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β-LAPACHONE ENHANCEMENT OF LIPID PEROXIDATION AND SUPEROXIDE ANION AND HYDROGEN PEROXIDE FORMATION BY SARCOMA 180 ASCITES TUMOR CELLS

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Abstract—Addition of β-lapachone, an *o*-naphthoquinone endowed with antitumor properties for Sarcoma 180 cells, induced the formation of a semiquinone radical. β-Lapachone was able to stimulate superoxide anion and hydrogen peroxide production by the mitochondrial fraction supplemented with NADH. β-Lapachone also increased O_2^- and H_2O_2 production by the microsomal fraction with NADPH as reductant. Cyanide-insensitive NADH and NADPH oxidations by the mitochondrial and microsomal fractions (quinone reductase activity) were stimulated to about the same extent by β-lapachone. Incubation of sarcoma cells with β-lapachone stimulated lipid peroxidation and resulted in a decrease in the viability of the cells. The toxicity of β-lapachone to tumor cells was reduced by incubation of the cells with the free radical scavenger, α-tocopherol. The basic mechanism of the biological action of β-lapachone in sarcoma cells seems to be: (a) reduction at the mitochondrial and microsomal membranes with generation of the semiquinone form, (b) autoxidation of the semiquinone free radical with primary production of O_2^-, (c) production of H_2O_2 via superoxide dismutase reaction and generation of HO· from the reaction of O^-_2 and H_2O_2 with subsequent stimulation of lipid peroxidation and decreased viability of the cells.

Intracellular reduction followed by autoxidation, yielding O_2^- and H_2O_2, has been suggested as the mode of action of several antitumor agents. Thus, streptonigrin [1], mitomycin C [2], platinum derivatives [3] and adriamycin [4–7], have been shown to act in cell extracts as electron carriers between NADH (or NADPH) and oxygen with concomitant production of either the superoxide anion or hydrogen peroxide.

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione), an antimicrobial [8] and antitrypanosomal [9] *o*-naphthoquinone, has been shown to possess similar O_2^- and H_2O_2 generating properties in mitochondrial and microsomal suspensions as well as in intact cells of *Trypanosoma cruzi* [10]. Since β-lapachone produces marked toxic effects on Yoshida sarcoma and Walker 256 carcinosarcoma cells, inhibiting their growth 40 per cent at a concentration of 7 mg/kg [11], it appeared possible that a reduction reoxidation cycle similar to that of streptonigrin, mitomycin C and other antitumor agents would play a role in the antitumor effect of β-lapachone. We tested this idea and report here our results.

MATERIALS AND METHODS

SW 55 male mice, 3- to 4-weeks-old and weighing 20–25 g, were used 10 days after i.p. inoculation of 1×10^6 Sarcoma 180 cells/mouse. Mice with hemorrhagic ascites were discarded. The ascites fluid drawn from inoculated mice contained mostly Sarcoma 180 cells. Cells were washed twice in 0.15 M NaCl.

Mitochondrial and microsomal preparations. Sarcoma cells were suspended in ice-cold 0.23 M mannitol, 0.07 M sucrose, 5 mM Tris–HCl (pH 7.4), and 1 mM EDTA (MSTE). Homogenization was performed in a Potter tissue grinder with a Teflon pestle until 90 per cent of the cells were disrupted or by ultrasonic treatment of cells for 1 min with a MSE ultrasonic disintegrator (Measuring and Scientific Equipment, London) operated at a power output of 90 W. The homogenate was diluted and centrifuged at 680 *g* for 10 min to remove whole cells, cell debris and nuclei. The supernatant fraction was then centrifuged at 12,000 *g* for 10 min to separate the mitochondrial fraction. The supernatant solution was centrifuged at 105,000 *g* for 60 min to separate the microsomal fraction.

Chemicals. Beef liver catalase, D-α-tocopherol (succinate), horseradish peroxidase (HRP), L-epinephrine and bovine erythrocyte superoxide dismutase (SOD) were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. and β-lapachone was a gift of Drs. A. V. Pinto and B. Gilbert.

