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INACTIVATION OF MICROSOMAL NADPH–CYTOCHROME *c* REDUCTASE BY SULFHYDRYL-REACTIVE DERIVATIVES OF MENADIONE*†

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Abstract—Five derivatives of menadione (2-methyl-1,4-naphthoquinone) having electronegative substituents on allylic carbons were prepared for study as sulfhydryl-reactive inactivators of mouse liver microsomal NADPH–cytochrome *c* reductase. Each of these naphthoquinones, incubated with dilute suspensions of microsomes, produced a loss of NADPH–cytochrome *c* reductase activity proportional to the initial naphthoquinone concentration. Each of the compounds also reacted with cysteine, as evidenced in the case of the halogenated compounds, by loss of reactive sulfhydryl groups and, in the case of 2-*p*-nitrophenoxymenadione, by the displacement of the leaving group, *p*-nitrophenol. Menadione, incubated under identical conditions, did not inactivate NADPH–cytochrome *c* reductase and was unreactive with cysteine. The requirement for a halogen or a nitrophenoxy substituent on at least one of the allylic carbons suggested that the mechanism of NADPH–cytochrome *c* reductase inactivation involves attack on critical microsomal nucleophiles, possibly sulhydryl groups. The possible significance of these findings is discussed in relation to the antitumor activity and bioactivation of the halomethyl naphthoquinones.

A series of menadione (2-methyl-1,4-naphthoquinone) derivatives with antitumor properties has been synthesized and investigated extensively by Sartorelli and his colleagues [1–4]. The active DNA alkylators in this series have electronegative substituents believed to serve as leaving groups when the quinones are reduced enzymatically to intermediates that form methides [1]. Prior to reduction, however, it is possible that these quinones may undergo SN_2 displacement reactions with tissue sulfhydryl groups and thus discharge their leaving groups. While such reactions might be DNA-sparing, they might also deplete cellular sulfhydryl groups, inactivate sulfhydryl-dependent enzymes, disrupt electron transport pathways, and, generally speaking, weaken cellular defenses against alkylating agents and/or radiation.

Our initial studies on the properties of these reactive menadione derivatives utilized liver microsomes and were carried out *in vitro* in aqueous mixtures maintained at physiologic pH and temperature. The liver microsomal NADPH–cytochrome *c* reductase was considered to be a possible target because of the likelihood that the hydrophilic portion of this enzyme contains several sulfhydryl residues [5, 6] that could react with menadione via Michael addition [7–9] or with its more reactive derivatives via SN_2 displacement. Early observations with 2-*p*-nitrophenoxymenadione, a compound that discharges a chromophore upon nucleophilic substitution, supported the possibility that liver microsomes contain nucleophilic moieties that react with this compound and prompted the studies described herein.

MATERIALS AND METHODS

Chemical synthesis. Five derivatives of menadione were prepared for study as sulhydryl-reactive NADPH–cytochrome *c* reductase inhibitors. Solvents and reagents required for the syntheses were obtained from commercial sources. The 3-chloromethyl, 3-bromomethyl, and 2,3-bischloromethyl derivatives of 1,4-naphthoquinone were prepared by reacting either, 1,4-naphthoquinone or menadione with formaldehyde and HCl (or HBr) gas as previously described [1, 10, 11]. The 2,3-bisbromomethyl-1,4-naphthoquinone was prepared by refluxing 1 equivalent of 2,3-dimethyl-1,4-naphthoquinone with 2.1 equivalents of *N*-bromosuccinimide for 3 hr in carbon tetrachloride containing a trace of benzoylperoxide as catalyst [11]; the 2,3-dimethyl-1,4-naphthoquinone had been prepared by chromic acid oxidation of the corresponding naphthalene [1]. The melting points of the four halogenated menadione derivatives agreed with published values [1, 10, 11], and mass spectra of the compounds supported the assigned structures. The 2-*p*-nitrophenoxy substituted derivative was prepared as follows: 2-bromomethyl naphthalene was dissolved in acetonitrile to which a slight molar excess of sodium *p*-nitrophenoxide was added. The suspension was stirred overnight, filtered, and concentrated to dryness, affording 2-*p*-nitrophenylmethyl naphthalene (90–95%), which was converted

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to the 1,4-naphthoquinone (m.p. 222–225°, yield 20–30%) by chromic acid oxidation [1]. The mass spectrum of the product displayed the expected fragmentation and the correct molecular ion; halogenated fragments (suggestive of impurities) were not detected.

