Biochemical Pharmacology, Vol. 52, pp. 1875–1882, 1996.

ISSN 0006-2952/96/$17.00 + 0.00
PII S0006-2952(96)00601-6

Redox Cycling of *o*-Naphthoquinones in Trypanosomatids

SUPEROXIDE AND HYDROGEN PEROXIDE PRODUCTION

*Maria P. Molina Portela,**
Silvia H. Fernandez Villamil,† Luis J. Perissinotti and Andres O. M. Stoppani‡§
BIOENERGETICS RESEARCH CENTRE, SCHOOL OF MEDICINE, UNIVERSITY OF BUENOS AIRES, BUENOS AIRES; AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MAR DEL PLATA, MAR DEL PLATA, ARGENTINA

ABSTRACT. β-Lapachone and structurally related lipophilic *o*-naphthoquinones, namely, CG 8-935, CG 9-442, CG 10-248 and mansonones A, C, E, and F, were investigated for redox cycling, production of reactive oxygen species, and cytotoxicity in the trypanosomatids *Crithidia fasciculata* and *Leptomonas seymouri*. Structural analysis of the assayed quinones indicated that a tricyclic structure, including a naphthalene ring, a 1,2*b* or 1,8*bc* pyran ring, and two *ortho*-carbonyl groups were required for quinone activities. The contribution of oxygen radical production to quinone cytotoxicity was supported by: (a) spectroscopic observation of quinone redox cycling; (b) production of the semiquinone radical; (c) H_2O_2 and O_2^- production; (d) the effect of β-lapachone on thiol pools in *C. fasciculata*; (e) the effect of quinones on cell respiration; (f) superoxide dismutase inactivation after incubation of *C. fasciculata* with CG 8-935; and (g) the effect of quinones on cell growth. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1875–1882, 1996.

KEY WORDS. *o*-naphthoquinones; lapachones; mansonones; superoxide; hydrogen peroxide; oxidative damage; *Crithidia fasciculata*; *Leptomonas seymouri*.

Naphthoquinones are biologically important compounds, especially because of their pharmacological and cytotoxic action. The lipophilic *o*-naphthoquinone β-lapachone‖ and related quinones (a) inhibit tumor cell growth [1–7]; (b) suppress HIV-1 replication in both acute and chronic infection [8]; (c) prevent oncogenic transformation [5]; (d) inhibit growth, DNA, RNA, and protein synthesis in *Trypanosoma cruzi*, the agent of Chagas' disease [9]; (e) produce strand breaks in the parasite DNA [9]; (f) induce production of reactive oxygen species in *T. cruzi* and related trypanosomatids [10–16]; (g) induce chromosomal alterations [17]; (h) modify topoisomerase I activity [18]; (i) have potential clinical utility against human leukemia and prostate cancer [7]; and (j) inhibit microsomal electron transfer and lipid peroxidation [19].

To (a) establish structure–activity relationships, and (b) ascertain the role of oxygen radicals for *o*-naphthoquinone toxicity in trypanosomatids, we have compared in the present study the effects of a series of β-lapachone analogues, namely, CG 8-935, CG 9-442, CG 10-248, and mansonones A, C, E, and F, using as assay organisms the trypanosomatids *Crithidia fasciculata* and *Leptomonas seymouri*. These organisms are useful experimental systems for studying the factors involved in determining drug susceptibility [20–23]. Moreover, *C. fasciculata* and *L. seymouri* have the advantage of their relatively rapid growth and their lack of pathogenicity for humans. Catalase content was relatively high in *C. fasciculata*, a feature that allowed Gutteridge *et al.* [24] to examine this enzyme function in trypanosomatids. Figure 1 shows the structures of the quinones investigated, which included: (a) quinones with two carbonyls in C1 and C2, one pyranic oxygen in C4, and one 1,2*b* pyran ring (β-lapachone and CG quinones) [4]; (b) quinones with two carbonyls in C1 and C2, one pyranic oxygen in C4, and one 1,8*bc* pyran ring (mansonones E and F) [25]; (c) one quinone with two carbonyls in C1 and C2 (mansonone C) [25]; and (d) an *o*-tetrahydronaphthoquinone

* Research Fellow, National Research Council and National Academy of Sciences, Argentina.
† Research Assistant, National Research Council, Argentina.
‡ Emeritus Investigator, National Research Council, Argentina.
§ Corresponding author: Professor A. O. M. Stoppani, Centro de Investigaciones Bioenergéticas, Facultad de Medicina (UBA), Paraguay 2155, (1121) Buenos Aires, Argentina. TelFax (054 1) 961-6521.

‖ *Abbreviations:* GSH, reduced glutathione; SOD, superoxide dismutase; 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; mansonone A, 5,6,7,8-tetrahydro-3,8-dimethyl-5-isopropyl-1,2-naphthoquinone; mansonone C, 3,8-dimethyl-5-isopropyl-1,2-naphthoquinone; mansonone E, 2,3-dihydro-3,6,9-trimethyl-naphtho[1,8-*bc*]pyran-7,8-dione; mansonone F, 3,6,9-trimethyl-naphtho[1,8-*bc*]pyran-7,8-dione; DMFA, dimethylformamide; MP-11, microperoxidase, the heme-portion of the cytochrome *c* molecule, with amino acids 11–21 still attached; and MOPS, 3-[*N*-morpholino]propanesulfonic acid.

Received 6 May 1996; accepted 28 June 1996.

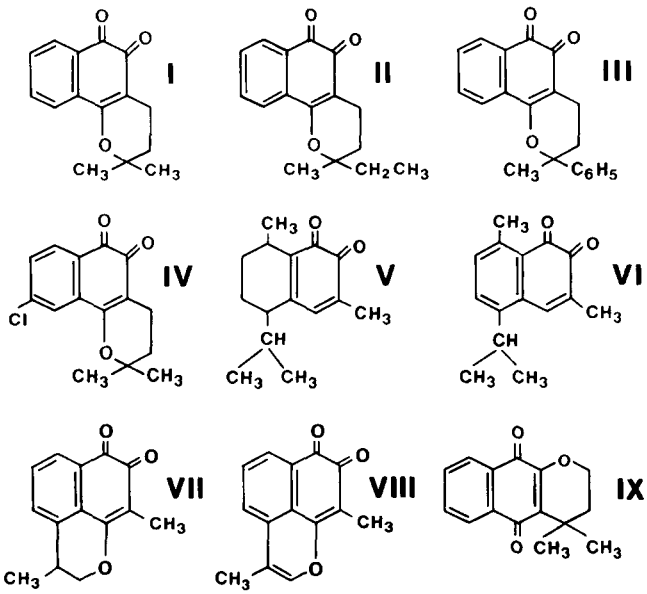

FIG. 1. Structures of lipophilic *o*-naphthoquinones. (I) β-lapachone; (II) CG 8-935; (III) CG 9-442; (IV) CG 10-248; (V) mansonone A; (VI) mansonone C; (VII) mansonone E; (VIII) mansonone F; and (IX) α-lapachone.

with two carbonyls in C1 and C2 (mansonone A) [25]. For comparative purposes, α-lapachone, a *p*-naphthoquinone with two carbonyls in C1 and C4 and one pyranic oxygen in C2, was assayed.

