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INHIBITION OF MICROSOMAL LIPID PEROXIDATION AND CYTOCHROME P-450-CATALYZED REACTIONS BY β-LAPACHONE AND RELATED NAPHTHOQUINONES

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Abstract—The lipophilic *o*-naphthoquinones β-lapachone, 3,4-dihydro-2-methyl-2-ethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione (CG 8-935), 3,4-dihydro-2-methyl-2-phenyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione (CG 9-442), and 3,4-dihydro-2,2-dimethyl-9-chloro-2*H*-naphtho[1,2*b*]pyran-5,6-dione (CG 10-248) (a) inhibited NADPH-dependent, iron-catalyzed microsomal lipid peroxidation; (b) prevented NADPH-dependent cytochrome P-450 destruction; (c) inhibited microsomal aniline 4-hydroxylase, aminopyrine *N*-demethylase and 7-ethoxycoumarin deethylase; (d) did not inhibit the ascorbate- and *tert*-butyl hydroperoxide-dependent lipid peroxidation and the cumenyl hydroperoxide-linked aniline 4-hydroxylase reaction; and (e) stimulated NADPH oxidation, superoxide anion radical generation and Fe(III)ADP reduction by NADPH-supplemented microsomes. In the presence of ascorbate, the same *o*-naphthoquinones stimulated oxygen uptake and semiquinone formation, as detected by ESR measurements. The *p*-naphthoquinones α-lapachone and menadione were relatively less effective than the *o*-naphthoquinones. These observations support the hypothesis that, in the micromolar concentration range, *o*-naphthoquinones inhibit microsomal lipid peroxidation and cytochrome P-450-catalyzed reactions, by diverting reducing equivalents from NADPH to dioxygen.

Quinones may exert different actions on lipid peroxidation. On the one hand, redox cycling of anthracycline quinones, such as Adriamycin®, results in generation of reactive oxygen species, which are capable of initiating lipid peroxidation [1, 2]. On the other hand, several anthracenediones [3], naphthoquinones [4–7] and benzoquinones [8–10] are effective antioxidants and prevent lipid peroxidation. As regards the naphthoquinones, Wills [5] reported that menadione inhibits both NADPH and ascorbate-dependent lipid peroxidation in rat liver microsomes; other quinones such as 1,2- and 1,4-naphthoquinone showed antioxidant properties, but to a lesser extent than menadione. Studies by Högberg *et al.* [6] demonstrated that menadione can act as an inhibitor of iron-catalyzed lipid peroxidation in isolated hepatocytes. Menadione was more effective at inhibiting iron-catalyzed lipid peroxidation than peroxidation initiated by cumenyl hydroperoxide. These effects led Högberg *et al.* [6] to support the conclusion of Wills [5] that quinones may inhibit lipid peroxidation by diverting equivalents from NADPH away from the peroxidation process. Studies by Kostyuk and Lunets [9] and Talcott *et al.* [7] suggested, however, that electron diversion *per se* cannot account for the antioxidant effect of quinones on lipid peroxidation.

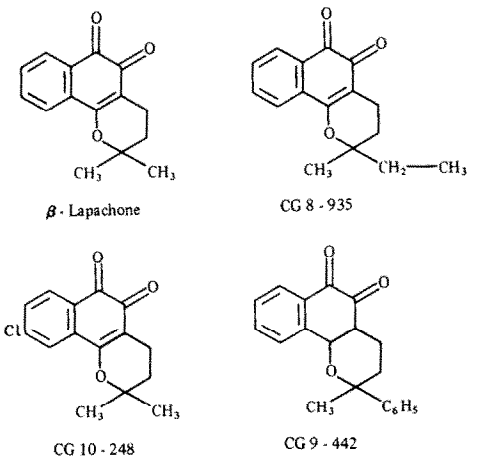

Fig. 1. Structures of *o*-naphthoquinones.

In the present study, we examined several β-lapachone§ derivatives (Fig. 1) for their action on microsomal lipid peroxidation and other reactions

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§ Abbreviations and chemical terms: β-lapachone, 3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; α-lapachone, 3,4-dihydro-2,2-dimethyl-2*H*-naphtho-[2,3*b*]-pyran-5,10-dione; CG 8-935, 3,4-dihydro-2-methyl-2-ethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; CG 9-442, 3,4-dihydro-2-methyl-2-phenyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; CG 10-248, 3,4-dihydro-2,2-dimethyl-9-chloro-2*H*-naphtho[1,2*b*]pyran-5,6-dione; menadione, 2-methyl-1,4-naphthoquinone; *t*-BuOOH, *tert*-butyl hydroperoxide; CuOOH, cumenyl hydroperoxide; DMFA, dimethyl formamide; SOD, superoxide dismutase; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; NADPH-generating system, 0.55 mM $NADP^+$, 5.5 mM $MgCl_2$, 5.5 mM G6P and G6PD (1.2 units/mL); MDA, malondialdehyde; and DETAPAC, diethylenetriaminepentaacetic acid.

involving the microsomal NADPH–cytochrome P-450 reductase, namely, superoxide anion radical generation and the cytochrome P-450-catalyzed aminopyrine *N*-demethylase, 7-ethoxycoumarin *O*-deethylase and aniline 4-hydroxylase. Comparative experiments were performed with β-lapachone and the *p*-naphthoquinones α-lapachone and menadione. The β-lapachone derivatives, which were synthesized as possible antitumoral agents [11], inhibit RNA-dependent DNA-polymerase and prolong the mean survival time of mice infected with Rauscher leukemia virus [11]. β-Lapachone is a trypanocidal quinone active *in vitro* on *Trypanosoma cruzi* [12–16].

