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RELATIONSHIP BETWEEN INHIBITION OF MITOCHONDRIAL RESPIRATION BY NAPHTHOQUINONES, THEIR ANTITUMOR ACTIVITY, AND THEIR REDOX POTENTIAL

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Abstract—The physicochemical properties of a series of 1,4-naphthoquinones were correlated with their activities against Sarcoma-180 by Hodnett *et al.* [*J. med. Chem.* **26**, 570 (1983)]. Redox potential was the most important molecular parameter determining antitumor activity in this series of compounds, suggesting that interference with electron transport contributes to their cytotoxicity. We evaluated this same series of quinones for their abilities to inhibit the beef heart mitochondrial succinoxidase and NADH-oxidase enzyme systems. They exhibited a broad range of inhibitory potencies. There was a strong relationship between succinoxidase inhibition, antitumor activity (T/C ratio), and redox potential. The redox potentials of the quinones which inhibited succinoxidase lay within the narrow range of endogenous components of the respiratory chain. In contrast, inhibition of NADH-oxidase was related to redox potential but did not significantly predict antitumor acitivity. These results suggest that inhibition of mitochondrial succinoxidase may be a useful preliminary screen for antitumor activity.

Various antineoplastic agents, including the clinically effective drugs daunorubicin and mitomycin, contain a quinone structure [1, 2]. It has been shown for adriamycin that the quinone moiety is essential for cytotoxic activity [3, 4]. For mitomycin C, it has been reported that reduction of the benzoquinone ring to a dihydroquinone is essential for alkylation and inhibition of nucleic acid synthesis [5–7]. In fact, Kinoshita *et al.* [8, 9] proposed that the carbonyl groups of the aziridine portion of the mitomycins are not strictly essential for alkylating activity. Antineoplastic quinones inhibit mitochondrial electron transport [10–13]. Thus, mitochondria may be a major target of the cytotoxic activity of antineoplastic quinones.

Many 1,4-naphthoquinones with bioreductive alkylation capability are antineoplastic [8, 12, 14]. Two mechanisms of action have been proposed for them. One requires the formation of an alkylating intermediate (a quinone methide) which alkylates important biomolecules such as nucleic acids and proteins. The other involves repeated bioreductions and spontaneous oxidations (redox cycling) of the quinone, resulting in the formation of toxic oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot$ OH), that cause oxidative stress [15–17].

To elucidate the mechanism of action of antineoplastic quinones, Hodnett *et al.* [18] studied a series of 2- and 2,3-substituted 1,4-naphthoquinones, including the natural products lapachol and menadione and the fungicide dichlone (CNQ; 2,3-dichloro-1,4-naphthoquinone). They evaluated the toxicity of these quinones as the LD_{50} in Swiss mice and the antitumor activity as the T/C ratio against the ascites tumor Sarcoma-180 (S-180) in Swiss mice [18]. They next correlated the toxicities and antineoplastic activities of this series of compounds with their physicochemical properties, including lipophilicity (log P), water solubility, melting point, half-cell potential (E_t) and electronic and steric substituent parameters [19]. Redox potential was the most important physicochemical property determining the antitumor activity of these quinones [18]. These findings support the hypothesis that quinone cytotoxicity involves oxidation–reduction reactions, since the redox potential of the active compounds falls within a range comparable to that in which biological reductions occur.

In this study we evaluated the effects of the Hodnett series of quinones on mitochondrial succinoxidase and NADH-oxidase, two major enzyme systems of the mitochondrial electron transport chain, and relate their effects on these enzymes to their previously reported antitumor activities *in vivo* and their redox potentials.

MATERIALS AND METHODS

Materials. Trizma (Tris) base, sucrose, cytochrome *c*, succinate, ethylenediamine tetraacetic acid (EDTA) disodium salt, and NADH were purchased from the Sigma Chemical Co., St. Louis, MO. Dimethyl sulfoxide (DMSO) (HPLC-grade) was purchased from Burdick & Jackson, Muskegon,

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MI. The twelve substituted 1,4-naphthoquinones were a gift from Ernest M. Hodnett.