MATERIALS AND METHODS

Culture and Treatment of Trypanosomatids

C. fasciculata ATCC 11745 and the *L. seymouri* strain supplied by Dr. S. H. Hutner were used in most of the experiments reported here. *C. fasciculata* ATCC 12858 and *L. seymouri* ATCC 30220 were purchased from the American Type Culture Collection, Rockville, MD, U.S.A. *C. fasciculata* and *L. Seymouri* were grown in a culture medium containing (in g/L): NaCl, 5.0; KCl, 2.0; NaH_2PO_4, 0.5; sucrose, 2.5; sodium-ascorbate, 0.2; MOPS, 0.5; desiccated liver powder, 3.0; pluripeptone, 7.5; Brain–Heart infusion, 7.5; hemin [dissolved in 1:1 (v/v) triethanolamine:H_2O], 0.02. Cultures were incubated at 28° for 48 hr (*C. fasciculata*), or at 26° for 72 hr (*L. seymouri*) in a model G-25 New Brunswick Gyratory Shaker (New Brunswick Scientific Co., Inc., Edison, NJ, U.S.A.), at 100 rpm. Cell growth was measured by the increase of culture turbidity, at fixed incubation times. The cell concentration/turbidity ratio was determined using cell suspensions, whose concentration was predetermined by cell counting in a Neubauer chamber.

To assay quinone inhibition of trypanosomatid growth, quinones were dissolved in ethanol or DMFA, and aliquots of these solutions were added to duplicate 10-mL samples of sterile culture medium, before the inoculum. To the control samples, the corresponding volume of solvent was added. Incubations were performed in the New Brunswick Gyratory Shaker as indicated above. Three or more quinone concentrations were assayed in each experiment and from the corresponding inhibition/concentration plots, the quinone concentration producing 50% inhibition of growth (IC_{50}) was calculated.

Cell Disruption and Homogenization

Cultured cells were collected by centrifugation at 750 *g* and 4°, for 10 min. The pellet was washed twice with 10–30 mL of 154 mM NaCl, and after centrifugation the cells were subjected to four cycles of freeze–thawing, using liquid nitrogen. The homogenate was centrifuged at 9800 *g* for 30 min, and the supernatant ("cell extract") was collected and used for enzyme measurements.

Analytical Procedures

Superoxide anion generation was measured by the adrenochrome method [26, 27]. The reaction mixture contained 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, 1.0 mM epinephrine, quinone, and cells as indicated under Results; total volume, 3.0 mL. Adrenochrome production was measured spectrophotometrically at 485–575 nm (ϵ = 2.95 $mM^{-1} \cdot cm^{-1}$). Addition of 6 U/mL SOD verified that the absorbance increase was due to O_2^-. H_2O_2 generation was measured by the MP-11 method [28]. The reaction mixture contained 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, 3.3 μM MP-11, quinone and cells as indicated under Results; total volume, 3.0 mL. MP-11 absorbance was measured spectrophotometrically, at 419–407 nm (ϵ = 78 $mM^{-1} \cdot cm^{-1}$). Addition of 60 U/mL catalase confirmed that the absorbance variation was due to H_2O_2.

Thiol compounds were determined using the *o*-phthalaldehyde reaction [29]. Briefly, cell samples were collected by centrifugation and subjected to four freeze–thawing cycles, using liquid nitrogen. The homogenate was diluted with 0.1 M Na_2HPO_4, pH 8.0, 5.0 mM EDTA (phosphate–EDTA buffer). One milliliter of 25% (w/v) HPO_3 was added to 3.7 mL of cell extract, and the precipitated protein was discarded by centrifugation. The supernatant was diluted 10-fold with the phosphate–EDTA buffer, and the diluted samples were used for analysis. The assay mixture contained 0.1 mL of cell extract or GSH standard solution, 0.1 mL of *o*-phthalaldehyde solution (1 mg/mL methanol) and 1.8 mL of phosphate–EDTA buffer. After a 15-min incubation at 20–25° in the dark, fluorescence was measured at 350 nm (excitation) and 420 nm (emission) in an Aminco–Bauman (Urbana, IL, U.S.A.) spectrofluorometer.

Oxygen uptake was measured at 30° with a Gilson (Middleton, WI, U.S.A.) Oxygraph, model 5/6, using a Clark electrode. Spectrophotometric measurements were performed with an Aminco DW-2™ UV/VIS, or an Aminco Chance spectrophotometer.

HPLC analysis of quinones was performed using a Spherisorb, 5 μm, 250 × 4 mm reverse-phase C18 column (from Pharmacia-LKB, Piscataway, NJ, U.S.A.) attached to a

Pharmacia-LKB system constituted by UV Detector VWM 214, HPLC Pump 2248, and LKB 2221 Integrator. The mobile phase was methanol–H_2O (30:70, v/v), and the flow rate was 0.8 mL/min. Quinones were dissolved in the mobile phase (1 μg/mL), and the injection volume was 10 μL. The UV detector was set at wavelengths selected from the quinone ultraviolet spectra.

Enzyme Assays

Catalase activity was determined at 22–25° by the rate of H_2O_2 decomposition, measured spectrophotometrically at 240 nm (ϵ = 40 $mM^{-1} \cdot cm^{-1}$) [30]. The reaction mixtures contained cell extract, 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, and 154 mM NaCl. The reaction was started by adding 10 mM H_2O_2. Activities were expressed in *k* U (sec^{-1}/mL · (mg protein)$^{-1}$). SOD activity was determined at 30° by the inhibition of adrenochrome production [31], measured spectrophotometrically at 480 nm. The reaction mixture contained 1.0 mM epinephrine, 50 mM glycine-NaOH, pH 10.2, and 1.0 mM EDTA. The reaction was started by adding the cell extract. SOD activity is expressed in units, one unit being the amount of enzyme that inhibited by 50% the rate of epinephrine oxidation. Protein concentration was determined by the biuret method [32].