MATERIALS AND METHODS

Microsomal preparations. Microsomes were obtained from the livers of 20-hr fasted, male Wistar rats, 240–280 g, fed a Purine-like rat chow. The homogenate and the microsomal fraction were prepared as described in Ref. 17. Microsomes were washed twice in the centrifuge with 150 mM KCl, for 1 hr at 105,000 *g*. The pellet was resuspended in 150 mM KCl and either used immediately or stored in liquid nitrogen. No superoxide dismutase or catalase activities were found in the microsomal suspension (data not shown).

Assay of lipid peroxidation. For the assay of NADPH-dependent lipid peroxidation, the incubation mixture consisted of liver microsomes (1.5 mg protein/mL), the NADPH-generating system (0.55 mM $NADP^+$, 5.5 mM G6P, 1.2 units/mL G6PD, 5.5 mM $MgCl_2$), 1.7 mM ADP, 0.1 mM $FeCl_3$, 130 mM KCl and 23 mM Na_2HPO_4–KH_2PO_4 buffer, pH 7.4. The $FeCl_3$–ADP mixture was prepared separately before beginning the experiments. After thermal equilibration at 37°, the reaction was started by the addition of G6PD. Aliquots (1.0 mL) were withdrawn at the times indicated and chilled to 0°, and MDA formation was measured. For the ascorbate-iron-induced lipid peroxidation, the incubation mixture (ascorbate system) consisted of microsomes (1.5 mg protein/mL, preheated in a water bath at 100° for 8 min), 2.0 mM ADP, 67 μM $FeCl_3$, 0.5 mM sodium ascorbate, 130 mM KCl and 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4. The reaction was started by adding ascorbate. The ascorbate solution was prepared immediately before use and kept under a stream of nitrogen. For the *t*-BuOOH-Fe or CuOOH-Fe-induced peroxidation, the incubation mixture consisted of 0.11 mM EDTA, 0.10 mM $FeSO_4$, 2.6 mM *t*-BuOOH (or 0.15 mM CuOOH), microsomes and phosphate buffer as with the NADPH-generating system. EDTA and $FeSO_4$ were added as an EDTA–$FeSO_4$ complex, which was prepared immediately before use and kept under a stream of nitrogen. The quinones were added dissolved in DMFA. Controls received the same volume of solvent whose concentration never exceeded 1% (v/v); DMFA failed to affect the rate of lipid peroxidation. The final volume of the incubation mixture was always 3.0 mL. Incubations were performed in a New Brunswick gyratory shaker, at 60 cycles/min and 37° under air. Other experimental conditions are described in the Results.

Enzyme assays. Aminopyrine *N*-demethylase activity was measured by determining formaldehyde production, using the Nash reagent [18]. The experimental conditions are described in the legend to Table 5. Aniline 4-hydroxylase activity was measured by determining 4-aminophenol production, which was estimated colorimetrically by the indophenol reaction [19]. For the assay using NADPH as electron donor, the reaction mixture contained microsomes (1.7 mg protein/mL), 1.25 mM aniline, 6.25 mM $MgCl_2$, 2.5 mM G6P, G6PD (0.5 units/mL), 0.125 mM $NADP^+$, 0.1 M KH_2PO_4-Na_2HPO_4, pH 7.4, preflushed with O_2. Incubation was for 20 min at 37°. For the assay using CuOOH, the reaction mixture contained microsomes (2.0 mg protein/mL), 3.0 mM aniline, 0.25 mM CuOOH, 80 mM Tris–HCl buffer, pH 7.6. Incubation was for 30 min at 37°. 7-Ethoxycoumarin *O*-deethylase activity was measured by determining 7-hydroxycoumarin, which was estimated fluorometrically [20] using an Aminco–Bowman spectrofluorometer. The reaction mixture contained microsomes (2.0 mg protein/mL), 0.5 mM 7-ethoxycoumarin, 0.75 mM $NADP^+$, 5.0 mM $MgCl_2$, 7.5 mM G6P, G6PD (1.0 units/mL), 50 mM Tris–HCl buffer, pH 7.8. After preincubation for 5 min, 7-ethoxycoumarin was added and the mixture was incubated for another 10 min at 37°. NADPH–cytochrome P-450 reductase activity was measured with NADPH as substrate and cytochrome *c* as artificial electron acceptor [21]. The reductase-catalyzed transfer of electrons to cytochrome *c* was measured spectrophotometrically at 550–540 nm. NADH–cytochrome b_5 reductase was estimated by the rate of reduction of potassium fericyanide followed spectrophotometrically at 420 nm [22]. Spectrophotometric measurements were performed at 30° using the Aminco DW 2a^{TM}UV/VIS spectrophotometer. Other experimental conditions are described in the Results. Enzyme activities are expressed in nanomoles substrate transformed per minute per milligram protein.