Determination of NADH-oxidase activity and succinoxidase activity. Heavy beef heart mitochondria were isolated by differential centrifugation as described by Smith [20]. The mitochondria were subjected to repeated freeze–thaw cycles to thoroughly uncouple them. The mitochondria were intentionally uncoupled because we wanted to investigate the direct interaction between the naphthoquinones and the mitochondrial electron chain. Activities of the succinoxidase and NADH-oxidase enzyme systems were determined manometrically at 30° in the presence of 33 mM Tris–HCl, (pH = 7.5), 166 mM sucrose, and 6 mg/100 ml cytochrome *c*, with either 50 mM succinate or 4 mM NADH in 27 μM EDTA as substrate. Cytochrome *c* was added to replenish the cytochrome *c* lost during isolation. KOH (0.2 ml, 20%) was added to a separate chamber in the reaction flask to absorb the CO_2 produced. Incubations were typically conducted for 20 min with manometric readings taken at 5-min intervals, after a 10-min thermal equilibration period to ensure linearity of the reaction. The quinones to be tested were dissolved in DMSO which was present at an equal concentration in all assay flasks (0.1 ml DMSO/3 ml reaction mixture). Mitochondrial protein was measured by the method of Lowry *et al.* [21]. Naphthoquinones that did not inhibit at a concentration of 3.3×10^{-4} M were arbitrarily classified as non-inhibitory.

RESULTS AND DISCUSSION

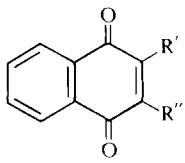
The abilities of twelve structurally-related 1,4-naphthoquinones to inhibit mitochondrial electron transport (succinoxidase and NADH-oxidase) were determined and compared to their previously reported [18] antineoplastic activities, toxicities and physicochemical characteristics. Several measures of antineoplastic activity against S-180 in Swiss mice had been used previously [18], including maximum survival time ratio (T/C), ED_{25} (effective dose 25) and optimum dose. Toxicity in Swiss mice was estimated as the LD_{50}. The physical and chemical properties reported [18] were lipophilicity (log P), water solubility, melting point, half-cell potential ($E_{\frac{1}{2}}$), and the lipophilic, electronic and steric substituent parameters described by Hansch *et al.* [19].

We determined the effects of the quinones on heavy beef heart mitochondrial succinoxidase and NADH-oxidase over a wide range of concentrations and estimated the I_{50} from each dose–response curve by interpolation (Table 1). Several of the quinones (compounds 2, 3, 5, 9 and 11) were inactive against both NADH-oxidase and succinoxidase. Only one (compound 6) was active against succinoxidase but completely inactive against NADH-oxidase.

Five of the quinones (compounds 1, 4, 7, 8 and 12) inhibited both succinoxidase and NADH-oxidase, several showing selectivity for one enzyme or the other. Their inhibition curves are shown in Figs. 1–5.

Certain naphthoquinones (compounds 6, 7, and 12, which are 2-chloro-3-methoxy, 2-chloro-3-ethoxy-, and 2-chloro-3-*n*-propyl-1,4-naphthoquinone respectively) selectively inhibited succinoxidase activity in a dose-dependent manner. These compounds depressed succinoxidase activity below 10% of control at higher concentrations (see Fig. 3a); data for compounds 6 and 12 not shown). Compounds 1 (Fig. 1a) and 8 (Fig. 4a), though more

Table 1. Structures of the naphthoquinones and comparison of their biological activities and physicochemical properties



Compound	R′	R″	I_{50}* (nmoles/mg protein) Succinoxidase	I_{50}* (nmoles/mg protein) NADH-oxidase	LD_{50}† (mg/kg i.p.)	$-E_{\frac{1}{2}}$‡ (V)	log P§	Max T/C‖ (%)
1	H	H	45	25	5.5	0.164	1.74	347
2	OH	C_5H_9	NA¶	NA	400.0	0.347	2.69	116
3	Cl	NHC_6H_5	NA	NA	1600.0	0.272	4.04	117
4	Cl	Cl	18	9	30.0	0.146	3.16	189
5	Cl	NH_2	NA	NA	800.0	0.382	2.08	112
6	Cl	OCH_3	65	NA	14.0	0.184	2.15	315
7	Cl	OC_2H_5	60	80	16.0	0.192	2.61	230
8	Cl	$NHCOCH_3$	45	40	11.0	0.165	1.42	284
9	OH	H	NA	NA	100.0	0.330	1.07	104
10	CH_3	H	NA	600	150.0	0.224	2.25	126
11	OCH_3	H	NA	NA	320.0	0.283	1.59	154
12	Cl	OnC_3H_7	60	80	20.0	0.195	2.95	271

* Estimated dose (nmoles/mg mitochondrial protein) producing 50% inhibition of oxygen consumption using NADH or succinate as substrate.

