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COMPARISON OF THE EFFECTS ON MITOCHONDRIAL FUNCTION OF A SERIES OF 2-METHYL SUBSTITUTED 1,4-NAPHTHOQUINONES TO THEIR 6-METHYL COUNTERPARTS

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Abstract—Seven derivatives of 2-methyl-1,4-naphthoquinone were compared to an equivalent series of 6-methyl-1,4-naphthoquinone derivatives for their abilities to interact with mitochondrial membranes and enzyme systems. The substituents on the methyl groups included various electron-withdrawing and -donating groups. The 6-methyl quinones were, in general, more potent inhibitors of NADH-oxidase and succinoxidase, depleted sulfhydryl groups more completely, produced greater rates of cyanide (CN^-)-insensitive respiration (respiratory bursts), and induced more severe large-amplitude mitochondrial swelling than the analogous 2-methyl quinones. The NADH-oxidase system was more sensitive than succinoxidase to inhibition by both series, suggesting that Complex I is the primary site of inhibition by these quinones.

The therapeutic action of the antineoplastic agent mitomycin C results from its ability to act as a bifunctional alkylating agent which cross-links the strands of the DNA double helix [1, 2]. The alkylating activity of the drug requires reduction of its benzoquinone moiety to the corresponding benzoquinol. This reduction is catalyzed by a quinone reductase requiring NADPH as the reductant [1–4]. Kinoshita *et al.* [5, 6] proposed that the carbamyl group and aziridine ring of the mitomycins are not essential for alkylation activity. Accordingly, a series of benzoquinone and naphthoquinone analogues of mitomycin C (lacking carbamyl groups and aziridine rings) was synthesized; they were shown to have anti-neoplastic activity against Adenocarcinoma 755 and Sarcoma 180 (S-180) and to inhibit beef heart mitochondrial NADH-oxidase and succinoxidase [7–10].

A representative naphthoquinone from this series of compounds, 2,3-(chloromethyl)-1,4-naphthoquinone (CMNQ), was reported to inhibit DNA, RNA and protein synthesis in S-180 cells *in vitro* [11], to depress the intracellular levels of ATP in S-180 cells *in vitro* [11], to inhibit S-180 tumor cell respiration *in vivo* [12, 13], to release mitochondrial respiratory control [13], and to inhibit the respiratory chain in beef heart mitochondria *in vitro* [11]. CMNQ was also shown to inhibit S-180 mitochondrial electron transport, to bind to S-180 mitochondrial proteins, to uncouple S-180 mitochondria and to stimulate S-180 mitochondrial ATPase *in vitro* [12, 13]. In addition, CMNQ induces osmotic swelling of rat liver mitochondrial suspensions in a cation non-specific, non-energy-linked and oxygen-dependent process [14]. Thus, a representative of these naphthoquinone anti-tumor agents disrupts a variety of bioenergetic functions. A structure–activity study of a series of 1,4-naphthoquinones incapable of forming alkylating quinone methide intermediates revealed that inhibition of beef heart mitochondrial succinoxidase activity is associated with redox potential and anti-neoplastic activity [15].

To extend the structure–activity investigations of potential bioreductive alkylating agents, we evaluated a series of 2-methyl and 6-methyl substituted 1,4-naphthoquinones for their abilities to alter mitochondrial functions. These naphthoquinones had previously been prepared and assessed for anti-neoplastic activity [16]. In this study, we investigated the relationship between the location of ring substitutions of the 1,4-naphthoquinones and their effects on mitochondrial membranes and enzyme systems. We also relate these effects on mitochondria to previous findings on anti-neoplastic activity. We report on inhibition of beef heart mitochondrial NADH-oxidase and succinoxidase activities, mitochondrial sulfhydryl depletion, mitochondrial swelling, and the production of a cyanide (CN^-)-insensitive respiratory burst in isolated mitochondria.