Enzyme assays. Twenty-four male Swiss–Webster mice, 4- to 6-weeks-old, were purchased from Simonsen, Inc., Gilroy, CA, and were used as the source of liver microsomes. Perfused livers were pooled and homogenized in 1.15% KCl (1:10, w/v), and the microsomal membranes were isolated by differential centrifugation. The protein content of the microsomes were measured by a modified Lowry method [12]. The sulfhydryl content was estimated by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) titration [13]. The stock microsomal suspension (12.5 mg protein/ml) was divided into several portions and stored at −70°.

Biochemicals required for assays of microsomal electron transport pathways were purchased from the Sigma Chemical Co., St. Louis, MO. NADPH–cytochrome *c* reductase, 2,6-dichloroindophenol (DCIP) reductase, and ferricyanide reductase activities were measured as previously described [14]. NADPH-dependent paraquat reductase was measured spectrophotometrically by monitoring the rate of NADPH disappearance following the addition of paraquat (final concentration, 200 μM) to the sample cuvette. Both sample and reference cuvettes contained 0.5 mM NADPH and 0.12 mg microsomal protein/ml in 50 mM phosphate buffer, pH 7.4. A Kontron Uvikon 810 spectrophotometer was used for this and all other spectrophotometric assays. Reductase activities were measured at 37°.

Reaction of menadione and derivatives with cysteine. Cysteine was dissolved in 50 mM potassium phosphate buffer, pH 7.4, to a final concentration of 100 μM. Menadione (25 μl of 5 mM solution in acetone) was added to 2.5 ml of the cysteine solution, warmed to 37°. The cysteine sulfhydryl content remaining after 15 min was measured by titration with DTNB. The four halogenated derivatives of menadione (see "Chemical synthesis" above) were tested in the same manner. The 2-*p*-nitrophenoxymenadione was not tested because of spectral overlap between *p*-nitrophenol and the product of the DTNB reaction [13]. Loss of reactive sulhydryl groups due to autoxidation of cysteine was negligible under these conditions.

p-*Nitrophenol displacement measurements.* The half-life of 2-*p*-nitrophenoxymenadione in the presence of excess sulfhydryl was estimated by measuring the rate of *p*-nitrophenol displacement as follows: cysteine, glutathione, mouse liver cytosol, or mouse liver microsomes were dissolved or suspended in 50 mM potassium phosphate buffer, pH 7.4, to yield a sulfhydryl content of 200 μM. The reference cuvette was filled with 2.5 ml of the sulfhydryl solution or suspension, and the empty sample cuvette was "loaded" by adding 25 μl of 2-*p*-nitrophenoxymenadione solution (2 mM in dimethyl sulfoxide). The displacement was initiated by adding 2.5 ml of the buffered sulfhydryl mixture (warmed to 37°) to the sample cuvette, with the monochromator set at 420 nm. The absorbance was recorded for 2 min after sulfhydryl addition. The concentration of *p*-nitrophenol was calculated at selected time points using an experimentally determined molar extinction coefficient of 10^4 A/cm^{-1}. The pseudo first-order rate constants and corresponding $T_{\frac{1}{2}}$ values were calculated from plots of $\log (C_0/C_0 - X)$ vs t where C_0 = initial concentration of 2-*p*-nitrophenoxymenadione (20 μM) and X = concentration of *p*-nitrophenol at t.

