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Preclinical Genotoxicology of Nor-β-lapachone in Human Cultured Lymphocytes and Chinese Hamster Lung Fibroblasts

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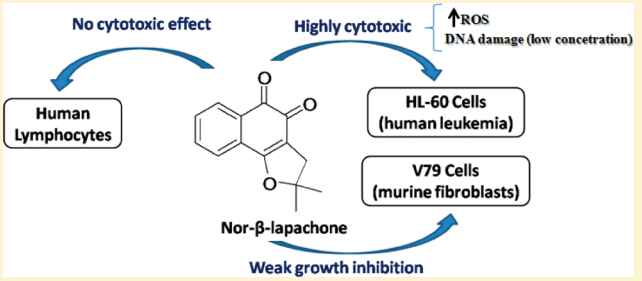
ABSTRACT: Nor-β-lapachone has shown several biological properties. Regarding cytotoxic activity against cancer cell lines, it has been recognized as an important prototype. However, quinonoid drugs present a major challenge because of their toxicity. In this study, we evaluated the cytotoxicity and genetic toxicity of nor-β-lapachone in human lymphocytes and HL-60 leukemia cells and murine V79 fibroblasts, to shed some light on its selectivity toward cancer cells. As measured by MTT test, exposure of V79 cells to nor-β-lapachone resulted in a weak cytotoxicity (IC_{50} = 13.41 μM), and at a concentration up to 21.9 μM, no cytotoxic effect was observed in lymphocytes, while in HL-60 cells, nor-β-lapachone elicited significantly greater cytotoxicity (IC_{50} = 1.89 μM). Cultures coexposed to GSH-OEt showed an increased viability, which may indicate a neutralization of ROS generated by quinonoid treatment. In fact, only the highest concentrations of nor-β-lapachone (10 or 20 μM) caused an increase in oxidative stress in nontumor levels cells as measured by TBARS and nitrite/nitrate detection. This was accompanied by an alteration in intracellular thiol content. However, NAC pre-exposure restored the redox equilibrium of the cells and the concentration of thiol levels to control values. Nor-β-lapachone at 2.5 and 5 μM failed to induce DNA damage in nontumor cells, but at the highest concentrations tested, it induced single and double DNA strand breaks and increased the frequency of chromosomal aberrations. Interestingly, these damages were prevented by NAC pretreatment or exacerbated by prior exposure to the GSH-depleting agent 1-bromoheptane. In electrochemical experiments, nor-β-lapachone at the same concentrations as those used in genotoxic tests did not damage DNA directly, but at the highest concentration tested (200 μM), it caused a very weak DNA interaction. Corroborating electrochemical data, oxidative modifications of DNA bases were observed, as checked by DNA repair enzymes EndoIII and FPG, which reinforced the indirect actions caused by nor-β-lapachone through ROS generation and not via DNA intercalation. The DNA repair capacities were higher for nontumor cells than for leukemia cells, which may be related to the selective cytoxicity of nor-β-lapachone toward cancer cells. Our data suggest that ROS play an important role in nor-β-lapachone toxicity and that its DNA-damaging effect occurs only at concentrations several times higher than that needed for its antiproliferative effect on cancer cells.



1. INTRODUCTION

Naphthoquinones (NQs) are considered privileged structures in medicinal chemistry due to their biological activities and structural properties.[1] In folk medicine, plants containing NQs have been employed for the treatment of various diseases,[2] including cancer.[3,4] Among the several quinonoid compounds with pharmacological activity, lapachol and β-lapachone, which are isolated from the heartwood of trees of the family Bignoniaceae (*Tabebuia* sp.), are examples of important bioactive NQs.[5,6]

As reviewed by Pinto and de Castro and by Hillard et al.,[7] quinones are oxidants and electrophiles, and the relative contribution of these properties to both toxic and therapeutic activities is influenced by their chemical structure, particularly substituent effects and the characteristics of the quinone nucleus.[8] Two major mechanisms of quinone cytotoxicity have been proposed: stimulation of oxidative stress and alkylation of cellular nucleophiles, which encompass a large range of biomolecules.[9] Reactive oxygen species (ROS) may react directly with DNA, lipids, and proteins, leading to cell damage[10−14] and shunting electrons toward oxygen, a futile pathway for reduction equivalents otherwise used for cytochrome P450 reductase-dependent

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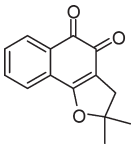

Figure 1. Chemical structure of nor-β-lapachone.

reactions. Cellular damage can also occur through the alkylation of crucial proteins and nucleic acids.[7,9]

Nor-β-lapachone (Figure 1), an inferior homologue of β-lapachone, has been recognized as an important prototype with activity against cancer,[15] and consequently, we have studied this substance against several cancer cell lines with activity at concentrations in the range of 0.3−1.75 μM (IC_{50}).[16] Recently, on the basis of the prototype nor-β-lapachone, our research group has described the antitumor activity of the 3-arylamino and 3-alkoxy-nor-β-lapachone derivatives and nor-β-lapachone-based 1,2,3-triazoles.[16−18] In addition, naphthoquinonoid compounds with the dihydrofuran ring, such as nor-β-lapachone, represent a group of substances with great potential for screening against cancer cell lines.[19−21]

In the development of new chemicals, it is necessary to have early knowledge of their potential genotoxic and mutagenic effects. Thus, to determine the safety of a possible pharmacological application of nor-β-lapachone and to explore other biological properties, the purpose of this study was to evaluate its cytotoxicity and genetic toxicity (DNA breaks and chromosomal damage) in mitogenically activated human lymphocytes and Chinese hamster lung fibroblasts (V79 cells). Also, to provide a greater understanding of the mechanism of interaction between nor-β-lapachone and DNA, its in situ DNA-damaging capacity was investigated by an electrochemical approach.

2. MATERIALS AND METHODS

2.1. Chemicals. Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Cultilab (Campinas, SP). RPMI 1640 medium, trypsin-EDTA, glutamine, penicillin and streptomycin, phytohemagglutinin (PHA), low-melting point agarose, and agarose were purchased from Gibco (Invitrogen, Carlsbad, CA). Colchicine, sulfanilamide, reduced glutathione (GSH), GSH ethyl ester (GSH-OEt), NADPH, glutathione reductase, *N*-acetylcysteine (NAC), 1-bromoheptane (BH), 5,5′-dithionitrobenzoic acid (DTNB), 4-vinypyridine, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich Co. (St. Louis, MO). Formamidopyrimidine DNA-glycosylase (FPG) and endonuclease III (ENDOIII) were obtained from NewEngland BioLabs (United States). *N*-(1-Naphthyl)-ethylenediamine dihydrochloride, acetic acid, and methanol were purchased from Merck (United States). Hydrogen peroxide was obtained from Vetec (Brazil). Doxorubicin (Doxolem) was purchased from Zodiac Produtos Farmacêuticos S/A, Brazil. All other chemicals and reagents used were of analytical grade.

Nor-β-lapachone was synthesized by cyclization of nor-lapachol in the presence of H_2SO_4 and purified by a series of recrystallizations using the appropriate solvent. Nor-lapachol [2-hydroxy-3-(2-methylpropenyl)-1,4-naphthoquinone] was synthesized using the Hooker oxidation method.[22]

2.2. Cells and Cultures. Heparinized blood (from healthy, nonsmoker donors who had not taken any medication at least 15 days prior to sampling) was collected, and peripheral blood lymphocytes were isolated by a standard method of density-gradient centrifugation on Histopaque-1077, and the Chinese hamster lung fibroblasts (V79 cells) were kindly provided by Dr. JAP Henriques (Federal University of Rio Grande do Sul, Porto Alegre, Brazil). HL-60 cells were obtained from the National Cancer Institute (Bethesda, MD).