Electron spin resonance spectroscopy. Sarcoma cells were suspended in MSTE at a concentration of 10^9 cells/ml. Solutions of β-lapachone in ethanol were made. Ethanol itself did not produce measurable free radicals when mixed with the cell suspension. Also, the MSTE solution alone did not produce free radicals when mixed with β-lapachone. Electron spin resonance spectra were obtained in a Varian E-9 spectrometer using the conditions described in the figures. A Varian

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aqueous sample cell was used. The β-lapachone solution, as well as the cell suspension mixed with β-lapachone, had been saturated previously with nitrogen.

Determination of the superoxide anion and hydrogen peroxide production. O_2^- production was determined by the adrenochrome assay [12], measuring the absorption at 485–575 nM and using an absorption coefficient (ε) of 2.96 mM^{-1} cm^{-1}. The reaction mixture contained 1 mM epinephrine. H_2O_2 generation in sarcoma fractions was determined by the HRP assay [13], measuring the absorption at 417–402 nM (ε: 50 mM^{-1} cm^{-1}). Reactions mixtures contained 0.3 to 0.8 μM HRP. An Aminco–Chance double beam spectrophotometer (American Instrument Co., Silver Springs, MD, U.S.A.) was utilized. All determinations were made at 30°. Protein was determined by the biuret method [14].

Lipid peroxidation. Lipid peroxidation *in vivo* was estimated by measuring diene conjugates by ultraviolet absorption of lipid extracts of the microsomal fraction of sarcoma cells, as described by Klaassen and Plaa [15]. The mean difference spectrum, β-lapachone-treated minus control, was recorded in a Beckman Acta III spectrophotometer.

Determination of enzymes. Catalase activity was determined spectrophotometrically by the decrease in absorbance of a hydrogen peroxide-containing reaction mixture at 240 nM [16]. The reaction mixture contained 50 mM Tris–HCl buffer, pH 7.3, 2.0 mM H_2O_2, and the sarcoma homogenate (0.5 mg protein/ml), in a final volume of 3 ml. Samples were placed in the spectrophotometer cuvette positioner and scanned automatically at 240 nm for 30 min.

Superoxide dismutase was determined in sarcoma homogenates by comparing the inhibition of adrenochrome formation by the homogenate with that produced by bovine superoxide dismutase [17]. The reaction mixture contained 0.23 M mannitol, 0.07 M sucrose, 30 mM Tris–morpholinopropane sulfonate buffer (pH 7.9), 1 mM epinephrine, 0.5 mM xanthine, and 1 μg/ml of xanthine oxidase (specific activity: 0.61 mU/mg of protein).

Quinone reductase activity was measured by following either NADH or NADPH oxidation at 340 nm in a Gilford 2000 spectrophotometer. Mitochondrial and microsomal fractions (0.2 to 0.5 mg protein/ml) were suspended in 50 mM phosphate buffer (pH 7.4), 1 mM KCN, 100 μM of either NADH or NADPH, and a variable (0–40 μM) amount of quinone. All enzyme reactions were measured in a thermostatically controlled cell compartment at 30°. Quinone concentrations were determined by recording the ultraviolet absorption spectra in a Beckman DK-2 spectrophotometer. β-Lapachone has an ε_{258} of 40 litres $nmole^{-1}$ cm^{-1} and an ε_{440} of 3.1 litres $m\text{-}mole^{-1}$ cm^{-1}.

RESULTS

Evidence for semiquinone radical generation. β-Lapachone is capable of accepting a single electron to form a semiquinone radical. Evidence for this radical intermediate was confirmed in the experiments recorded in Figs. 1 and 2.

When β-lapachone was added to a suspension of sarcoma cells saturated previously with nitrogen, the signal of the semiquinone derivative was evident (Fig.