METHODS

Determination of NADH-oxidase activity and succinoxidase activity. Heavy beef heart mitochondria were isolated by differential centrifugation as described by Smith [17]. The activities of the succinoxidase and NADH-oxidase enzyme systems were determined manometrically in the presence of 33 mM Tris–HCl (pH = 7.5), 166 mM sucrose, 6 mg/100 ml cytochrome *c*, with either 50 mM succinate or 4 mM NADH in 27 μM EDTA as substrate. KOH

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(20%, 0.1 ml) was added to a separate chamber in the reaction flask to absorb the CO_2 produced. The various naphthoquinones were dissolved in dimethyl sulfoxide (DMSO) which was present at an equal concentration in all assay flasks (0.1 ml of DMSO in 3 ml reaction buffer). Mitochondrial protein was measured by the method of Lowry *et al.* [18]. Titration curves (naphthoquinone concentration vs percent inhibition) were constructed from quadruplicate assays, and the I_{50} values were determined by interpolation.

Preparation of rat liver mitochondria and respiratory control ratio determination. Livers of female Wistar white rats were used as a source of coupled mitochondria. Mitochondria were judged coupled if their respiratory control ratios (RCRs) were at least 3.0; preparations with lower RCRs were discarded. The mitochondria were isolated by differential centrifugation by the method of Lardy and Johnson [19]. Oxygen consumption was measured polarographically on a Gilson Oxygraph using a Clark-type electrode, as described by Estabrook [20]. The respiration buffer consisted of 0.25 M sucrose, 7.5 mM $MgCl_2$ and 15 mM Tris–HCl (pH = 7.4). To start the reaction, 3.3 mM succinate was added to 0.2–0.3 mg of mitochondrial protein in 2 ml of the reaction buffer. ADP (final concentration 10 mM) was then added to initiate state 3 respiration. The steady rate of respiration (state 3) was monitored as the rate of oxygen consumption. Upon depletion of ADP, a slower rate of respiration (state 4) returned and was measured. This process was repeated, and the second state 3 to 4 transition was used for calculating the respiratory control ratio (RCR), the ratio of the state 3 to state 4 rates.

Measurement of mitochondrial swelling. Freshly isolated rat liver mitochondria were added to 3.0 ml of medium consisting of 150 mM KCl and 20 mM Tris–HCl (pH = 7.4). Sufficient mitochondria to cause an absorbance of 0.9 O.D. at 520 nm were added (0.1–0.6 mg/ml protein). The swelling process was monitored continuously on a Cary 14 spectrophotometer by measuring the decrease in absorbance as described by Koch [21]. The naphthoquinones were added in DMSO. The rates and total amount of swelling caused by the naphthoquinone were determined by subtracting the solvent control from the total.

Measurement of sulfhydryl depletion. Thiol groups were measured spectrophotometrically by the procedure of Ellman [22]. The absorption maximum of the colored product was measured at 412 nm as in the standard 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay. Since the substances tested were expected to cause mitochondrial swelling, and since mitochondrial swelling decreases absorbance, Ellman's method was modified as follows. The difference in absorbance between 412 and 520 nm was recorded using the dual wavelength mode of an Aminco DW-2 spectrophotometer. The 520 nm wavelength was found to be an isobestic point for the DTNB anion and swollen mitochondria; therefore, the absorbance at 520 nm was subtracted from the absorbance at 412 nm to correct for the decrease in absorbance caused by the swelling of the intact mitochondria under the conditions of the assay.

Measurement of respiratory burst in mitochondria. The respiratory burst in heavy beef heart mitochondria was measured as oxygen consumption of uncoupled mitochondria in the presence of 454 μM KCN (CN^--insensitive respiration). The measurement was performed on a Gilson Oxygraph equipped with a Clark-type electrode. Oxygen consumption was determined in the presence of 33 mM Tris–HCl pH 7.5, 166 mM sucrose, 6 mg/100 ml cytochrome *c* and 13 mM succinate. KCN was then added, and the reaction was monitored until a steady baseline rate of CN^--insensitive oxygen consumption was established. The naphthoquinones were added in absolute ethanol, and the respiratory burst was determined as the initial increase in rate of oxygen consumption.