Lymphocytes and HL-60 cells were maintained in RPMI 1640 medium, and V79 cells were cultivated under standard conditions in MEM with Earle's salts. All culture media were supplemented with 20 (lymphocytes) or 10% (HL-60 and V79 cells) fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. For evaluation of cytotoxic and genotoxic activities, cells were grown for 2 days prior to treatment with the test substance, and afterward, the medium was replaced with fresh medium containing the test substance or DMSO solution for control. The final concentration of DMSO in the culture medium was kept constant, less than 0.1% (v/v).

2.3. Inhibition of Cell Proliferation—MTT Test. Cell growth was quantified by the ability of living cells to reduce the yellow dye MTT to a purple formazan product.[23] For the experiments, lymphocytes, V79 fibroblasts, and HL-60 cells were plated in 96-well plates (1×10^6 cells/well for lymphocytes and 0.3×10^6 cells/well for fibroblasts and leukemia cells), and nor-β-lapachone (0.04−21.9 μM), dissolved in DMSO (0.1%), was then added to each well, followed by incubation for 24 h. In some experiments, the contribution of ROS to the cytotoxicity of nor-β-lapachone was assessed by cells cotreated with GSH-OEt (15 mM). Our preliminary experiments showed that the ethyl ester of GSH was not cytotoxic and provided a more efficient protection than GSH at the same concentration (15 mM). In fact, GSH is not readily transported into most cells. Thus, in the MTT experiments, we used GSH ethyl ester, which is more lipophilic, readily taken up by cells and hydrolyzed to GSH by cellular nonspecific esterases.[24]

Afterward, the plates were centrifuged, and the medium was replaced by fresh medium (150 μL) containing 0.5 mg/mL MTT. Three hours later, the MTT formazan product was dissolved in 150 μL of DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The effect of the test substances was quantified as the percentage of control absorbance of the reduced dye at 595 nm. Experiments were carried out in duplicate and repeated at least three times. Doxorubicin (0.001−1.06 μM) was used as a positive control.

2.4. Treatment Protocols. For oxidative stress evaluation, treatments were performed with different concentrations of nor-β-lapachone (2.5, 5, 10, or 20 μM) or H_2O_2 (10 μM) for 4 and 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 in the presence or not of 5 mM NAC. For genetic toxicology evaluation, cells were exposed to increasing concentrations (2.5, 5, 10, or 20 μM) of nor-β-lapachone for 4 h. In addition, the contribution of ROS to the cytotoxicity (mitotic index), genotoxicity, and mutagenicity of the test NQ was determined in cells pretreated for 24 h with NAC (5 mM) or BH (50 μM), before exposure to the test compound for 24 h. All experiments were performed in triplicate in three independent experiments.

2.5. Lipid Peroxidation—TBARS Assay. The extent that test compounds induced lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed by lipid peroxidation.[25] The assays were performed according to Salgo and Pryor,[26] with minor modifications. Cells were incubated with the test compounds for 4 and 24 h and then lysed with 15 mM Tris-HCl for 1 h. Two milliliters of trichloroacetic acid (0.4 mg/mL) and HCl (0.25 M) was added to the lysate, which was then incubated with 6.7 mg/mL TBA for 15 min at 100 °C. The mixture was centrifuged at 750*g* for 10 min. As TBA reacts with other products of lipid peroxidation in addition to MDA, results are expressed in terms of thiobarbituric reactive species (TBARS), which are determined by absorbance at 532 nm. Hydrolyzed 1,1,3,3-tetramethoxypropane was used as the standard. The results were normalized by protein content.[27]

2.6. Nitrite/Nitrate Production. The production of nitrite/nitrate as a result of nitric oxide (NO) release was measured according

to Green et al.[28] After cell treatments (4 and 24 h), 100 μL of the cell culture supernatant was added to 100 μL of the Griess reagent [1% sulfanilamide in 1% H_3PO_4/ 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/1% H_3PO_4/distilled water, 1:1:1:1], and the mixture was incubated at room temperature for 10 min. A standard curve was prepared with several concentrations of $NaNO_2$ (ranging from 0.75 to 100 μM) under the same conditions. Blanks were prepared by adding 100 μL of the Griess reagent to 100 μL of the culture medium. The absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada) at 560 nm. Experiments were performed in triplicate in three independent experiments.

2.7. Determination of Reduced (GSH) and Oxidized (GSSG) Glutathione and GSG/GSSG Ratio. Total glutathione (GSH + GSSG) was determined by a spectrophotometric assay based on the formation of 5-thio-2-nitrobenzoate (TNB) from DTNB, according to Akerboom and Sies[29] with minor modification. Briefly, cells were incubated with nor-β-lapachone (10 and 20 μM) for 4 h. The cells were then washed with ice-cold PBS and resuspended in 0.1 M sodium phosphate-5 mM EDTA, pH 8.0, and sonicated to obtain the cell homogenate. An equal volume of 2 M $HClO_4$—4 mM EDTA was added to the cell extract, and the precipitated proteins were pelleted by centrifugation at 8000*g* for 15 min at 4 °C. The supernatant was neutralized with 2 M KOH, and the insoluble residue was removed by centrifugation under the same conditions. For spectrophotometric determination, 910 μL of the cell extract supernatant or of a standard glutathione solution, in the same phosphate-EDTA buffer, was mixed with 50 μL of 4 mg/mL NADPH in 0.5% (w/v) $NaHCO_3$, 20 μL of 6 U/mL glutathione reductase in phosphate-EDTA buffer, and 20 μL of 1.5 mg/mL DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. The results were normalized by protein content.[27] The total glutathione content was determined as μg/mg protein. For GSSG determination, 4-vinypyridine was added to a final concentration of 0.1% (v/v) and then incubated for 1 h at room temperature. At this concentration, 4-vinypyridine is able to react with all GSH without interfering with GSSG determination. GSH was determined based on the total glutathione and GSSG concentration results.

2.8. Comet Assay (Alkaline and Neutral Conditions). The alkaline comet assay was performed as described by Singh et al.[30] with minor modifications. At the end of treatment (4 or 24 h), cells were washed with ice-cold PBS, trypsinized with 100 μL of trypsin (0.15%) and resuspended in complete RPMI or DMEM medium. Next, 20 μL of cell suspension ($\sim 10^6$cells/mL) was mixed with 0.75% low melting point agarose and immediately spread onto a glass microscope slide precoated with a layer of 1% normal melting point agarose. The agarose was allowed to set at 4 °C for 5 min. The slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10.0) at 4 °C for a minimum of 1 h to remove cellular proteins, leaving the DNA as "nucleoids". After the lysis procedure, the slides were placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13.0) to cover the slides for 20 min at 4 °C to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V and 300 mA (0.86 V/cm). The neutral pH protocol was carried out following essentially the same procedure as the alkaline version except for the pH value. In the neutral version, the electrophoretic gel was run in buffer consisting of 100 mM Tris and 300 mM sodium acetate at pH 8.5. Electrophoresis was performed for 60 min, after a 60 min equilibrium period in neutral electrophoresis buffer, at 12 mA and 14 V (0.5 V/cm).

In both versions of the comet assay, the slides were next neutralized (0.4 M Tris, pH 7.5), stained with ethidium bromide (20 μg/mL), and analyzed using a fluorescence microscope. All of the above steps were conducted under yellow light or in the dark to prevent additional DNA damage. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each concentration of test substance. Cells were scored visually and placed in five classes, according to tail size (from undamaged-0 to maximally damaged-4), and a value (damage index) was assigned to each comet according to its class. The damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4).[31] The vehicle was used as negative control, and doxorubicin (0.6 μM) was used as positive control.