† Estimated single intraperitoneal dose which kills 50% of mice within 5 days, as previously published [18].

‡ Half-cell potential against a calomel electrode, as previously published [18].

§ Octanol–water partition coefficient, as previously published [18].

‖ The maximum increase in survival of mice treated with any dose of the quinone, as previously reported [18].

¶ NA = not active; less than 50% inhibition of enzyme activity at 1000 nmoles/mg mitochondrial protein.

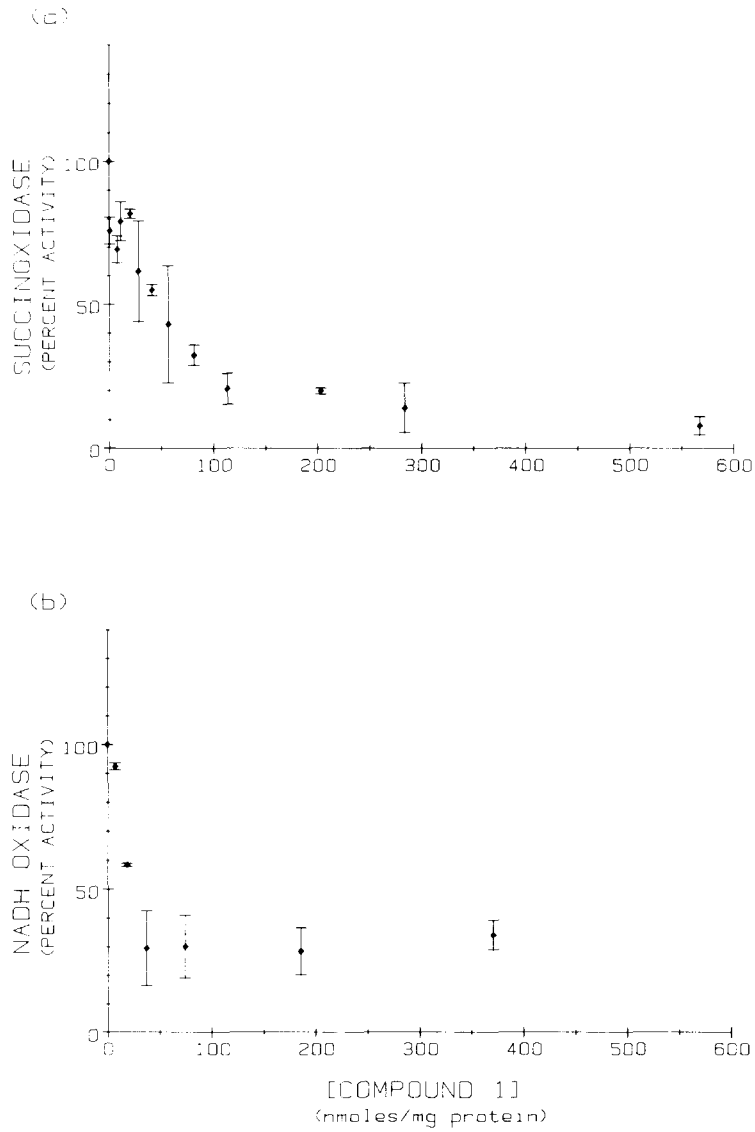


Fig. 1. Inhibition of succinoxidase (a) and NADH-oxidase (b) by compound 1. Ordinate: percent of enzyme activity compared to control (absence of compound 1) (means ± S.E.M.). Abscissa: concentration of compound 1 in nmoles/mg mitochondrial protein. In Figs. 1–5, control (100%) is the average of 6–14 replicates and there were usually 6–8 replicates for each concentration of quinone (range 2–8). Specific activity was 0.17 to 0.39 and 0.30 to 0.36 μatoms oxygen/min/mg protein for the control succinoxidase and NADH-oxidase activities respectively.

potent inhibitors of NADH-oxidase, also produced nearly total inhibition of succinoxidase activity at higher doses.

