performed in the laboratory of Dr. Ian A. Blair at the Center for Cancer Pharmacology, University of Pennsylvania School of Medicine. Many PAH metabolites were made available from the National Cancer Institute Chemical Carcinogen Standard Reference Repository, and the work was supported by grants R01 CA39505 and P01 CA092537 awarded to TMP.

[4] Redox Cycling of β-Lapachone and Structural Analogues in Microsomal and Cytosol Liver Preparations

By SILVIA FERNÁNDEZ VILLAMIL, ANDRÉS O. M. STOPPANI, and MARTA DUBIN

Introduction

Quinones are widely distributed in nature and make up an important group of substrates for flavoenzymes. Lipophilic *o*-naphthoquinones possess antibacterial, antifungal, trypanocidal, and cytostatic effects. Among those quinones, β-lapachone[1,2] (3,4-dihydro-2,2-dimethyl-2*H*-naphtho [1,2*b*]pyran-5,6-dione) isolated from the lapacho tree *(Tabebuia avellanedae)* has proved to be an effective cytostatic agent in different human tumor cells, such as murine leukemia, melanoma, hepatoma, human leukemia, colon carcinoma, lymphoma, and glioma, as well as epidermoid laryngeal, ovarian, breast, lung, and prostate cancer.[3–14] On these grounds, β-lapachone has

[1] I. L. D'Albuquerque, M. C. N. Maciel, A. R. Schuler, M. do C. de Araujo, G. Medeiros Maciel, M. da S. B. Cavalcanti, D. Gimino Martins, and A. Lins Lacerda, *Revta. Inst. Antibiot. Univ. Recife* **12,** 31 (1972).
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been suggested for clinical use; its effects have often been described as apoptosis or necrosis, depending on target cells, time, and drug dose.

β-Lapachone redox cycling in the presence of reductants and oxygen yields[15] reactive oxygen species (ROS), including superoxide anion radical O_2^-, hydroxyl radical $^\bullet OH$, hydrogen peroxide H_2O_2, and singlet oxygen $^1O_2$, whose cytoxicity explains β-lapachone activity in cells.

Quinones can undergo enzymatic one-electron reduction catalyzed by microsomal reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase to the semiquinone.[16] In the presence of molecular oxygen, the semiquinone radical can transfer an electron and generate the O_2^-. This reaction results in shunting electrons toward oxygen, as a futile pathway for reduction equivalents otherwise used for cytochrome P450 reductase-dependent reactions.[15] Superoxide can dismutate, by a superoxide dismutase (SOD)-catalyzed reaction to H_2O_2, and $^\bullet OH$ would then be formed by the iron-catalyzed reduction of peroxide by means of the Fenton reaction. All these highly reactive species may react directly with DNA or other cellular macromolecules, such as lipids and proteins, causing cell damage.

Unlike most other cellular reductases, two-electron reduction of quinone can also be catalyzed by cytosolic and mitochondrial DT-diaphorase (DTD), quinone oxidoreductase, E.C. 1.6.99.2 (NQO1), directly to the hydroquinone.[17–19] DTD-mediated production of the hydroquinone, which can be readily conjugated and excreted from the cell, constitutes a protective mechanism against these types of damage. It has been suggested that the reducing activity of DTD protects cells from the toxicity of naturally occurring xenobiotics containing quinone moieties. In addition to its protective effects, DTD can also reduce certain quinones to more reactive forms.[20,21] Interestingly, DTD is overexpressed in a number of tumors, including breast, colon, and lung cancers, compared with surrounding normal tissue.[22] This observation, more than any other, suggests that drugs that are

[13] S. M. Planchon, S. M. Wuerzberger-Davis, J. J. Pink, K. A. Robertson, W. G. Bornmann, and D. A. Boothman, *Oncol. Rep*. **6,** 485 (1999).
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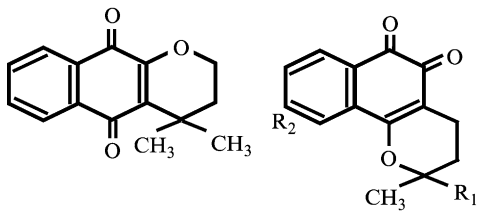

Quinone	R_1	R_2
CG 8-935	C_2H_5	H
CG 9-442	C_6H_5	H
CG 10-248	CH_3	Cl

FIG. 1. Structures of α- and β-lapachone and β-lapachone analogues.

activated by DTD (e.g., mitomycin C, streptonigrin) should show significant tumor-specific activity. DTD expression has been proposed as a major determinant of β-lapachone–mediated apoptosis and lethality. On the other hand, redox cycling of β-lapachone catalyzed by DTD could also be a futile cycle and a possible component of the cytotoxic mechanism.[14] Because DTD can use either NADPH or reduced nicotinamide adenine dinucleotide (NADH) as electron donors, this cycle would lead to a substantial loss of NADH and NADPH, with a concomitant rise in NAD^+ and $NADP^+$ levels. Such depletion of reduced enzyme cofactors may be a critical stage for the activation of the apoptotic pathway after β-lapachone treatment. One novel aspect of β-lapachone toxicity is the apparent activation of calpain followed by NAD(P)H depletion, showing an atypical cleavage pattern of poly(ADP-ribose)polymerase (PARP).[14]

Recently, it was reported that β-lapachone selectively induces apoptosis in transformed cells but not in proliferating normal cells, which is an unusual property not shared by conventional chemotherapeutic agents. It activates checkpoints in the absence of DNA damage. This selective induction of apoptosis is preceded by the rapid and sustained increase in the E2F1 level and activity in cancer cells. Taken jointly, the preceding results suggest direct checkpoint activators as selective agents against transformed cells.[23]

β-Lapachone cytotoxicity prompted the synthesis of a number of *o*-naphthoquinones, to establish the structural requirements for optimal therapeutic use. Several of those quinones (CG 10–248, 3,4-dihydro-2,2-dimethyl-9-chloro-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; and CG 8–935, 3,4-dihydro-2-methyl-2-ethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione) (Fig. 1)

[23] Y. Li, X. Sun, J. T. LaMont, A. B. Pardee, and Ch. J. Li, *Proc. Natl. Acad. Sci. USA* **100,** 2674 (2003).

proved in some assay systems to be more effective than β-lapachone itself.[24]