2.9. Measurement of Oxidized Purines and Pyrimidines. The alkaline comet assay was performed as described above. At the end of treatment (20 μM for 4 h), the slides were removed from the lysis solution and washed three times in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM Na_2EDTA, and 0.2 mg/mL BSA, pH 8.0), drained, and incubated with 70 μL of FPG (30 min 37 °C) or ENDOIII (45 min at 37 °C). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were visually analyzed per group. The amount of oxidized purines (FPG-sensitive sites) and pyrimidines (ENDOIII-sensitive sites) was then determined by subtracting the amount of strand breaks (samples incubated with buffer alone) to the total amount of breaks obtained after incubation with FPG and ENDOIII according to da Silva Júnior et al.[14]

2.10. Rejoining Kinetics of DNA Strand Breaks. The alkaline version of the comet assay was applied to assess whether DNA damage induced by nor-β-lapachone could be repaired. Briefly, lymphocytes, V79, and HL-60 cells were exposed to nor-β-lapachone (20 μM) for 24 h. After treatment, the compound was removed, and the cultures were then reincubated. Samples of treated cells were collected at different times (1, 3, 6, 12, and 24 h), and the comet assay was performed as described above.

2.11. Chromosomal Aberrations (CAs) Test. After the end of treatment (4 or 24 h), cells were washed with ice-cold PBS and recultivated in complete RPMI medium for 48 h. Colchicine (0.0016%) was added 2 h before fixation (72 h). Chromosomes were prepared according to standard procedures.[32] Hypotonic treatment with KCl (0.75 M, 37 °C) was applied for 15 min. The cells were fixed with methanol and acetic acid (3:1), and the fixative solution was changed twice. Air-dried slides were stained with Giemsa (5%, pH 6.8) for 7 min and scored for CAs according to Savage.[33] Gap cells were also recorded but not considered for the evaluation of mutagenicity. MMS (4×10^{-5} M) was used as a positive control. Only well-spread metaphases were examined. One hundred fifty metaphases per culture were analyzed for the presence of CAs. The mitotic index (MI) was determined for 2000 cells and given as the number of mitoses per 100 cells (%).

2.12. Electrochemical Approaches. Electrochemical experiments (differential pulse voltammetry, DPV) were performed using an Autolab (Echo-Chemie, Utrecht, Netherlands) PGSTAT-20 or PGSTAT-30. The working electrode was a BAS (Bioanalytical Systems, West. Lafayette, IN) GC electrode of 3 mm diameter, the counter electrode was a platinum coil, and the reference electrode was Ag/AgCl, Cl^- (0.1 M), with all of them contained in a single-compartment electrochemical cell with 10 mL capacity. The glassy carbon electrode was polished with alumina on a polishing felt (BAS polishing kit). After mechanical cleaning, the electrochemical pretreatment of the glassy carbon electrode consisted of a sequence of five cyclic potential scans from 0 to +1.4 V, in acetate buffer, pH 4.5.

2.12.1. Preparation of the dsDNA-GC Biosensor. The electrochemical procedure for the investigation of nor-β-lapachone—dsDNA interaction involved three steps: preparation of the electrode surface, immobilization of dsDNA gel, and voltammetric transduction. The GC electrode was first polished with alumina, using a Metrohm felt-polishing pad, until the surface displayed a mirrorlike appearance. The electrode was then electrochemically pretreated with a sequence of five cyclic potential scans from 0 to +1.4 V versus Ag/AgCl, Cl^- (0.1 M) in acetate buffer, washed thoroughly with distilled/deionized water, dried, and placed in

an upright position in the cell. To immobilize the dsDNA (calf thymus, type 1), the surface of the electrode was coated with 10 μL of calf thymus DNA solution (containing 12.0 mg of dsDNA in 1.0 mL of acetate buffer). The gel was allowed to dry at room temperature under a stream of nitrogen, and the biosensor was subsequently immersed in 5 mL of aqueous acetate buffer. The quantity (0.36 mg) of dsDNA employed was estimated to be sufficient to cover the entire surface of the GC electrode. For each series of experiments, an identical dsDNA-GC electrode was prepared as a reference blank serving as a control. This electrode was not treated with substrate but received the same pre- and post-treatments as the test electrode. The procedure produced a thick-layer dsDNA-modified electrode. Since uniform coverage of the electrode surface had been achieved, any new peaks observed in the presence of additives were due solely to analyte interaction with the DNA film, without any contribution from the diffusion process in the solution.[34]

2.12.2. Interaction of dsDNA-GC Biosensor with Nor-β-lapachone. The biosensor was immersed in solutions of nor-β-lapachone (5, 10, and 20 μM) for 15 min, after which electrochemical measurements were carried out immediately. The same procedure was also applied to the biosensor immersed only in acetate buffer.

2.12.3. Interaction of ssDNA with Nor-β-lapachone and Doxorubicin. Single-stranded DNA (ssDNA) was prepared by dissolving 3.0 mg of dsDNA in 1.0 mL of HCl (1 M) and heating for 1 h until complete dissolution. This treatment was followed by neutralizing the solution with 1.0 mL of NaOH (1 M) and finally adding 9 mL of acetate buffer. Freshly prepared ssDNA solution was added to the cell, and single-scan DPV experiments were conducted in the range of 0 to +1.4 V vs Ag/AgCl, Cl^- (0.1 M). Two peaks corresponding to the oxidation of guanine and adenine appeared at potentials of +0.815 and +1.131 V, respectively. After the first run, the electrode was washed, polished, and returned to the ssDNA solution. To ensure reproducibility, this assay format was repeated at least three times, and the oxidation current and potential of the bases were very similar (RSD of 5%). For the analysis of nor-β-lapachone and ssDNA, a stock solution (1 mM) of the quinone was prepared in acetate buffer/ethanol (4:1) with an apparent pH of 4.5. Adequate amounts of the quinone were added to the electrochemical cell containing ssDNA to obtain concentrations in the range of 10−200 μM. DPVs were obtained in the potential range of 0−1.4 V, using ν = 10 mV s^{-1}, amplitude = 50 mV, pulse width of 70 ms, and scan rate of 5 mV s^{-1} [using a step potential (ΔE values) of 0.002 V]. For doxorubicin, similar conditions were used with concentrations in the range of 10−200 μM.

2.13. Statistical Analysis. All experiments were independently performed three times. For the comet assay, TBARS, and nitrite/nitrate analysis, data are presented as means ± SEMs and compared by analysis of variance (ANOVA) followed by Tukey's test. For induction of CAs, data are presented as means ± SEMs and were compared by ANOVA followed by Student's t test. The significance level was set at $p < 0.05$. GRAPHPAD program (Intuitive Software for Science, San Diego, CA) was used for all statistical analyses.

3. RESULTS

3.1. Cytotoxic Activity of Nor-β-lapachone. After 24 h of exposure, the test compound did not exhibit a significant cytotoxic effect in human lymphocytes (IC_{50} > 21.9 μM). Nor-β-lapachone showed weak cytotoxicity against V79 fibroblasts (IC_{50} = 13.41 μM) when compared to cultures treated with known chemotherapeutic agent doxorubicin (IC_{50} = 0.42 μM). Interestingly, GSH cotreatment abolished the even weak cytotoxicity of nor-β-lapachone in V79 cultured cells (Table 1 and Figure 2), suggesting a pronounced ROS contribution to the cytotoxic mechanism of nor-β-lapachone in this cell line. Corroborating our previous data,[14,16,17] nor-β-lapachone exhibited strong cytotoxicity in HL-60 cells after 24 h, but exogenous GSH-OEt added to leukemia cultures, in part, protected but did not abolish nor-β-lapachone's cytotoxicity (Table 1 and Figure 2).

3.2. Oxidative Stress Induced by Nor-β-lapachone. To determine the oxidative damage triggered by nor-β-lapachone, we examined the TBARS formation and the production of nitrite/nitrate as a result of NO release after exposure to it. As shown in Figures 3 and 4, after 4 h of exposure, nor-β-lapachone-treated V79 cells showed a significant ($p < 0.05$) increase in TBARS and nitrite/nitrate levels, respectively, which started at a concentration near the IC_{50} (13.41 μM), while the increased levels in oxidative stress for human cultured lymphocytes were observed only at the highest concentration (20 μM). For a period of 24 h, the increase in lipid peroxidation and nitrite/nitrate levels was observed in both cell types only at the highest concentrations (10 and 20 μM).