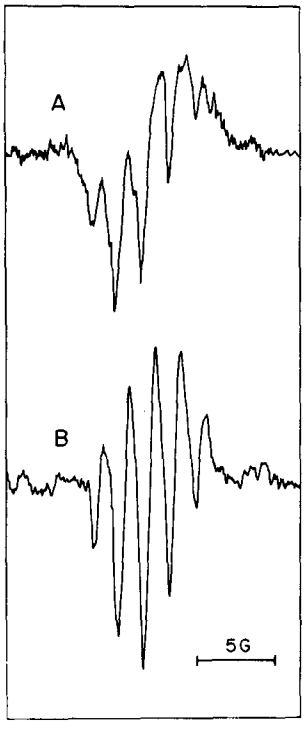

Fig. 1. X-band (3 cm) e.s.r. spectra of β-lapachone free radical. (A) Biologically reduced: to a suspension of Sarcoma 180 cells in MSTE buffer, β-lapachone was added to a final concentration of 2 mg/ml and was deaerated with nitrogen. The spectrum was recorded at 0.66 gauss/min. The modulation amplitude was 0.63 gauss at a frequency of 100 (k)Hz. (B) Chemically reduced: β-lapachone at a concentration of 2 mg/ml in MSTE buffer was deaerated with nitrogen and reduced by addition of sodium borohydride at 40 μg/ml. The spectrum was recorded at 0.1 gauss, with high gain, high time constant and slow sweep.

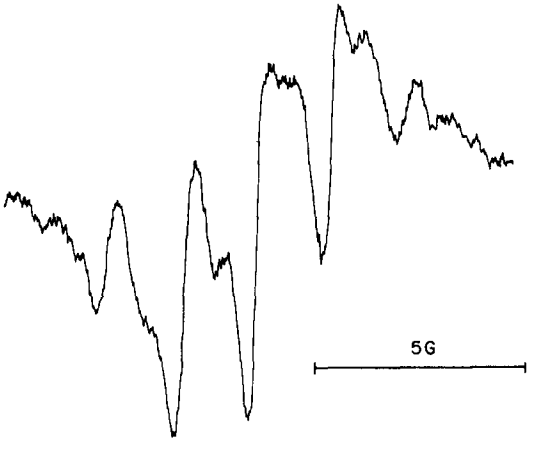

Fig. 2. X-band (3 cm) e.s.r. spectra of β-lapachone free radical recorded 6 hr after mixing Sarcoma 180 suspension and β-lapachone. The spectrum was recorded at 0.66 gauss/min; the modulation amplitude was 0.5 gauss at a frequency of 100 (k)Hz. By comparison with the spectrum shown in Fig. 1, the 5-line hyperfine structure was broader.

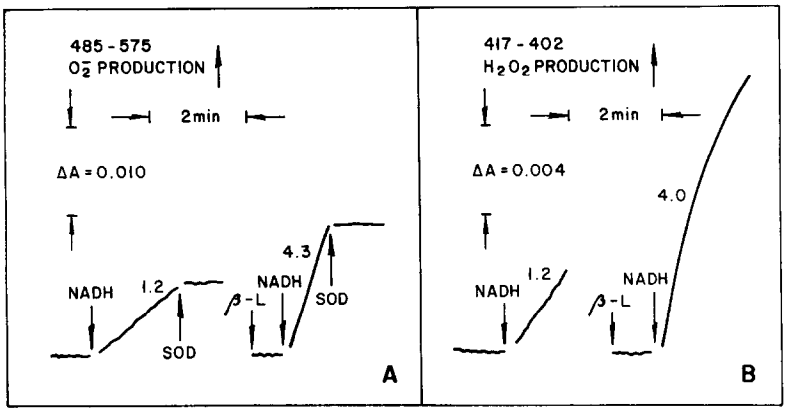

Fig. 3. O_2^- and H_2O_2 generation in the mitochondrial fraction from Sarcoma 180. The mitochondrial fraction (0.12 mg protein/ml for O_2^- determination and 0.66 mg protein/ml for H_2O_2 determination) was suspended in MSTE and 1 mM epinephrine (for O_2^- determination) or 0.5 μM HRP (for H_2O_2 determination). Forty μM NADH, and 20 μM β-lapachone and 30 μg/ml of superoxide dismutase (SOD) were added as indicated. Values indicate O_2^- generation (A) or H_2O_2 production (B) in nmoles/min/mg of protein.

1B). The nature of the β-lapachone free radical which arose in the biological system using sarcoma cells was confirmed by comparison with the spectrum of the chemically reduced β-lapachone (Fig. 1A). The characteristics of the signal suggest a semiquinone radical. The 5-line hyperfine structure of the β-lapachone semiquinone radical was clearly discernible in both chemically and biologically reduced samples. The signal appeared immediately upon addition of β-lapachone, grew in intensity in about half an hour and thereafter remained constant. Further observations were made about 6 hr later, the sample being kept at 20°. After this period there were marked modifications in the spectrum (Fig. 2), the 5-line hyperfine signal being maintained but with broader lines. The spectrum obtained by chemical reduction was unchanged except for a decline in intensity.

