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POTENT AND SELECTIVE HYDROXYNAPHTHOQUINONE INHIBITORS OF MITOCHONDRIAL ELECTRON TRANSPORT IN *EIMERIA TENELLA* (APICOMPLEXA: COCCIDIA)

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Abstract—Novel hydroxynaphthoquinones have been shown to be potent and selective inhibitors of mitochondrial electron transport in the protozoan *Eimeria tenella*, inhibiting at concentrations of 10^{-10} to 10^{-11} M. The primary site of electron transport inhibition has been localized to the ubiquinol–cytochrome *c* reductase span of the respiratory chain, whereas a secondary site of inhibition occurs in the NADH- and succinate-ubiquinone reductase complexes. Inhibition at the primary site is selective for the *E.tenella* enzyme; inhibition at the secondary sites is comparable in both *E.tenella* and chick (*Gallus gallus*) liver mitochondria. Hydroxynaphthoquinone inhibition of chick liver succinate–cytochrome *c* reductase was fully reversible by addition of the exogenous ubiquinone-2 analogue, 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone; inhibition of the corresponding *E.tenella* enzyme was not reversed by this ubiquinone. *E.tenella* lines made resistant to the anticoccidial agents decoquinate or clopidol showed no cross-resistance to the hydroxynaphthoquinones, either at the level of electron transport or *in vivo*.

The first observation that certain 2-hydroxy-3-alkyl-naphthoquinones exerted an inhibitory action on respiratory processes was made by Wendel in 1946 [1], during a study of the action of these compounds on malarial parasites. Various hydroxynaphthoquinones were studied as respiratory inhibitors by Ball *et al.* [2] who showed certain of these compounds to inhibit completely beef-heart succinate oxidase at concentrations of about 3×10^{-6} M; the site of inhibition was assessed as lying between cytochromes *b* and *c* of the respiratory chain. Fieser and Richardson [3] and Fieser *et al.* [4] showed a number of naphthoquinones to have considerable antimalarial activity against *Plasmodium lophurae* and *P. vivax*. In later work Fieser *et al.* [5] synthesized additional 2-hydroxy-3-(*w*-cyclohexyl-alkyl)-1,4-naphthoquinones, including 3-*w*-cyclohexyl-octyl-2-hydroxy-1,4-naphthoquinone (menoctone). This latter compound was shown to be a potent inhibitor of the NADH–cytochrome *c* reductase of yeast submitochondrial particles, and the succinate–cytochrome *c* reductase of both yeast and beef-heart submitochondrial particles, inhibition being reversed by the addition of exogenous ubiquinone-6 [6, 7]. Inhibition of succinate oxidase [8] and succinate–cytochrome *c* reductase [9] by 3-substituted-2-hydroxy-1,4-naphthoquinones was also shown to be reversed by the addition of ubiquinone. The obvious structural similarities between the naphthoquinones and ubiquinone prompted Porter and Folkers [10] to synthesize a wide variety of heterocyclic analogues of the 2-hydroxy-3-alkyl-1,4-naphthoquinones as putative antagonists of ubiquinone function in the respiratory chain. Recent reviews by Olenick [11] and Hudson [12] have summarized the literature on the antibacterial and antimalarial properties of these compounds.

Our interest in the antiprotozoal properties of the hydroxynaphthoquinones was stimulated by the discovery that menoctone was highly active against the previously incurable tick-borne cattle disease East Coast fever (ECF) caused by the protozoan *Theileria parva* [13, 14]. This interest has led to the development of 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone (BW 993C—parvaquone, 'CLEXON') as an effective antitheilerial agent [15–17] now being used for the treatment of ECF. Novel hydroxynaphthoquinones that show high efficacy against *Plasmodium*, *Eimeria* and *Theileria* species have now been synthesized [18].

The hydroxynaphthoquinones have been assumed to owe their antiprotozoal activity to inhibition of the parasite respiratory chain reputedly via ubiquinone antagonism [19]. Evidence for this mode of action has been based mainly on studies using mitochondria derived from mammalian sources. In a study of electron transport in mitochondria isolated from the coccidian *Eimeria tenella*, Wang [20] identified a number of quinolone anticoccidial agents as potent respiratory inhibitors; they were, however, only poor inhibitors of electron transport in chick liver mitochondria. In this same study, 2-hydroxy-3-(4-phenoxy-*o*-phenyl) propyl-1,4-naphthoquinone and 2-hydroxy-3-(4-*trans*-cyclohexyl)cyclohexyl-1,4-naphthoquinone, were also shown to be good inhibitors of *E.tenella* electron transport but, unlike the

quinolones, were equally effective against chick liver mitochondria. We have since confirmed decoquinate (a quinolone) and clopidol (a pyridone) to be potent inhibitors of *E.tenella* mitochondrial electron transport [21].

The present work extends our studies on *E.tenella* mitochondrial electron transport [21]. Novel hydroxynaphthoquinones are reported that show high efficacy and selectivity as electron transport inhibitors in *E.tenella*.

MATERIALS AND METHODS

Chemicals

Respiratory inhibitors and substrates, including cytochrome *c* (Type VI: horse heart), were obtained from Sigma Chemical Co. Cholate was obtained from Aldrich Chemical Co. 6-Decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone (DB), an analogue of ubiquinone-2, was synthesized as described by Wan *et al.* [22]. For use as substrate in the ubiquinol–cytochrome *c* reductase assay, DB was reduced to DBH by the method of Trumpower and Edwards [23].