ESR

Measurements were performed with a Bruker (Bruker Analytische Messtechnik GMBH, Rheinstellen, Karlsruhe, Germany) ER 200 tt x-band ESR spectrometer equipped with a TE_{102} cavity, at room temperature (28°). The general instrumental conditions were: microwave power, 21 mW; modulation frequency, 100 KHz; microwave frequency, 9.72 GHz; time constant, 0.5 sec; scan rate, 0.24 mT/min; gain, 1.0×10^6. In all measurements the field was centered at 0.349 T. With *C. fasciculata* and CG 10-248, the assay mixture contained *Crithidia* (12.4 mg protein/mL) in 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, and 154 mM NaCl (gassed with nitrogen for 5 min in the spectrometer cell). The quinone pregassed with nitrogen was added (final quinone concentration, 2 mM), and signals were recorded with modulation amplitude of 0.04 mTpp. Controls without *Crithidia* or quinone were examined negatively for ESR.

Chemicals

β-Lapachone and α-lapachone were supplied by the Program for the Synthesis of Antiparasitic Drugs, Universidad Federal de Rio de Janeiro, Brazil; CG quinones were supplied by CIBA-GEIGY, Basel, Switzerland; and mansonones A, C, E, and F were isolated and supplied by Dr. C. Galeffi, Istituto Superiore di Sanitá, Rome, Italy. Quinols were prepared as described [33]. Other reagents were purchased from the following suppliers: antimycin A, D-glucose, sucrose, $NaBH_4$, MP-11 (prepared by enzymatic degradation of equine heart cytochrome *c*), SOD (from bovine erythrocytes), glucose oxidase, catalase, EDTA, MOPS, bovine serum albumin, sodium ascorbate, sodium dithionite, hemin from the Sigma Chemical Co., St. Louis, MO, U.S.A.; DMFA from the T. Baker Chemical Co., Phillisburg, NJ, U.S.A.; argon from Matheson, East Rutherford, NJ, U.S.A.; Brain–Heart infusion and Bacto^R Desiccated Powdered Beef Liver, from Difco Laboratories, Detroit, MI, U.S.A.; Pluripeptone from Laboratorio Britania, Buenos Aires, Argentina.

o-Naphthoquinones are unstable [7] and degrade over time. Accordingly, the available compounds were examined for purity by HPLC. β-Lapachone, α-lapachone, CG quinones, and mansonone F were 100% pure; mansonones A, C, and E were 98.2, 98.4, and 97.3% pure, respectively.

Expression of Results

Unless otherwise indicated, means ± SEM are given. When values were the average of duplicate measurements, the experimental values deviated from the mean by < 5%. Statistical analysis was performed using ANOVA or Student's *t*-test. Assays were systematically repeated in *C. Fasciculata* and *L. seymouri*, but only typical experiments are illustrated.

RESULTS

Quinone Redox Cycling

Quinone redox cycling in *C. fasciculata* and *L. seymouri* was demonstrated by absorption measurements at specific wavelengths. Figure 2 illustrates typical results obtained with CG 8-935, CG 9-442, and *C. fasciculata* at 450 nm. Experiments A and C show that (a) addition of quinone to the anaerobic cell suspension caused an initial increase of absorption, due to the quinone absorbance; (b) immediately thereafter, a rapid decrease followed, due to quinone reduction by cellular electron donors; (c) a relatively stable redox state was then reached, in which about 90% of the quinone was reduced; (d) oxygenation of the anaerobic cell suspension produced a rapid increase in absorption, especially with CG 8-935, indicating quinol oxidation; (e) a new redox state was then reached, in which about 23% of CG 8-935 or 47% or CG 9-442 remained reduced; (f) finally, sodium dithionite re-reduced the quinone to quinol. Omission of the anaerobic step (experiments B and D) resulted in a simpler reaction sequence in which, after several minutes of incubation, 93% of CG 8-935 or 78% of CG 9-442 remained in the oxidized state. Similar results were obtained with mansonones E and F (tracings not shown). Different kinetics were observed with mansonones A and C, as illustrated by a typical experiment with *L. seymouri* (Fig. 3). The kinetics of the anaerobic reduction of these mansonones resembled that of the CG quinones (Fig. 2) but, after oxygenation, the rate of quinol oxidation was negligible (with mansonone A) or very slow (with mansonone C). Moreover, reduction also occurred aerobically, in con-

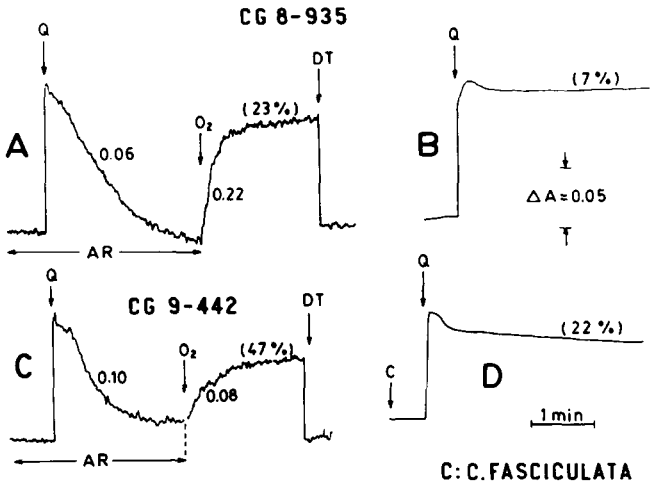

FIG. 2. CG 8-935 and CG 9-442 redox cycling in *C. fasciculata*. The reaction mixture contained *Crithidia* (0.96 mg/mL), 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, 50 mM glucose, glucose oxidase (1.0 μg/mL) and catalase (283 U/mL); total volume, 3.0 mL. Experiments A and C: The reaction mixture and the quinone solution were pre-gassed with argon (AR) and continuously stirred with a magnetic bar at 30°. Q indicates the addition of 50 μM quinone; O_2 indicates gassing with pure oxygen, and DT indicates addition of 1.2 mM sodium dithionite. The numbers near the tracings indicate rate of quinone reduction or quinol oxidation, in nmol/min/mg cell protein; values in parentheses indicate total quinol production, as a percentage of the initial quinone concentration. Experiments B and D: conditions were as in A and C, except for the gassings with argon and oxygen, which were omitted. Other experimental conditions were as described under Materials and Methods.