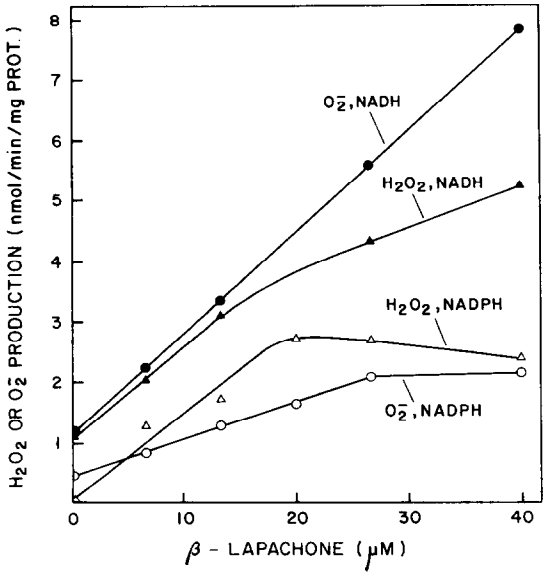

Fig. 4. Effect of β-lapachone concentration on O_2^- and H_2O_2 formation by the mitochondrial fraction of Sarcoma 180 in the presence of 40 μM NADH or 40 μM NADPH. Experimental conditions were as in Fig. 3.

Generation of the superoxide anion and hydrogen peroxide in the mitochondrial fraction. The failure to detect a metabolically reduced β-lapachone derivative in the presence of oxygen may indicate either that cellular metabolism under aerobic conditions is not capable of reducing the drug or that reduced intermediates are re-oxidized rapidly. Quinols in oxygenated solutions are known to undergo spontaneous oxidation leading to O_2^- and H_2O_2 [12]. One way of demonstrating whether reduced β-lapachone undergoes this autoxidation is to examine its effects on the rates of O_2^- and H_2O_2 production by the subcellular fractions.

The rate of formation of O_2^- by the mitochondrial fraction of Sarcoma 180 as detected by adrenochrome formation is illustrated in Fig. 3A. Upon addition of 20 μM β-lapachone alone no significant rate of O_2^- production was observed. Further addition of NADH caused a 3.5-fold stimulation of O_2^- production compared with the preparations without β-lapachone. This O_2^- production was specifically inhibited by superoxide dismutase. The inhibition could be reversed or prevented by the addition of cyanide. Heat-inactivated superoxide dismutase did not inhibit adrenochrome formation. An enzymatic reaction was apparently required in this system since no alteration in the absorbance, indicating O_2^- production, was observed in the absence of the mitochondrial fraction. Moreover, the rate of the reaction was directly proportional to the amount of protein. Similarly no significant H_2O_2 production was observed in the mitochondrial fraction upon addition of 20 μM β-lapachone (Fig. 3B). Further addition of NADH induced an increase in H_2O_2 production 3.5 times greater than that measured in the preparations without β-lapachone. Figure 4 shows a titration of the effect of β-lapachone concentration on O_2^- and H_2O_2 production by the mitochondrial fraction. In the presence of NADH, the rates of O_2^- and H_2O_2 formation were proportional to the amount of β-lapachone added. NADPH was less effective than NADH as reductant.

Generation of superoxide and hydrogen peroxide in the microsomal fraction. The rate of formation of O_2^- by the microsomes of Sarcoma 180 is illustrated in Fig. 5A. Twenty μM β-lapachone did not increase the rate of