The syntheses of hydroxynaphthoquinones used in this study are to be found in the following references: BW 59C [24], menoctone [5], BW 993C [25], BW 58C and BW 568C [26] and BW 720C [27]. BW 661C (2,hydroxy-3-(*trans*-4-cyclohexylcyclohexyl)-1,4 naphthoquinone) was purchased from the Aldrich Chemical Co. The structures of these compounds and their isomer ratios where appropriate are given in Fig. 1.

Biological materials and in vivo *sensitivity tests*

The strain and lines of *Eimeria tenella* used in these studies, and methods for *in vivo* sensitivity tests in chickens (*Gallus gallus*) and the collection of unsporulated oocysts were as previously described [21]. All lines of *E.tenella* were derived from the Weybridge strain: the sensitive (control) line is designated the Berkhamsted line; the decoquinate-resistant line, Berkhamsted decR; the clopidol-resistant line, Berkhamsted cloR.

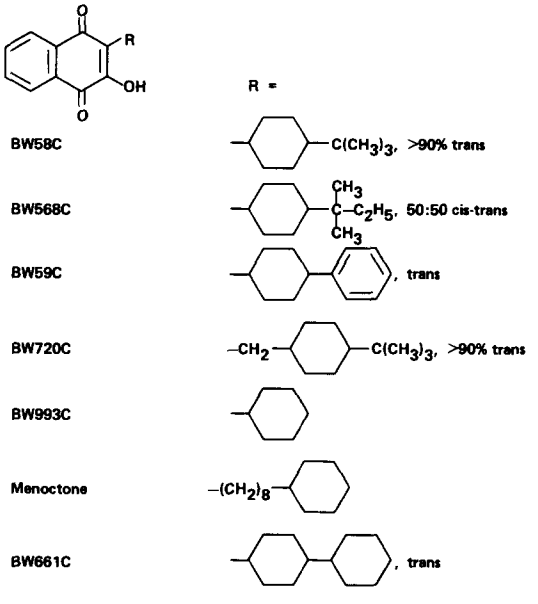

Fig. 1. Chemical formulae of hydroxynaphthoquinones.

Isolation of mitochondria. Mitochondria from unsporulated oocysts of *E.tenella* were isolated and assayed polarographically in the same medium as previously described [21]. Liver mitochondria from rat (Wistar) and chick (Brown Leghorn) were isolated and assayed polarographically in the same medium using established isolation procedures [28]. Submitochondrial particles were prepared by sonicating mitochondrial fractions at 6 μm (peak to peak amplitude) for 3 × 30 sec.

Measurement of respiration. Oxygen uptake by mitochondrial fractions was assayed polarographically using a closed system Clark-type oxygen electrode, as previously described [21]. Approximately 1 mg mitochondrial protein was used per assay (3 ml total volume), with either NADH (0.3 mM) or succinate (3.3 mM) as substrates. Hydroxynaphthoquinones were formulated and added in 'AnalaR' dimethylformamide. The final concentration of dimethylformamide in the assay medium never exceeded 0.33% v/v. The effect of inhibitors was expressed as a percentage inhibition of the respiratory rate (compared to controls with dimethylformamide added alone) and as an EC_{50} (that concentration required to give 50% inhibition of respiration). Assay of NADH- and succinate-oxidases was made on mitochondrial fractions (*E.tenella*) or submitochondrial fractions (rat or chick liver) (*E.tenella* mitochondria readily oxidise NADH without the need to invert by sonication [21]).

Assay of respiratory chain complexes. Isolated mitochondria (of either *E.tenella*, or chick or rat liver) were sedimented (10,000 *g*, 10 min) and resuspended to about 10 mg protein/ml in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.1% v/v potassium cholate. All enzyme assays were made in 3 ml of phosphate/cholate buffer, at 37°. In addition, medium for the assay of succinate–ubiquinone reductase contained succinate (16 mM), dichlorophenolindophenol (0.1 mM), KCN (1 mM), 1,2-di-(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, EGTA (1 mM), and DB (1 μM) and this reaction was followed by reduction of dichlorophenolindopenol at 600 nm. NADH-ubiquinone reductase was assayed in a similar medium containing in addition malonate (6 mM), and using NADH (0.3 mM) as substrate; the reaction was followed by oxidation of NADH at 340 nm. Succinate–cytochrome *c* reductase and NADH–cytochrome *c* reductase were assayed by modification of the above procedures, replacing DB and dichlorophenolindophenol with cytochrome *c* (33 μM) and the reaction followed by reduction of cytochrome *c* at 550 nm. Ubiquinol–cytochrome *c* reductase was assayed in medium containing 1 mM KCN, 60 μM DBH and 33 μM cytochrome *c*, and followed by reduction of cytochrome *c* at 550 nm. Cytochrome oxidase was assayed in medium containing reduced cytochrome *c* (50 μM) and followed by oxidation of reduced cytochrome *c* at 550 nm (cytochrome *c* was

reduced by ascorbate, followed by dialysis to remove excess ascorbate [29]).