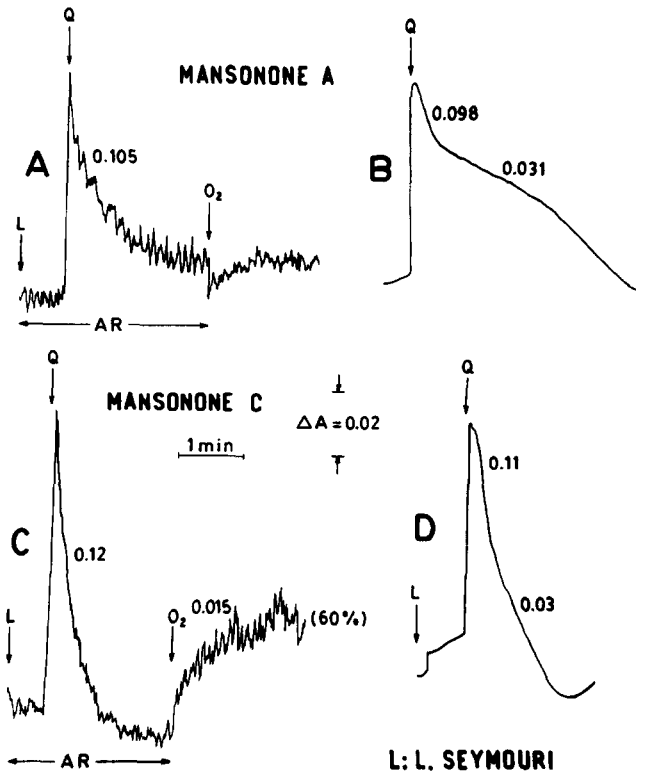

FIG. 3. Redox reactions of mansonones A and C in *L seymouri*. Experimental conditions were as in the legend of Fig. 2, except for the quinones. Quinone concentration, 50 μM (Q).

trast to the results obtained with the CG quinones (Fig. 2), experiments B and D).

Quinone reduction and quinol oxidation rates were calculated from the experiments described in Fig. 2 and 3, and similar ones were performed with other *o*-naphthoquinones. Table 1 shows that (a) with mansonones A and C, the rate of quinone reduction was greater than that of quinol oxidation; (b) with CG 8-935 and mansonones E and F, the rate of quinol oxidation was greater than the rate of quinone reduction; (c) with CG 9-442, the rates of quinone reduction and quinol oxidation were similar. The characteristic redox cycling of CG quinones and mansonones was confirmed by reduction with sodium dithionite, under air (spectra not shown).

Effect of Quinones on Cell Respiration

Table 2 shows the effect of quinones on C. *fasciculata* respiration. Quinones enhanced the rate of oxygen consumption, β-lapachone, CG quinones and mansonones E and F being the most effective. On the other hand, mansonone A and α-lapachone did not increase oxygen consumption above the control level, at least not to a significant degree. Quinone effects became more evident after inhibiting endogenous respiration with antimycin A, which is known to act upon C. *fasciculata* mitochondrial electron transfer [34].

Effect of Quinones on Thiol Pools

C. *fasciculata* contains soluble thiol compounds, namely, GSH, glutathionyl-spermidine, dihydrotrypanothione [35], and ovothiol A [36]. Total soluble thiols, therefore, were considered as interesting markers of oxidative damage and, accordingly, worth measuring. The corresponding values

TABLE 1. Rate of quinone reduction and quinol oxidation in *C. fasciculata* and *L. seymouri*

	Reaction rate (nmol/min · (mg protein)$^{-1}$)			
	C. fasciculata		*L. seymouri*	
Quinone (50 μM)	Quinone reduction	Quinol oxidation	Quinone reduction	Quinol oxidation
CG 9-442	93	80	100	110
CG 8-935	82	225	65	230
Mansonone A	151	10	96	16
Mansonone C	115	13	107	7
Mansonone E	43	1165	31	1950
Mansonone F	60	244	60	1950

Experimental conditions were as described in Fig. 2. Values are the averages of duplicate measurements.

TABLE 2. Effect of quinones on *C. fasciculata* respiration

Quinone (10 μM)	Oxygen consumption (nmol O_2/min · (mg protein)$^{-1}$) *C. fasciculata* Antimycin omitted	Antimycin added
None	17.1 ± 2.6	4.3 ± 0.5
β-Lapachone	36.1 ± 2.1*	22.5 ± 1.6†
CG 8-935	35.7 ± 2.2*	22.8 ± 2.2†
CG 9-442	33.7 ± 3.0*	15.3 ± 0.9†
CG 10-248	36.4 ± 2.3*	19.9 ± 2.2†
Mansonone E	33.1 ± 2.0*	19.9 ± 1.0†
Mansonone F	32.1 ± 3.8*	21.4 ± 1.4†
Mansonone A	18.4 ± 1.9	4.0 ± 0.2
α-Lapachone	18.1 ± 1.6	4.0 ± 0.2

The reaction mixture contained cells (0.5 to 0.7 mg protein/mL), 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, quinone, and antimycin (1 μg/mL) as indicated above. Other experimental conditions were as described under Materials and Methods. Values are means ± SEM (N = 4).

* $P < 0.003$.

† $P < 0.001$.

are presented as "XSH" in Table 3. The results obtained with β-lapachone and α-lapachone in C. *fasciculata* show that, after a 1-hr incubation, β-lapachone produced a significant decrease of XSH, whereas α-lapachone did not. Interestingly enough, glucose prevented quinone effects on XSH pools (Table 3). CG 9-442 and CG 10-248 behaved like β-lapachone (data not shown). Investigation of GSH–quinone adducts by HPLC yielded negative results (experimental data not shown).

