emulsion using a total lipid extract from *P. falciparum* mitochondria [24]. However, in none of these cases did [^{14}C]566C80 enter the gel. Excision of the appropriate area of the gel and quantitation by liquid scintillation counting gave approximately 950 dpm for 50 μg of labelled protein electrophoresed. If a 1 cm^2 strip of gel was excised, but excluding approximately 1 mm of gel above the dye front, then about 540 dpm were recovered. A [^{14}C]566C80 labelled mitochondrial preparation, prior to electrophoresis, contained ~1213 dpm/50 μg of protein.

In an attempt to further resolve the ^{14}C-labelled material, the low molecular mass fraction of *P. falciparum* mitochondria extracted from 12% SDS gels (see Materials and Methods) was electrophoresed on 3–27% polyacrylamide gels (Fig. 4A). In the molecular mass region of ~4000–17,000 Da, the extract was resolved into some seven faint bands,

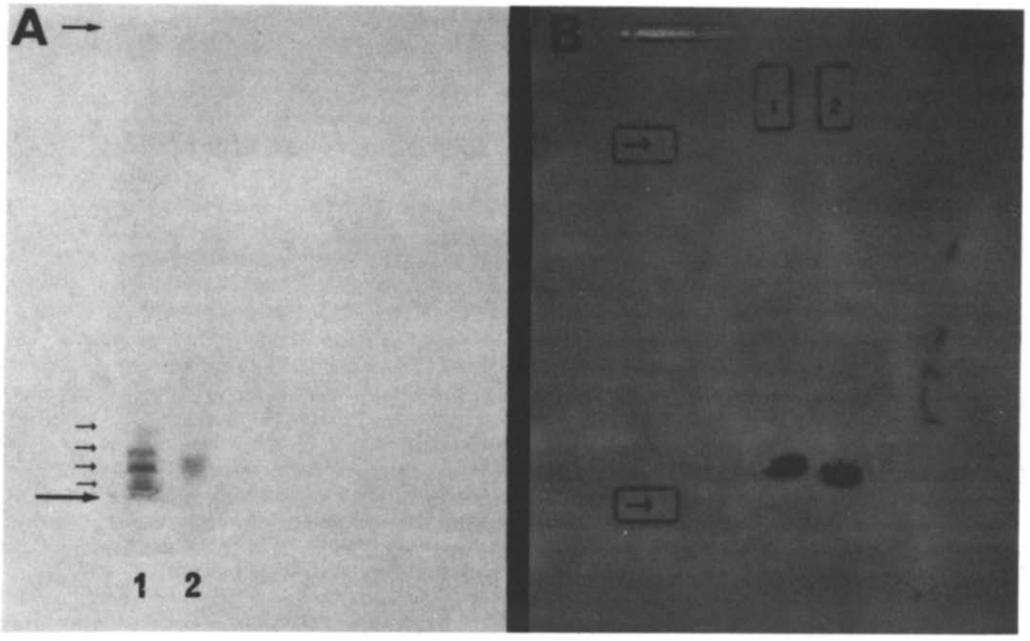

Fig. 4. (A) Acrylamide gel (3–27%) silver stained for molecular mass markers (column 1) and low molecular mass components of *P. falciparum* mitochondria (column 2) as extracted from 12% acrylamide gels (see Materials and Methods). Small arrows show positions of marker proteins in column 1, from top to bottom these being myoglobin (molecular mass 16,949 Da); myoglobin I & II (14,404); myoglobin I (8159); and myoglobin II (6214). Bold arrows show gel origin and tracking dye front. Mitochondrial protein (1 μg) extract was applied to the gel. (B) Autoradiograph of 3–27% acrylamide gel. Column 1 is ^{14}C-methylated cytochrome *c* marker (12,384 Da and column 2 is [^{14}C]566C80 radiolabelled low molecular mass. *P. falciparum* mitochondrial components; 1 μg of protein was applied to the gel in column 2 containing ~5150 dpm. The autoradiograph was developed for 8 days at −80°.

with approximate molecular masses of 16,900, 15,500, 13,000, 11,500, 7600, 6000 and 5000 Da; only those bands of 13,000, 11,500 and 7600 were readily visualized by Coomassie blue or silver staining (seen as a broad band in column 2 of Fig. 4A). Even Coomassie blue staining, followed additionally by silver staining, failed to further enhance the visibility of these components. Autoradiographs of electrophoresed [^{14}C]566C80-labelled material on 3–27% gels produced a well-defined radioactive band, corresponding to a molecular mass of approximately 11,500 Da (column 2 of Fig. 4B) as judged relative to the position of a ^{14}C-methylated cytochrome *c* marker of molecular mass 12,384 Da (column 1, Fig. 4B); no radiolabel was detectable by autoradiography at the tracking dye front. Excision of gel and quantitation of the radiolabel provided some 4200 dpm in a 1 cm^2 gel strip corresponding in position to the 11,500 Da region, for 1 μg of the protein extract electrophoresed on the gel. [^{14}C] 566C80 radiolabelled extract, prior to electrophoresis, contained approximately 5150 dpm/μg protein.