Reactions were started by addition of solubilized mitochondrial protein: about 0.1 mg protein/ml for assay of succinate–ubiquinone reductase and succinate–cytochrome *c* reductase, and about 0.5 mg protein/ml for other respiratory complexes. Specific activities were calculated after subtraction of non-enzymic oxidation–reduction reactions. Hydroxynaphthoquinones were added in dimethylformamide immediately following addition of mitochondrial protein and mixed by inversion of stoppered cuvettes. All assays were made at 37° in 3 ml quartz cuvettes of 1 cm light-path using a Beckman DU-8 spectrophotometer.

Protein estimation. Protein was estimated using a Bio-Rad Coomassie Blue protein assay kit (Bio-Rad Laboratories Ltd) employing bovine gammaglobulin as a standard.

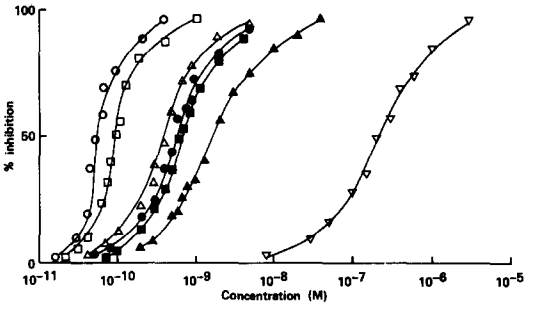

Fig. 2. Semi-logarithmic dose–response curves for hydroxynaphthoquinone inhibition of *E.tenella* mitochondrial NADH-oxidase. Results are from a typical experiment; points are single determinations on one preparation of *E.tenella* mitochondria. Hydroxynaphthoquinones were added in dimethylformamide (not exceeding 0.33% v/v) following addition of substrate; % inhibition was determined from single additions of hydroxynaphthoquinones per assay. BW 58C —○—, 568C —□—, 59C —△—, Menoctone —●—, 720 —■—, 661C —▲—, 993C —▽—.

RESULTS

The hydroxynaphthoquinones used in these studies were all potent inhibitors of NADH- and succinate-oxidase activity of respiring *E.tenella* mitochondria. Figure 2 exemplifies the typical dose–response curves obtained for hydroxynaphthoquinone inhibition of NADH-oxidase activity of *E.tenella* mitochondria. Dose–response curves were typically sigmoidal in appearance, 100% inhibition of respiration was achieved with all the hydroxynaphthoquinones tested, and preincubation of inhibitor and mitochondria was unnecessary for the full expression of inhibition. EC_{50} values were calculated from dose–response curves by unweighted linear regression analysis of points (minimum of 4 points) between 20 and 70% inhibition of respiration, i.e. the linear portion of the dose–response curve. Hydroxynaphthoquinone EC_{50}s against NADH- and succinate-oxidase of *E.tenella*, rat and chick liver mitochondria are compared in Table 1. The extreme potency of these compounds against the *E.tenella* electron transport system is exemplified by the two most active members of this series, BW 58C ($EC_{50} = 5.3 \times 10^{-11}$ M, NADH-oxidase) and BW 568C ($EC_{50} = 9.3 \times 10^{-11}$ M, NADH-oxidase). Hydroxynaphthoquinone inhibition of *E.tenella* mitochondrial electron transport was similar for both NADH- and succinate-oxidase, sug-

Table 1. Comparison of hydroxynaphthoquinone inhibition of electron transport in *E.tenella*, rat and chick liver mitochondria

Hydroxynaphthoquinone		EC_{50} (M)			Therapeutic index EC_{50} (chick)/ EC_{50} (*E.tenella*)
		E.tenella	Chick	Rat	
BW 58C	N	$5.3 \pm 2.6 \times 10^{-11}$ (6)	$5.4 \pm 2.2 \times 10^{-7}$ (2)	$5.3 \pm 1.7 \times 10^{-7}$ (3)	10,189
	S	$6.2 \pm 2.2 \times 10^{-11}$ (3)	$5.8 \pm 1.8 \times 10^{-7}$ (3)	$6.4 \pm 1.9 \times 10^{-7}$ (3)	9355
BW 568C	N	$9.3 \pm 1.8 \times 10^{-11}$ (6)	$2.1 \pm 2.8 \times 10^{-7}$ (2)	$7.0 \pm 2.1 \times 10^{-7}$ (3)	2258
	S	$1.3 \pm 3.3 \times 10^{-10}$ (3)	$4.2 \pm 2.9 \times 10^{-7}$ (3)	$8.1 \pm 3.4 \times 10^{-7}$ (3)	3231
BW 59C	N	$3.8 \pm 1.9 \times 10^{-10}$ (4)	$3.8 \pm 4.2 \times 10^{-7}$ (2)	$4.3 \pm 3.7 \times 10^{-7}$ (3)	1000
	S	$5.2 \pm 2.5 \times 10^{-10}$ (3)	$4.7 \pm 1.9 \times 10^{-7}$ (3)	$5.4 \pm 2.4 \times 10^{-7}$ (3)	904
Menoctone	N	$4.4 \pm 1.8 \times 10^{-10}$ (5)	$9.3 \pm 2.1 \times 10^{-8}$ (2)	$3.3 \pm 1.8 \times 10^{-8}$ (5)	211
	S	$6.2 \pm 2.8 \times 10^{-10}$ (4)	$1.3 \pm 3.1 \times 10^{-7}$ (3)	$5.8 \pm 2.6 \times 10^{-8}$ (4)	210
BW 720C	N	$5.0 \pm 2.2 \times 10^{-10}$ (4)	$3.2 \pm 1.7 \times 10^{-7}$ (3)	$1.3 \pm 0.9 \times 10^{-7}$ (3)	640
	S	$6.2 \pm 3.1 \times 10^{-10}$ (3)	$4.3 \pm 2.8 \times 10^{-7}$ (3)	$3.7 \pm 3.4 \times 10^{-7}$ (3)	694
BW 661C	N	$1.4 \pm 1.8 \times 10^{-9}$ (5)	$5.7 \pm 1.9 \times 10^{-8}$ (3)	$1.6 \pm 2.0 \times 10^{-7}$ (3)	41
	S	$3.2 \pm 2.3 \times 10^{-9}$ (3)	$6.2 \pm 1.8 \times 10^{-8}$ (3)	$3.4 \pm 3.1 \times 10^{-7}$ (3)	19
BW 993C	N	$2.2 \pm 0.8 \times 10^{-7}$ (5)	$1.8 \pm 1.3 \times 10^{-7}$ (3)	$3.3 \pm 1.5 \times 10^{-7}$ (4)	0.8
	S	$3.1 \pm 1.7 \times 10^{-7}$ (3)	$2.0 \pm 1.1 \times 10^{-7}$ (3)	$3.8 \pm 0.9 \times 10^{-7}$ (4)	0.6