NADPH–cytochrome c *reductase inactivation measurements.* Mouse liver microsomes (0.2 mg protein/ml in 50 mM potassium phosphate buffer, pH 7.4) were incubated with graded concentrations of menadione, 3-chloromethylmenadione, 3-bromomethylmenadione, 2-*p*-nitrophenoxymenadione, 2,3-bischloromethyl-1,4-naphthoquinone, or 2,3-bisbromomethyl-1,4-naphthoquinone for 60 min at 37°. The microsomes were resedimented, resuspended in naphthoquinone-free buffer, and assayed for residual NADPH–cytochrome *c* reductase activity. The concentration of each naphthoquinone required to inactivate 50% of the enzyme in the 60-min incubation period was estimated from a plot of log(% activity remaining) versus naphthoquinone concentration. In one experiment, microsomes treated with menadione (75 μM) and 3-bromomethylmenadione (75 μM) as described above were also assayed for residual paraquat reductase, DCIP reductase, and ferricyanide reductase activities. In this experiment, the effect of 3-bromomethylmenadione treatment on the kinetics of microsomal cytochrome *c* reductase was examined by estimating the apparent Michaelis constants of the enzyme for cytochrome *c*.

To study the relationship between leaving group displacement and enzyme inactivation, 2-*p*-nitrophenoxymenadione was utilized. In these experiments, the concentration of *p*-nitrophenol in the buffer and the loss of NADPH–cytochrome *c* reductase activity were studied as a function of incubation time and as a function of initial 2-*p*-nitrophenoxymenadione concentration. As described above, the

Table 1. Relative potencies of various menadione derivatives tested as inactivators of microsomal NADPH–cytochrome *c* reductase*

Compound	IC_{50} (μM)
Menadione	160†
3-Chloromethylmenadione	25
2,3-Bischloromethyl-1,4-naphthoquinone	9
3-Bromomethylmenadione	25
2,3-Bisbromomethyl-1,4-naphthoquinone	8
2-*p*-Nitrophenoxymenadione	23

* Mouse liver microsomes (0.2 mg protein/ml) in 50 mM potassium phosphate buffer, pH 7.4, were incubated at 37° for 60 min with graded concentrations of the six naphthoquinones tabulated above. The microsomes were then resedimented, resuspended in naphthoquinone-free buffer, and assayed for residual NADPH–cytochrome *c* reductase activity. The concentration of each naphthoquinone required to inactivate 50% of the enzyme in the 60-min incubation period (IC_{50}) was estimated from a plot of log(% activity remaining) versus concentration.

† Highest concentration tested; no inactivation was observed.

Table 2. Effects of menadione and 3-bromomethylmenadione on NADPH-dependent reductase activities in mouse liver microsomes*

Naphthoquinone added to buffer	NADPH reductase activities (μmoles/min/mg protein)			
	Cytochrome c	Paraquat	DCIP	Ferricyanide
None	140	72	188	434
Menadione, 75 μM	120	96	185	477
3-Bromomethylmenadione, 75 μM	12.9	12	64	87

* Mouse liver microsomes were suspended in 200 ml of 50 mM phosphate buffer, pH 7.4, to a final concentration of 0.2 mg protein/ml, and the suspension was divided evenly among three flasks. Menadione (final concentration, 75 μM) or 3-bromomethylmenadione (final concentration, 75 μM) was added to the experimental flask; the acetone vehicle (0.5 ml) was added to the control flasks. The flasks were incubated in a water bath at 37° for 1 hr without shaking after which the microsomes were resedimented, resuspended in naphthoquinone-free buffer, and assayed for reductase activities.

microsomes (0.2 mg protein/ml) were incubated with the naphthoquinone in 50 mM potassium phosphate buffer, pH 7.4, at 37°. Velocity constants for the displacement of *p*-nitrophenol and the inactiviation of NADPH–cytochrome *c* reductase were estimated by the method of Aldrich [15].