Materials and Methods

Microsomal and Cytosol Preparations

Microsomal and cytosol preparations were obtained from the livers of 20-h fasted, male Wistar rats, 240–280 g fed a Purine-like chow (A. C. A., Buenos Aires, Argentina), whose protein content was 23.4% and included all the essential amino acids. After the rats were rapidly decapitated, the liver was removed, weighed, washed, and homogenized in a Potter tissue grinder with a Teflon pestle, using 4 ml of homogenization medium per 2.5 g of tissue. All these steps were carried out in the cold. Following homogenization of the livers in TRIS-KCl buffer (50 m*M* TRIS-HCl, 150 m*M* KCl, pH 7.4) and centrifugation at 11,000*g* (15 min), the supernatant was centrifuged for 60 min at 105,000*g*. The microsomal pellet was washed twice with 150 m*M* KCl by centrifugation for 1 h at 105,000*g*, resuspended in 150 m*M* KCl, and either used immediately or stored in liquid nitrogen up to 3 months. The final supernatant named ''cytosol'' was used for DTD assays or stored at -70° for 15 days. No superoxide dismutase or catalase activities were found in the microsomal suspension.[15]

DTD activity was measured spectrophotometrically at 340 nm and 30° using NADPH as the immediate electron donor and menadione as the intermediate electron acceptor. The reaction mixture contained 100 μM NADPH, 100 μM menadione, 0.1 *M* K^+-phosphate, pH 7.4, and 10–30 μl cytosol for a total volume of 3.0 ml.

Each assay was repeated in the presence of 10 μM dicoumarol, and activity attributed to DTD was the inhibited by dicoumarol.[17]

The protein concentration was measured by the Biuret method.

Quinone Preparations

CG-quinones were supplied by Ciba Geigy-Novartis (Basel, Switzerland), and α-lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphthol[2,3*b*]-pyran-5,10 dione) and β-lapachone were obtained from a program for the synthesis of antiparasitic drugs at the Universidade Federal de Rio de Janeiro, Brazil. *o*-Naphthoquinones are unstable and degrade over time, so the compounds assayed were examined by HPLC, indicating more than

[24] K. Schaffner-Sabba, K. H. Schmidt-Ruppin, W. Wehrli, A. R. Schuerch, and J. W. F. Wasley, *J. Med. Chem.* **27,** 990 (1984).

98% purity. Quinones were dissolved in dimethylformamide (DMFA); the corresponding volume of DMFA was added to control samples.

Electron Spin Resonance (ESR) Measurements

ESR measurements for microsomal preparations were performed at room temperature using a Bruker ER 200 tt x-band ESR spectrometer (Bruker Analytische Messtechnic GMBH, Rheistellen, Karlsruhe, Germany) equipped with a TE 102 cavity. General instrumental conditions were microwave power, 21 mw; microwave frequency, 9.90 GHz; modulation frequency, 100 KHz; and time constant, 0.5 s. The field was centered at 3500*G*.

ESR measurements for cytosol preparations were performed using a Bruker ER 1100 ESR spectrometer. General instrumental conditions were microwave power, 20 mw; microwave frequency, 10 GHz; modulation frequency, 50 KHz; time constant, 0.2 s. The field was centered at 3490*G*. Modulation amplitude, gain, and scan rates were as indicated in legends to Figs. 2, 3, and 4.

Redox Cycle of β-Lapachone and Related *o*-Naphthoquinones Catalyzed by Microsomal NADPH Cytochrome P450 Reductase

ESR Study of Semiquinone Radical Formation by NADPH Cytochrome P450 Reductase

Principle. In the hepatic microsomal mono-oxygenase system, electrons donated from NADPH are transferred to the quinone (Q) through NADPH cytochrome P450 reductase. The semiquinone radical ($Q^{\bullet}$) generation can be detected by ESR.

$$Q + NADPH \xrightarrow{\textit{Cyt}\ \text{P450}\ \textit{reductase}} Q^{\bullet} + NADP^{+} + H^{+} \quad (1)$$

Procedure. The NADPH cytochrome P450 reductase assay medium contained liver microsomes (6 $mg \cdot ml^{-1}$ protein), 50 m*M* TRIS-HCl, pH 7.4, 10 m*M* NADPH and NADPH regenerating system (10 m*M* G_6P, 12 $U \cdot ml^{-1}$ G_6P dehydrogenase, and 3.0 m*M* $MgCl_2$). To obtain anaerobic conditions for the ESR measurements, the assay media (less quinone) were flushed with a nitrogen stream. At the same time, the quinone solution was also flushed, with nitrogen, as earlier. A sample of the deoxygenated, concentrated quinone solution was added to the deoxygenated assay medium, and the mixture was further flushed with nitrogen as earlier for 1 min. Last, the reaction medium was transferred to the spectrometer cell, flushed with

nitrogen, and the spectrum was recorded. The time elapsing from the reaction mixture preparation to ESR spectrum recording was roughly 10 min, a period considered as the incubation time.[16]

ESR Spectrum of β*-Lapachone–Related Semiquinones*

The ESR spectrum of CG 10–248 semiquinone, after quinone reduction by the microsomal NADPH cytochrome P450 reductase system, is shown in Fig. 2A. Hyperfine splittings (HFSC) indicate spin couplings at protons at C7, C8, and C10 of the naphthalene ring. The observed quadruplet indicates three nuclei of 0.5 spin. HFSC values varied between 0.140 and 0.150 mT. The chlorine atom has a 1.5 nuclear spin, and no splitting is observed, probably because of its very low HFSC value.

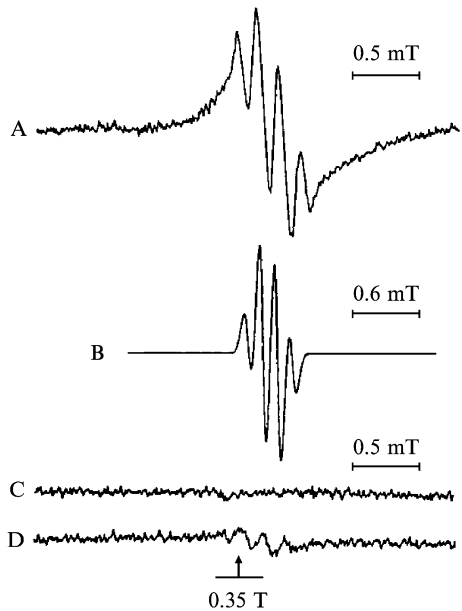

FIG. 2. ESR spectrum of CG 10–248 semiquinone after quinone reduction by the liver microsomal NADPH cytochrome P450 system. (A) The reaction mixture contained 5 m*M* CG, 10 m*M* NADPH, NADPH regenerating system, liver microsomes, 50 m*M* TRIS-HCl, pH 7.4, under nitrogen. (B) Computed simulation of the semiquinone spectrum using a spin-coupling constant value of 0.145 mT. (C) and (D) same as (A), except for quinone (C) or microsomes (D) omission. Instrumental conditions: modulation amplitude, 0.08 mTpp; gain 1.0×10^6, scan rate, 0.36 mT/min. Other experimental conditions were as described in "Materials and Methods" and in the text. From S. Fernandez Villamil, M. Dubin, M. P. Molina Portela, L. J. Perissinotti, M. A. Brusa, and A. O. M. Stoppani, *Redox Report* **3,** 245 (1997).