Effect of Quinones on H_2O_2 and O_2^- Production

Quinone addition to C. *fasciculata* and *L. seymouri* enhanced H_2O_2 and O_2^- production by these organisms. The effect of quinone was concentration dependent. Figure 4 shows the effect of CG 10-248 concentration on H_2O_2 and O_2^- production. Table 4 summarizes the effect of quinones on H_2O_2 and O_2^- production by *L. seymouri* and C. *fasciculata*. The results presented show that (a) β-lapachone, CG quinones, and mansonones E and F enhanced H_2O_2 and O_2^- production, without significant differences between quinones; (b) the rate of O_2^- production was greater than that of H_2O_2, in good agreement with the stoichiom-

TABLE 3. Effects of β-lapachone and α-lapachone on XSH in *C. fasciculata*

Quinone (10 μM)	Glucose (mM)	XSH (nmol/10^8 cells)
None	0	22.2 ± 0.6
β-Lapachone	0	13.4 ± 0.6*
α-Lapachone	0	24.0 ± 0.9
None	10	25.9 ± 0.2
β-Lapachone	10	20.8 ± 1.1
α-Lapachone	10	25.1 ± 0.3

The reaction mixture contained *Crithidia* (0.4 × 10^8 cells), 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, and additions as indicated above; total volume, 2.0 mL. Incubation was for 1 hr. Other conditions were as described under Materials and Methods. Values are means ± SEM (N = 3).

* $P < 0.05$.

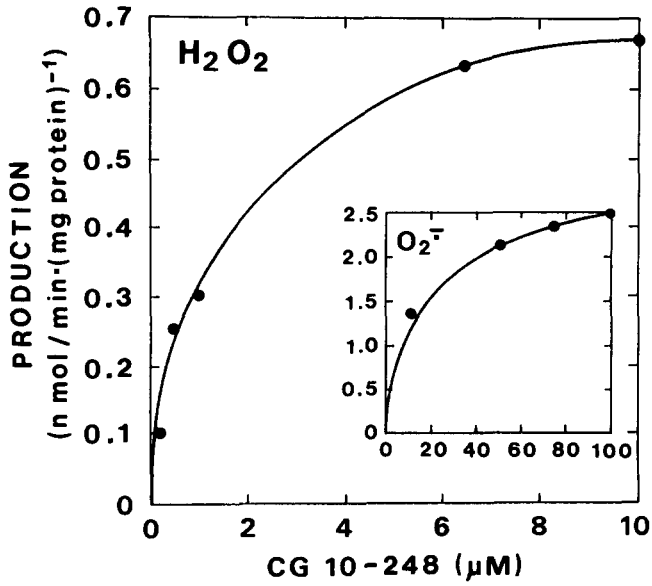

FIG. 4. Effect of CG 10-248 concentration on H_2O_2 and O_2^- production by *L. seymouri*. The reaction mixture contained *Leptomonas* (0.13 mg protein/mL), 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, and 3.3 μM MP-11 (H_2O_2 measurement) or 1.0 mM epinephrine (O_2^- measurement). Quinone concentration was as indicated on the abscissa. Other experimental conditions were as described under Materials and Methods. Values are the means of four or more experiments performed in duplicate.

TABLE 4. Effect of quinones on O_2^- and H_2O_2 production by *C. fasciculata* and *L. seymouri*

Quinone (10 μM)	O_2^- or H_2O_2 production (nmol/min · (mg protein)$^{-1}$) O_2^-	H_2O_2
	Organism: C. *fasciculata*	
None	0.03 ± 0.01	0.05 ± 0.01
CG 8-935	1.72 ± 0.51*	1.04 ± 0.04*
β-Lapachone	1.22 ± 0.10*	0.43 ± 0.04*
Mansonone E	2.20 ± 0.20*	0.48 ± 0.04*
Mansonone F	1.84 ± 0.10*	0.30 ± 0.14*
	Organism: *L. seymouri*	
None	0.03 ± 0.01	0.06 ± 0.01
CG 8-935	1.88 ± 0.82*	0.35 ± 0.08*
CG 10-248	1.08 ± 0.03*	0.65 ± 0.02*
Mansonone E	1.80 ± 0.05*	0.43 ± 0.2*
Mansonone F	2.03 ± 0.43*	0.23 ± 0.05*

The reaction mixture contained cells (0.1 to 0.5 mg protein/mL), 20 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 154 mM NaCl, 1.0 mM epinephrine (O_2^- measurements) or 3.3 μM MP-11 (H_2O_2 measurements), and quinone as indicated above; final volume, 3.0 mL. The reaction was initiated by the addition of quinone. Other conditions were as described under Materials and Methods. Values are means ± SEM ($N \geq 3$).

* $P < 0.05$.

etry of the SOD reaction; (c) no significant differences were observed between H_2O_2 or O_2^- production rates by C. *fasciculata* and L. *seymouri*; (d) mansonone A did not enhance the rate of O_2^- and H_2O_2 production by the assayed organisms (experimental data not shown). Addition of catalase (60 U/mL) prevented the formation of the MP-11–H_2O_2 complex completely.

Production of O_2^- by the quinone-supplemented cell might involve quinol oxidation by oxygen. This hypothesis is supported by O_2^- production by quinol oxidation. Thus, with 25 μM quinol in 20 mM potassium phosphate, pH 7.4, 154 mM NaCl, and 1.0 mM epinephrine, the rate of SOD (3.0 U/mL)-sensitive adrenochrome production was [in μmol/min · (μmol quinol)$^{-1}$]: 21.6 (CG 8-935), 19.0 (mansonone E), 0.01 (mansonone A), and 0.15 (mansonone C) (average of duplicate measurements). From the summarized results it may be concluded that CG 8-935 and mansonone E quinols were oxidized rapidly, producing O_2^-, whereas mansonone A and C quinols were not.

Semiquinone Production

The ESR spectrum of the CG 10-248 semi-quinone radical was observed after anaerobic incubation of the quinone with C. *fasciculata*. A similar spectrum was observed after CG 10-248 reduction with $NaBH_4$ (4.0 mg/mL; *Crithidia* omitted). The hyperfine splittings of 0.11, 0.13, and 0.15 mT indicated spin couplings at the protons at C5, C7, and C8 of the naphthalene ring. The semiquinone spectrum was confirmed by computational analysis using the coupling constants obtained experimentally (spectra not shown).

Effect of Quinones on Antioxidant Enzymes

Both C. *fasciculata* and L. *seymouri* contain catalase, but the enzyme activity in *Crithidia* is 5-fold greater than in *Leptomonas* [the specific activities in C. *fasciculata* and L. *seymouri* (*k* units per mg of protein) were (mean ± SEM): 0.64 ± 0.13 (N = 6) and 0.04 ± 0.01 (N = 4), respectively]. Catalase and SOD may be inhibited by oxygen radicals and H_2O_2 [37–40]. Therefore, these enzymes were measured in C. *fasciculata* subjected to quinone-induced oxidative stress. The results in Table 5 indicate that only SOD activity decreased significantly, despite the relatively long incubation time and the high concentration of quinone. Under similar experimental conditions, trypanothione reductase was not modified (experimental data not shown).