RESULTS

Five derivatives of menadione having one or two potential leaving groups on the allylic carbons were incubated with dilute suspensions of mouse liver microsomes for 60 min. The microsomes were then reisolated and resuspended in naphthoquinone-free buffer. Each compound produced a concentration-dependent loss in NADPH–cytochrome *c* reductase activity; the parent compound, menadione, had no effect in the concentration range that was tested (Table 1). Losses in reductase activity were also observed when other electron acceptors were substituted for cytochrome *c*. The results of a typical experiment, shown in Table 2, suggest that both FMN-dependent activities (cytochrome *c*, DCIP) and FAD-dependent activities (ferricyanide) are sensitive to 3-bromomethylmenadione exposure [16]. With cytochrome *c* as the substrate, the 3-bromomethylmenadione pretreatment reduced the apparent V_{max} to 15% of the control value but did not affect the apparent K_m (Fig. 1). Exposure of the microsomes to an equivalent concentration of menadione did not affect any of the NADPH-dependent reductase activities measured in this study (Table 2).

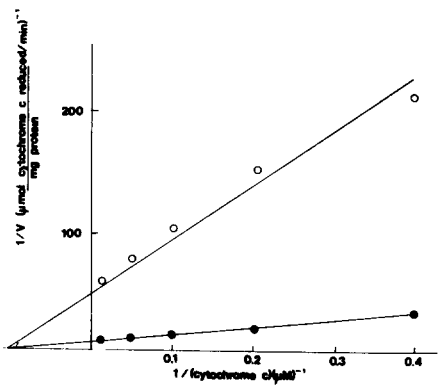

Fig. 1. Effect of 3-bromomethylmenadione pretreatment on the kinetics of microsomal NADPH–cytochrome *c* reductase. Mouse liver microsomes were incubated with either 3-bromomethylmenadione or the Me_2SO vehicle as described in Table 2. The cytochrome *c* reductase activity was measured in the reisolated microsomes using substrate concentrations ranging from 2.5 to 80 μM. The Lineweaver–Burk plots for the control microsomes (closed circles) and the naphthoquinone-treated microsomes (open circles) are shown above.

In the incubations done with 2-*p*-nitrophenoxymenadione, the release of *p*-nitrophenol was evident from the progressive change from colorless to yellow of the incubation mixture. Subsequently, 2-*p*-nitrophenoxymenadione was found to be unstable when added to buffered solutions or suspensions containing excess sulfhydryl in the form of cysteine, glutathione, mouse liver cytosol, or mouse liver microsomes (Table 3), indicating that soluble or membrane-bound cysteinyl residues may be nucleophilic targets for naphthoquinones of this type. In support of this possibility, halogenated naphthoquinones (but not menadione) reacted with cysteine in solution; monohalogenated compounds produced a near-stoichiometric loss of sulfhydryl in a 15-min incubation (Table 4). One equivalent of the bifunctional compounds caused a loss of more than one equivalent of sulfhydryl, suggesting that both halogens may be displaced.

When *p*-nitrophenol displacement and NADPH–

Table 3. Release of *p*-nitrophenol by 2-*p*-nitrophenoxymenadione in the presence of sulfhydryl groups*

Source of thiol	$T_{\frac{1}{2}}$ (sec)
Cysteine	11.6
Glutathione	9.6
Mouse liver cytosol	23.9
Mouse liver microsomes	38.1

* 2-*p*-Nitrophenoxymenadione (20 μM) was incubated with thiol (200 nmoles/ml) at 37° in 50 mM potassium phosphate buffer, pH 7.4, and the release of *p*-nitrophenol was recorded spectrophotometrically as described in Materials and Methods. The pseudo first-order rate constants were estimated graphically (Materials and Methods).

Table 4. Reaction of menadione and derivatives with cysteine*

Compound	Final free sulfhydryl concn (μM)
None	99
Menadione	96
3-Chloromethylmenadione	57
2,3-Bischloromethyl-1,4-naphthoquinone	42
3-Bromomethylmenadione	57
2,3-Bisbromomethyl-1,4-naphthoquinone	37

* Menadione and its derivatives were incubated at 37° for 15 min with 100 μM cysteine in 50 mM potassium phosphate buffer, pH 7.4. The initial naphthoquinone concentration was 50 μM. The final free sulfhydryl concentration was measured by titration with 5,5′-dithiobis-2-nitrobenzoic acid [13].