The kinetics of semiquinone formation shows that maximum values were reached after a 10-min incubation, scarcely varying thereafter (data not shown). The computed spectrum, using the average HFSC value (0.145 mT), fits well with the experimental results obtained (Fig. 2B). Omission of quinone or reductant system prevented the appearance of the semiquinone signal (Fig. 2C and D).

Figure 3A shows the CG 8–935 semiquinone spectrum after quinone reduction by the microsomal NADPH cytochrome P450 reductase system. With CG 8–935, a quintuplet signal indicates four spin couplings at protons at C7–C10 of the naphthalene ring. HFSC values were similar to those calculated for CG 10–248. Computational analysis reproduced the experimental spectrum (Fig. 3B). Similar results were obtained with CG 9–442 (data not shown), demonstrating that protons at the methyl, ethyl, and phenyl group or pyran ring failed to contribute to the ESR signal.

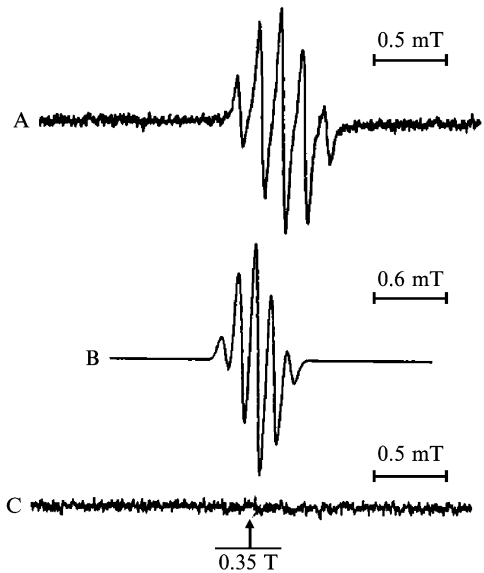

FIG. 3. ESR spectrum of CG 8–935 semiquinone after quinone reduction by the liver microsomal NADPH cytochrome P450 system. (A) The reaction mixture contained 5 m*M* CG, 10 m*M* NADPH, NADPH regenerating system, liver microsomes, 50 m*M* TRIS-HCl, pH 7.4, under nitrogen. (B) Computed simulation of the semiquinone spectrum. (C) Same as (A) except for microsomes omission. Instrumental conditions: modulation amplitude, 0.08 mTpp; gain 0.8×10^6; scan rate, 0.9 mT/min. Other experimental conditions were as described in "Materials and Methods" and in the text. From S. Fernandez Villamil, M. Dubin, M. P. Molina Portela, L. J. Perissinotti, M. A. Brusa, and A. O. M. Stoppani, *Redox Report* **3,** 245 (1997).

TABLE I
EFFECTS OF NAPHTHOQUINONES ON MICROSOMAL SUPEROXIDE ANION PRODUCTION

Quinone (5 μM)	Superoxide anion production (nmol min^{-1} mg^{-1} protein)
CG 10–248	27.4
CG 9–442	17.3
CG 8–935	14.7
β-Lapachone	17.9
α-Lapachone	3.4
None	1.9

Superoxide anion production by microsomes was measured by the adrenochrome method at 30°. Other experimental conditions are described in the text. Values are the means of triplicate measurements.

Effects of β-*Lapachone and Related* o-*Naphthoquinones on Superoxide Anion Production by Liver Microsomes*

Principle. Semiquinones in aerobic conditions are reoxydized, generating O_2^-.

$$Q^\bullet + O_2 \rightarrow Q + Q_2^- \tag{2}$$

$$\text{Adrenaline} + O_2^- \rightarrow O_2 + \text{Adrenochrome} \tag{3}$$

Production of O_2^- can be determined by the adrenochrome assay by measuring the absorption change at 485–575 nm ($\varepsilon = 2.96$ mM^{-1} cm^{-1}).[25]

Procedure. The reaction mixture consisted of microsomes (0.20 mg·ml^{-1} protein), 23 mM Na^+/K^+ phosphate buffer, pH 7.4, 130 mM KCl, 1 mM epinephrine, 5 μM quinone, and the NADPH generating system. Adrenochrome production was measured at 30°, using an Amino $DW2_a$ spectrophotometer (American Instrument Company, Silver Spring, MD) at 485–575 nm. Control samples were supplemented with DMFA.

The *o*-naphthoquinones redox cycling, in microsomal preparations, can readily be demonstrated by O_2^- generation, as shown in Table I, because *o*-quinones were about five-fold more effective O_2^- generators than the *p*-quinone, as exemplified by the β-lapachone/α-lapachone pair. Addition of SOD (6 U · ml^{-1}) confirmed that the absorbance increase was due to O_2^-.

[25] H. P. Misra and I. Fridovich, *J. Biol. Chem.* **247,** 188 (1972).

Effects of β-Lapachone and Related *o*-Naphthoquinones on Cytochrome P450-Catalyzed Reactions

In cytochrome P450-catalyzed reactions, electrons donated from NADPH are transferred to cytochrome P450 through NADPH cytochrome P450 reductase. Considering that the cytochrome P450 reductase catalyzes quinone redox-cycling, it was assumed that this reaction may lead to diversion of electrons from cytochrome P450, thereby inhibiting cytochrome P450-dependent reactions. To test this hypothesis, β-lapachone and related *o*-naphthoquinones were assayed on aniline 4-hydroxylase and aminopyrine *N*-demethylase activities, at fixed quinone concentrations.[15]

Aminopyrine N-*Demethylase Determination*

Principle. Many drugs are dealkylated by hepatic microsomal enzymes, and the method described here can be applied to such substrates.

$$\text{Aminopyrine} \xrightarrow[\text{NADPH}]{\text{Microsomes}} \text{Monomethyl-4-aminoantipyrine} \xrightarrow[\text{NADPH}]{\text{Microsomes}} \text{4-Aminoantipyrine} \quad (4)$$

$$\text{Monomethyl-4-aminoantipyrine} \searrow \text{Formaldehyde} \qquad \text{4-Aminoantipyrine} \searrow \text{Formaldehyde}$$

The preceding reaction indicates that dealkylation activity may be determined by measuring the formation of either formaldehyde or 4-aminoantipyrine[26]; in the following experiments we measured formaldehyde formation.