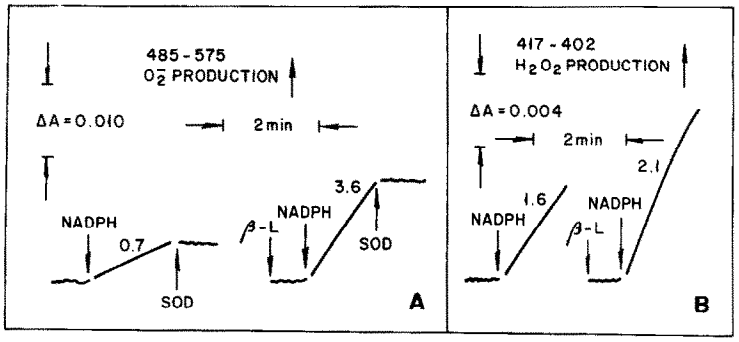

Fig. 5. O_2^- and H_2O_2 generation in the microsomal fraction from Sarcoma 180. This microsomal fraction (0.99 mg protein/ml for O_2^- determination and 0.03 mg protein/ml for H_2O_2 determination) was suspended in a medium containing 0.13 M KCl and 20 mM potassium phosphate (pH 7.4) and 1 mM epinephrine (for O_2^- determination) or 0.5 μM HRP (for H_2O_2 determination). Forty μM NADPH, and 20 μM β-lapachone and 30 μg/ml of SOD were added as indicated. Values indicate O_2^- generation (A) of H_2O_2 production (B) in nmoles/min/mg of protein.

O_2^- production. Upon addition of NADPH, O_2^- production was stimulated five times more than the rate measured in preparations without β-lapachone. This O_2^- production was specifically inhibited by superoxide dismutase. The rate of the reaction was directly proportional to the amount of protein. Similarly, no significant H_2O_2 production was observed in the microsomal fraction upon addition of 20 μM β-lapachone alone; NADPH was required. NADPH caused 40 per cent greater H_2O_2 production in the presence of β-lapachone than in the preparations without β-lapachone (Fig. 5B). Figure 6 shows a titration of the effect of β-lapachone on O_2^- and H_2O_2 production by the microsomal fraction of sarcoma. In the presence of NADPH the rates of O_2^- and H_2O_2 formation were two times greater than in the presence of NADH. This specificity for pyridine nucleotide of the microsomal fraction is in agreement with the more active reducing ability of NADPH compared with NADH.

β-Lapachone reductase activity of the mitochondrial and microsomal fractions. The β-lapachone reductase activities of the mitochondrial and microsomal fraction were determined with NADH and NADPH as electron donors in the presence of cyanide and quinone (Fig. 7). The rate of NADH oxidation by mitochondrial fragments was stimulated about 4-fold by 40 μM β-lapachone, whereas NADPH oxidation was less stimulated (data not shown). Similar effects of β-lapachone were found when the quinone reductase activity of microsomal preparations were determined. In this case, NADPH was more effective than NADH as reductant. β-Lapachone at 40 μM was able to increase the cyanide-insensitive NADPH oxidation by the microsomal fraction about 2.5-fold, whereas NADH oxidation was less stimulated (data not shown). The rates of pyridine nucleotide oxidation by the mitochondrial and microsomal fractions (Fig. 7) were 120–200 per cent higher than the rates of H_2O_2 production by the same fractions, irrespective of the addition of β-lapachone (Figs. 4 and 6). The relatively lower rates of H_2O_2 production are understandable, considering that (a) some reduced pyridine nucleotide oxidation does not lead to H_2O_2

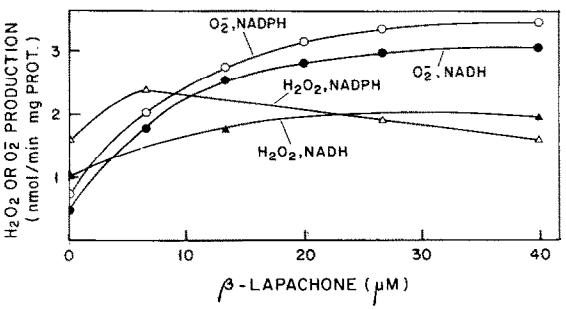

Fig. 6. Effect of β-lapachone concentration on O_2^- and H_2O_2 formation by the microsomal fraction of Sarcoma 180 in the presence of 40 μM NADH or 40 μM NADPH. Experimental conditions were as in Fig. 5.