In contrast, compounds that inhibited NADH-oxidase activity did not completely inhibit this activity at the highest doses tested (see Figs. 1b, 2b, 3b and 4b). Indeed, there was a trend toward reversal of inhibition at the higher doses. One possible explanation for this apparent anomaly is that the naphthoquinones "short circuit" the respiratory chain at the higher concentrations and cause a non-enzymatic consumption of oxygen. This possibility is currently under investigation.

Another interesting finding was that certain of these quinones appeared to stimulate enzyme activity (oxygen consumption) at low concentrations (typically, < 50 nmoles per mg protein). This occurred more frequently in succinoxidase assays: compounds 4, 7, 8 (Figs. 2a, 3a, 4a) and compounds 3 and 12 (data not shown) increased oxygen consumption from 3 to 30% more than control. In NADH-oxidase assays, compounds 3 and 10 increased oxygen consumption at low doses (data not shown). Only one compound (3) stimulated oxygen consumption during both succinoxidase and NADH-oxidase assays. These findings suggest a dual mechanism of action for these quinones. The mechanism of the apparent increase in enzyme activity at low doses is not known.

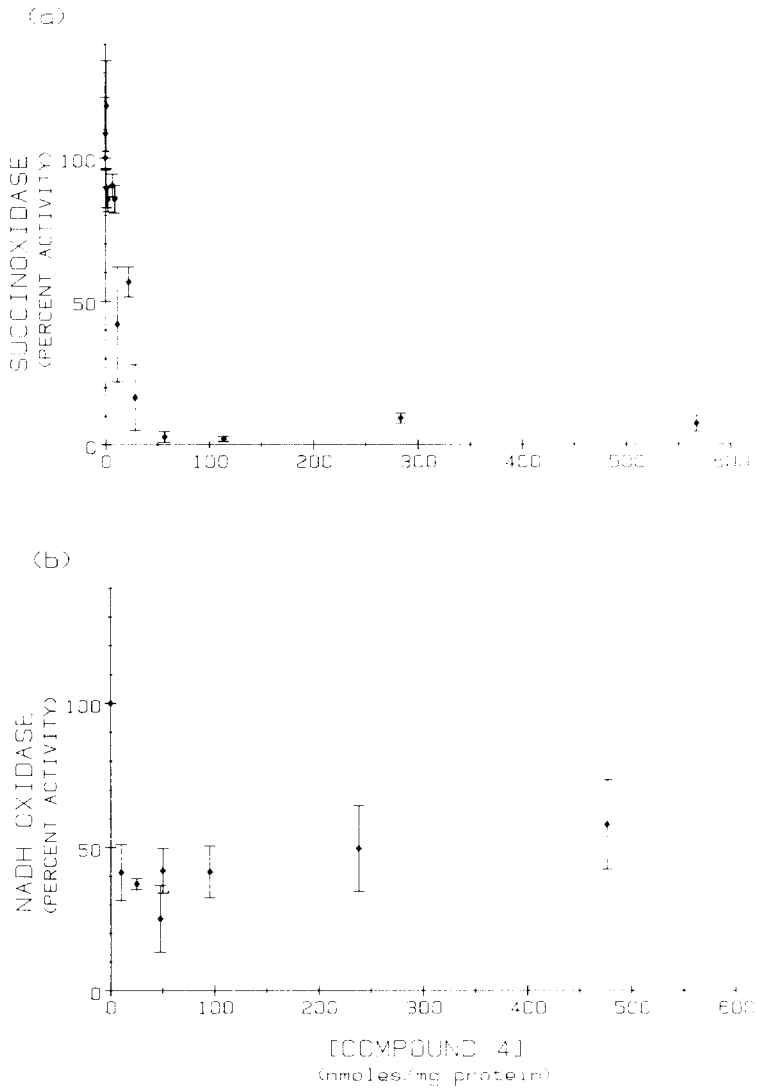


Fig. 2. Inhibition of succinoxidase (a) and NADH-oxidase (b) by compound 4. Ordinate: percent of enzyme activity compared to control (means ± S.E.M.). Abscissa: concentration of compound 4 in nmoles/mg mitochondrial protein. Specific activity was 0.30 to 0.38 and 0.26 μatoms oxygen/min/mg protein for control succinoxidase and NADH-oxidase respectively.