Assay of NADPH-oxidase activity. Quinones were assayed as a substrate for the microsomal NADPH–cytochrome P-450 reductase by measuring the rate of NADPH disappearance in the presence of oxygen, microsomes and quinone, at 30°. The reaction mixture consisted of microsomes (1.65 mg protein/mL), 0.165 mM NADPH, 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4. The reaction was started by adding the quinone, as stated in the Results. The rate of NADPH oxidation was measured fluorometrically using the fluorometer attachment of the Aminco–Chance spectrophotometer, equipped with adequate filters for excitation and emission. A calibration curve was constructed using a NADPH solution whose concentration was predetermined spectrophotometrically.

Reduction of Fe(III)ADP complex. The reduction was monitored at 37° in 0.15 M Tris–HCl buffer, pH 7.4, in the presence of microsomes [0.17 mg protein/mL, 0.10 mM NADPH, 5 mM G6P, G6PD (0.5 units/mL), 64 μM Fe(III)ADP (molar ratio Fe(III):ADP = 1:100)]. During reduction under air, the reoxidation of ferrous iron was inhibited by 0.3 mM 2,2′-dipyridyl and the formation rate of the stable ferrous-(bipyridyl)$_3$ complex was measured at

Table 1. Effects of *o*-naphthoquinones on lipid peroxidation by liver microsomes incubated with the NADPH-generating system, ascorbate or *t*-BuOOH

o-Naphthoquinone (5 μM)	MDA equivalents (nmol/mg protein)		
	NADPH-generating system	Ascorbate	*t*-BuOOH
None	30.7 ± 1.7	39.4 ± 2.8	9.5 ± 0.3
CG 8-935	3.2 ± 1.9* (90)	39.3 ± 4.5†	8.8 ± 0.6†
CG 9-442	1.8 ± 0.6* (94)	37.7 ± 4.7†	8.7 ± 0.8†
CG 10-248	2.3 ± 0.7* (92)	38.6 ± 8.0†	8.8 ± 1.1†
β-Lapachone	3.5 ± 0.8* (88)	37.9 ± 1.8†	9.1 ± 0.1†

Microsomes were incubated with reaction mixtures containing lipid peroxidation inducers, as indicated above. Their composition was as described in Materials and Methods. Incubation time (min): 60, 90 or 20, with the NADPH-generating system, ascorbate or *t*-BuOOH respectively. Values are means ± SEM (N = 6). Numbers in parentheses: percent inhibition of lipid peroxidation.

*† Analysis of variance: *$P < 0.001$; and †$P > 0.05$ (treated vs control).

510 nm [23]. The absorbance change was calibrated by the addition of excess dithionite at the end of each experiment and was taken as the internal standard. Spectrophotometric measurements were performed using the Perkin Elmer 5505 UV/VIS spectrophotometer.

Determination of O_2^- *and* H_2O_2 *generation.* Production of O_2^- was determined by the adrenochrome assay [24] by measuring the absorption change as 480–575 nm ($\varepsilon = 2.96$/mM/cm), using the Aminco DW-2^{TM}UV/VIS spectrophotometer, at 30°. The reaction mixture consisted of microsomes (0.20 mg protein/mL), 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4, 130 mM KCl, 1 mM epinephrine and the NADPH-generating system. Control samples were supplemented with SOD (6.0 units/mL). Production of H_2O_2 was determined by the microperoxide assay by measuring the absorption change at 419–407 nm ($\varepsilon = 78$/mM/cm). The reaction mixture consisted of microsomes (62 μg protein/mL), 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4, 130 mM KCl, 3 μM microperoxidase (MP-11) and the NADPH-generating system. The spectrophotometric measurements were performed with the Aminco–Chance spectrophotometer. H_2O_2 production was measured at 30°.

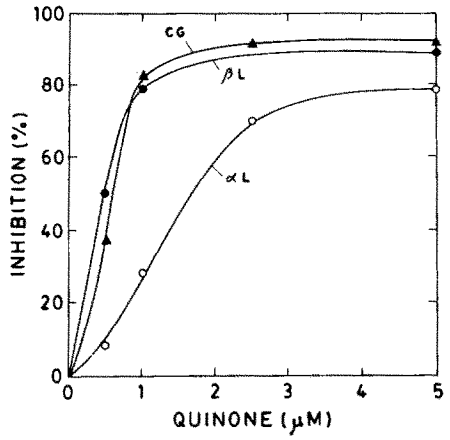

Fig. 2. Inhibition of NADPH-induced lipid peroxidation by *o*-naphthoquinones. Experimental conditions are described in Materials and Methods and in the legend of Table 1. Points are the means of two or more independent measurements. Abbreviations: βL, β-lapachone; αL, α-lapachone, and CG, CG 10-248.

Analytical methods. MDA and protein content of microsomal membranes were determined as indicated in Refs. 25 and 26 respectively. The assayed quinones did not interfere with the MDA assay. The cytochrome P-450 content of microsomes was estimated as described by Omura and Sato [27], and cytochrome b_5 was estimated from its redox spectrum of NADH-reduced versus oxidized cytochrome [28]. Spectra were recorded using the DW-2^{TM}UV/VIS spectrophotometer. Oxygen uptake was measured with a Gilson Oxygraph, model 5/6, using the Clark electrode.