Table 5. Effect of 2-*p*-nitrophenoxymenadione concentration on the loss of NADPH–cytochrome *c* reductase activity in mouse liver microsomes*

2-*p*-Nitrophenoxymenadione concn (μM)	Nitrophenol displaced (nmoles/60 min/mg protein)	Residual cytochrome *c* reductase activity (μmoles/min/mg protein)
0	0	112.6
10	70	84.0
20	120	60.5
40	170	45.4
80	230	37.0
160	315	28.6

* Mouse liver microsomes were suspended in 17.5 ml of 50 mM potassium phosphate buffer, pH 7.4, to a final concentration of 0.2 mg protein/ml. Aliquots of the suspension (2.5 ml) were pipetted into glass cuvettes; six cuvettes were placed in the sample chamber of a Kontron model 810 recording spectrophotometer equipped with an automatic sample changer; one cuvette served as the reference. An external circulating water bath maintained the temperature at 37°. 2-*p*-Nitrophenoxymenadione was added to the sample cuvettes to the final concentrations indicated above. The absorbance increases at 420 nm, proportional to the increases in free nitrophenol concentrations, were recorded for 1 hr. The contents of the sample cuvettes were then centrifuged, the pellets were resuspended in fresh buffer, and the residual enzyme activities were measured.

Table 6. Release of *p*-nitrophenol and loss of NADPH–cytochrome *c* reductase activity during incubation of mouse liver microsomes with 2-*p*-nitrophenoxymenadione*

Incubation time (min)	*p*-Nitrophenol released (nmoles/mg protein)	NADPH–cytochrome *c* reductase activity (μmoles/min/mg protein)
0.5	141	154
3	210	177
8	267	172
16	290	107
34	310	91
60	329	62

* Mouse liver microsomes were suspended in 50 mM potassium phosphate buffer, pH 7.4, and the suspension (0.2 mg protein/ml) was divided between the sample and reference cuvettes in the spectrophotometer (T = 37°). At $t = 0$, 2-*p*-nitrophenoxymenadione (initial concentration, 160 μM or 800 nmoles/mg protein) was added to the sample cuvette. The release of *p*-nitrophenol was monitored at 420 nm for 1 hr. At the times indicated, 50 μl aliquots were withdrawn from the sample cuvette and transferred to NADPH-free cytochrome *c* reductase assay buffer. The assays for NADPH–cytochrome *c* reductase were done after 60-min aliquot was taken.

cytochrome *c* reductase loss were studied as functions of the initial 2-*p*-nitrophenoxymenadione concentration, the final concentration of *p*-nitrophenol and the final specific activity of the enzyme were found to be inversely proportional (Table 5). When the initial naphthoquinone concentration was fixed (800 nmoles/mg microsomal protein) and the incubation time was varied, NADPH–cytochrome *c* reductase activity was found to be stable for the first 8 min, during which 267 nmoles of *p*-nitrophenol were released per mg of microsomal protein (Table 6). During the remaining 52 min of incubation time, 64% of the initial reductase activity was lost (k_1 = 0.017 min^{-1} for t = 8 min to t = 60 min) and an additional 62 nmoles of *p*-nitrophenol were released per mg of microsomal protein (k_1 = 0.0026 min^{-1} for t = 8 min to t = 60 min).

DISCUSSION

The results of this study show conclusively that menadione derivatives with leaving groups attached to the allylic carbons are sulfhydryl reactive and can diminish the microsomal NADPH-dependent cytochrome *c* reductase activity during the course of *in vitro* incubations. Menadione, incubated under identical conditions, was not sulfhydryl reactive and did not diminish reductase activity, demonstrating that the presence of a potential leaving group is essential. The involvement of the leaving groups was evident in the experiments performed with 2-*p*-nitrophenoxymenadione; *p*-nitrophenol was released when this compound was added to buffers containing cysteine, glutathione, cytosol, or microsomes, indicating that sulfhydryl groups may displace the leaving group from the allylic carbon.