Anaerobiosis in rat liver mitochondrial suspensions, oxidizing succinate, produced cytochrome difference spectra characteristic of reduced cytochromes *c* (550 nm) and *b* (564 nm), shown as trace *b* in Fig. 5A. Addition of antimycin A to mitochondria utilizing succinate generated a reduced cytochrome *b* spectra, but with the absorbance maximum shifted to the slightly longer wavelength of 566 nm, and with oxidation of the cytochrome *c* (trace c). Similarly, addition of 566C80 also caused a reduction of *b* cytochromes (but without any apparent "red shift") and oxidation of cytochrome *c* (trace d). For *P. yoelii* mitochondria, respiring with α-glycerophosphate (Fig. 5B), addition of azide (1 mM) to the sample cuvette was necessary to provide a significant reduction of *b*- and *c*-type cytochromes (trace a). In the absence of azide, addition of antimycin A (trace b) or 566C80 (trace c) caused a partial reduction in *b*-cytochromes and oxidation of *c*-cytochromes.

DISCUSSION

The results presented herein have shown that 566C80 is a highly potent and selective inhibitor of the *Plasmodium* mitochondrial respiratory chain; compared to its effect on isolated rat liver mitochondria, 566C80 inhibition of the parasites mitochondria was some 1000 times more effective, providing for a high therapeutic index. The inhibition of cytochrome *c* reductase activity with different substrates, and the inhibition of the individual respiratory chain complexes, has conclusively shown the cytochrome bc_1 complex (Complex III) to be a prime site of action of 566C80. These findings are very

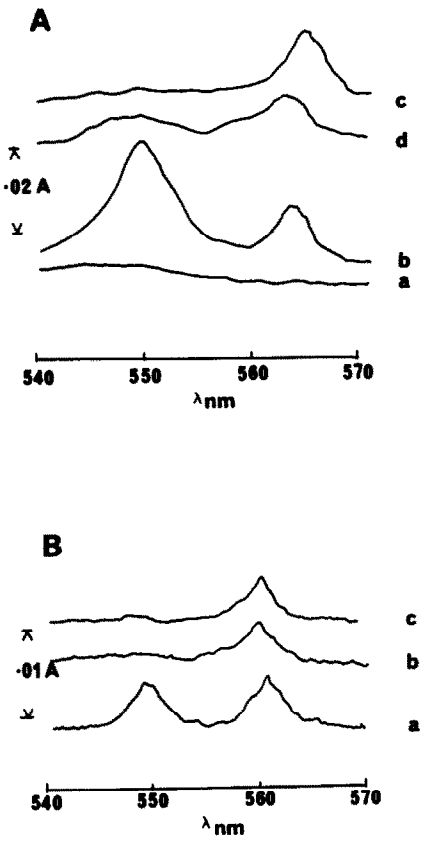

Fig. 5. Cytochrome difference spectra of mitochondria. (A) Rat liver mitochondria oxidizing succinate, (a) baseline, oxidized minus oxidized, (b) succinate reduced (anaerobic) minus oxidized, (c) succinate + antimycin A (5 μM) minus oxidized, (d) succinate + 566C80 (1 μM) minus oxidized. (B) *P. yoelii* mitochondria oxidizing α-glycerophosphate (αGP), (a) α-GP + 1 mM azide minus oxidized, (b) α-GP + antimycin A (5 μM) minus oxidized, (c) α-GP + 566C80 (1 μM) minus oxidized.

similar to those with other hydroxynaphthoquinones shown to be active against the protozoan *Eimeria tenella* [8]. At higher concentrations, 566C80 appeared to act as a more general ubiquinone antagonist, also inhibiting the substrate-ubiquinone reductases of the respiratory chain. Although 566C80 was primarily active against the cytochrome bc_1 complexes of either rat liver or *Plasmodium* mitochondria, a high degree of specificity for the *Plasmodium* complex was clearly evident. Indeed, a comparison of the inhibition between the *trans*- and *cis*-isomers of 566C80 (296C85) demonstrated the marked stereospecificity of the 566C80 interaction with the *Plasmodium* respiratory chain (but not so with the mammalian respiratory chain). Lack of inhibition of the NADH-cytochrome *c* reductase by 566C80, is consistent with the lack of effect of other cytochrome bc_1 inhibitors on this enzyme in *Plasmodium* [12].