Table 1. Cytotoxicity of Nor-β-lapachone after 24 h of Exposure

	GSH-OEt[a]	lymphocytes	V79 cells	HL-60
nor-β-lapachone	−	>21.9	13.41 ± 0.56	1.89 ± 0.21
	+	>21.9	>21.9	8.43 ± 0.10
doxorubicin[b]	−	0.77 ± 0.11	0.42 ± 0.10	0.09 ± 0.01

[a] Ethyl ester of reduced glutathione (15 mM). [b] Positive control.

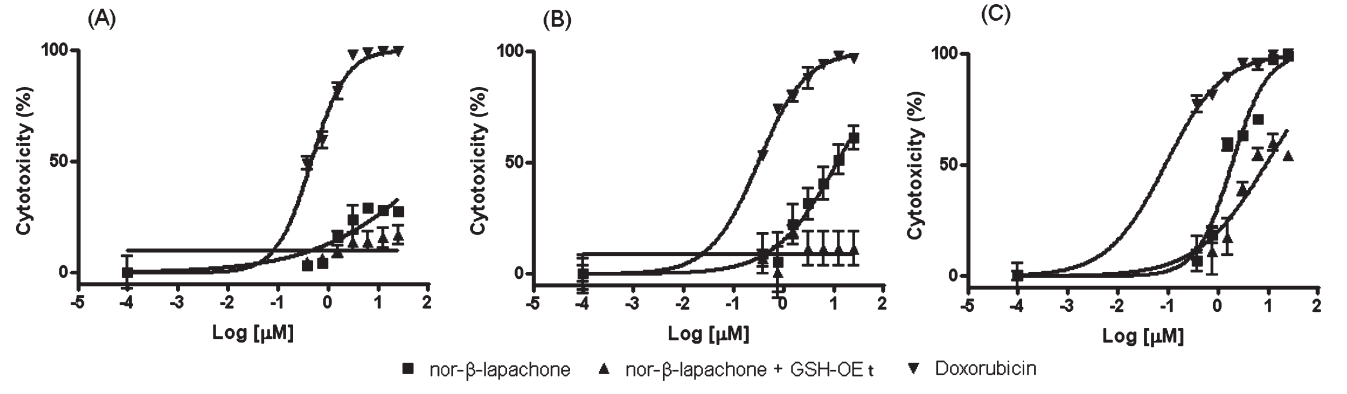

Figure 2. Concentration−response curve of nor-β-lapachone cytotoxicity (%) in the presence or absence of GSH-OEt (15 mM). Cells were treated for 24 h: human lymphocytes (A), V79 (B), and HL-60 (C). Doxorubicin (0.6 μM) was used as a positive control.

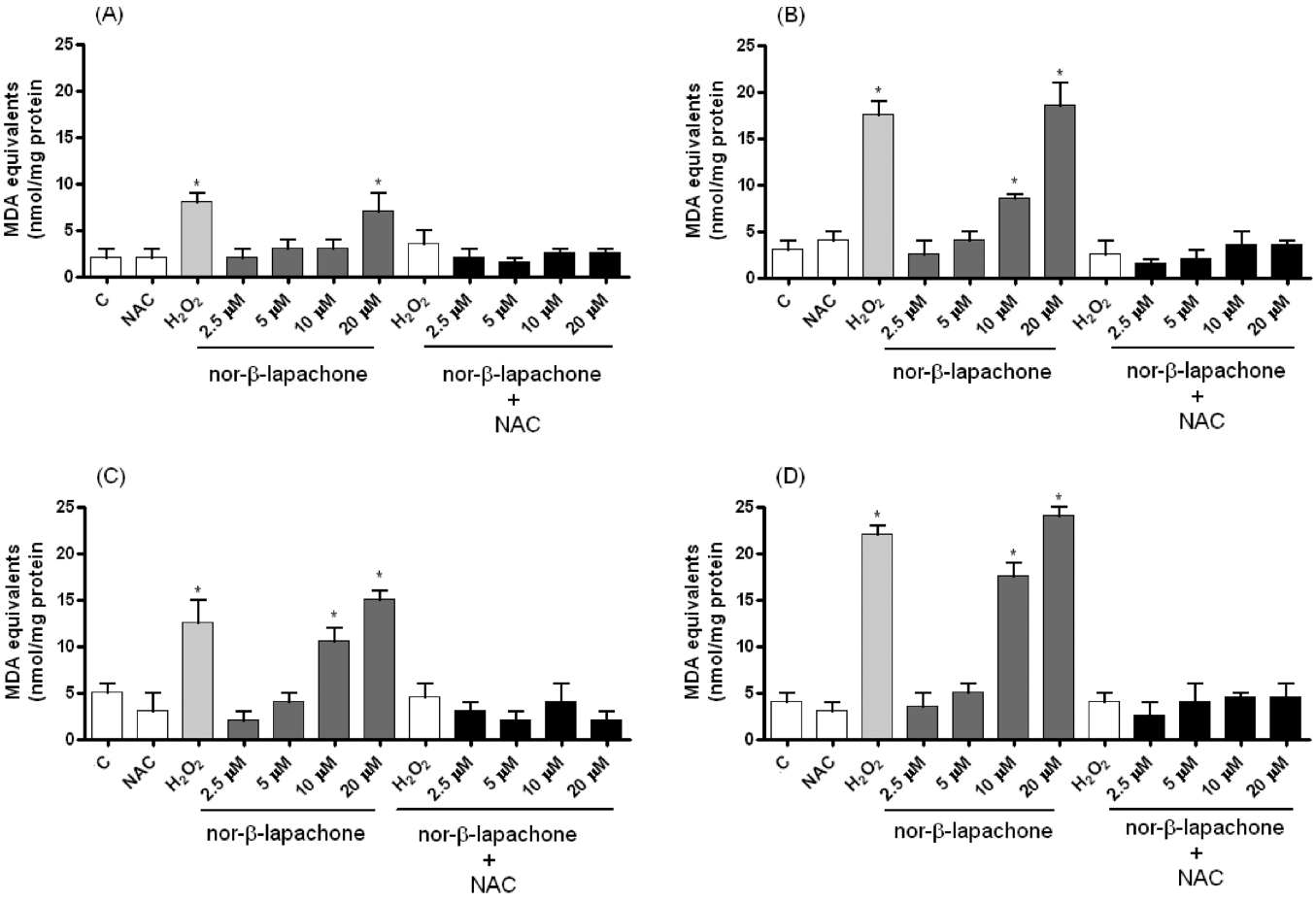

Figure 3. Determination of TBARS in cells after nor-β-lapachone treatment in the presence or absence of NAC (5 mM). Lymphocytes were treated for (A) 4 and (B) 24 h; V79 cells were treated for (C) 4 and (D) 24 h. Control (C) was treated with the vehicle (DMSO) used for diluting the test substance, and H_2O_2 (10 μM) was used as a positive control. *$p < 0.05$ as compared to control by ANOVA followed by Tukey's test. Data are presented as mean values ± SEMs for three independent experiments in triplicate.

3.3. Effect of Nor-β-lapachone on Intracellular Thiols. As seen in Table 2, nor-β-lapachone caused a statistically significant decrease in intracellular thiols levels after 4 h of incubation. The effect on total glutathione was more pronounced in V79 cultures, in which at both concentrations tested (10 and 20 μM), a reduction in GSH level followed by an increase in GSSG level, while in lymphocytes, a change in the balance of intracellular thiols was observed only at the highest concentration (20 μM). NAC preincubation followed by nor-β-lapachone treatment restored the GSH/GSSG ratio to control values due to a significant decrease in the GSSG level to reach the control value. NAC alone elevated GSH levels, keeping the ratio of GSH/GSSG at the control value.