Electron spin resonance (ESR). Measurements were performed with a Varian 45020 spectrometer at room temperature (23°), equipped with a rotatory cavity V-4533. The modulation amplitude was 2×10^3 (12 G). A 3-mL sample of quinone solution in 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.4, was flushed with a stream of nitrogen, then 15 mM sodium ascorbate was added, and the ESR spectrum was recorded.

Chemicals. Quinones CG 8-935, CG 9-442 and CG 10-248 were supplied by CIBA-GEIGY (Basle, Switzerland). β-Lapachone and α-lapachone were supplied by the Programme for the Synthesis of Antiparasitic Drugs of the Universidade Federal do Rio de Janeiro. G6P, G6PD (from Baker's yeast), SOD (from dog erythrocytes), microperoxidase (MP-11), cytochrome *c* (Type VI), $NADP^+$, NADPH, EDTA, DETAPAC, ADP (Grade IV), sodium ascorbate, *t*-BuOOH, CuOOH, DMFA, thiobarbituric acid, aminopyrine, aniline, 7-ethoxycoumarin, umbelliferone, 4-aminophenol, formaldehyde, epinephrine, menadione, sodium dithionite, bovine serum albumin (A6003) and Trizma were purchased from the Sigma Chemical Co. (St Louis, MO). 2,2′-Dipyridyl was purchased from C. Erba

(Milan, Italy). Carbon monoxide was purchased from Matheson (East Rutherford, NJ). Semicarbazide, phenol, ferric chloride, ferrous sulfate and DMFA were purchased from Mallinckrodt Chemical Works (New York, NY). Other reagents were of analytical grade.

Statistical analysis. This was performed using Student's *t*-test for paired values. The values presented are the means ± SEM of three or more independent experiments. When duplicate samples were measured, the experimental values deviated from the mean by less than 5%.

RESULTS

Effects of naphthoquinones on lipid peroxidation. Table 1 shows the effects of several quinones on MDA production by liver microsomes. Quinones were assayed at a fixed concentration (5 μM), and lipid peroxidation was initiated by NADPH, ascorbate, or *t*-BuOOH. It should be noted that the first two systems contained the $FeCl_3$–ADP mixture, whereas *t*-BuOOH was supplemented with $FeSO_4$-EDTA. The results presented here show that *o*-naphthoquinones inhibited the NADPH-induced lipid peroxidation by 88–94% but no significant effects were observed with the other systems. The negative results with ascorbate and *t*-BuOOH rule out quinone interference with the thiobarbituric acid reaction.

To determine quinone inhibition parameters, titration experiments were performed. Figure 2 shows typical curves obtained with two *o*-quinones (β-lapachone and CG 10-248) and one *p*-quinone (α-lapachone). Half-maximal inhibition ($I_{0.5\,max}$) values were calculated from these curves (or similar ones), and the results obtained (in μM) were as follows: 0.4 (β-lapachone); 0.6 (CG 10-248); 0.7 (CG 9-442); 0.9 (CG 8-935); 1.4 (α-lapachone); and 4.7 (menadione). These values show that *o*-quinones, as exemplified by β-lapachone, were several-fold more active than *p*-quinones, as exemplified by α-lapachone or menadione. Maximal inhibition values ranged between 88 and 94%, the quinone concentration required to obtain these effects being relatively higher with the *p*-quinones. It should be noted that modifications at position 2 in the pyran ring (CG 8-935 and CG 9-442) or position 9 in the benzene ring (CG 10-248) scarcely affected the inhibitory potency of β-lapachone derivatives.

The results in Table 1 show that *o*-naphthoquinone inhibition of MDA production occurred solely in the presence of NADPH. This was confirmed by adding a relatively small amount of NADPH to microsomes incubated with the *t*-BuOOH system and quinone (Table 2). Thus, addition of NADPH and quinone inhibited the *t*-BuOOH-induced lipid peroxidation by 31–41% but on omitting NADPH no inhibition was observed. Under these experimental conditions, NADPH stimulated the *t*-BuOOH-induced lipid peroxidation by 17% which leads one to assume that, in the presence of NADPH, quinone inhibition involved to some extent the NADPH-dependent lipid peroxidation. Nevertheless, considering the quinone concentration used in this experiment (10 μM), a direct antioxidant effect cannot be ruled out.

Table 2. Effects of *o*-naphthoquinones and NADPH on *t*-BuOOH-dependent microsomal lipid peroxidation

Quinone (10 μM)	MDA production (nmol equivalent/mg protein)	
	+NADPH	−NADPH
None	6.06	5.17
CG 10-248	3.60 (41)*	4.95 (4)
β-Lapachone	4.16 (31)	5.20 (0)

The incubation mixture consisted of microsomes (1.4 mg protein/mL), 2.6 mM *t*-BuOOH, 130 mM KCl, 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4, and 0.5 mM NADPH as indicated above. Incubation was for 20 min at 37°. Other experimental conditions were described in Materials and Methods. Values are averages of duplicate samples.

* Values in parentheses: percent inhibition of lipid peroxidation.