Formaldehyde generated during incubation is trapped as the semicarbazone (by semicarbazide in the incubation mixture) and measured by the spectrophotometric procedure of Nash,[27] based on the Hantzsch reaction. The Nash reaction has been widely used, because it is simple, fast, and accurate. The Hantzsch reaction requires a β-diketone (acetylcetone), an aldehyde (formaldehyde), and an amine (NH_2 from ammonium acetate) as shown in Eq. 5. With the indicated reactants, the product formed is 3,5-diacetyl-1,4-dihydrolutidine (DDL), which can be monitored by its absorption at 415 nm.

$$\text{Acetylacetone} + \text{Ammonia} + \text{Formaldehyde} \rightarrow \text{DDL} + H_2O \quad (5)$$

[26] P. Mazel, *in* "Fundamentals of Drug Metabolism and Drug Disposition" (B. N. LaDu, H. Mandel, and E. Way, eds.), p. 546. Williams & Wilkins, Baltimore, 1971.
[27] T. Nash, *J. Biol. Chem*. **55,** 416 (1953).

Procedure. Into a tube immersed in ice add the following solutions in order: 1.7 m*M* aminopyrine, 4.2 m*M* $MgCl_2$, 7.5 m*M* semicarbazide, NADPH generating system containing 0.10 m*M* $NADP^+$, 1.7 m*M* G_6P, and 0.4 $U \cdot ml^{-1}$ G_6P dehydrogenase in 0.05 *M* Na^+/K^+ phosphate buffer, pH 7.4, and 10 or 5 μM β-lapachone or related *o*-naphthoquinones preflushed with oxygen. The reaction was started by adding microsomal suspension equivalent to 0.7 $mg \cdot ml^{-1}$ protein. Total volume was 6.0 ml. Blanks carried incubates through the procedure without substrate, whereas controls received the same volume of quinone solvent (DMFA). DMFA fails to interfere with the assay up to 5%.

Incubate with shaking for 30 min at 37°. At the end of the incubation period, remove the tubes and add 2.0 ml of 15% zinc sulfate to each one to stop the reaction. Mix well and wait 5 min. Add 2.0 ml of saturated barium hydroxide to each tube. Again, mix well and wait 5 min. Centrifuge for 10 min using a high speed to completely settle the precipitate. Transfer 5.0 ml of the supernatant to a test tube. Add 2.0 ml of Nash reagent (30% ammonium acetate, 0.4% v/v acetylacetone), mix well, and place in a water bath (60°) for 20 min. Filter if the solutions are cloudy. Measure the absorbance spectrophotometrically at 415 nm. A small amount of endogenous formaldehyde must be subtracted from the total amount of formaldehyde formed by setting the blanks to zero absorbance. Determine the quantity of formaldehyde formed from the standard curve.

Preparation of Standard Formaldehyde Curve. A standard formaldehyde curve may be prepared using formaldehyde solution (40%). Although formaldehyde solution contains 12% methanol, the final dilutions are such that methanol does not interfere in the reaction. Dilute the formaldehyde solution in water so as to obtain the following concentrations: 4, 2, 1, and 0.5 $\mu g \cdot ml^{-1}$.

Comments

It is essential to use proper concentrations of barium and zinc. Barium hydroxide is much more soluble in boiling water.

The Nash reagent is quite stable and may be kept in the refrigerator for many weeks.

The optimal pH for the Hantzsch reaction ranges from 5.5–6.5.

Large amounts of acetaldehyde interfere with the measurements of formaldehyde, but at molar formaldehyde concentration acetaldehyde produces only 1% interference. Drug de-ethylation to acetaldehyde could be a source of the latter aldehyde under certain experimental conditions. Acetone, chloral, and glucose do not interfere, but microsomes and soluble

fractions obtained in sucrose solutions yield lower values of formaldehyde. Amines compete with ammonia in the reaction.

The reaction product is relatively stable but is affected by prolonged exposure to light and oxidizing agents.

Color development is faster at 60° (10–20 min) than at 37° (60 min).

Aniline Hydroxylase Determination

Principle. Hydroxylation of aniline by cytochrome P450 can be investigated in the presence of liver microsomes in NADPH- or cumene hydroperoxide (CHP)–dependent systems.[28]

The rate of aniline metabolism *in vitro* may be determined by measuring the quantity of *p*-aminophenol formed according to the following reaction:

$$\text{Aniline} \xrightarrow[\text{NADPH or CHP}]{\text{Microsomes}} p\text{-aminophenol} \qquad (6)$$

In this procedure, trichloroacetic acid (TCA) is used to precipitate the protein, and the quantity of *p*-aminophenol produced by aniline hydroxylation is determined in an aliquot of the TCA supernatant fraction. Phenol is then added to form the blue phenol–indophenol complex, which is measured at 640 nm.[29]

Procedure

Method 1 (NADPH-Dependent System). Into a series of flasks, immersed in ice, add the following components: 1.25 m*M* aniline hydrochloride, NADPH generating system (1.2 m*M* $NADP^+$, 2.5 m*M* G_6P, 0.5 U ml^{-1} G_6P dehydrogenase, 6.0 m*M* $MgCl_2$, dissolved in 0.1 *M* Na^+/K^+ phosphate buffer, pH 7.4), and β-lapachone or related quinones preflushed with O_2. The reaction was started by adding 1.0-ml microsomes suspension equivalent to 1.7 $mg \cdot ml^{-1}$ protein. Total incubation volume was 4.0 ml. Blanks carried incubates through the procedure without substrate. Controls received the same volume of quinone solvent (DMFA). DMFA interferes with the reaction, so that, depending on the volume to obtain the appropriate quinone concentration to be tested, the corresponding control with DMFA must be included. Incubate the flasks with rapid shaking for 20 min at 37°. At the end of the incubation period, add 2.0 ml of 20% TCA to the incubation flasks. Mix well and centrifuge. To a 2.0-ml aliquot

[28] I. I. Karuzina, A. I. Varenitsa, and A. I. Archakov, *Biokhimiya* **48,** 1788 (1983).