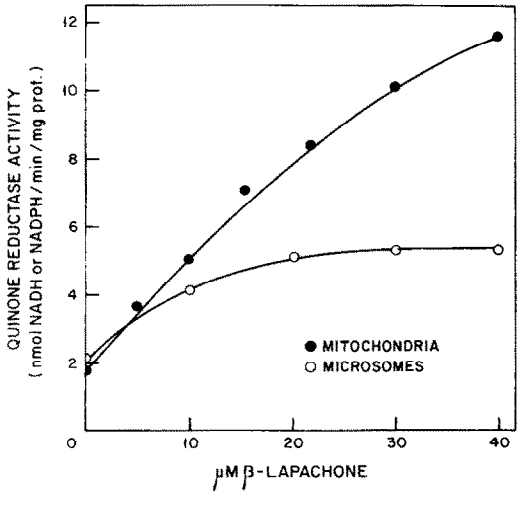

Fig. 7. Effect of β-lapachone on the cyanide-insensitive NADH and NADPH-oxidase activity of the mitochondrial and microsomal fraction of Sarcoma 180. Experimental conditions: 50 mM phosphate buffer, pH 7.2, 0.1 to 0.2 mg protein/ml; other experimental conditions were as indicated in Materials and Methods. Key: (●) mitochondria, NADH and β-lapachone; and (○) microsomes, NADPH and β-lapachone.

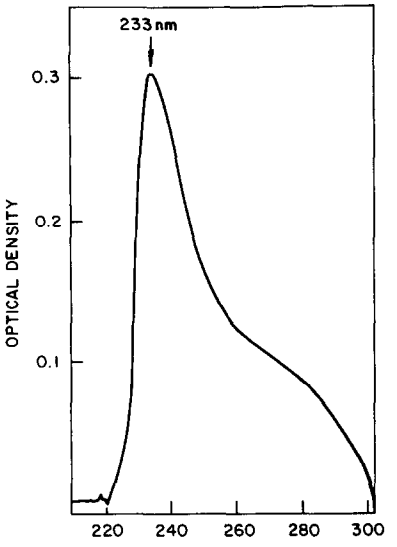

Fig. 8. Diene conjugation absorption of Sarcoma 180 microsomal lipids 3 hr after β-lapachone treatment. Sarcoma cells (10^9 cells/ml) were suspended in MSTE and 0.6 mM NADPH with or without 40 μM β-lapachone and incubated during 3 hr at 37°. After this period, the cells were centrifuged, washed, suspended in MSTE and homogenized by ultrasonic treatment. The homogenates were then centrifuged at 12,000 *g* for 20 min and at 105,000 *g* for 60 min. The supernatant solutions were decanted and the microsomal fractions were resuspended in MSTE solution. Aliquots of the microsomal fractions (15 mg of total protein) were extracted by the method of Klaassen and Plaa [15]. Methanol was added to the recovered microsomal lipids and the difference spectrum (β-lapachone-treated minus control) was read in the spectrophotometer. The spectrum of peroxidized lipids was characterized by an intense K-band at 233 nm with a shoulder, due to ketone dienes in the region from 260 to 280 nm.

formation and (b) the HRP–H_2O_2 assay slightly underestimates H_2O_2 production [13].

It has been shown previously [18] that reduced β-lapachone is a more effective H_2O_2 than O_2^- generator. The redox steady state of the β-lapachone system was calculated by comparison of the rates of quinol production by sarcoma mitochondria and autoxidation (see Ref. 18 for details). In the steady state, the β-lapachone couple could be 2.4 per cent reduced and 98.6 per cent oxidized. This calculation was confirmed by measuring the lack of effect of NADH on the bleaching of the 258 nm and the 440 nm absorption bands of β-lapachone in the presence of the mitochondrial and microsomal fractions.

Catalase and superoxide dismutase content of sarcoma cells. No catalase activity could be detected in the sarcoma homogenate. The possible existence of catalase inhibitors in the homogenate could be excluded by comparing the activity of beef liver catalase in homogenate-containing and homogenate-free reaction mixtures respectively (data not shown). In contrast to these negative results, superoxide dismutase activity could be demonstrated in the sarcoma homogenate and was equivalent to 0.37 μg bovine superoxide dismutase/mg of homogenate protein.

Lipid peroxidation. Sarcoma 180 cells incubated in the presence of β-lapachone, as indicated in Fig. 8, were washed, suspended in MSTE and then were disrupted by ultrasonic treatment as described under Materials and Methods. The microsomal fraction obtained was then extracted by the method of Klaassen and Plaa [15]. The difference spectrum (β-lapachone-treated minus control) of the microsomal extracts (Fig. 8) shows the existence of substantial amounts of diene conjugate.