The antioxidant activity of a given quinone may bear a relation to its activity as a substrate for microsomal "quinone reductase" or, in other words, to the possibility of quinone redox-cycling, which can easily be demonstrated by $O_2^{\cdot-}$ generation. The results in Table 3 support this hypothesis since *o*-quinones were about 5-fold more effective $O_2^{\cdot-}$ generators than *p*-quinones, as emplified by the β-lapachone/α-lapachone pair. Similar results were obtained by measuring microsomal NADPH-oxidase activity (Table 3). Spontaneous dismutation of $O_2^{\cdot-}$ yielded small amounts of H_2O_2. Thus, the rate of H_2O_2 generation by microsomes supplemented with the NADPH-generating system and 5 μM β-lapachone was 1.7 nmol/min/mg protein (control value, 1.16) that is, 10% of the $O_2^{\cdot-}$ production rate under the same experimental conditions (Table 3).

Effect of quinones on loss of cytochrome P-450. The hepatic microsomal monooxygenase system, which catalyzes monooxygenation of xenobiotics as well as endogenous lipids, consists of the terminal enzyme cytochrome P-450 and NADPH–cytochrome P-450 reductase. This multienzyme system is unique in that its component proteins are all tightly bound to the endoplasmic reticulum membrane and electron transfer between them involves direct protein–protein interactions [29]. Loss of microsomal cytochrome P-450 has been associated with both NADPH- and hydroperoxide-induced lipid peroxidation [30–33]. Accordingly, naphthoquinone inhibition of lipid peroxidation prevented cytochrome P-450 destruction in microsomes incubated with the NADPH-generating system. Figure 3 illustrates the results of a typical experiment, using CG 10-248 as inhibitor. It can be seen that the cytochrome P-450 content in the microsomes incubated with the NADPH-generating system decreased by 50%, this effect being counteracted by the quinone. Cytochrome P-450 loss did not represent conversion into cytochrome P-420 as was to be expected from the observations of Lame and Segall [32]. Table 4 summarizes the results obtained with several naphthoquinones, using the NADPH- and *t*-BuOOH systems to produce lipid peroxidation and hemoprotein

Table 3. Effects of naphthoquinones on superoxide anion production and NADPH oxidation by liver microsomes

Quinone (5 μM)	Superoxide anion production (nmol/min/mg protein)		NADPH oxidation (nmol/min/mg protein)
	−SOD	+SOD	
CG 10-248	27.4	0	12.8
CG 9-442	17.3	0	10.7
CG 8-935	14.7	0	—
β-Lapachone	17.9	0	—
α-Lapachone	3.4	0	1.1
Menadione	2.1	0	2.4
None	1.9	0	3.1

O_2^- production was measured by the adrenochrome method; NADPH oxidation was measured fluorometrically, at 30°. Other experimental conditions are described in Materials and Methods. Values are the means of duplicate measurements.

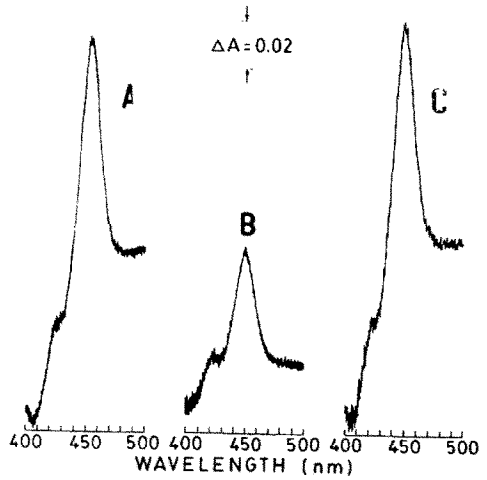

Fig. 3. Effect of *o*-naphthoquinones on cytochrome P-450 destruction after incubation with the NADPH-generating system. Samples contained microsomes (1.5 mg protein/mL), 130 mM KCl, 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4, and the following additions: sample A, none; sample B, 0.1 mM Fe(III)ADP (molar ration 1:17), NADPH-generating system and 5 μL DMFA; sample C, same as B, except for DMFA which was replaced by the same volume of CG 10-248 (final concentration, 5 μM); total vol., 6 mL. After a 1-hr incubation at 37°, the suspensions were centrifuged at 105,000 *g* for 30 min, the microsomes were suspended in 6.0 mL of 0.1 M phosphate buffer, pH 7.4, and cytochrome P-450 was measured in duplicate 3.0-mL samples. Other experimental conditions are described in Materials and Methods.

destruction. The data presented show that the *o*-naphthoquinones prevented the NADPH-dependent destruction of cytochrome P-450 but did not interfere with *t*-BuOOH action. Measurement of cytochrome b_5 in the NADPH-treated microsomes revealed the expected [34] diminution of this hemoprotein but no significant alterations were observed as regards the NADH–cytochrome b_5 reductase (control activity, 4.71 units/mg protein; experimental conditions omitted) and NADPH–cytochrome *c* (P-450) reductase (control activity 0.12 units/mg protein; experimental conditions omitted). The quinones prevented cytochrome b_5 destruction but failed to affect the reductase activities (experimental data omitted).