3.4. DNA Damage Induced by Nor-β-lapachone. Table 3 shows the DNA damage induced by nor-β-lapachone tested in human lymphocytes and V79 cells. In both cell lines treated with nor-β-lapachone, an increased DNA damage was observed in the alkaline (pH >13) and neutral (pH 8.5) comet tests at the highest concentrations. No concentration-dependent increase in DNA damage index was noted for the test compounds for both cell lines. Lymphocytes treated with 2.5−10 μM nor-β-lapachone and V79 cells treated with 2.5−5 μM nor-β-lapachone did not show any significant DNA damage (single and double strand breaks). Increased DNA damage was observed only at the highest concentration (20 μM) for lymphocytes, while for V79 cells, the increase in the migration pattern of DNA started at 10 μM.

3.5. Electrochemical DNA Interaction Studies. The interaction between nor-β-lapachone and dsDNA was analyzed using thick film dsDNA biosensors in which undesirable binding of drug molecules to the electrode surface was prevented by completely covering the electrode surface with DNA.[34] Figure 5 displays the effects of nor-β-lapachone on dsDNA and ssDNA. In this case, like the behavior of β-lapachone,[35] there was no evidence of interaction with dsDNA (Figure 5A), by the absence of the diagnostic oxidation peaks of the nucleobases.[7,34] In terms of ss-DNA (Figure 5B), signals associated with oxidation of guanine (G) and adenine (A) (+0.83 and +1.16 V vs Ag/AgCl 0.1 M, respectively) in ssDNA were very intense and were kept at, practically, the same peak height. At the highest concentration of nor-β-lapachone (200 μM), a 10% decrease was demonstrated, which is indicative of a very weak interaction. Similar behavior was observed for β-lapachone, and these results suggest that these compounds do not damage DNA directly.[7,35,36] For comparison purposes, in literature reports, a positive interaction can be observed in the case of berenil[34] and some quinones, including adriamycin.[7,37,38] Doxorubicin (Dox), the positive control, showed an interaction with ssDNA, where there was a

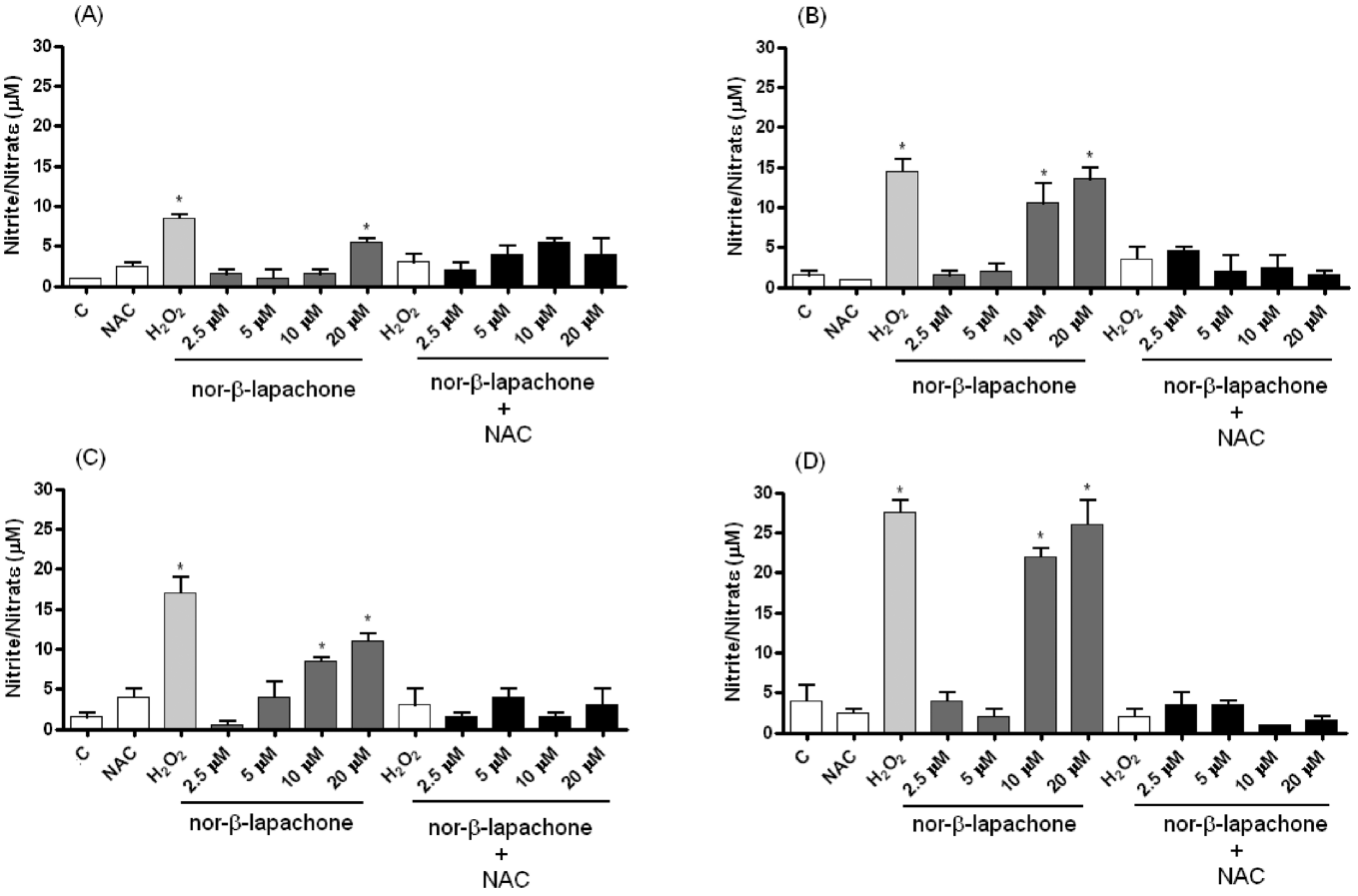

Figure 4. Nitrite−nitrate formation after treatment of cells with nor-β-lapachone in the presence or absence of NAC (5 mM). Lymphocytes were treated for (A) 4 and (B) 24 h; V79 cells were treated for (C) 4 and (D) 24 h. Control (C) was treated with the vehicle (DMSO) used for diluting the test substance. H_2O_2 (10 μM) was used as a positive control. *$p < 0.05$ as compared to control by ANOVA followed by Tukey's test. Data are presented as mean values ± SEMs for three independent experiments in triplicate.

Table 2. Effects of Nor-β-lapachone on Intracellular Thiols after 4 h of Exposure with or without NAC Pretreatment

		intracellular thiols (μg/mg protein)			
compds	treatment	total	GSH	GSSG	GSH/GSSG
		lymphocytes			
NC[a]		4.27 ± 0.05	3.05 ± 0.21	1.14 ± 0.05	2.52 ± 0.10
NAC[b]	5 mM	5.53 ± 0.16[c]	4.32 ± 0.15[c]	1.20 ± 0.21	3.79 ± 0.33[c]
nor-β-lapachone	10 μM	4.98 ± 0.01	3.61 ± 0.21	1.25 ± 0.01	2.63 ± 0.21
	20 μM	3.19 ± 0.25[c]	0.68 ± 0.01[c]	2.48 ± 0.40[c]	0.24 ± 0.03[c]
nor-β-lapachone plus NAC	10 μM	6.22 ± 0.10[c]	4.85 ± 0.75[c]	1.36 ± 0.01	3.95 ± 0.40[c]
	20 μM	6.07 ± 0.10[c]	4.65 ± 0.33[c]	1.32 ± 0.11	3.61 ± 0.10[c]
		V79 cells			
NC[a]		5.12 ± 0.19	3.14 ± 0.75	1.91 ± 0.56	1.61 ± 0.16
NAC[b]	5 mM	6.38 ± 1.16[c]	5.12 ± 0.81[c]	1.04 ± 0.33	5.23 ± 1.05[c]
nor-β-lapachone	10 μM	3.07 ± 0.21[c]	0.72 ± 0.01[c]	2.29 ± 0.15[c]	0.31 ± 0.02[c]
	20 μM	2.45 ± 0.10[c]	0.34 ± 0.10[c]	2.07 ± 0.01[c]	0.17 ± 0.01[c]
nor-β-lapachone plus NAC	10 μM	5.47 ± 0.15	3.98 ± 0.21	1.42 ± 0.51	2.83 ± 0.75[c]
	20 μM	4.97 ± 0.05	3.82 ± 0.17	1.15 ± 0.81	3.44 ± 0.25[c]

[a] Negative control was treated with the vehicle (0.1% DMSO) used for diluting the test substances. [b] NAC. [c] $p < 0.05$ as compared to control by ANOVA followed by Tukey's test. Data are presented as means ± SEMs for three independent experiments in triplicate.