Hydroxynaphthoquinones were tested for inhibition of oxygen uptake by *E.tenella* mitochondria and rat or chick liver submitochondrial particles. N = NADH-oxidase, S = succinate oxidase. Rat and chick liver submitochondrial particles were used to (a) provide an NADH-oxidase assay for direct comparison with *E.tenella* mitochondria, and (b) to avoid problems introduced with possible uncoupling of State 3 respiring rat and chick liver mitochondria. (*E.tenella* mitochondria isolated as described are uncoupled [21].) Results are ± standard deviation; figures in brackets denote number of different mitochondrial preparations studied. EC_{50} values were calculated from linear regression analysis of dose response curves (see Fig. 2) for points (minimum of 4) between 20 and 70% inhibition.

gesting a common site of inhibition on the electron transport chain. Although the hydroxynaphthoquinones, particularly menoctone and BW 661C, were relatively potent inhibitors of rat and chick liver mitochondrial electron transport, they display an obvious selectivity for the *E.tenella* system. An indication of the therapeutic indices of these compounds is given by consideration of the ratio EC_{50} (chick): EC_{50} (*E.tenella*) (Table 1); ratios as high as 10,000 were obtained (for BW 58C).

Individual respiratory chain complexes were assayed spectrophotometrically in solubilized mitochondrial preparations from *E.tenella* and chick liver (Table 2). Measureable specific activities were present for all respiratory chain complexes in *E.tenella* mitochondria, although these were lower than the corresponding enzyme complexes of chick liver mitochondria.

The inhibition of respiratory chain complexes in *E.tenella* mitochondria by BW 58C is exemplified by Fig. 3. Electron transport from NADH or succinate to cytochrome *c*, and from ubiquinol to cytochrome *c*, was particularly sensitive to disruption, 100% inhibition being achieved at concentrations of about 10^{-9} M. Higher concentrations of BW 58C also inhibited electron transport from succinate or NADH to ubiquinone, 100% inhibition being achieved at concentrations of about 10^{-4} M. Cytochrome oxidase activity was inhibited to some 20% at millimolar concentrations of BW 58C.

In Table 3 are listed the EC_{50} values for hydroxynaphthoquinone inhibition of the various respiratory chain complexes of *E.tenella* and chick liver mitochondria. EC_{50} values for NADH- and succinate-oxidase are included in Table 3 for comparison. It is clear from Fig. 3 and Table 3 that the hydroxynaphthoquinones exert their primary inhibition on the ubiquinol–cytochrome *c* reductase segment of the respiratory chain, in both *E.tenella* and chick liver mitochondrial electron transport chains. The extent and pattern of hydroxynaphthoquinone inhibition of the ubiquinol to cytochrome *c* span of the respiratory chain, for either *E.tenella* or chick liver mitochondria, parallels that obtained in the overall polarographic assay of NADH- and succinate-oxidases (Table 1). Similarly, the selectivity of the hydroxynaphthoquinones for the *E.tenella*

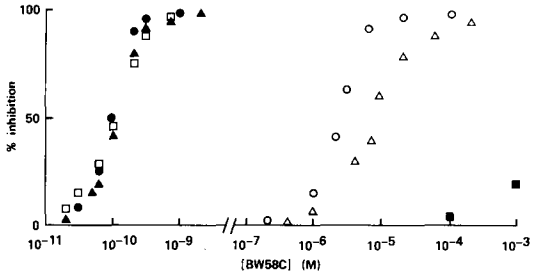

Fig. 3. Semi-logarithmic dose–response curves for BW 58C inhibition of *E.tenella* mitochondrial respiratory chain complexes. Results are from a typical experiment; points are single determinations on one preparation of *E.tenella* mitochondria. BW 58C was added in dimethylformamide (not exceeding 0.5% v/v); % inhibition was determined from single additions of BW 58C per assay. NADH–ubiquinone reductase (○), succinate–ubiquinone reductase (△), NADH–cytochrome *c* reductase (□), succinate–cytochrome *c* reductase (▲), ubiquinol–cytochrome *c* reductase (●), cytochrome oxidase (■).