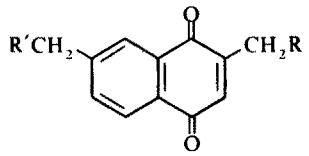
RESULTS AND DISCUSSION

To elucidate the biological activities of the series of 2- and 6-methyl substituted naphthoquinones, their effects on a variety of bioenergetic processes were compared. Based on previous observations that CMNQ inhibits mitochondrial NADH-oxidase and succinoxidase activities [11], induces a respiratory burst in isolated mitochondria [11] and in S-180 tumor cells [13], and causes swelling of isolated mitochondria from rat liver [14], we used these five tests to assess the effects of a series of 2-methyl naphthoquinones and their 6-methyl counterparts on mitochondrial function. The results of these experiments *in vitro* were compared to the previously reported [16] anti-neoplastic activity of the naphthoquinones towards Sarcoma 180 and their relative toxicity as measured by loss of body weight during treatment.

Our results (Table 1) demonstrate that the 6-methyl naphthoquinones were generally more potent inhibitors of beef heart mitochondrial succinoxidase activity than the analogous 2-methyl naphthoquinones. Every 6-methyl naphthoquinone tested was an equally, or more, potent inhibitor of the mitochondrial NADH-oxidase system than its 2-methyl counterpart. The 6-methyl group increased the electron transport inhibitory potency of this series of naphthoquinones. The two potent inhibitors of NADH-oxidase were 6-bromomethyl-1,4-naphthoquinone (compound 3) and 6-*N*-methyl carbamyl-1,4-naphthoquinone (compound 5) with I_{50} values of 16 nmoles/mg mitochondrial protein. In addition, since all of the 6-methyl substituted naphthoquinones and most of the 2-methyl substituted naphthoquinones tested were more potent inhibitors of the NADH-oxidase enzyme system than succinoxidase (Table 1), the primary site of respiratory chain inhibition was in Complex I (NADH–coenzyme Q reductase). These findings are consistent with findings on other quinones [23], CNQ [24], CMNQ [11], and various potential bioreductive alkylating agents [7–10].

All of the 6-methyl substituted naphthoquinones were more potent thiol depleters than the corresponding 2-methyl substituted naphthoquinones: their I_{50} values for thiol depletion were lower in all cases except one (Table 1). The 6-chloromethyl, 6-(*N*-methylcarbamyl) methyl and 6-acetoxymethyl substituted quinones were the most potent sulfhydryl depleters with I_{50} values of 8 μM.

Table 1. Comparison of the effects of 2- and 6-methyl substituted naphthoquinones on mitochondrial function



	2-Methyl substituted R	6-Methyl substituted R′	Inhibition of succinoxidase activity I_{50} (nmoles/mg protein)	Inhibition of NADH-oxidase activity I_{50} (nmoles/mg protein)	Sulfhydryl depletion* I_{50} (μM)	Cyanide-insensitive respiration† (natoms oxygen/min)	Swelling		Maximum T/C ratio against Sarcoma 180§	Average weight change‖ (%)
							Initial rate‡ (O.D./min)	Maximum swelling‡ (O.D.)		
1	H	—	296	98	83	68.9	0.004	0.050	1.34	+17.3
	—	H	60	42	25	95.0	0.039	0.385	1.09[b]	−11.1
2	Cl	—	92	84	17	5.5	0.186	0.355	1.47[c]	−9.8
	—	Cl	75	21	8	14.2	0.072	0.330	1.38[b]	−13.5
3	Br	—	296	98	25	154.4	0.022	0.481	1.73[b]	−7.7
	—	Br	92	16	17	18.9	0.006	0.338	2.07[d]	−11.3
4	O—C(=O)—CH_3	—	75	42	12	19.5	0.006	0.444	1.66[b]	−14.4
	—	O—C(=O)—CH_3	100	42	8	130.7	0.019	0.639	1.35[d]	−7.4
5	O—C(=O)—NH—CH_3	—	296	982	12	11.4	0.004	0.056	1.51[c]	−10.5
	—	O—C(=O)—NH—CH_3	100	16	8	69.1	0.026	0.356	1.71[d]	−10.8
6	O—C(=O)—NH—CH_2—CH_2—Cl	—	92	42	10	16.3	0.030	0.302	2.08[b]	−11.4
	—	O—C(=O)—NH—CH_2—CH_2—Cl	85	20	10	158.5	0.090	0.383	1.47[b]	−11.7
7	O—CH_3	—				127.9	0.007	0.046	1.38[c]	+1.6
	—	O—CH_2—CH_3				244.8	0.043	0.331	1.32[d]	−10.3