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Table 3. Effects of Nor-β-lapachone on DNA Damage Index after 4 h of Exposure Using Alkaline and Neutral Versions of Comet Assay

		pH conditions	
		pH > 13	pH 8.5
compds	treatment	damage index ± SEM	damage index ± SEM
		lymphocytes	
NC[a]		6.64 ± 0.17	2.19 ± 0.11
doxorubicin[b]	0.6 μM	153.28 ± 6.33[c]	57.39 ± 3.15[c]
nor-β-lapachone	2.5 μM	5.08 ± 1.16	1.15 ± 0.10
	5 μM	8.24 ± 0.75	1.27 ± 0.33
	10 μM	6.15 ± 2.45	2.33 ± 0.55
	20 μM	23.84 ± 1.12[c]	10.33 ± 0.21[c]
		V79 cells	
NC[a]		12.33 ± 1.45	5.33 ± 1.45
doxorubicin[b]	0.6 μM	178.22 ± 4.50[c]	63.19 ± 2.75[c]
nor-β-lapachone	2.5 μM	9.33 ± 2.60	3.00 ± 1.15
	5 μM	13.33 ± 1.20	6.33 ± 0.88
	10 μM	48.33 ± 2.33[c]	23.66 ± 2.33[c]
	20 μM	71.37 ± 3.15[c]	29.82 ± 0.10[c]

[a] Negative control was treated with the vehicle (0.1% DMSO) used for diluting the test substances. [b] Positive control. [c] $p < 0.05$ as compared to control by ANOVA followed by Tukey's test. Data are presented as means ± SEMs for three independent experiments in triplicate.

substantial decrease in the oxidation waves of the bases guanine and adenine, as displayed in Figure 5C,D.

3.6. DNA Repair Enzymes Recognize Oxidative DNA Damage Induced by Nor-β-lapachone. Figure 6 shows mean DNA damage caused by nor-β-lapachone (20 μM after 4 h of exposure), expressed as DNA damage index after treatment with DNA repair enzymes ENDOIII and FPG. It can be noted that the postincubation with the enzymes clearly increased DNA migration of the positive control H_2O_2. It suggests that the enzyme activity was appropriate. Both enzymes were able to detect nor-β-lapachone-induced oxidative DNA damage. However, the results indicate that the extent of oxidative DNA damage caused by nor-β-lapachone, as recognized by ENDOIII and FPG in V79 cells, was significantly higher when compared to exposed lymphocytes.

3.7. Rejoining Kinetics of Nor-β-lapachone-Induced DNA Strand Breaks Is Cell-Specific. Figure 7 shows the rejoining kinetics of DNA breaks measured by the alkaline comet assay in human lymphocytes, V79, and HL-60 cells (for the purpose of comparison) treated with the highest concentration of nor-β-lapachone (20 μM). The kinetics of DNA rejoining was measured 1, 3, 6, 12, and 24 h after treatment. Comparing the kinetic curves obtained between the different cell lines, we observed that the rates of the repair of DNA lesions were higher for nontumor cells (especially for lymphocytes) than for HL-60 leukemia cells. After 6 h of rejoining time, almost 70% of DNA strand breaks were repaired in lymphocytes, and over 40% of DNA damages induced in V79 cells were rejoined. The percentages of DNA break rejoining after 12 and 24 h were very similar for lymphocytes (~80%) and V79 cells (~60%). In contrast, for HL-60 leukemia cells, only 28% of DNA strand breaks was repaired after 6 h of rejoining time, with a peak at 45% after 24 h of repair time.

3.8. Structural CAs Induced by Nor-β-lapachone. The induction of structural CAs and MI in human peripheral lymphocytes and V79 Chinese hamster cells by nor-β-lapachone is summarized in Tables 4 and 5, respectively. The percentages of aberrant metaphases and numbers of individual types of structural aberrations were statistically compared with those in untreated control. Data in Tables 4 and 5 show an increase in the percentages of aberrant metaphases and in the total numbers of aberrations in both human lymphocytes and V79 cells, only at the highest concentrations ($p < 0.05$). V79 cells exposed to nor-β-lapachone (10 and 20 μM) showed a higher number of structural aberrations when compared to lymphocyte cultures treated at the same concentrations. The nor-β-lapachone-treated V79 cells showed a significant decrease in proliferation ratio, as evidenced by a decrease in the MI at the highest concentrations (10 and 20 μM). On the other hand, only at the higher concentration (20 μM) was there an interference with lymphocyte proliferation.

3.9. Modulation of Thiol Content in Cytotoxicity and Genetic Toxicity Induced by Nor-β-lapachone. To understand the mechanism underlying the effects of ROS on the cytotoxicity and genetic toxicity induced by nor-β-lapachone (20 μM), concerning DNA damage, cell cultures were pretreated with NAC, a widely used thiol antioxidant that is a precursor of GSH,[39] or to BH, a nontoxic GSH-depleting agent.[40] As seen in Table 6, NAC had a positive effect in protecting both cell lines against damage to DNA molecule (strand breaks and mutations) as well as growth inhibition (mitotic index). In contrast, in GSH-depleted cell cultures, an increase in DNA breakage levels and frequency of CAs was observed. Also, a reduction in MI was observed.

4. DISCUSSION

During the last decades, a large number of natural and synthetic NQs (including nor-β-lapachone) and its derivatives have been extensively studied as antitumor,[14,16,41] antiparasitic,[42−45] and antimicrobial[46−48] compounds.

Toxicity represents an important obstacle in drug development; consequently, a balance between the therapeutic and the toxic effects of a given compound is important when determining its applicability in patients. To obtain information on nor-β-lapachone's cytotoxicity in vitro, the MTT test, which assesses the functional intactness of mitochondria based on the enzymatic reduction of a tetrazolium salt by the mitochondrial dehydrogenase of viable cells,[23,49] was applied. Studies with rat hepatocytes indicated that MTT reduction is predominantly an assessment of cytosolic NAD(P)/NAD(P)H redox balance.[50] Also, the level of MTT reduction is, therefore, directly related to the level of metabolic activity.[51] Thus, the decrease in cell viability indicated by MTT reduction assays, observed only in nor-β-lapachone-treated V79 (low activity) and HL-60 cells (strong activity) and not in cells after GSH-OEt coincubation [cotreatment could lower (in HL-60 cells) or even abolish (in V79 cells) the observed effects (Table 1)], may be expected to be a result of the amount of cytosolic NAD(P)H levels, in which the NAD(P)H formed reduces the MTT reagent to formazan, suggesting that the treatment can interfere in the cellular redox status.

The differences in the sensitivity of V79 cells and human lymphocytes to nor-β-lapachone, as demonstrated by the IC_{50} values (Table 1), appear to reflect differences in cell cycle times.