[29] B. G. Lake, *in* "Biochemical Toxicology" (K. Snell and B. Mullock, eds.), p. 206. IRL Press, Oxford, 1987.

of the TCA supernatant add 1.0 ml of 10% Na_2CO_3 and mix well. Add 2.0 ml of 2% phenol in 0.2 *N* NaOH and allow the color to develop for 30 min at 37°. Read the absorbance in a spectrophotometer at 640 nm. Determine the micrograms of *p*-aminophenol formed from a previously plotted standard curve. Multiply this number by 3 to obtain the total amount of *p*-aminophenol in the incubation flasks.

Preparation of Standard p-*Aminophenol Curve*

Prepare a series of tubes containing *p*-aminophenol HCl (1–4 $\mu g \cdot ml^{-1}$) in 6.67% TCA (2.0 ml), because this is the final concentration of TCA after adding 20% TCA to the incubate. To 2.0 ml of each concentration in TCA (in duplicate) add 1.0 ml of 10% Na_2CO_3 followed by 2.0 ml of 2% phenol in 0.2 *N* NaOH. Allow color to develop and read the absorbance in a spectrophotometer at 640 nm.

Method 2 (Cumene Hydroperoxide-Dependent System)

It is known that cytochrome P450 can catalyze oxidation reactions of many substrates, using organic hydroperoxides as active oxygen donors. In this method hydroxylation of aniline in the microsomes was measured in the presence of CHP.[28]

Procedure. For the assay using CHP, the reaction mixture contained 3.0 m*M* aniline, 0.25 m*M* CHP, 80 m*M* TRIS-HCl buffer, pH 7.6, and microsomes (2.0 $mg \cdot ml^{-1}$ protein). Incubation was for 30 min at 37°. Total incubation volume was 4.0 ml. Blanks carried incubates through the procedure without substrate, whereas controls received the same volume of quinone solvent (DMFA). DMFA interferes with the reaction, so that controls with the same volume of DMFA must be included. At the end of the incubation period, add 7.7% TCA to the incubation flasks. Mix well and centrifuge. To a 2.0-ml aliquot of the TCA supernatant add 1.0 ml of 10% Na_2CO_3 and mix well. Add 2.0 ml of 2% phenol in 0.2 *N* NaOH and allow the color to develop for 30 min at 37°. Read the absorbance in a spectrophotometer at 640 nm.

Comments

The procedures described for the determination of microsomal aniline formation by hydroxylase activity are both simple and sensitive.

The cofactor mixture must be preincubated briefly before adding enzyme.

Hydrogen peroxide (100 m*M*) can be used instead of CHP, but the latter is better as a cosubstrate than the former. Because the presence of residual hydrogen peroxide may prevent the development of color in the

following reaction, it must be previously decomposed by adding 8000 U · ml^{-1} catalase.

The incubation period should be as short as possible, because microsomes incubation with *p*-aminophenol for about 30 min leads to its loss.

Inhibition of Cytochrome P450-Catalyzed Reaction by β-*Lapachone and Related* o-*Naphthoquinones*

A comparative effect of *o*-naphthoquinones on microsomal aminopyrine *N*-demethylase and aniline hydroxylase activities is shown in Table II.

With 10 μM quinone, β-lapachone and CG quinones inhibited the demethylase activity by 70–77%, lesser effects being obtained with the 5 μM concentration. The naphthoquinones also inhibited the aniline 4-hydroxylase activity using NADPH as electron donor by 66–77% (Table II). However, for aniline hydroxylase activity with CHP, which also supports cytochrome P450-catalyzed reactions by a different mechanism (Table II), no significant inhibition was observed. Inhibition of cytochrome P450

TABLE II
EFFECTS OF *o*-NAPHTHOQUINONES ON MICROSOMAL AMINOPYRINE *N*-DEMETHYLASE (A) AND ANILINE 4-HYDROXYLASE (B) ACTIVITIES

		Inhibition of enzyme activities (%)		
			B	
Quinone	Quinone concentration (μM)	A	NADPH	CHP
CG 10–248	5	52	10	1.4
	10	76	77	2.8
CG 9–442	5	46	19	ND
	10	77	66	3.6
CG 8–935	5	21	10	5.6
	10	74	71	7.0
β-Lapachone	5	51	26	4.2
	10	70	68	2.8

Experiment A: the reaction mixture contained microsomes (0.7 mg · ml^{-1} protein), 1.7 mM aminopyrine, 7.5 mM semicarbazide, 4.2 mM $MgCl_2$, 0.10 mM $NADP^+$, 1.7 mM G_6P, 0.4 U · ml^{-1} G_6P dehydrogenase, 50 mM KH_2PO_4-Na_2HPO_4, pH 7.4, flushed with O_2 before use. Incubation was for 30 min at 37°. Experiment B: the reaction mixture contained microsomes (~2 mg · ml^{-1} protein), aniline, and NADPH generating system (NADPH) or cumene hydroperoxide (CHP) as indicated earlier. Control activity (nmol substrate min^{-1} mg $protein^{-1}$): 0.99 (A), 0.31 (B, NADPH), and 0.71 (B, CHP). Values are the means of triplicate measurements. Other experimental conditions are described in the text. ND: not done.

reactions may imply inhibitor binding, which can be monitored by spectral changes in the Soret absorption of cytochrome P450. Under standard experimental conditions and using 5–25 μM quinone, however, no spectral changes could be observed (data omitted).