We are investigating the possibility that these naphthoquinones consume oxygen non-enzymatically at low doses.

Because the electron transport chain is branched, comparison of the effects of a compound on succinoxidase and NADH activities may pinpoint the site at which it inhibits the respiratory chain. A compound that specifically inhibits NADH-oxidase activity, but not succinoxidase activity, must act at complex I; conversely, a compound which specifically inhibits succinoxidase must act at complex II. For compound 8 (2-acetamido-3-chloro-1,4-naphthoquinone) in Hodnett's series of quinones, which inhibits NADH-oxidase and succinoxidase equally, two possibilities exist: either the compound inhibits at a site common to both substrates (i.e. complex III, coenzyme Q–cytochrome *c* reductase or IV, cytochrome oxidase), or it inhibits complexes I and II equally. Compounds which inhibit NADH-oxidase and succinoxidase unequally, including naphthoquinone, 2,3-dichloro-1,4-naphthoquinone, 2-chloro-3-methoxy-1,4-naphthoquinone, 2-chloro-3-ethoxy-1,4-naphtoquinone, 2-methyl-1,4-naphthoquinone and 2-chloro-3-propoxy-1,4-naphthoquinone (compounds 1, 4, 6, 7, 10 and 12 respectively), must have at least two sites of action. For the compounds which are more inhibitory toward succinoxidase (compounds 7 and 12), complex II is likely to be the primary site of inhibition, with a secondary site in complex I, III or IV. Compounds 1 and 4 are more potent inhibitors of NADH-oxidase; therefore, their primary site of inhibition is complex I with a secondary site in complex II, III or IV.