system is underlined by consideration of their inhibition of NADH- or succinate–cytochrome *c* reductases and ubiquinol–cytochrome *c* reductase. A secondary hydroxynaphthoquinone inhibition is observed for the NADH- and succinate–ubiquinone reductases of both *E.tenella* and chick liver mitochondria. Selective inhibition of these latter respiratory complexes in *E.tenella* is not nearly so marked, and the pattern of hydroxynaphthoquinone inhibition differs from that observed against the ubiquinol to cytochrome *c* span of the respiratory chain. Thus, menoctone is the best inhibitor of NADH–ubiquinone reductase and succinate–ubiquinone reductase in both *E.tenella* ($EC_{50} = 1.7 \times 10^{-6}$ M and 5.5×10^{-5} M, respectively) and chick liver mitochondria ($EC_{50} = 5.3 \times 10^{-5}$ M and 6.5×10^{-5} M, respectively). In general, the hydroxynaphthoquinones were rather better inhibitors of NADH- and succinate–ubiquinone reductases of *E.tenella* than of chick liver mitochondria, and also gave consistently better inhibition of the NADH- rather than the succinate–ubiquinone reductase (of *E.tenella* and chick liver mitochondria).

Hydroxynaphthoquinones were tested against mitochondria from lines of *E.tenella* that showed

Table 2. Specific activity of respiratory chain complexes in *E.tenella* and chick liver mitochondria

Respiratory complex	Specific activity (nmole substrate oxidized/min/mg protein)	
	E.tenella	Chick liver
NADH–ubiquinone reductase	402 ± 21 (4)	820 ± 29 (3)
NADH–cytochrome *c* reductase	386 ± 15 (4)	810 ± 28 (3)
Succinate–ubiquinone reductase	423 ± 18 (4)	843 ± 31 (3)
Succinate–cytochrome *c* reductase	410 ± 24 (4)	823 ± 24 (3)
Ubiquinol–cytochrome *c* reductase	316 ± 11 (4)	786 ± 23 (3)
Cytochrome oxidase	512 ± 28 (4)	982 ± 38 (3)

Respiratory chain complexes were assayed in phosphate–cholate medium, as described in Methods. Results are given ± standard deviation; figures in brackets denote number of different mitochondrial preparations studied.

Table 3. Hydroxynaphthoquinone inhibition of respiratory chain complexes in chick and *E.tenella* mitochondria