Effect of Quinones on Trypanosomatid Growth

Table 6 summarizes the effect of quinones on the growth of C. *fasciculata* and L. *seymouri*. It is to be observed that both organisms were sensitive to the assayed quinones. β-Lapachone, CG quinones, and mansonones E and F were very effective, showing IC_{50} values in the 0.1 to 0.7 μM range. Mansonones A, C, and α-lapachone were less cytotoxic.

TABLE 5. Effect of CG 8-935 on catalase and SOD in C. *fasciculata*

CG 8-935 (μM)	Enzyme activity	
	SOD (U · (mg protein)$^{-1}$)	Catalase (*k*)*
0	17.0 ± 3.1	0.64 ± 0.13
100	7.5 ± 1.9†	0.51 ± 0.17

C. *fasciculata* (73 × 10^8 cells) were incubated in 20 mM KH_2PO_4-K_2HPO_4, pH 7.4, 154 mM NaCl, containing CG 8-935 as indicated above; total volume, 10 mL. After 3 hr of incubation, cells were collected by centrifugation and homogenized, and enzyme activities in the supernatant were measured. Other experimental conditions were as described in Materials and Methods. Values are means ± SEM (N ≥ 3).

* *k*, sec^{-1}/mL · (mg protein)$^{-1}$.

† $P < 0.05$.

DISCUSSION

The biological activity of quinones (Tables 2, 3 and 6) allows one to establish major differences, with maximum activities for the CG quinones and mansonones E and F, and minimum activities for α-lapachone and mansonone A. These differences fit in well with the pro-oxidant activity of quinones, using dihydrolipoamide as reductant [41], and can be explained in terms of electronic symmetry and polarity [42]. Comparison of quinone activities with the rate of quinone reduction or quinol oxidation (Table 1 and text) indicates a better correlation with the latter parameter.

Taken together, our observations support the hypothesis that, in the assayed trypanosomatids, redox cycling and oxygen radical production contributed in a significant degree to *o*-naphthoquinone cytotoxicity. This conclusion is borne out by: (a) spectroscopic demonstration of quinone redox cycling (Fig. 2 and 3; Table 1); (b) H_2O_2 and O_2^- production (Fig. 4; Table 4); (c) the capability of quinols for O_2^- production (quoted in text); (d) the effect of quinones on trypanosomatid growth (Table 6); (e) semiqui-

TABLE 6. Effects of naphthoquinones on C. *fasciculata* and L. *seymouri* growth

Quinone	IC_{50} (μM)	
	C. *fasciculata*	L. *seymouri*
CG 8-935	0.79 ± 0.02	0.49 ± 0.06
CG 9-442	0.72 ± 0.14	0.64 ± 0.08
CG 10-248	0.76 ± 0.02	0.55 ± 0.08
α-Lapachone	27.0	4.1
Mansonone A	15.0	8.5
Mansonone C	6.0 (11.7)	4.0 (6.5)
Mansonone E	0.6 (0.9)	0.4 (0.4)
Mansonone F	0.3 (1.2)	0.1 (0.1)

C. *fasciculata* strain ATCC 11745, except for values in parentheses which were obtained with strain ATCC 12858. L. *seymouri* strain provided by Dr. S. H. Hutner, except for values in parentheses which were obtained with strain ATCC 30220. Other experimental conditions were as described in Materials and Methods. Values for CG 8-935, CG 9-442, adn CG 10-248 are presented as means ± SEM (N ≥ 3). Other values represent averages of duplicate IC_{50} determinations.

none radical production (quoted in text); (f) the effect of β-lapachone on thiol pools (Table 3); (g) the effect of quinones on cell respiration (Table 2); and (h) SOD inactivation (Table 5). The latter effect fits in well with SOD inactivation by H_2O_2 [37–40]. The variety of effects supports the role of "reactive oxygen species" in quinone cytotoxicity.

Catalase content in C. *fasciculata* was about 5-fold greater than in *L. seymouri* (see Results). Despite these differences, (a) the rates of quinone-dependent H_2O_2 production by these organisms were about the same (Table 4), and (b) quinones were equally cytotoxic to C. *fasciculata* and *L. seymouri* (Table 6). It seemed, therefore, that in the assayed organisms, catalase would not play a protective role against H_2O_2. This hypothesis agrees with the notion that, in trypanosomatids, catalase acts essentially as a heme store [24]. On the other hand, the discrepancy between the stimulated O_2 consumption and $O_2^{\cdot-}/H_2O_2$ production suggests that a large proportion of reactive oxygen species was scavenged by defense mechanisms, in close agreement with effects observed in several catalase- and peroxidase-deficient trypanosomatids [43–45]. In some parasites (*T. cruzi*), a non-enzymatic trypanothione-dependent peroxide metabolism has been detected [46].

Thiol oxidation and depletion by quinones may contribute to quinone cytotoxicity. In fact, intracellular thiol compounds, such as GSH, contribute to the regulation of DNA and protein synthesis and are essential cofactors for many enzymes [47, 48]. Interaction of quinones with a thiol compound would determine, among other effects, quinone reduction and one- or two-electron thiol oxidation [48–50]. The results in Table 3 fit in well with such an effect of quinones. On the other hand, the anti-oxidant effect of glucose in Table 3 may be explained by the coupling of the pentose phosphate cycle and the GSH redox cycle [51, 52].

DNA strand breakage seems to play an important role in quinone-induced cytotoxicity, as previously observed in *T. cruzi* exposed to low concentrations of β-lapachone, not α-lapachone [9]. It should be noted, however, that strand breakage and cell death induced by quinones can occur by mechanisms excluding oxygen radicals [53]. The very low IC_{50} values in Table 6 suggest that, in addition to oxygen radicals, other effects may be involved in quinone cytotoxicity, especially in actively dividing cells. Inactivation of topoisomerase I [18] may be one of these mechanisms. This enzyme inhibition would explain the almost equal activity of quinones on C. *fasciculata* and *L. seymouri* (Table 6).