* The concentration of the drug that depletes 50% of the detectable sulfhydryls. Mitochondrial protein was from 1.0 to 1.8 mg/flask. Measurements were done in triplicate at each concentration.

† The initial rate of the respiratory burst caused by the drug at a concentration of 296 nmoles/mg protein.

‡ The change in optical density caused by the drug at a 152 μM concentration. This dose represents an excess of the I_{50} for thiol depletion which should result in extensive swelling. The initial rate of swelling and total amount of swelling are presented to compare the rate of swelling and the total extent of swelling. Values are the mean of duplicate determinations. Mitochondrial protein content was 1.2 mg/assay.

§ From Antonini *et al.* [16]. The maximum T/C is the maximum ratio of the survival time of the treated to the control animals, among T/Cs obtained for daily doses of (a) 20, (b) 5, (c) 10 and (d) 2.5 mg/kg.

‖ From Antonini *et al.* [16]. The average weight change from onset to termination of drug treatment (+16.9% for untreated control tumor bearing animals).

In general, the 6-methyl substituted naphthoquinones were also more potent inducers of mitochondrial swelling then their corresponding 2-methyl analogues (Table 1). In all cases except two, the 6-methyl substituted quinones gave a faster initial rate of swelling and, with two exceptions, the 6-methyl series induced a greater maximum extent of swelling then their 2-methyl counterparts. 6-Acetoxymethyl-1,4-naphthoquinone (compound 4) induced the most swelling with a total O.D. change of 0.639 at 520 nm. However, the initial rate of swelling was greatest in the presence of 2-chloromethyl-1,4-naphthoquinone (compound 2), 6-[*N*-(chloroethyl)carbamoylmethyl-1,4-naphthoquinone (compound 6), and 6-chloromethyl-14-naphthoquinone (compound 2), with initial rates of 0.186, 0.090 and 0.072 O.D. units/min respectively.

In this investigation, the abilities of the various naphthoquinones to induce mitochondrial swelling were determined at a single concentration of 152 μM which was significantly above the I_{50} values for thiol depletion shown in Table 1 (8–83 μM). This high concentration was selected to ensure that the thiol pool was depleted and to determine whether or not the 2-methyl and 6-methyl substituted naphthoquinones induced swelling under these conditions. The 2-methyl substituted compounds 1, 5, and 7 (2-methyl-, 2-(*N*-methyl carbamyl)- and 2-methoxynaphthoquinones, respectively) did not cause mitochondrial swelling at this concentration. This finding was unexpected in light of the previous finding that suggested that 15% of the total mitochondrial sulfhydryl groups were important in maintaining the integrity of the mitochondrial inner membrane, preventing swelling [25] and lipid peroxidation [26]. The concentration of these compounds in the swelling experiment was greater than their I_{50} values for thiol depletion. This discrepancy may indicate that these compounds partition differently in the mitochondria than those that caused swelling, and react with a different pool of thiols.