These observations support the hypothesis that, in the 0–10 μM concentration range, β-lapachone and related CG quinones inhibit cytochrome P450-catalyzed reactions by diverting reduction equivalents from NADPH to dioxygen.[15]

Redox Cycle of β-*Lapachone and Related* o-*Naphthoquinones Catalyzed by Cytosol DT-Diaphorase*

Principle. In the NAD(P)H/*o*-naphthoquinone/DTD/oxygen system, the redox cycle involves two phases, namely: (1) the reductive phase and (2) the oxidative phase. The former is represented by Eq. 7 involving Q and QH_2, which are the *o*-naphthoquinone and the corresponding hydroquinone, respectively:

$$2NAD(P)H + Q \rightarrow 2NAD(P)^{+} + QH_2 \tag{7}$$

Concerning the hydroquinone formed in Eq. 7, it autoxidizes with formation of ROS in a process inhibited by SOD. Ionization of the QH_2 is a necessary prerequisite for the reaction, and oxidation is initiated by reaction of anion QH^{-} with molecular oxygen forming superoxide and semiquinone ($Q^{\bullet}$) (Eq. 8).

$$H^{+} + QH^{-} + O_2 \leftrightarrow Q^{\bullet} + O_2^{-} + 2H^{+} \tag{8}$$

The oxidative phase of the NAD(P)H/*o*-naphthoquinone/DTD/oxygen system redox cycle involves three reactions termed initiation, propagation, and termination.[30–32] The initiation reaction (Eq. 8) can be demonstrated by semiquinone production by ESR, oxygen uptake, and O_2^{-} production.

The propagation reaction of hydroquinone oxidation proceeds by way of O_2^{-} in agreement with Eq. 9 and Eq. 10.

$$H^{+} + QH^{-} + O_2^{-} \leftrightarrow Q^{\bullet} + H_2O_2 \tag{9}$$

$$Q^{\bullet} + O_2 \leftrightarrow Q + O_2^{-} \tag{10}$$

[30] T. Ishii and I. Fridovich, *Free Radic. Biol. Med*. **8,** 21 (1990).
[31] R. Munday, *Free Radic. Biol. Med*. **26,** 1475 (1999).
[32] R. Munday, *Free Radic. Res*. **35,** 145 (2001).

Finally, SOD-catalyzed O_2^- disproportionation (Eq. 11) and semiquinone disproportionation (Eq. 12) terminate the naphthoquinone redox cycle in cytosol.

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (11)$$

$$Q^\bullet + Q^\bullet + H^+ \rightarrow Q + QH^- \quad (12)$$

ESR Study of Semiquinone Radical Formation after CG Reduction by the NADPH/DT-Diaphorase System

Procedure. The reaction mixture contained 21 m*M* NADPH, 8.4 m*M* CG 10–248, 360 μl cytosol (1.6 $U \cdot ml^{-1}$ DTD), 0.1 *M* K^+ phosphate, pH 7.4, under air, total volume, 0.47 ml. Spectra were recorded immediately after completing the reaction mixture.

Semiquinone is an immediate product of hydroquinone oxidation by one-electron transfer to dioxygen. Figure 4A shows the ESR signal of CG semiquinone after CG reduction by the NADPH/DTD system. Hyperfine splittings indicate spin couplings at protons at C7, C8, and C10 of the naphthalene ring, as described previously. No signals were observed when cytosol or CG was omitted (Figs. 4B, 4C).

Effect of CG 10–248 Addition on Oxygen Uptake by Liver Cytosol Fraction

Principle. o-Naphthoquinones redox cycling in the presence of NAD(P)H and DTD consumed oxygen, a reaction used for monitoring the redox-cycle operation. Oxygen uptake results from hydroquinone oxidation according to Eq. 8.

Procedure. Oxygen uptake rate was polarographically measured with a Model 5/6 Oxygraph (Gilson Medical Electronics, Madison, WI), fitted with a Clark-type oxygen electrode.[33] The reaction mixture is placed in the electrode chamber, and the solution is stirred by a Teflon-coated magnetic bar driven from a stirrer located under the electrode. The chamber should be maintained at 30° by circulating water. *o*-Naphthoquinone solution was injected through the small opening in the top of the chamber, using a Hamilton-type syringe. Continuous measurements were taken on a chart recorder.

[33] I. Stadler, *in* "Free Radical and Antioxidant Protocols" (D. Armstrong, ed.), p. 3. Humana Press, Totowa, New Jersey, 1988.

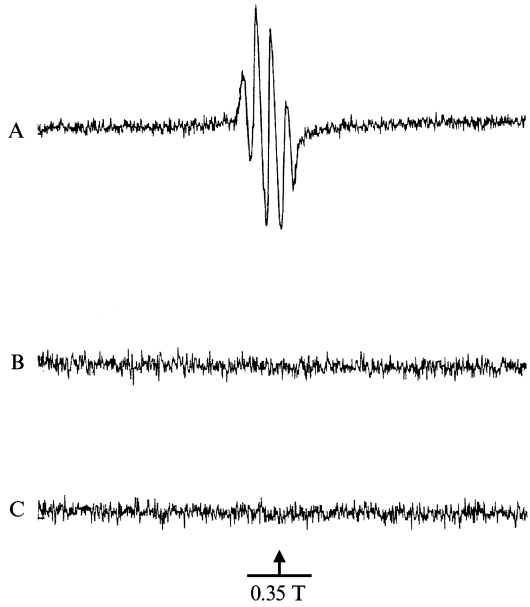

FIG. 4. ESR spectrum of CG 10–248 semiquinone after quinone reduction by the NADPH/DTD system. The reaction mixture contained 21 m*M* NADPH, 8.4 m*M* CG (A) or 1% DMFA (B), 360 μl cytosol (1.6 U $\cdot$ ml^{-1} DTD), 0.1 *M* K^+ phosphate buffer, pH 7.4. (C) Same condition as in (A) except for cytosol omission. Instrumental conditions: modulation amplitude, 0.946 G; gain 1.0×10^5. Other experimental conditions were as described in "Materials and Methods" and in the text.

Comment

The Teflon electrode membrane should be replaced daily.

The effect of CG 10–248 (CG) on oxygen uptake by cytosol fraction is shown in Fig. 5. In experiment A, the reaction mixture contained 300 μM NADPH, 100 μM CG 10–248 and cytosol (153 mU $\cdot$ ml^{-1} DTD), 0.1 *M* K^+ phosphate buffer, pH 7.4, total volume 1.8 ml. In experiment B, the same experimental conditions were maintained, except that 25 μM dicoumarol (DC) (a DTD inhibitor) was added as indicated in Fig. 5.

Figure 5A shows that CG addition to the NADPH/DTD system increased several fold the rate of oxygen consumption, compared with the one depending on endogenous substrate oxidation (150/6.7 $\mu M \cdot$ min^{-1}). Oxygen uptake ceased when approximately 50% of the reaction mixture

oxygen had been consumed. A second addition of NADPH re-established the oxidation rate although at a lower value (89 $\mu M \cdot \text{min}^{-1}$) than during the first NADPH oxidation. Finally, the reaction stopped when all the reaction mixture oxygen was consumed. The addition of dicoumarol (Fig. 5B) produced significant inhibition of oxygen uptake, in the same system, after the first and the second NADPH addition compared with experiment A. The dicoumarol effect was not immediate as shown by the kinetics of the oxidation inhibition.