This work was aided by grants from the University of Buenos Aires, National Academy of Sciences (Buenos Aires), CEDIQUIFA, and the Swedish Agency for Research and Cooperation with Developing Countries (SAREC) through Dr. M. Paulino, Universidad de la Republica, Montevideo, Uruguay; Professor S. H. Hutner, Pace University, New York, supplied C. fasciculata, *ATCC 11745, and the* L. seymouri *strains used in most of the experiments described herein; Dr. C. Galeffi, Istituto Superiore di Sanitá, Rome, prepared the mansonones used; Dr. E. M. de Pahn lent able assistance for the quinone toxicity assays; Dr. A. M. Biscardi performed the HPLC analysis of quinones; and Drs. M. A. Brusa and S. N. Mendiara collaborated in the ESR experiments; M. G. Gutierrez and M. A. E. Veron lent able technical assistance.*

References

1. Santana CF, Lima OG, D'Albuquerque IL, Lacerda AL and Martins DG, Observaçoes sôbre as propriedades anti-tumorais e toxicológicas do extrato do líber e de alguns componentes do cerne do pau d'arco (*Tabebuia avellanedae*). *Rev Inst Antibiot Univ Recife* **8:** 89–94, 1968.
2. D'Albuquerque IL, Maciel MCN, Schuler AR, de Araujo M do C, Medeiros Maciel G, Cavalcanti M da SB, Gimino Martins D and Lins Lacerda A, Preparaçao e primeiras observaçoes sobre as propriedades antibióticas e antineoplásicas das naftoquinonas homólogos inferiores na série da 2-hidróxi-3-(3-metil-2-butenil)-1,4-naftoquinona (lapachol). *Rev Inst Antibiot Univ Recife* **12:** 31–40, 1972.
3. Docampo R, Cruz FS, Boveris A, Muniz RPA and Esquivel DMS, β-Lapachone enhancement of lipid peroxidation and superoxide anion and hydrogen peroxide formation by Sarcoma 180 ascites tumor cells. *Biochem Pharmacol* **28:** 723–728, 1979.
4. Schaffner-Sabba K, Schmidt-Ruppin KH, Wehrli W, Schuerch AR and Wasley JWF, β-Lapachone: Synthesis of derivatives and activities in tumor models. *J Med Chem* **27:** 990–994, 1984.
5. Boothman DA and Pardee AB, Inhibition of radiation-induced neoplastic transformation by β-lapachone. *Proc Natl Acad Sci USA* **86:** 4963–4967, 1989.
6. Li CJ, Wang C and Pardee AB, Induction of apoptosis by β-lapachone in human prostate cancer cells. *Cancer Res* **55:** 3712–3715, 1995.
7. Planchon SM, Wuerzberger S, Frydman B, Witiak DT, Hutson P, Church DR, Wilding G and Boothman DA, β-Lapachone-mediated apoptosis in human promyelocytic leukemia (HL-60) and human prostate cancer cells: A p53-independent response. *Cancer Res* **55:** 3706–3711, 1995.
8. Li CJ, Zhang LJ, Dezube BJ, Crumpacker CS and Pardee AB, Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication. *Proc Natl Acad Sci USA* **90:** 1839–1842, 1993.
9. Goijman SG and Stoppani AOM, Effects of β-lapachone, a peroxide-generating quinone, on macromolecule synthesis and degradation in *Trypanosoma cruzi*. *Arch Biochem Biophys* **240:** 273–280, 1985.
10. Boveris A, Docampo R, Turrens JF and Stoppani AOM, Effect of β-lapachone on superoxide anion and hydrogen peroxide production by *Trypanosoma cruzi*. *Biochem J* **175:** 431–439, 1978.
11. Boveris A, Stoppani AOM, Docampo R and Cruz FS, Superoxide anion production and trypanocidal action of naphthoquinones on *Trypanosoma cruzi*. *Comp Biochem Physiol* [C] **61:** 327–329, 1978.
12. Cruz FS, Docampo R and de Souza W, Effect of β-lapachone on hydrogen peroxide production in *Trypanosoma cruzi*. *Acta Trop (Basel)* **35:** 35–40, 1978.
13. Lopez JN, Cruz FS, Docampo R, Vasconcellos ME, Sampaio MCR, Pinto AV and Gilbert B, *In vitro* and *in vivo* evaluation of the toxicity of 1,4-naphthoquinone and 1,2-naphthoquinone derivatives against *Trypanosoma cruzi*. *Ann Trop Med Parasitol* **72:** 523–531, 1978.
14. Docampo R, Cruz, FS, Boveris A, Muniz RPA and Esquivel DMS, Lipid peroxidation and the generation of free radicals, superoxide anion and hydrogen peroxide in β-lapachone-treated *Trypanosoma cruzi* epimastigotes. *Arch Biochem Biophys* **186:** 292–297, 1978.
15. Molina Portela MP, Pahn EM de, Galeffi C and Stoppani AOM, Efecto de *orto*-naftoquinonas lipofílicas sobre el crecimiento y la producción de peróxidos por *Leptomonas seymouri*