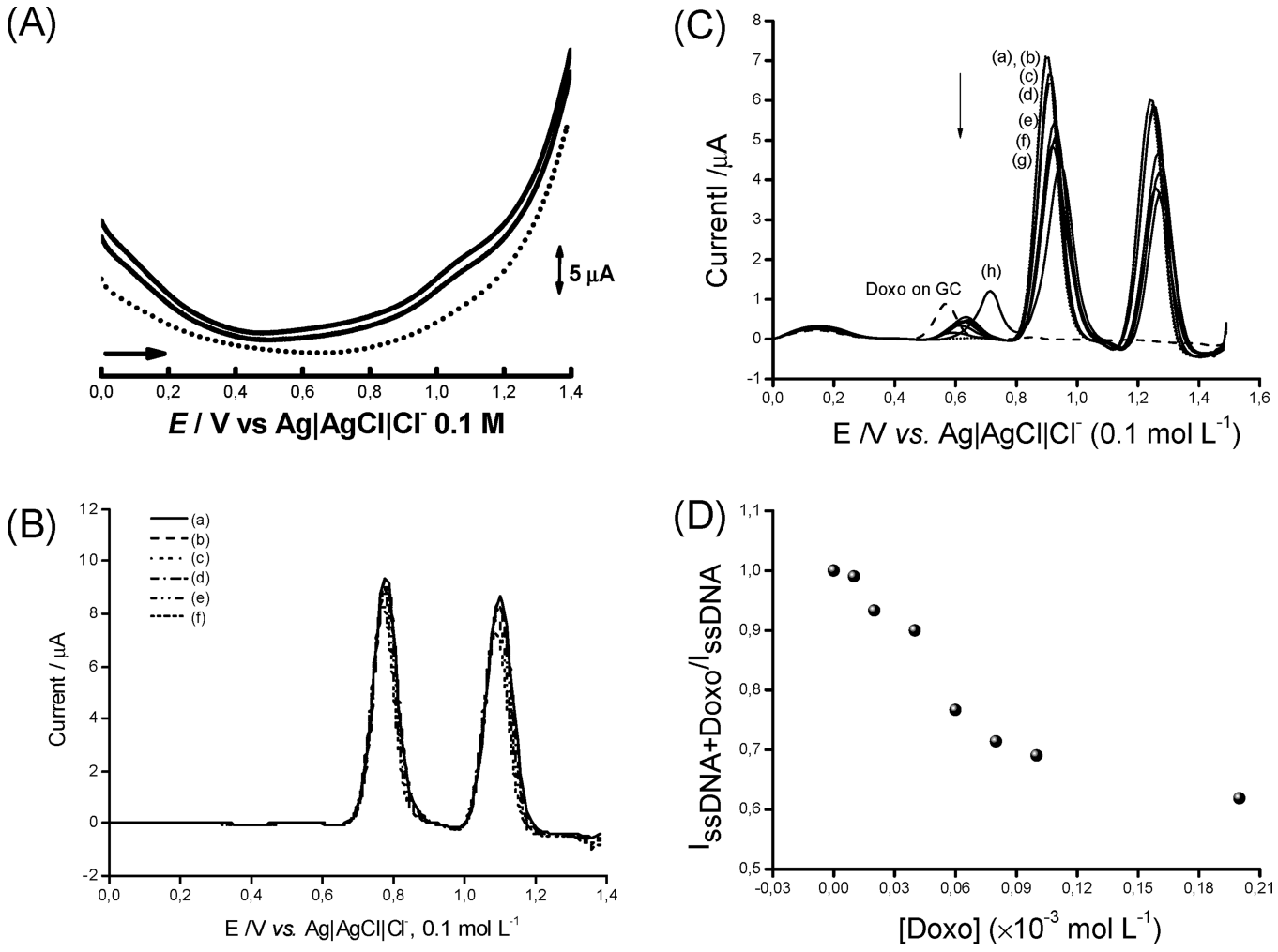

Figure 5. Differential pulse voltammograms (DPV) in aqueous ethanol (27%)—acetate buffer (0.1 M), pH 4.5. Glassy carbon electrode vs Ag|AgCl|Cl^- (0.1 M), $\nu = 0.010$ V s^{-1}. Pulse amplitude = 50 mV, and pulse width = 70 ms. (A) DPV of nor-β-lapachone, $c_1 = 0.2 \times 10^{-6}$ M, in aqueous acetate buffer (0.2 M; pH 4.5); ds-DNA (···), ds-DNA + nor-β-lapachone (—) (after 1 and 48 h of contact). The absences of the diagnostic peaks of guanine and adenine are indicative of absence of interaction. (B) Effects of increasing concentrations of nor-β-lapachone on ssDNA: 10 (b), 20 (c), 60 (d), 100 (e), and 200 μM (f) and (a) only ssDNA solution. (C) Effects of increasing concentrations of doxorubicin (positive control) on ssDNA: 10 (b), 20 (c), 40 (d), 60 (e), 80 (f), 100 (g), and 200 μM (e) and (a) only ssDNA solution. For comparison, cDox = 200 μM, in the absence of ssDNA. (D) Graphs of peak current of guanine oxidation in ssDNA, depending on the concentration of doxorubicin, showing a decrease in current, indicative of positive interaction.

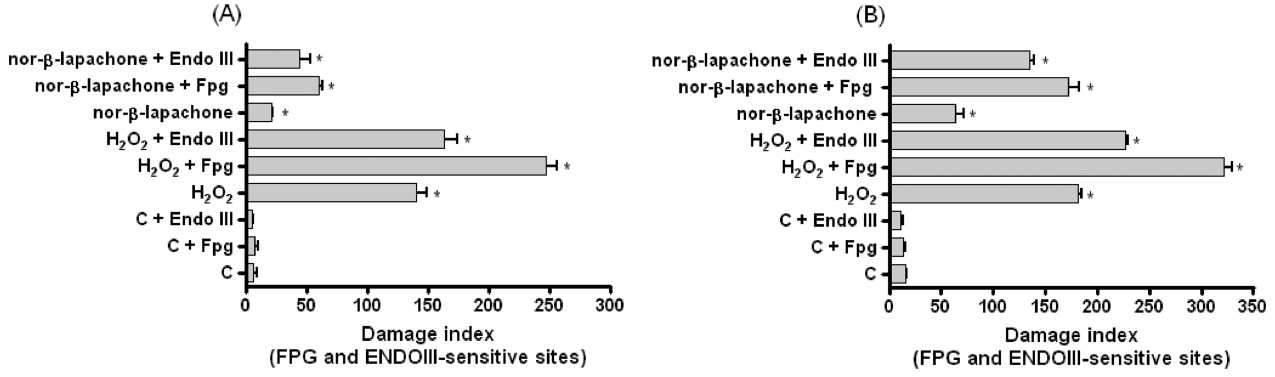

Figure 6. Effects of FPG and ENDOIII on nor-β-lapachone (10 μM)-induced oxidative DNA damage after 4 h of exposure. DNA damage was assessed by the alkaline version of the comet assay: lymphocytes (A) and V79 cells (B). Control (C) was treated with the vehicle (DMSO) used for diluting the test substance. H_2O_2 (10 μM; 5 min) was used as a positive control. Data are presented as mean values ± SEMs from three independent experiments in triplicate.

Most of mitosis seen in 48 h PHA-stimulated human lymphocytes in culture represents cells that have undergone one division, and most in the 72 h cultures have completed the second division,[52] while the doubling time for V79 cells is approximately 12 h.[53] Our data suggest that cytotoxicity may be cell-specific, and also, the lack of cytotoxic activity of nor-β-lapachone against lymphocytes is consistent with our previous reports,[14,16] where it was shown that nor-β-lapachone did not exert antiproliferative effects on PHA-stimulated human lymphocytes after 72 h of exposure.

The current study showed that in the presence of nor-β-lapachone the content of intracellular GSH significantly decreased in both human lymphocytes and V79 cells (Table 2).