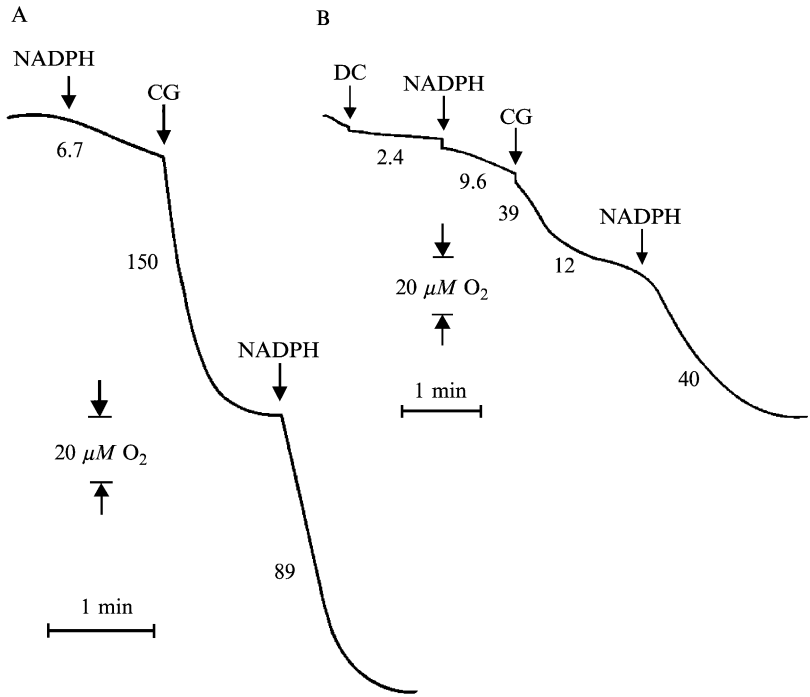

FIG. 5. Oxygen uptake by the NADPH/*o*-naphthoquinone/DTD system. (A) The reaction mixture contained 300 μM NADPH, 100 μM CG 10–248, cytosol (153 $\text{mU} \cdot \text{ml}^{-1}$ DTD), 0.1 M K^+ phosphate buffer, pH 7.4, total volume 1.8 ml. (B) Same conditions as in (A), except that 25 μM dicoumarol (DC) was added. Oxygen uptake was measured polarographically. The number near each tracing indicates oxygen uptake rate ($\mu M \cdot \text{min}^{-1}$).

Effect of o*-Naphthoquinones Addition on Superoxide Anion Formation by Cytosol Fraction*

Principle. In liver cytosol fraction, superoxide anion production was measured by the acetyl–cytochrome *c* reduction according to Eq. 13.[34]

$$O_2^- + \text{Ac cyt } c(3+) \rightarrow O_2 + \text{Ac cyt } c(2+) \tag{13}$$

Procedure. The reaction mixture contained quinone, 30 μM acetyl-cytochrome *c*, 0.1 mM K^+ phosphate buffer, pH 7.4, and cytosol (19 mU $\cdot$ ml^{-1} DTD), total volume 3.0 ml. The reaction was started by adding 300 μM NADPH, prepared immediately before use, and the rate of reaction in the linear phase was calculated. Reduced acetyl-cytochrome *c* produced during the reaction was monitored spectrophotometrically at 550–540 nm at a constant temperature (30°) in an Aminco Chance $DW2_a$ spectrophotometer (American Instrument Company, Silver Spring, MD) ($\varepsilon = 19.1$ mM^{-1} cm^{-1}). The addition of SOD (150 U $\cdot$ ml^{-1}) confirmed that absorbance increase was due to O_2^-.

β-Lapachone and related quinones may be included in the group of quinones generating redox labile hydroquinones that autoxidize to produce O_2^-.

Table III shows the effect of several naphthoquinones on O_2^- production by the NADPH/*o*-naphthoquinone/DTD system. At 100 μM concentration, *o*-naphthoquinones were more effective than α-lapachone (*p*-naphthoquinone). Assayed *o*-naphthoquinones, including β-lapachone, showed similar activities, despite structural differences. β-Lapachone was somewhat more effective than the other *o*-naphthoquinones, but differences were not significant. With the 10 μM quinone concentration, O_2^- production was still effective. Dicoumarol inhibited 86–94% O_2^- production.

Figure 6 shows the effect of increasing concentrations of CG 10–248 on O_2^- production by the NADPH/*o*-naphthoquinone/DTD system. In the 0–10 μM range, O_2^- production increased almost linearly as a function of CG concentration, but in the 10–50 μM range, O_2^- production failed to vary to a significant degree, apparently as a result of substrate saturation of DTD active site. SOD inhibition confirmed the O_2^- production by the quinone redox cycling.

[34] A. Azzi, C. Montecucco, and C. Richter, *Biochem. Biophys. Res. Commun.* **65,** 597 (1975).

TABLE III
SUPEROXIDE PRODUCTION BY THE NADPH/NAPHTHOQUINONE/DTD SYSTEM: EFFECT OF NAPHTHOQUINONE STRUCTURE AND DICOUMAROL

	Superoxide anion production ($\mu M \cdot min^{-1}$)	
	Quinone: 100 μM	Quinone: 10 μM
CG 10–248	39.89 ± 0.36 (92)	26.05 ± 0.78 (87)
CG 9–442	41.05 ± 0.20 (88)	25.26 ± 0.0 (86)
CG 8–935	38.68 ± 0.78 (87)	28.00 ± 0.3 (92)
β-Lapachone	42.00 ± 0.36 (94)	25.52 ± 0.78 (91)
α-Lapachone	5.84 ± 0.78	ND
None	1.10 ± 0	1.10 ± 0

The reaction mixture contained 300 μM NADPH, 19 $mU \cdot ml^{-1}$ DTD, 30 μM acetyl-cytochrome *c*, 0.1 *M* K^+ phosphate, pH 7.4, naphthoquinone as stated previously. Values in parentheses indicate inhibition of O_2^- production by 15 μM dicoumarol. Values represent means ± SD (n = 3). ND: not done.