y *Crithidia fasciculata*. *Rev Argent Microbiol* **23:** 1–14, 1991.
16. Schwarcz de Tarlovsky MN, Goijman SG, Molina Portela MP and Stoppani AOM, Effect of isoxazolyl naphthoquinoneimines on growth and oxygen radical production by *Trypanosoma cruzi* and *Crithidia fasciculata*. *Experientia* **46:** 502–505, 1990.
17. Degrassi F, De Salvia R and Berghella L, The production of chromosomal alterations by β-lapachone, an activator of topoisomerase I. *Mutat Res* **288:** 263–267, 1993.
18. Li CJ, Averboukh L and Pardee AB, β-Lapachone, a novel DNA topoisomerase I inhibitor with a mode of action different from camptothecin. *J Biol Chem* **268:** 22463–22468, 1993.
19. Dubin M, Fernandez Villamil SHF and Stoppani AOM, Inhibition of microsomal lipid peroxidation and cytochrome P-450-catalyzed reactions by β-lapachone and related naphthoquinones. *Biochem Pharmacol* **39:** 1151–1160, 1990.
20. Bacchi CJ, Lambros C, Goldberg B, Hutner SH and De Carvallo GDF, Susceptibility of an insect *Leptomonas* and *Crithidia fasciculata* to several established antitrypanosomatid agents. *Antimicrob Agents Chemother* **6:** 785–790, 1974.
21. Bacchi CJ, Lambros C, Ellenbogen BB, Penkovsky LN, Sullivan W, Eyinna EE and Hutner SH, Drug-resistant *Leptomonas*: Cross-resistance in trypanocide-resistant clones. *Antimicrob Agents Chemother* **8:** 688–692, 1975.
22. Shapiro A, Katz LH, Hutner SH, Harracksingh C and Bacchi CJ, *Crithidia* media as guide to antihaemoflagellate chemotherapy aimed at porphyrin and transition-metal metabolism. In: *The* In Vitro *Cultivation of the Pathogens of Tropical Diseases* (Ed. WHO: Trop. Dis. Res. Ser.), pp. 401–415. Schwabe & Co., Basel, Switzerland, 1980.
23. Coolbear KP and Midgley M, Characteristics of ethidium uptake by the trypanosomatid flagellates *Crithidia fasciculata* and *Leptomonas seymouri*. *Antimicrob Agents Chemother* **29:** 258–262, 1986.
24. Gutteridge WE, Ross J, Hargadon MRJ and Hudson JE, *Crithidia fasciculata*: A catalase-containing trypanosomatid sensitive to nitroheterocyclic drugs. *Trans R Soc Trop Med Hyg* **76:** 493–496, 1982.
25. Galeffi C, Casinovi CG, Miranda Delle Monache E and Marini-Bettolo GB, Chinoni sesquiterpenici della *Mansonia Altissima* Chev. - Nota II. *Ann Ist Super Sanita* **4:** 305–316, 1968.
26. Misra HP and Fridovich I, The univalent reduction of oxygen by reduced flavins and quinones. *J Biol Chem* **247:** 188–192, 1972.
27. McCord JM and Fridovich I, Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244:** 6049–6055, 1969.
28. Paget TA, Fry M and Lloyd D, Effect of inhibitors on the oxygen kinetics of *Nippostrongylus brasiliensis*. *Mol Biochem Parasitol* **22:** 125–133, 1987.
29. Hissin PJ and Hilf R, A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* **74:** 214–226, 1976.
30. Aebi H, Catalase. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), 2nd Ed., pp. 673–684. Verlag Chemie International, Deerfield Beach, FL, 1981.
31. Misra HP and Fridovich I, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* **247:** 3170–3175, 1972.
32. Gornall AG, Bardawill CJ and David MM, Determination of serum proteins by means of the biuret reaction. *J Biol Chem* **177:** 751–766, 1949.
33. Cadenas E, Boveris A, Ragan CI and Stoppani AOM, Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* from beef heart mitochondria. *Arch Biochem Biophys* **180:** 248–257, 1977.
34. Toner JJ and Weber MM, Respiratory control in mitochondria from *Crithidia fasciculata*. *Biochem Biophys Res Commun* **46:** 652–660, 1972.
35. Fairlamb AH and Cerami A, Metabolism and functions of trypanothione in the kinetoplastida. *Annu Rev Microbiol* **46:** 695–729, 1992.
36. Steenkamp DJ and Spies HSC, Identification of a major low-molecular-mass thiol of the trypanosomatid *Crithidia fasciculata* as ovothiol A. Facile isolation and structural analysis of the bimane derivative. *Eur J Biochem* **223:** 43–50, 1994.
37. Sinet P-M and Garber P, Inactivation of the human CuZn superoxide dismutase during exposure to O_2^- and H_2O_2. *Arch Biochem Biophys* **212:** 411–416, 1981.
38. Le Trant N, Meshnick SR, Kitchener K, Eaton JW and Cerami A, Iron-containing superoxide dismutase from *Crithidia fasciculata*. *J Biol Chem* **258:** 125–130, 1983.
39. Yamakura F and Suzuki K, Inactivation of *Pseudomonas* iron-superoxide dismutase by hydrogen peroxide. *Biochim Biophys Acta* **874:** 23–29, 1986.
40. Beyer WF and Fridovich I, Effect of hydrogen peroxide on the iron-containing superoxide dismutase of *Escherichia coli*. *Biochemistry* **26:** 1251–1257, 1987.
41. Molina Portela MP and Stoppani AOM, Redox cycling of β-lapachone and related *o*-naphthoquinones in the presence of dihydrolipoamide and oxygen. *Biochem Pharmacol* **51:** 275–283, 1996.
42. Paulino M, Hansz M, Hikichi N, Tabares G, Molina Portela MP, Fernandez Villamil SH, Sreider CM and Stoppani AOM, Propiedades electronicas y actividad redox de naftoquinonas. *An Asoc Quim Argent* **82:** 371–389, 1994.
43. Penketh PG and Klein RA, Hydrogen peroxide metabolism in *Trypanosoma brucei*. *Mol Biochem Parasitol* **20:** 111–121, 1986.
44. Penketh PG, Kennedy WPK, Patton CL and Sartorelli AC, Trypanosomatid hydrogen peroxide metabolism. *FEBS Lett* **221:** 427–431, 1987.
45. Penketh PG, Kennedy WPK, Patton Cl and Sartorelli AC, Competent metabolic utilization of hydrogen peroxide by trypanosomes. *Acta Trop (Basel)* **44:** 461–462, 1987.
46. Carnieri EGS, Moreno SNJ and Docampo R, Trypanothione-dependent peroxide metabolism in *Trypanosoma cruzi* different stages. *Mol Biochem Parasitol* **61:** 79–86, 1993.
47. Ross D, Glutathione, free radicals and chemotherapeutic agents. *Pharmacol Ther* **37:** 231–249, 1988.
48. Biaglow JE, Varnes ME, Epp ER, Clark EP, Tuttle SW and Held KD, Role of glutathione and other thiols in cellular response to radiation and drugs. *Drug Metab Rev* **20:** 1–12, 1989.
49. Wardman P, Bioreductive activation of quinones: Redox properties and thiol reactivity. *Free Radic Res Commun* **8:** 219–229, 1990.
50. Miura T, Muraoka S and Ogiso T, Generation of semiquinone and oxygen radicals by the reaction of menadione with reduced glutathione at various pH. *Chem Pharm Bull (Tokyo)* **40:** 709–712, 1992.
51. Averill-Bates DA and Przybytkowski E, The role of glucose in cellular defences against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. *Arch Biochem Biophys* **312:** 52–58, 1994.
52. Toxopeus C, van Holsteijn I, De Winther MPJ, van den Dobbelsteen D, Horbach GJMJ, Blaauboer BJ and Noordhoek J, Role of thiol homeostasis and adenine nucleotide metabolism in the protective effects of fructose in quinone-induced cytotoxicity in rat hepatocytes. *Biochem Pharmacol* **48:** 1683–1692, 1994.
53. Morgan WA, DNA single-strand breakage in mammalian cells induced by redox cycling quinones in the absence of oxidative stress. *J Biochem Toxicol* **10:** 227–232, 1995.