Differences in the time–course of cytochrome P-450 destruction produced by NADPH and CuOOH afforded another procedure for determining the site of action of the *o*-naphthoquinones in lipid peroxidation [33]. Figure 4 shows that when NADPH was used to initiate lipid peroxidation, the time course of hemoprotein loss involved two phases: (a) a slow initial one of about 5 min (maximal rate 0.16 nmol/min/mL), and (b) a faster terminal one (maximal rate, 0.63 nmol/min/mL). The latter phase was in all probability due to exaggerated lipid peroxidation catalyzed by iron made available by the destruction of cytochrome P-450 during the first phase [33]. In contrast to these results, the kinetics of the CuOOH-induced hemoprotein destruction followed a monotonic linear course, at a rate of 0.059 nmol/min/mL, that is to say, 30% of the value observed during the first phase of cytochrome P-450 destruction. In good agreement with the results in Fig. 3 and Table 4, 5 μM β-lapachone and CG 8-935 almost completely inhibited the NADPH-induced but not the CuOOH-dependent cytochrome P-450 loss (Fig. 4). At the same concentration, α-lapachone also prevented cytochrome P-450 destruction but less than β-lapachone. A better defined difference between β-lapachone and α-lapachone effects was established by using quinone concentrations lower than 1.0 μM. Thus, 0.50 μM α-lapachone afforded partial protection to cytochrome P-450 but the 0.25 μM concentration was ineffective. In contrast to these results, the same concentrations of β-lapachone afforded total protection (data omitted). The latter incubations were performed in the presence of ADP-iron, in order to reproduce the conditions prevailing in the lipid peroxidation experiments (Table 1 and Fig. 2).

Effects of naphthoquinones on cytochrome P-450 catalyzed reactions. In cytochrome P-450-catalyzed reactions, electrons donated from NADPH are transferred to cytochrome P-450 through NADPH–cytochrome P-450 reductase [35]. Considering that the latter enzyme catalyzes quinone redox-cycling,

Table 4. Effects of *o*-naphthoquinones on cytochrome P-450 and cytochrome b_5 loss, after incubation of microsomes with the NADPH-generating system or *t*-BuOOH

Quinone (5 μM)	Hemoprotein loss after incubation (% of control)		
	Cytochrome P-450		Cytochrome b_5
	NADPH-system	*t*-BuOOH	NADPH-system
None	62	87	25
CG 10-248	16	80	8
CG 9-442	26	96	14
β-Lapachone	28	87	8

Microsomes were incubated with the NADPH-generating system or *t*-BuOOH as indicated above. The experimental conditions were as described in the legend of Fig. 3 and in Materials and Methods. Control microsomes were incubated without the NADPH-generating system or *t*-BuOOH; cytochrome content (nmol/mg protein): 0.85 (P-450) and 0.36 (b_5). Values are the means of duplicate measurements.

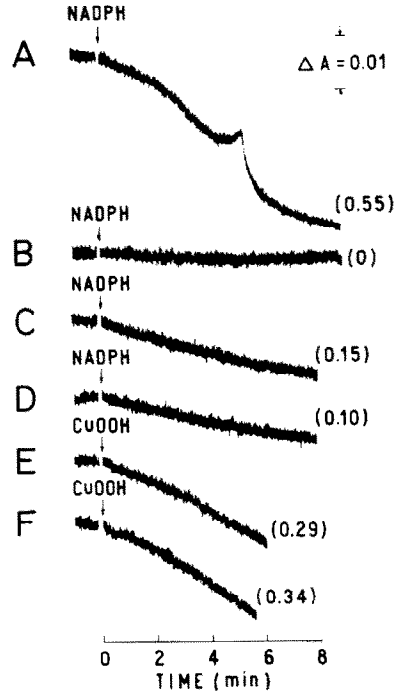

Fig. 4. Effect of *o*-naphthoquinones on the time-course of NADPH- and CuOOH-induced losses of microsomal cytochrome P-450. A 5.0-mL microsomal suspension (1.0 mg protein/mL) in 0.1 M KH_2PO_4–Na_2HPO_4, pH 7.4, containing 150 μM NADH was divided equally between two cuvettes and placed in the sample and reference cuvette chambers of the spectrophotometer, at 30°. Additions were as follows: samples A and E, 5.0 μL DMFA; B and F, 5.0 μM β-lapachone; C, 5 μM α-lapachone; and D, 5.0 μM CG 8-935. Destruction of cytochrome P-450 was initiated by adding either NADPH (final concentration 600 μM, samples A–D) or CuOOH (final concentration 2.6 mM, samples E and F). Absorption at 418 nm was measured for the time indicated on the abscissa. Values on the figure represent the total loss of cytochrome P-450 (in nmol/mL) and are the means of duplicate measurements. Other experimental conditions are described in Materials and Methods.

it was assumed that this reaction may determine diversion of electrons from cytochrome P-450, thereby inhibiting cytochrome P-450 dependent reactions. To test this hypothesis, quinones were assayed on aniline 4-hydroxylase, aminopyrine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase activities. Table 5 shows the demethylase and deethylase inhibitions, at fixed quinone concentrations. With 10 μM quinone, β-lapachone and the CG quinones inhibited the demethylase activity by 70–77%, lesser effects being obtained with the 5 μM concentration. Under the same experimental conditions, the *p*-naphthoquinones were weaker inhibitors, as exemplified by menadione (Table 5). The naphthoquinones also inhibited the deethylase activity and, once again, menadione and α-lapachone (data omitted) were less active than β-lapachone and the other *o*-naphthoquinones, especially at the 10 μM concentration. Similar results were obtained when aniline 4-hydroxylase activity was measured using NADPH as electron donor (Table 5). However, with CuOOH, which also supports cytochrome P-450-catalyzed reactions [36], no significant inhibitions were observed (Table 5). Inhibition of cytochrome P-450 reactions may imply inhibitor binding which can be monitored by spectral changes in the Soret absorption of cytochrome P-450 [35, 37, 38]. Under standard experimental conditions [37] and using 5–25 μM quinone, however, no spectral changes could be observed (data omitted). Negative results were also obtained when 10 μM *o*-naphthoquinones were added to a suspension of microsomes pre-equilibrated with aniline as described in Ref. 38.