Their selectivity for inhibiting NADH-oxidase and

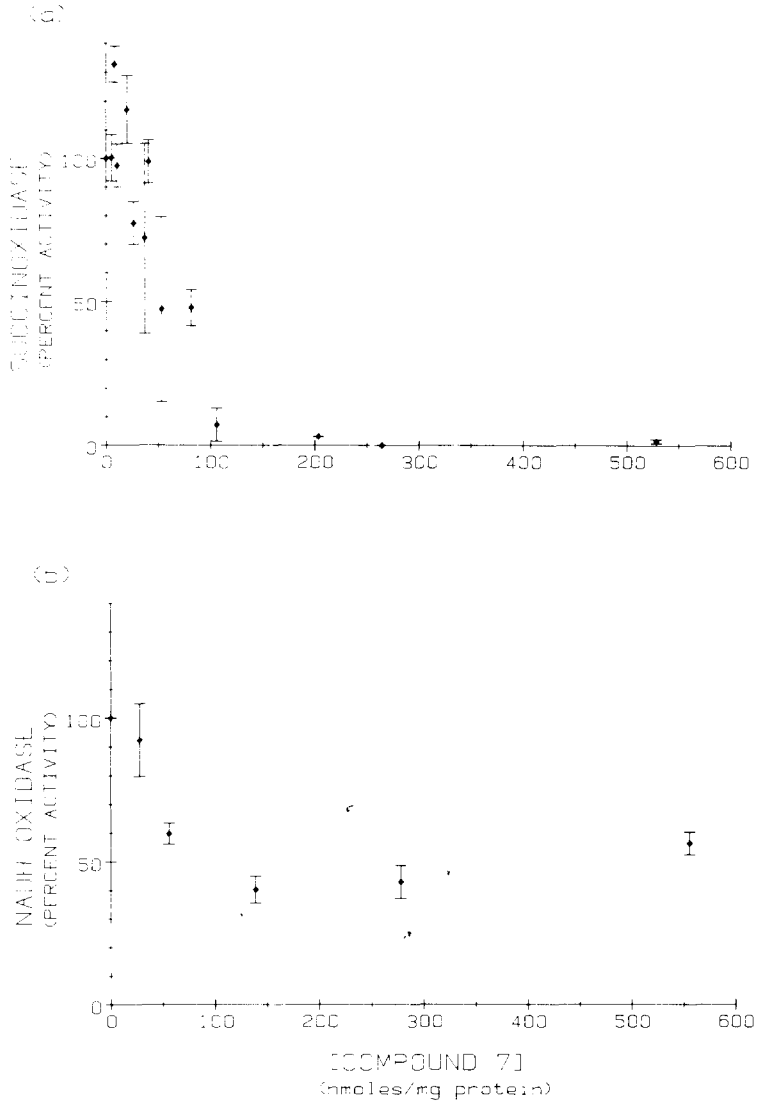


Fig. 3. Inhibition of succinoxidase (a) and NADH-oxidase (b) by compound 7. Ordinate: percent of enzyme activity compared to control (means ± S.E.M.). Abscissa: concentrations of compound 7 in nmoles/mg mitochondrial protein. Specific activity was 0.35 to 0.54 and 0.59 to 0.67 μatoms oxygen/min/mg protein for control succinoxidase and NADH-oxidase respectively.

succinoxidase (Table 1) demonstrates that certain of these naphthoquinones act at distinctly different sites in complexes I (NADH-coenzyme Q reductase) and II (succinate-coenzyme Q reductase) of the respiratory chain. This conclusion is further supported by the incomplete inhibition of NADH oxidase at higher naphthoquinone concentrations and the apparent stimulatory effect of low concentrations of the naphthoquinones on one or the other enzyme system but, usually, not both.

In Table 1 our results are compared to the antineoplastic activities, toxicities and the physicochemical properties of these quinones as previously determined by Hodnett's group [18]. The mean $E_{\frac{1}{2}}$ of the quinones that inhibited succinoxidase ($I_{50} < 1$ μmole/mg protein) was less negative than that of the inactive quinones; this difference was significant (Table 2, $P < 0.05$). Similarly, the average $E_{\frac{1}{2}}$ of the active NADH inhibitors was significantly different than that of the inactive quinones (Table 2).

The active succinoxidase inhibitors increased survival time (T/C) more than the quinones that were inactive in the succinoxidase assay (Tables 1 and 2), but there was no significant difference between the antineoplastic activities of the active NADH-oxidase inhibitors and the quinones which were inactive in the NADH-oxidase assay (Table 2). Whole animal toxicity expressed as LD_{50} appeared to relate to the ability of the quinones to inhibit succinoxidase *in vitro* but not to NADH-oxidase (Table 1). The effects of these quinones on succinoxidase and