The principle mechanism underlying the loss of microsomal NADPH–cytochrome *c* reductase activity remains to be established. It is unlikely that the loss of activity results from a competition between naphthoquinone and cytochrome *c* because, if such a mechanism were operating, the apparent K_m of the reductase for the its substrate would have been increased (Fig. 1). Moreover, since competitive inhibition was not observed, and since the parent compound, menadione, was not inhibitory, it is unlikely that traces of dissolved naphthoquinone in the washed microsomes could have contributed significantly to the loss of cytochrome *c* reductase activity. It is also unlikely that the loss resulted strictly from a loss of the loosely bound prosthetic group FMN [16], because ferricyanide reductase activity, dependent only on FAD [16], was also reduced. It seems reasonable to postulate that the loss of reductase activities is related in some way to the displacement of the electronegative group attached to the allylic carbon of the naphthoquinone derivative by microsomal nucleophiles.

Most of the sulfhydryl groups in mouse liver microsomes are associated with peptides other than the NADPH–cytochrome *c* reductase. Assuming that critical sulfhydryl groups are involved in the reductase inactivation mechanism, the non-critical sulfhydryl would be expected to have a sparing effect. Such an effect is suggested by the observation that the microsomal reductase activity was stable during the initial 8 min of an incubation with 2-*p*-nitrophenoxymenadione (Table 6).

The chemical reactivity of halogenated menadione derivatives is of interest in relation to the antitumor activities of these compounds [1–4]. It is believed that the active antitumor species are generated by enzymatic two-electron reductions and spontaneous rearrangements to the DNA-reactive quinone methides [1]. It is also possible that various quinones may generate cytotoxic free radicals by facilitating the transfer of electrons to oxygen. Recent evidence indicates that the NADPH-dependent microsomal cytochrome *c* reductase may catalyze one-electron reductions of oxygen when quinones, as intermediate electron carriers, are available [17].

In view of the results presented above, it seems likely that the toxicity of a given intracellular concentration of a halomethyl naphthoquinone may be limited by the concentration of sulfhydryl groups available to react with it. Sulfhydryl adduct formation would be expected to reduce the amount of substrate available for either one-electron or two-

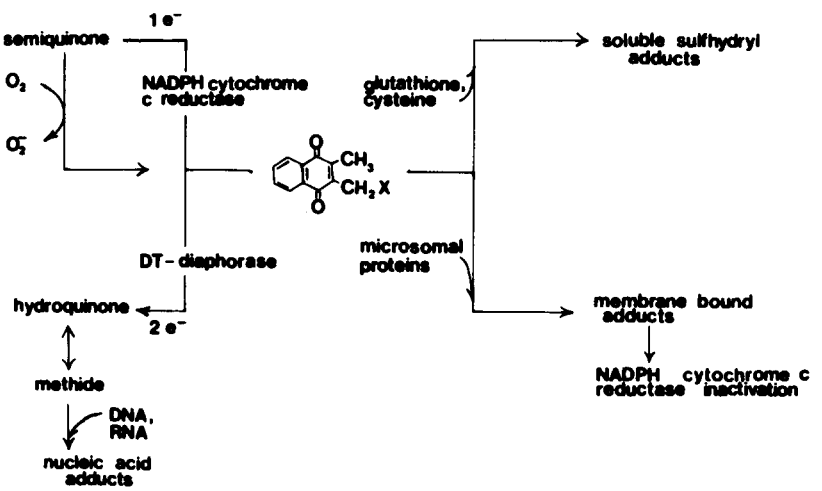

Fig. 2. Activation and inactivation of halomethyl naphthoquinones.

electron reduction, and may also, directly or indirectly, produce a loss in cellular quinone reductase activity (Fig. 2).

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