With the exception of 2-bromomethyl-1,4-naphthoquinone (compound 3), all of the 6-methyl substituted 1,4-naphthoquinones induced a greater CN^--insensitive respiratory burst in isolated mitochondria than their corresponding 2-methyl substituted analogues. The 6-ethoxymethyl-, 6[*N*-(2-chloroethyl)carbamoyl]methyl, and 2-bromomethyl-1,4-naphthoquinones (compounds 7, 6 and 3, respectively) caused the greatest rate of CN^--insensitive respiration, consuming 245, 159 and 154 natoms O_2/min respectively. In previous reports, the CN^--insensitive respiratory burst induced in mitochondria by 2,3-dichloro-1,4-naphthoquinone (CNQ) resulted in O_2^- and H_2O_2 production, lipid peroxidation, thiol depletion [27] and generalized oxidative stress at the organelle level *in vitro*. We reported previously that chronic feeding of CNQ increases activities of superoxide dismutase and catalase in rat heart and liver [28], an observation substantiating the role of this naphthoquinone in inducing generalized oxidative stress *in vivo*. In α-tocopherol-deficient animals, CNQ feeding depresses the respiratory control ratio of isolated cardiac mitochondria, a finding consistent with production of oxidative stress at the organelle level *in vivo* [28]. Thus, by analogy, the greater ability of the 6-methyl substituted naphthoquinones to induce a CN^--insensitive respiratory burst in the present study implies that they also produce larger quantities of O_2^- and hydrogen peroxide. This, in turn, would cause greater oxidative stress at the organelle level by such mechanisms as membrane lipid peroxidation and depletion of the mitochondrial thiol pool.

In general, it is difficult to make specific comparisons between our thiol depletion data and our swelling or CN^--insensitive respiration data since I_{50} values were determined for thiol depletion over a range of concentrations, but swelling and CN^--insensitive respiration were determined at single concentrations (152 μM and 296 nmoles/mg protein respectively). Such comparisons would erroneously compare potencies in the form of I_{50} values to efficacy in the form of maximum effect of a single high dose. These experiments were conducted to make qualitative comparisons on the abilities of the 2-methyl and 6-methyl substituted naphthoquinones to cause CN^--insensitive respiration and induce mitochondrial swelling. Further experiments are needed to determine the I_{50} values for these effects for correlation with the I_{50} values for thiol depletion.

Consistent with the observed greater biochemical toxicities of the 6-methyl substituted naphthoquinones is the observation that the 6-methyl series generally caused a greater loss of body weight during the experiment than the 2-methyl derivatives (Table 1). Thus, the structural selectivity for disruption of bioenergetic functions of isolated mitochondria parallels that causing whole animal toxicity at 10 mg/kg body wt and implicates generalized oxidative stress in the mitochondria in whole animal toxicity. In an analogous manner, mitochondrial aberrations [29] and lipid peroxidation via redox cycling [30] have been implicated in the cardiotoxicity of the anthroquinone anti-tumor agent adriamycin.

The 2-methyl substituted naphthoquinones showed less mitochondrial toxicity in this study than their 6-methyl counterparts, but they are generally more potent anti-neoplastic agents against S-180 [16]. These results contrast to those of Hodnett *et al.* [31] and Pisani *et al.* [15]. Hodnett *et al.* [31] showed that the anti-neoplastic activity of a series of naphthoquinones were related most closely to their midpoint redox potential. Further investigation of this Hodnett series of quinones [15] revealed an association among their inhibition of the beef heart mitochondrial succinoxidase system, their anti-tumor activity, and their redox potential. Thus, in the Hodnett series of quinones, the respiratory chain inhibitors were the best anti-neoplastic agents against S-180, whereas with the bioreductive alkylating quinones studied herein the opposite was observed. This discrepancy suggests that the 2-methyl and 6-methyl substituted naphthoquinones exert their anti-cancer effects by a different mechanism than the Hodnett series of naphthoquinones. The 2-methyl and 6-methyl substituted naphthoquinones have been proposed to exert their effects by bioreductive alkylation, whereas our data imply that the Hodnett series of naphthoquinones exert their effects independently of the alkylation effects, perhaps by producing toxic oxygen radicals.

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