Effects of o-naphthoquinones on reduction of Fe(III)ADP complex. In the presence of NADPH, liver microsomes rapidly reduced the Fe(III)ADP complex [23]. Since this reduction may be essential for initiating lipid peroxidation it seemed of interest to examine the effect of naphthoquinones on Fe(III)ADP reduction. The results are presented in Fig. 5 and deserve the following comments: (a) reduction values for control samples fit in well with those previously reported [23]; (b) β-lapachone

Table 5. Effects of naphthoquinones on microsomal aminopyrine *N*-demethylase (A), 7-ethoxycoumarin *O*-deethylase (B) and aniline 4-hydroxylase (C) activities

Quinone	Quinone concentration (μM)	Inhibition of enzyme activities (%) A	B	C "NADPH"	C CuOOH
CG 8-935	5	21	13	10	5.6
	10	74	61	71	7.0
CG 9-442	5	46	—	—	—
	10	77	—	—	—
CG 10-248	5	52	−2.7	10	1.4
	10	76	53	77	2.8
β-Lapachone	5	51	45	26	4.2
	10	70	84	68	2.8
Menadione	5	5.1	—	6.6	0
	10	31	—	42	0

Experiment A: the reaction mixture contained microsomes (0.7 mg protein/mL), 1.7 mM aminopyrine, 7.5 mM semicarbazide, 4.2 mM $MgCl_2$, 0.10 mM $NADP^+$, 1.7 mM G6P, G6PD (0.4 units/mL), 50 mM KH_2PO_4–Na_2HPO_4, pH 7.4, flushed with O_2 before use. Incubation was for 30 min at 37°. The other experimental conditions are described in Materials and Methods. Experiments B and C: the experimental conditions are described in Materials and Methods. In Expt. C, microsomes were incubated with the NADPH-generating system ("NADPH") or with CuOOH, as indicated above. Control activity (nmol substrate/min/mg protein): 0.99 (A), 0.38 (B), 0.31 (C, "NADPH") and 0.71 (C, CuOOH). Values are the means of duplicate measurements.

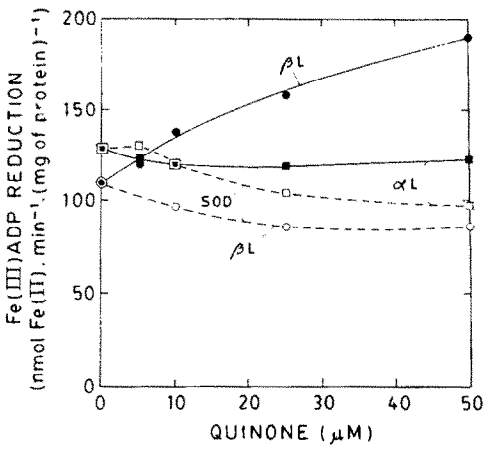

Fig. 5. Effects of β- and α-lapachone on the reduction rate of the Fe(III)ADP complex. The experimental conditions are described in Materials and Methods. Fe(III) reduction was monitored continuously by the rate of formation of the Fe(II)-(dipyridyl) complex, at 510 nm. Points are the means of duplicate measurements.

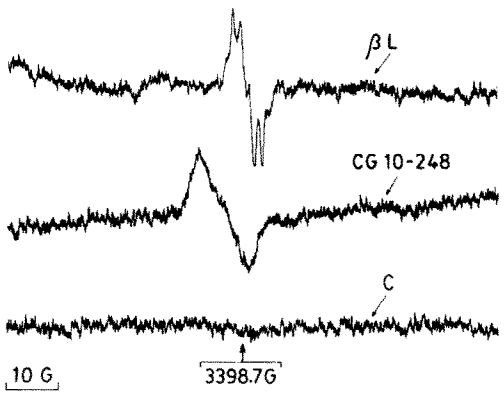

Fig. 6. ESR signals of quinone–ascorbate mixtures. The incubation mixture containing 15 mM ascorbate, 1.5 mM quinone, 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4, was flushed with nitrogen for 5 min, placed in the spectrometer cell (1 mm o.d. and 0.7 mm i.d.) and left for 20–30 min before recording the spectra. Other experimental conditions are described in Materials and Methods. Abbreviations: βL, β-lapachone; and C, control sample without quinone.

stimulated Fe(III)ADP reduction as a function of the quinone concentration; (c) SOD abolished the effect of β-lapachone; (d) α-lapachone did not inhibit Fe(III)ADP reduction. These results were confirmed using CG 10-248, under the same experimental conditions (data omitted). The effect of SOD with β-lapachone (Fig. 5) leads one to conclude that O_2^- generated by quinone redox-cycling reduced ferric iron in accordance with the reaction $Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$ [39], thus masking a possible inhibition of the iron complex reduction by the NADPH–cytochrome P-450 reductase.