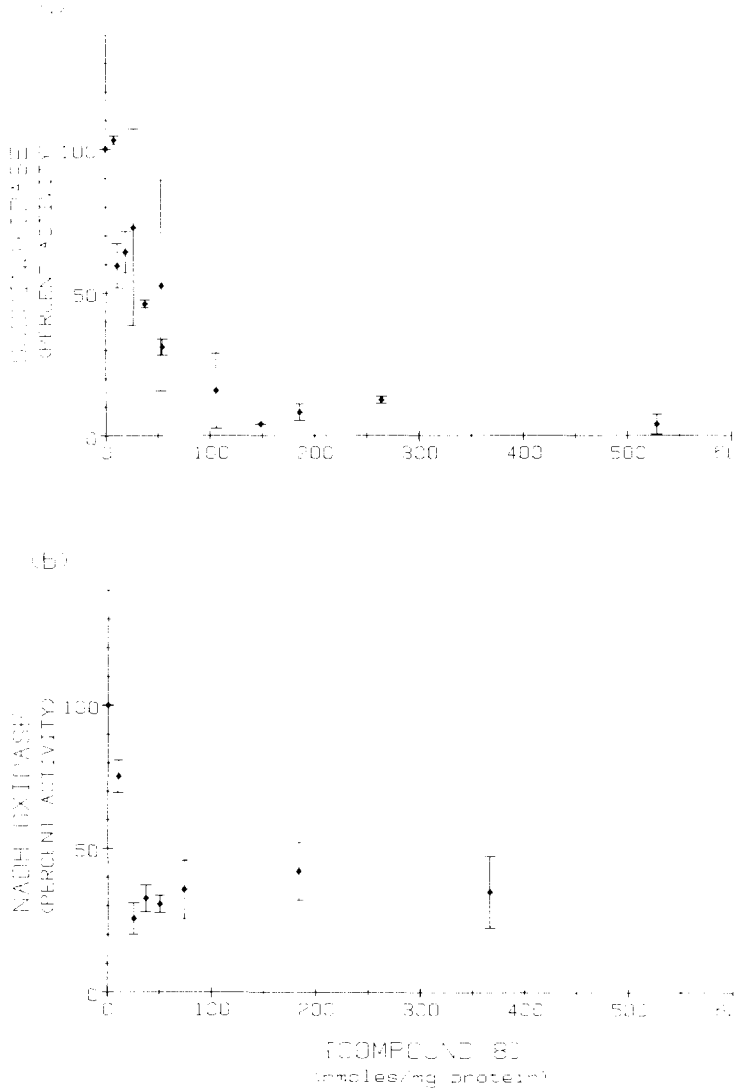


Fig. 4. Inhibition of succinoxidase (a) and NADH-oxidase (b) by compound 8. Ordinate: percent of enzyme activity compared to control (means ± S.E.M.). Abscissa: concentration of compound 8 in nmoles/mg mitochondrial protein. Specific activity was 0.34 to 0.75 and 0.54 to 0.84 μatoms oxygen/min/mg protein for control succinoxidase and NADH-oxidase respectively.

NADH-oxidase were not apparently related to their other physicochemical or biological properties studied by Hodnett *et al.* [18].

Powis and Appel [22] reported that the metabolism of quinones by several flavoenzymes that catalyze single-electron reductions is more closely related to the single-electron reduction potential of the quinones than to other structural or physical parameters, including lipophilicity. They found that the lower limit of reduction potential for metabolism by NADH: ubiquinone oxidoreductase is −0.170 V and reported that the two quinones that reacted most rapidly with this enzyme had potentials of +0.023 and −0.060 V. These redox potentials are close to the potentials of the iron–sulfur centers FeS_{N-2}, ubiquinone and cytochrome *b* [23], endogeneus carriers of the mitochondrial electron transport chain. This implies that, like duroquinone and juglone [24, 25], exogenous quinones can intercept electrons from the electron transport chain. Under our experimental conditions, this mechanism can lead either to apparent inhibition of respiratory chain enzyme activity or non-enzymatic oxygen consumption, depending on whether the intercepted electrons eventually reduce oxygen and how quickly.

Our results demonstrate that quinone inhibitors of mitochondrial succinoxidase and NADH-oxidase activities were within the midpoint potential ranges of −0.146 to −0.195 V [18] and −0.146 to −0.224 V respectively. Quinones with redox potentials lower than −0.195 and −0.224 were inactive against succinoxidase and NADH-oxidase respectively. The similarity of the midpoint potential range of the electron transport carriers in NADH: ubiquinone