Cytotoxic effect of β-lapachone. Sarcoma cells were incubated in MSTE in the presence of 40 μM and 80 μM β-lapachone. After 60 min of incubation with the drug the cells were swollen. Lysis followed with prolonged incubation. The cells incubated during 60 min in the absence or in the presence of 40 and 80 μM β-lapachone were injected i.p. in mice and the survival time was recorded (Table 1). All the mice inoculated with β-lapachone-treated sarcoma cells survived. The addition of tocopherol (10^{-3}–10^{-4} M) to the incubation medium protected the sarcoma cells from the cytotoxic effect of β-lapachone.

DISCUSSION

β-Lapachone is capable of accepting a single electron to form a semiquinone radical (Figs. 1 and 2). As this radical is readily autoxidable, its determination requires an N_2 atmosphere. The initial reduction of the β-lapachone appears to be initiated by the mitochondrial and microsomal quinone reductases (Fig. 7). Autoxidation of semiquinones causes the univalent reduction of oxygen generating O_2^- which in turn produces H_2O_2, either spontaneously or by the action of the enzyme superoxide dismutase [12]. In addition, O_2^- and H_2O_2 react nonenzymatically to form the hydroxyl radical [19] and these products of the partial reduction of oxygen can initiate free-radical reactions which lead to

Table 1. Effect of β-lapachone on Sarcoma 180 inoculated mice*

Addition to the incubation medium (mM)	Average survival time (days)	Long-term survivors (number alive at 60 days/number tested)
None	13.5	0/10
β-Lapachone (0.04)	> 60	10/10
β-Lapachone (0.08)	> 60	10/10
β-Lapachone (0.1) + tocopherol (1)	14	0/10

* Sarcoma cells suspended in MSTE at a concentration of 1×10^7 cells/ml were incubated during 60 min at 37°. After washing in 0.15 M NaCl, they were inoculated i.p. in mice as described under Materials and Methods.

the peroxidation of polyunsaturated fatty acids of membrane lipids [6, 7, 20, 21].

A rapid O_2^- and H_2O_2 formation was observed in the mitochondrial fraction of Sarcoma 180 in the presence of β-lapachone and NADH. NADH was more effective than NADPH as electron donor for β-lapachone reduction and O_2^- and H_2O_2 formation. Comparison of the rate of H_2O_2 generation and O_2^- production in the mitochondrial fraction suggested that approximately 1 mole of O_2^- is produced for each mole of H_2O_2. Similar results were obtained with the isolated microsomal fraction. In this case, NADPH was more effective than NADH as electron donor for β-lapachone reduction and O_2^- and H_2O_2 generation. In addition, lipids extracted from microsomes of β-lapachone-treated cells exhibited typical diene conjugation absorption, indicating a significant peroxidation, whereas lipids extracted from microsomes of control cells did not. Accordingly, the free radical scavenger, tocopherol, protected the sarcoma cells from the cytoxic effect of β-lapachone.

Modifications in the e.s.r. spectrum of the biological system are possibly due either to free radicals attached to the membrane or to some other radicals arising in the system, such as those produced after the direct reaction of oxygen and lipids [21].

It is of interest that Risse and Tiedemann [22] showed that phenanthraquinone was involved in a cyclic process of oxidation and reduction in Erlich ascites tumor cells under aerobic conditions. NADH was the hydrogen donor for the reduction. During the autoxidation of the corresponding quinol by atmospheric oxygen, radicals and H_2O_2 were formed. These radicals and H_2O_2 were regarded as responsible for the inhibition of glycolysis [23].

Intracellular (or *in vivo*) formation of O_2^- and H_2O_2 may be considered as the cause of β-lapachone toxicity against tumor cells [11] since such intermediates are highly toxic to biological systems. In addition, tumor cells have little superoxide dismutase activity, compared with cells from normal tissues [24, 25]. The levels of this enzyme may thus be insufficient for detoxifying unusually high concentrations of O_2^-. Since O_2^- is converted to H_2O_2 either spontaneously or by SOD, H_2O_2 may actually be more important in terms of tumor toxicity, especially since tumor cells have little or no catalase [24, 26]. In the tumor cells investigated in this work, no catalase activity could be detected. The concentration of superoxide dismutase was lower as compared to normal tissues [24]. The values obtained confirm previous reports on catalase and superoxide dismutase activities in different ascites tumor cells [24].

The reduced level of enzymes that protect against the toxicity of the intermediates of oxygen reduction may provide a rational approach for chemotherapy by using O_2^- and H_2O_2 generators.

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