	Hydroxynaphthoquinone EC_{50}(M)						
	BW 58C	BW 568C	BW 59C	Menoctone	BW 720C	BW 661C	BW 993C
E.tenella							
NADH oxidase	$5.3 \pm 2.6 \times 10^{-11}$ (6)	$9.3 \pm 1.8 \times 10^{-11}$ (6)	$3.8 \pm 1.9 \times 10^{-10}$ (4)	$4.4 \pm 1.8 \times 10^{-10}$ (5)	$5.0 \pm 2.2 \times 10^{-10}$ (4)	$1.4 \pm 1.8 \times 10^{-9}$ (5)	$3.3 \pm 2.1 \times 10^{-7}$ (4)
NADH-QR	$2.4 \pm 3.2 \times 10^{-6}$ (3)	$4.3 \pm 3.6 \times 10^{-6}$ (3)	$2.3 \pm 3.3 \times 10^{-5}$ (3)	$1.7 \pm 3.0 \times 10^{-6}$ (3)	$4.8 \pm 3.6 \times 10^{-5}$ (3)	$5.3 \pm 3.4 \times 10^{-5}$ (3)	$5.6 \pm 2.6 \times 10^{-5}$ (3)
NADH-CR	$9.0 \pm 1.8 \times 10^{-11}$ (3)	$9.8 \pm 2.5 \times 10^{-11}$ (3)	$4.5 \pm 1.7 \times 10^{-10}$ (3)	$5.6 \pm 2.1 \times 10^{-10}$ (3)	$6.3 \pm 2.4 \times 10^{-10}$ (3)	$3.2 \pm 1.6 \times 10^{-9}$ (3)	$4.8 \pm 1.5 \times 10^{-7}$ (3)
Succinate oxidase	$6.2 \pm 2.2 \times 10^{-11}$ (3)	$1.3 \pm 3.3 \times 10^{-10}$ (3)	$5.2 \pm 2.5 \times 10^{-10}$ (3)	$6.2 \pm 2.8 \times 10^{-10}$ (4)	$6.2 \pm 3.1 \times 10^{-10}$ (3)	$3.2 \pm 2.3 \times 10^{-9}$ (3)	$3.1 \pm 1.7 \times 10^{-7}$ (3)
Succ-QR	$7.4 \pm 3.7 \times 10^{-6}$ (3)	$5.8 \pm 4.1 \times 10^{-6}$ (3)	$5.3 \pm 3.6 \times 10^{-5}$ (3)	$5.5 \pm 3.2 \times 10^{-6}$ (3)	$6.6 \pm 2.8 \times 10^{-5}$ (3)	$8.4 \pm 4.1 \times 10^{-5}$ (3)	$9.6 \pm 1.6 \times 10^{-5}$ (3)
Succ-CR	$1.2 \pm 2.1 \times 10^{-10}$ (3)	$1.4 \pm 1.8 \times 10^{-10}$ (3)	$5.2 \pm 2.4 \times 10^{-10}$ (3)	$6.8 \pm 1.6 \times 10^{-10}$ (3)	$7.2 \pm 3.0 \times 10^{-10}$ (3)	$4.1 \pm 1.4 \times 10^{-9}$ (3)	$3.8 \pm 0.9 \times 10^{-7}$ (3)
Ubiquinol-R	$1.0 \pm 1.7 \times 10^{-10}$ (3)	$1.2 \pm 0.8 \times 10^{-10}$ (3)	$3.9 \pm 1.9 \times 10^{-10}$ (3)	$4.9 \pm 1.8 \times 10^{-10}$ (3)	$5.8 \pm 1.8 \times 10^{-10}$ (3)	$1.8 \pm 0.8 \times 10^{-9}$ (3)	$3.8 \pm 0.8 \times 10^{-7}$ (3)
Cyt. oxidase	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$
Chick liver							
NADH oxidase	$5.4 \pm 2.2 \times 10^{-7}$ (2)	$2.1 \pm 2.8 \times 10^{-7}$ (2)	$3.8 \pm 4.2 \times 10^{-7}$ (2)	$9.3 \pm 2.1 \times 10^{-8}$ (3)	$3.2 \pm 1.7 \times 10^{-7}$ (3)	$5.7 \pm 1.9 \times 10^{-8}$ (3)	$1.8 \pm 1.3 \times 10^{-7}$ (3)
NADH-QR	$3.2 \pm 1.6 \times 10^{-4}$ (2)	$3.8 \pm 3.1 \times 10^{-4}$ (2)	$8.9 \pm 1.8 \times 10^{-5}$ (3)	$5.3 \pm 1.8 \times 10^{-5}$ (3)	$6.8 \pm 2.3 \times 10^{-5}$ (2)	$5.5 \pm 1.8 \times 10^{-5}$ (2)	$5.8 \pm 1.7 \times 10^{-5}$ (2)
NADH-CR	$5.9 \pm 1.1 \times 10^{-7}$ (3)	$2.7 \pm 2.2 \times 10^{-7}$ (3)	$3.9 \pm 2.4 \times 10^{-7}$ (3)	$9.8 \pm 3.1 \times 10^{-8}$ (2)	$3.9 \pm 1.8 \times 10^{-7}$ (3)	$6.3 \pm 1.2 \times 10^{-8}$ (3)	$2.3 \pm 0.8 \times 10^{-7}$ (2)
Succinate oxidase	$5.8 \pm 1.8 \times 10^{-7}$ (3)	$4.2 \pm 2.9 \times 10^{-7}$ (3)	$4.7 \pm 1.9 \times 10^{-7}$ (3)	$1.3 \pm 3.1 \times 10^{-7}$ (3)	$4.3 \pm 2.8 \times 10^{-7}$ (3)	$6.2 \pm 1.8 \times 10^{-8}$ (3)	$2.0 \pm 1.1 \times 10^{-7}$ (3)
Succ-QR	$4.1 \pm 2.3 \times 10^{-4}$ (2)	$5.2 \pm 1.8 \times 10^{-4}$ (2)	$1.2 \pm 0.8 \times 10^{-4}$ (2)	$6.5 \pm 1.7 \times 10^{-5}$ (2)	$8.4 \pm 1.8 \times 10^{-5}$ (2)	$7.1 \pm 1.8 \times 10^{-5}$ (2)	$4.9 \pm 2.2 \times 10^{-5}$ (2)
Succ-CR	$6.6 \pm 1.6 \times 10^{-7}$ (3)	$5.1 \pm 2.1 \times 10^{-7}$ (2)	$5.4 \pm 1.4 \times 10^{-7}$ (3)	$2.1 \pm 2.6 \times 10^{-7}$ (2)	$5.1 \pm 0.9 \times 10^{-7}$ (2)	$6.6 \pm 1.7 \times 10^{-8}$ (3)	$3.1 \pm 1.8 \times 10^{-7}$ (2)
Ubiquinol-R	$5.4 \pm 2.3 \times 10^{-7}$ (3)	$2.8 \pm 2.4 \times 10^{-7}$ (3)	$4.1 \pm 0.8 \times 10^{-7}$ (3)	$1.1 \pm 0.5 \times 10^{-7}$ (3)	$3.3 \pm 0.5 \times 10^{-7}$ (3)	$5.8 \pm 1.1 \times 10^{-8}$ (3)	$2.2 \pm 0.9 \times 10^{-7}$ (5)
Cyt. oxidase	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$

Respiratory chain complexes were assayed in cholate-solubilized mitochondrial preparations from *E.tenella* and chick liver mitochondria. Hydroxynaphthoquinones were added in dimethylformamide (not exceeding 0.5% v/v final concentration) following addition of substrate. Results were adjusted for endogenous oxidation–reduction activity in the absence of substrate, and control activity in the presence of dimethylformamide alone. Results are given ± standard deviation; figures in brackets denote number of different mitochondrial preparations studied. Succ (NADH)-QR = succinate (NADH)–ubiquinone reductase, Succ (NADH)-CR = succinate(NADH)–cytochrome *c* reductase, Ubiquinol-R = ubiquinol–cytochrome *c* reductase, and Cyt.oxidase = cytochrome oxidase.