Although gel elecrophoretic profiles of rat liver and *Plasmodium* mitochondrial preparations showed some obvious differences, there were also similarities, not least of which was a significant contribution by low molecular mass components running at or close to the tracking dye front. Initial studies, using 12% acrylamide gels, only poorly resolved those components. However, to our surprise, autoradiographs of such gels run with *Plasmodium* mitochondria that had been incubated with [^{14}C]-566C80, indicated that some low molecular mass component(s) had become labelled. Since Sudan black B staining of such gels indicated the presence of lipoprotein material running at the dye front, it was considered possible that the [^{14}C]566C80-labelled material, particularly that running at the dye front, represented non-specific hydrophobically bound 566C80. Extraction of the low molecular mass components from 12% acrylamide gels, taking care to avoid material at the dye front, allowed a better resolution with electrophoresis on 3–27% acrylamide gels (although the resulting bands were poorly stained with silver or Coomassie blue). Furthermore, autoradiography of such gels run with [^{14}C]566C80-labelled *Plasmodium* material indicated the presence of a radiolabelled component with an estimated molecular mass of 11,500 Da. The radiolabelled band in Fig. 4B was relatively broad and may have indicated more than one component with similar molecular mass had been labelled, although no radiolabelled material was present at the dye front. It was apparent therefore that incubation of *Plasmodium* mitochondria with [^{14}C]566C80 resulted in the radiolabelling of at least one component with an approximate molecular mass of 11,500 Da. The possibility that other lower molecular mass components, not fully resolved with these gel systems, were also labelled by 566C80 could not be discounted. However, pre-incubation of *Plasmodium* mitochondria with "cold" 566C80 or inactivation by heat treatment effectively negated the presence of radiolabelled product running close to the dye front while also diminishing that detectable at the dye front (Fig. 3B); this would suggest that actively respiring mitochondria were required to mediate the labelling of the 566C80-binding component(s). We would suggest that the radiolabelled 566C80 running with the dye front was most likely the result of non-specific 566C80–lipoprotein association, although the presence of lytic fragments of the 566C80-binding component cannot be discounted. Since there was no evidence of 566C80 labelling of high molecular mass mitochondrial components, it was assumed that the 11,500 Da-labelled component was not a breakdown product. In the absence of mitochondria, 566C80 was either insoluble or did not migrate in these gel systems, when applied from a DMSO solution, in detergent (SDS) or in a *Plasmodium* mitochondrial lipid extract.

About 4200 dpm were associated with the 11,500 Da component(s) as resolved on 3–27% acrylamide gels, for 1 μg of protein applied. If only one component was actually being labelled with 566C80, and this was assumed to represent approximately one-seventh of the total protein applied to the gel (assuming the labelled component to be a polypeptide), then with a molecular mass of 11,500 Da it could be calculated that 12.4 pmol of

this component were binding approximately 6.9 pmol of 566C80. We would speculate that this was approaching a 1:1 binding with the 11,500 Da component being about 55.6% labelled under the conditions employed.

A hypothesis, developed from these studies, would be that the 11,500 Da component, the "566C80-binding protein", was in fact a constituent of the *Plasmodium* mitochondrial Complex III. Das Gupta and Rieske [25], using a deformamide azido antimycin A (DAA), showed that [^{3}H]DAA bound to a single 11,500 Da component of Complex III (beef-heart), while Wilson *et al.* [26] identified an 11,000 Da polypeptide in the bc_1 complex of *Rhodopseudomonas* van Keulen and Berden [27] identified a 12,000 Da polypeptide in bovine heart cytochrome reductase as an antimycin-binding protein, while Berden *et al.* [28] reported binding of azido derivatives of ubiquinone to this subunit in centre i of the complex. The 566C80-binding component might well be analogous to the antimycin-binding protein, although the binding sites (on this polypeptide) for these two inhibitors would not necessarily be identical. In rat liver mitochondria, although both 566C80 and antimycin A led to a reduction in cytochrome *b* and concomitant oxidation of cytochrome *c*, only the latter caused the characteristic "red shift" in the cytochrome *b* absorbance maximum. The 4-hydroxyquinoline *N*-oxides are also believed to inhibit Complex III in the antimycin A region, although apparently not at an identical binding site [29]. Possibly the binding site for 566C80 is more akin to that of 4-hydroxyquinoline *N*-oxides.

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