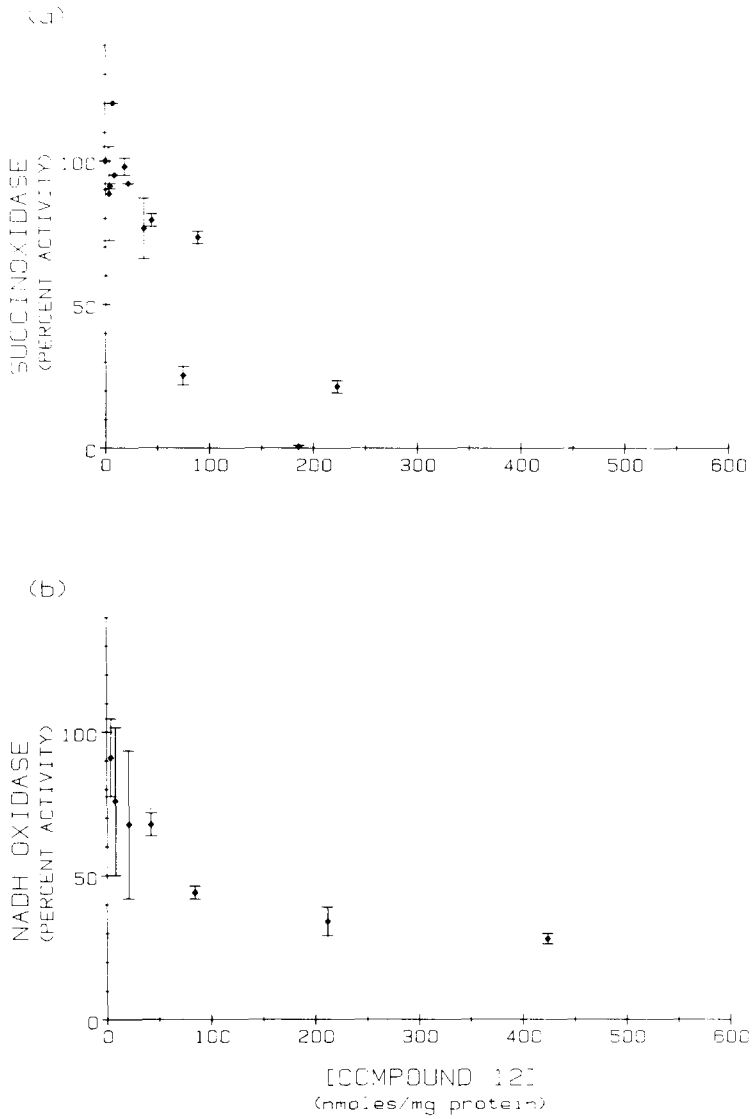


Fig. 5. Inhibition of succinoxidase (a) and NADH-oxidase (b) by compound 12. Ordinate: percent of enzyme activity compared to control (means ± S.E.M.). Abscissa: concentration of compound 12 in nmoles/mg mitochondrial protein. Specific activity was 0.27 to 0.33 and 0.20 to 0.23 μatoms oxygen/min/mg protein for control succinoxidase and NADH-oxidase respectively.

reductase (−0.340 to 0.020 V) [23] and the $E_{\frac{1}{2}}$ of quinone inhibitors of NADH-oxidase (Table 1) implicates an oxidation–reduction reaction in the quinones' inhibition of the mitochondrial electron transport chain. This same argument applies to the succinate-ubiquinone reductase (Complex II) portion of the respiratory chain which spans the midpoint potential range of −0.260 to +0.065 V. Consistent with this hypothesis is the observation that 2,3-dichloro-1,4-naphthoquinone (compound 4) undergoes redox cycling in mitochondrial membranes, with concomitant production of O_2^-, H_2O_2 and organelle oxidative stress [15–17].

Our findings are also consistent with those of Ball *et al.* [26] who reported that lapachol (compound 2) does not inhibit succinoxidase at a concentration of 100 mg/l. In our systems lapachol did not inhibit either NADH-oxidase or succinoxidase at the equivalent concentration of 3.3×10^{-4} M (Table 1).

In this series of quinones, compounds that inhibited beef heart mitochondrial succinoxidase had antitumor activity towards S-180. In conjunction with previous reports that antineoplastic quinones inhibit the mitochondrial respiratory chain [4, 10–13], this finding suggests that beef heart mitochondrial succinoxidase be evaluated as a preliminary screen for potential antineoplastic activity of quinones and other redox-active compounds.

Table 2. Comparison of half-wave potential and antitumor activities with inhibition of mitochondrial respiration

	$-E_t$* (V)	T/C†
Inhibitors of succinoxidase		
Active	0.17 ± 0.02	273 ± 57
Inactive	0.31 ± 0.06	122 ± 17
Inhibitors of NADH-oxidase		
Active	0.18 ± 0.03‡	241 ± 77
Inactive	0.30 ± 0.07	153 ± 81

Values shown are means ± S.D., N = 6. "Active" and "Inactive" compounds: inactive compounds produced less than 50% inhibition at 1000 nmoles/mg mitochondrial protein, active compounds produced more than 50% at that concentration.

* Half-wave potential (data from Hodnett *et al.* [18]).

† Antitumor activity (maximum T/C: see text): data from [18].

‡ Active compounds were significantly different than inactive compounds ($P < 0.05$, Student's *t*-test).

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