Table 4. Hydroxynapthoquinone inhibition of sensitive, and decoquinate and clopidol resistant *E.tenella* mitochondria

Hydroxynaphthoquinone	EC_{50} (M) against lines of Weybridge strain		
	Sensitive line (Berkhamsted)	Decoquinate resistant line (Berkhamsted decR)	Clopidol resistant line (Berkhamsted cloR)
BW 58C	$5.3 \pm 2.6 \times 10^{-11}$ (6)	$6.8 \pm 1.8 \times 10^{-11}$ (3)	$5.5 \pm 3.1 \times 10^{-11}$ (3)
BW 568C	$9.3 \pm 1.8 \times 10^{-11}$ (6)	$1.6 \pm 2.1 \times 10^{-10}$ (3)	$1.2 \pm 1.7 \times 10^{-10}$ (3)
BW 59C	$3.8 \pm 1.9 \times 10^{-10}$ (4)	$5.2 \pm 1.8 \times 10^{-10}$ (3)	$3.9 \pm 2.1 \times 10^{-10}$ (2)
Menoctone	$4.4 \pm 1.8 \times 10^{-10}$ (5)	$6.6 \pm 1.2 \times 10^{-10}$ (3)	$4.2 \pm 1.3 \times 10^{-10}$ (3)
BW 720C	$5.0 \pm 2.2 \times 10^{-10}$ (4)	$7.4 \pm 1.6 \times 10^{-10}$ (3)	$4.7 \pm 2.6 \times 10^{-10}$ (2)
BW 661C	$1.4 \pm 1.8 \times 10^{-9}$ (5)	$3.2 \pm 2.2 \times 10^{-9}$ (3)	$1.8 \pm 1.7 \times 10^{-9}$ (2)
BW 993C	$3.3 \pm 1.5 \times 10^{-7}$ (4)	$5.3 \pm 0.9 \times 10^{-7}$ (3)	$3.6 \pm 1.8 \times 10^{-7}$ (2)

Hydroxynaphthoquinones were tested for inhibition of oxygen uptake by respiring *E.tenella* mitochondria (NADH-oxidase) derived from either sensitive, decoquinate or clopidol-resistant lines [21]. Results are ± standard deviation; figures in brackets denote number of different preparations studied.

resistance to anticoccidial agents of different chemical types, either decoquinate or clopidol. The decR line of *E.tenella* tested shows an almost 80-fold resistance to decoquinate at the level of electron transport, and the cloR line about a 3-fold resistance to clopidol at the level of electron transport [21]. From Table 4 it can be seen that the EC_{50} values for all hydroxynaphthoquinones were essentially similar for either sensitive, or decoquinate or clopidol resistant *E.tenella* lines. There would therefore appear to be little or no cross-resistance at the level of electron transport between the hydroxynaphthoquinones and quinolone or pyridone anticoccidial agents. This was confirmed *in vivo*; representative results are shown in Tables 5 and 6.

Addition of 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone (DB) to chick liver submitochondrial particles was successful in reversing the inhibition caused by the hydroxynaphthoquinones. Double reciprocal plots of data showed that hydroxynaphthoquinone inhibition was competitive with respect to ubiquinone. Figure 4 shows a double reciprocal plot for DB reversal of menoctone inhibition of chick liver succinate cytochrome *c* reductase. Hydroxynaphthoquinones were added at concentrations approximating to their EC_{50} levels for inhibition of chick liver succinate–cytochrome *c* reductase, and the concentration of DB required to give 50% reversal of inhibition was assessed (Table 7). The 50% reversal value was calculated from linear regression analysis of points (minimum of 4 points) corresponding to 20–80% DB reversal of hydroxynaphthoquinone inhibition. A DB:hydroxynaphthoquinone ratio of 21:1 was required to partly reverse (50%) inhibition by the most active member of the series (BW 661C); less active members of the series were more readily reversed by DB.

Attempts to reverse the hydroxynaphthoquinone

Table 5. Responses of sensitive or decoquinate-resistant lines of *E.tenella* in chickens to BW58C (50:50 *cis–trans*)

BW58C concentration (mg/kg in diet)	Chickens surviving infection (out of 15)	
	Sensitive line (Berkhamsted)	Decoquinate-resistant line (Berkhamsted decR)
200	13	14
150	12	14
100	10	11
50	2	6
0	3	2

Table 6. Responses of sensitive or clopidol-resistant lines of *E.tenella* in chickens to BW568C (50:50 *cis–trans*)

BW568C concentration (mg/kg in diet)	Chickens surviving infection (out of 10)	
	Sensitive line (Berkhamsted)	Clopidol-resistant line (Berkhamsted cloR)
150	10	10
100	10	10
50	10	8
25	7	5
0	4	2

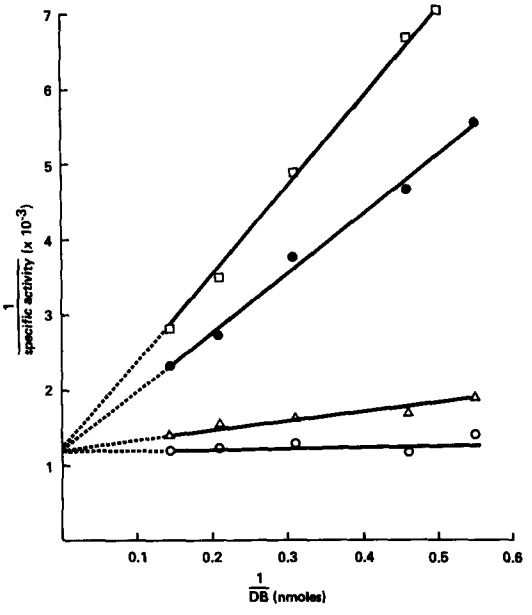

Fig. 4. Double reciprocal plot of ubiquinone (DB) reversal of menoctone inhibition of chick liver succinate–cytochrome *c* reductase. Nanomoles of menoctone present were ○, none; △, 0.29, ●, 0.43, □, 0.66.