Quinone redox-cycling in the presence of ascorbate. Quinones are reduced by ascorbate to semiquinone [40] and one electron transfer from the semiquinone to dioxygen yields the superoxide anion radical [4]. The resulting oxygen uptake then constitutes a measure of semiquinone production [4, 41, 42]. ESR measurements after mixing β-lapachone and CG 10-248 with sodium ascorbate revealed a semiquinone signal (Fig. 6), but under similar experimental conditions, α-lapachone and menadione did not (tracings omitted).

Table 6. Naphthoquinone effect on ascorbate oxidation

Quinone (25 μM)	Oxygen uptake (nmol/min/mL)
CG 10-248	57
CG 9-442	41
β-Lapachone	22
CG 8-935	20
Menadione	6.7
α-Lapachone	3.9
None	0

Oxygen uptake was measured polarographically, at 30°. The reaction mixture contained 5.5 mM ascorbate, 1.0 mM DETAPAC, 100 mM Tris–HCl buffer, pH 7.5, and quinone as stated above. The other experimental conditions are described in Materials and Methods.

Values are the means of duplicate measurements.

In order to compare the capability of the assayed naphthoquinones for redox-cycling, their action on ascorbate oxidation was measured. The results in Table 6 indicate that α-lapachone was the least reactive, in good agreement with results in Fig. 2. The β-lapachone group, however, showed differences in individual activities that could not be detected when these quinones were tested for their effect on lipid peroxidation or cytochrome P-450-dependent reactions.

DISCUSSION

Quinone inhibition of lipid peroxidation is a well known phenomenon, although its precise mechanism has not yet been determined [4, 7]. The present study demonstrates that *o*-naphthoquinones (a) inhibited NADPH-dependent, but not hydroperoxide- or ascorbate-dependent microsomal lipid peroxidation (Tables 1 and 2 and Fig. 2); (b) prevented the NADPH-dependent but not the hydroperoxide-induced destruction of cytochrome P-450 (Figs 3 and 4 and Table 4); (c) inhibited NADPH-dependent but not hydroperoxide-dependent cytochrome P-450 catalyzed reaction (Table 5); (d) generated superoxide anion radicals and stimulated microsomal NADPH oxidation (Table 3); and (e) stimulated Fe(III)ADP reduction when incubated with microsomes and NADPH (Fig. 5). These effects were either of lesser magnitude or were not observed with α-lapachone and menadione (Figs 2 and 4 and Table 5), in close agreement with the lesser ability of *p*-quinones for redox-cycling (Table 6). Taken together, our observations indicate that quinone redox-cycling, as catalyzed by the NADPH–cytochrome P-450 reductase, plays an essential role for *o*-naphthoquinone inhibitory effects. According to Wills [5] and Högberg *et al.* [6], quinone reduction by NADPH and semiquinone oxidation by dioxygen divert electrons away, thus preventing lipid peroxidation and cytochrome P-450-dependent reactions respectively. The negative results obtained with the ascorbate-iron system (Table 1) deserve special comment. In fact, ascorbate reduces quinones to the semiquinone and hydroquinone [40] and, accordingly, with the ascorbate-iron system (Table 1), quinones and/or their reduction products should inhibit lipid peroxidation, as suggested by Talcott *et al.* [7] and Kostyuk and Lunets [9]. The results obtained in the present study (Table 1) are at variance with a direct antioxidant activity of *o*-naphthoquinones. However, it is possible that at relatively high quinone concentrations additional effects, not excluding direct antioxidant activity, may complicate the mechanism of quinone inhibitions. The results presented in Table 2 lend support to this hypothesis.

o-Naphthoquinone redox-cycling generates superoxide anion radical (Table 3) and hydrogen peroxide (text), which, according to the Fenton reaction, generate more toxic species [43] capable of initiating lipid peroxidation. It is then remarkable that the *o*-naphthoquinones assayed in the present study, at variance with Adriamycin® [1, 2], did not stimulate either lipid peroxidation (Table 1 and Fig. 2) or enzyme inactivation (Table 5 and text). In this context, it should be noted that NADPH–cytochrome P-450 reductase, the enzyme catalyzing quinone reduction, is anchored in the endoplasmic reticulum membrane solely by a short hydrophobic segment, the conformation of the enzyme hydrophylic catalytic domain being independent of the hydrophobic membrane-bound domain [29]. The membrane-bound enzyme can interact with relatively large molecules in the aqueous phase, such as cytochrome *c* [29] and, obviously, quinones. Quinone redox-cycling and related reactions forming toxic oxy-radicals would then occur in the aqueous phase, far removed from targets such as lipid chains and membrane-bound protein molecules. Furthermore, ·OH radicals resulting from the iron-catalyzed Fenton reaction, because of their extraordinarily high reactivity, would not survive for more than a few collisions after their formation [29]. In support of this hypothesis stands the observation that with lipid vesicles, Fenton reactions forming ·OH from iron and H_2O_2 in the aqueous phase do not induce lipid peroxidation and may even be protective if the concentration of H_2O_2 is rate-limiting [39]. The latter condition prevails in the quinone, NADPH-supplemented microsomal suspension used in the present study (see Results).

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