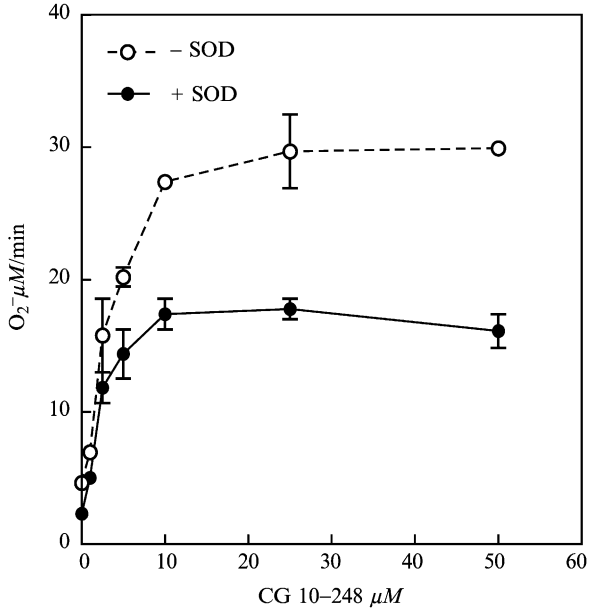

FIG. 6. Effect of CG 10–248 (CG) concentration and SOD on O_2^- production by the NADPH/*o*-naphthoquinone/DTD system. The reaction mixture contained 300 μM NADPH, cytosol (19 $mU \cdot ml^{-1}$ DTD), 30 μM acetyl-cytochrome *c*, 0.1 *M* K^+ phosphate buffer, pH 7.4, and CG as indicated on the abscissa, total volume, 3.0 ml. – SOD: sample without SOD; + SOD: sample containing 150 $U \cdot ml^{-1}$ SOD. Values are means ± SD. ($n = 3$) and represent O_2^- production μM min^{-1}.

CG 10–248 Redox Cycling by Cytosol Fraction

Reduction of naphthoquinones can also be monitored by absorption measurements at characteristic wavelengths.

Procedure. The reaction mixture contained 0.1 *M* K^+ phosphate buffer, cytosol (95 mU ml^{-1} DTD), and 100 μM CG 10–248 (CG); final volume 3.0 ml. The effect of NADPH on CG redox cycling was monitored by absorbance variation at 448 nm, a wavelength at which the hydroquinone does not interfere.

Figure 7A illustrates typical results obtained with CG 10–248 and rat liver cytosol. The initial rate of CG reduction by the NADPH/DTD system, which generates CG hydroquinone, was relatively fast (180 μM min^{-1}) but decreased to such an extent that CG reduction ceased when the level of CG concentration was about 35% of its initial value. Such minimum value was observed for a few seconds, and then absorbance increased at a rate of 99 $\mu M \cdot min^{-1}$, with semiquinone and/or original quinone generation, at approximately half the rate of CG reduction. Finally, the

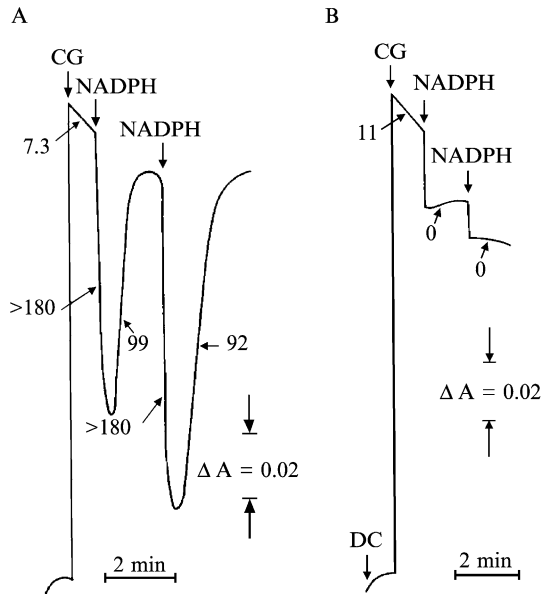

FIG. 7. CG 10–248 redox cycling in the presence of the NADPH/DTD/oxygen system. Cytosol was incubated with 100 μM CG 10–248 (CG) in the absence (A) or presence (B) of 15 μM dicoumarol (DC). Arrows indicate NADPH addition. The number near each tracing indicates the rate of quinone reduction or quinol oxidation.

CG redox level stabilized at approximately 80% of the initial value. It should be noted that the spectroscopic method failed to distinguish semiquinone from quinone, but, nevertheless, for kinetic reasons the quinone was assumed to be the main contributor to absorbance.

Figure 7B shows the effect of dicoumarol on CG 10–248 redox cycling in cytosol fraction. Experimental conditions were as indicated in Fig. 7A, except for dicoumarol addition as indicated in Fig. 7B. No redox cycling was observed under these experimental conditions.

Conclusions

The lipophilic *o*-naphthoquinones β-lapachone and structural analogues quinones (CG quinones) are proposed as cytostatic, trypanocidal, and antiviral agents. With rat liver microsomal NAD(P)H cytochrome P450 reductase or cytosol flavoenzyme DTD, these quinones constitute redox systems, which in the presence of oxygen generate ROS. *o*-Naphthoquinones redox cycling, catalyzed by the NADPH cytochrome P450 reductase, generate in microsomal liver preparations: (1) semiquinone free radicals, (2) ROS, and (3) inhibition of cytochrome P450-dependent reactions, exerting cytotoxic effects. Hydroquinones are the immediate products of quinones reduction by the DTD-dependent systems (Eq. 7). Three types of hydroquinones formed by that reaction have been proposed by Cadenas[21]: (1) redox-stable hydroquinones; (2) redox-labile hydroquinones that subsequently reoxidize, with formation of semiquinone and ROS; and (3) redox-labile semiquinones that immediately rearrange to potent electrophils undergoing biological alkylating reactions.[21] Our observations with β-lapachone and related *o*-naphthoquinones indicate that the corresponding hydroquinones must be included in the second group in agreement with (1) the semiquinone spectrum, demonstrated by ESR spectroscopy; (2) semiquinone (or quinone) production, demonstrated by optical spectroscopy; and (3) the effect of dicoumarol on the quinone redox cycling and oxygen consumption by the NADPH/*o*-naphthoquinone/DTD system. These reactions associated with DTD activity seem to rule out the contention proposing DTD as an antioxidant enzyme protecting against quinone toxicity.

Acknowledgments

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