inhibition of *E.tenella* electron transport by the addition of either DB or ubiquinone-10 were unsuccessful. Hydroxynaphthoquinone inhibition of *E.tenella* NADH– or succinate–oxidase, or succinate–cytochrome *c* reductase, was unaffected by high concentrations of DB; ratios of DB: BW 58C as high as 10,000:1 were without effect. Inhibition by even the least active member of the series reported here, BW 993C, was not reversible by DB addition. Addition of DB alone neither increased nor decreased oxygen uptake of respiring *E.tenella* mitochondria.

DISCUSSION

Hydroxynaphthoquinones BW 58C, 568C, menoctone, 720C, 661C and 993C, have been shown to be highly potent and selective inhibitors of *E.tenella* mitochondrial electron transport. BW 58C and 568C are respectively 10,000 and 2000 times better inhibitors of the *E.tenella* respiratory system than the corresponding chick liver system. Assay of the individual electron transport complexes has localized this potent and selective inhibition at the ubiquinol–cytochrome *c* reductase (Complex III; b–c_1 complex) span of the respiratory chain in *E.tenella* mitochondria. The primary site of inhibition of the hydroxynaphthoquinones against chick liver mitochondria was also found to reside within the ubiquinol–cytochrome *c* reductase, although the efficacy and specificity of inhibition by this series of compounds differed markedly from that observed for the corresponding *E.tenella* respiratory complex. At higher concentrations, the hydroxynaphthoquinones also inhibit the NADH– and succinate–ubiquinone reductases of both *E.tenella* and chick liver mitochondria; in this case selectivity for either the *E.tenella* or chick liver enzymes was less marked.

Hydroxynaphthoquinone inhibition of ubiquinone-mediated electron transfer reactions lends support to the notion that such compounds inhibit by virtue of their antagonism of ubiquinone function [10]. However, whereas hydroxynaphthoquinone inhibition of chick liver succinate–cytochrome *c* reductase was fully reversible by addition of exogenous ubiquinone (DB), inhibition of the corresponding *E.tenella* enzyme was essentially irreversible. Assuming a ubiquinone involvement in the *E.tenella* electron transport chain (and certainly exogenous ubiquinone mediates the ubiquinone and ubiquinol reductase activities of *E.tenella* mitochondria), a lack of ubiquinone reversibility would suggest a basic difference in the mechanism of hydroxynaphthoquinone inhibition of *E.tenella* elec-

Table 7. Ubiquinone reversal of hydroxynaphthoquinone inhibition of succinate–cytochrome *c* reductase of chick liver mitochondria

Hydroxynaphthoquinone	nmoles HNQ added to give 50% inhibition	nmoles DB required to give 50% reversal of inhibition	DB:HNQ (for 50% reversal of inhibition)
BW 661C	0.065	1.4	21:1
Menoctone	0.22	3.4	15:1
BW 993C	0.31	2.8	9:1
BW 720C	0.51	2.3	4.5:1
BW 568C	0.48	2.3	4.8:1
BW 59C	0.52	2.2	4.2:1
BW 58C	0.62	1.9	3.1:1

Hydroxynaphthoquinones were added in dimethylformamide to mitochondrial suspensions and the reaction started by addition of succinate. After attainment of a linear absorbance change, generally within 20 sec of adding succinate, DB in dimethylformamide was added and the increase in the absorbance rate measured. No more than two consecutive additions of DB were made per assay, and the final concentration of dimethylformamide never exceeded 0.5% v/v. Results were corrected for endogenous reduction of cytochrome *c* in the absence of succinate, and the rate of reduction in the presence of DB and dimethylformamide alone. Results given above are the mean of duplicate determinations made on a single preparation of chick liver mitochondria. HNQ = hydroxynaphthoquinone; DB = 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone.

tron transport compared to that in the host. Hydroxynaphthoquinone inhibition of *E. tenella* electron transport might result in an irreversible binding of a transition state of these compounds (e.g. a semiquinone form) or binding to a site with no ubiquinone involvement. Whatever the molecular mechanism of inhibition, the efficacy and selectivity of these compounds for the *E. tenella* electron transport chain, and their ease of reversibility in the host system, provides a sound basis for their anticoccidial and low host toxicity effects. This is supported in full by *in vivo* studies against various *Eimeria* species [18].

Quinolone derivatives (e.g. buquinolate, amquinate, methyl benzoquate and decoquinate) are effective anticoccidial agents [30], although high frequencies of resistance in coccidia have developed against these drugs [31]. Quinolone derivatives [20, 21] as well as the pyridone clopidol [21], have been shown to be potent inhibitors of *E. tenella* electron transport, probably acting at a site near cytochrome *b* [20]. The site of inhibition of these compounds is therefore probably in close proximity to that of the hydroxynaphthoquinones, although it is apparently not identical; *E. tenella* lines made resistant to either decoquinate or clopidol manifest that resistance at the level of electron transport [20, 21] but, as is shown here, are not cross-resistant to the hydroxynaphthoquinones, either *in vitro* or *in vivo*.

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