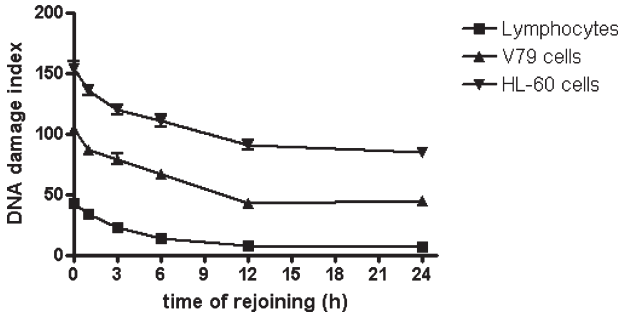

Figure 7. Rejoining kinetics of DNA strand breaks, assayed by the alkaline comet assay in human lymphocytes and V79 and HL-60 cells treated for 24 h with nor-β-lapachone (20 μM) and analyzed 1, 3, 6, 12, and 24 h after exposure. Data are presented as mean values ± SEMs from three independent experiments in triplicate.

The measured GSH pool size confirms a direct relation of cellular GSH content and cytotoxic activity of nor-β-lapachone. This could explain its pro-oxidant effects, as demonstrated before for β-lapachone.[54] Recently, we reported that nor-β-lapachone selectively inhibited the proliferation of several human and murine cancer cell lines.[16,17] In different human cancer cell lines (leukemia, colon, breast, and glioblastoma), nor-β-lapachone has been shown to cause cell death as a consequence of oxidative stress leading to DNA strand breaks and oxidized bases,[14] which is considered to be the most serious ROS-induced cellular modifications.[55] Corroborating the above-reported data, the increased levels of TBARS (Figure 3) and nitrite/nitrate (Figure 4) observed after treatment of cells (lymphocytes and V79 cells) suggest that ROS play an important role in nor-β-lapachone antiproliferative activities.

A characteristic of bioreductive compounds such as quinones is that they are designed to require reduction for activation to a cytotoxic species.[56−58] Several enzymes are known to be involved in the reduction of quinone compounds.[59] These include those catalyzing one-electron reduction to the semiquinone (i.e., NADPH: cytochrome P450 reductase and cytochrome *b*5 reductase) as well as NAD(P)H:quinone oxidoreductase-1 (NQO1), also known as DT-diaphorase, which carries out two-electron reduction[57] to the hydroquinone. However, it is not clear which metabolic pathway is responsible for the generation of the ultimate cytotoxic species.

There is minimal information regarding the metabolism of bioreductive agents by human NQO1.[60,61] NQO1 plays a prominent role in the detoxification of quinones, primarily because of its ability to reduce quinone substrates directly to

Table 4. Mitotic Index, Frequency of CAs, and Numeric Changes in Human Lymphocytes in Culture after Nor-β-lapachone Treatment

			mitotic index[c]		no. of aberrations[d]				aberrant cells[e]	
compds	treatment	exp.	%	mean ± SEM	G	CtR	ChR	E	%	mean ± SEM
DMSO[a]	0.1%	1	8.24	7.66 ± 0.29	3	1	0	0	0.60	0.40 ± 0.20
		2	7.46		2	0	0	0	0.0	
		3	7.28		2	1	0	0	0.60	
doxorubicin[b]	0.6 μM	1	4.12	3.52 ± 0.30[f]	5	13	11	3	12.66	13.21 ± 2.80[f]
		2	3.25		8	18	9	1	8.66	
		3	3.19		3	17	2	3	18.33	
lymphocytes	2.5 μM	1	7.63	7.41 ± 0.16	1	0	1	0	0.60	0.63 ± 0.37
		2	7.09		1	2	0	0	1.30	
		3	7.51		0	0	0	0	0.0	
	5 μM	1	7.60	7.22 ± 0.21	1	0	0	0	0.0	0.43 ± 0.43
		2	6.85		1	0	0	0	0.0	
		3	7.22		1	0	2	0	1.30	
	10 μM	1	6.44	6.65 ± 0.22	3	0	0	0	0.00	0.86 ± 0.43
		2	7.11		5	1	1	0	1.30	
		3	6.42		8	1	0	1	1.30	
	20 μM	1	4.37	3.9 ± 0.28[f]	4	12	3	0	8.66	12.88 ± 2.32[f]
		2	3.41		1	19	7	0	16.66	
		3	4.12		5	15	4	2	13.33	

[a] Vehicle (negative control). [b] Positive control. [c] Frequency per experiment, mean, and standard error of the mean in 2000 cells. [d] Number of aberrations per 150 metaphases analyzed. [e] Frequency per experiment, mean, and standard deviation of aberrant cells excluding gaps; G, gaps (chromosome and chromatid); CtR, chromatid ruptures; ChR, chromosome ruptures; and E, endoreduplication. [f] Data significant in relation to control group (vehicle) at $p < 0.05$/ANOVA followed by Student's *t* test.

Table 5. Mitotic Index, Frequency of CAs, and Numeric Changes in V79 Cells in Culture after Nor-β-lapachone Treatment

			mitotic index[c]		no. of aberrations[d]				aberrant cells[e]	
compds	treatment	exp.	%	mean ± SEM	G	CtR	ChR	E	%	mean ± SEM
DMSO[a]	0.1%	1	7.40	7.17 ± 0.50	2	0	0	1	0.66	1.10 ± 0.22
		2	6.22		0	0	1	1	1.33	
		3	7.91		0	1	1	0	1.33	
doxorubicin[b]	0.6 μM	1	2.11	2.31 ± 0.21[f]	3	19	12	0	13.66	13.55 ± 1.82[f]
		2	2.75		5	14	8	0	16.66	
		3	2.08		2	11	10	0	10.33	
V79 cells	2.5 μM	1	7.04	7.22 ± 0.11	0	2	1	0	1.33	1.10 ± 0.22
		2	7.20		0	0	1	0	0.66	
		3	7.43		3	1	1	0	1.33	
	5 μM	1	6.24	6.02 ± 0.16	1	3	0	0	2.00	1.77 ± 0.22
		2	5.71		0	1	0	1	1.33	
		3	6.12		1	1	2	0	2.00	
	10 μM	1	4.81	4.32 ± 0.30[f]	8	7	9	1	6.60	8.42 ± 1.47[f]
		2	4.39		11	14	3	1	7.33	
		3	3.77		6	12	6	0	11.33	
	20 μM	1	1.23	2.10 ± 0.44[f]	5	15	7	0	15.33	12.99 ± 1.71[f]
		2	2.46		9	16	5	0	9.66	
		3	2.63		13	14	10	0	14.00	

[a] Vehicle (negative control). [b] Positive control. [c] Frequency per experiment, mean, and standard error of the mean in 2000 cells. [d] Number of aberrations per 150 metaphases analyzed. [e] Frequency per experiment, mean, and standard deviation of aberrant cells excluding gaps; G, gaps (chromosome and chromatid); CtR, chromatid ruptures; ChR, chromosome ruptures; and E, endoreduplication. [f] Data significant in relation to control group (vehicle) at $p < 0.05$/ANOVA followed by Student's t test.

Table 6. Effects of Pretreatment of NAC or BH on DNA Damage, CAs Induction, and Cell Proliferation after 24 h of Exposure to Nor-β-lapachone (20 μM)

	Comet assay (pH >13)	CAs test	
treatment	damage index ± SEM	% aberrant cells ± SEM	% mitotic index ± SEM
	lymphocytes		
NC[a]	17.43 ± 3.25	1.20 ± 0.18	5.86 ± 0.18
H_2O_2[b]	208.19 ± 1.15[e]	5.25 ± 0.21[e]	2.98 ± 0.01[e]
H_2O_2 plus NAC[c]	21.54 ± 2.70	0.51 ± 0.50	6.12 ± 0.10
H_2O_2 plus BH[d]	257.73 ± 5.33[e]	13.19 ± 1.75[e]	1.35 ± 0.45[e]
nor-β-lapachone	45.19 ± 0.21[e]	16.54 ± 0.20[e]	2.75 ± 0.15[e]
nor-β-lapachone plus NAC	12.07 ± 1.25	1.10 ± 0.01	6.87 ± 0.33
nor-β-lapachone plus BH	103.28 ± 0.56[e]	23.55 ± 0.75[e]	1.08 ± 0.10[e]
	V79 cells		
NC[a]	9.14 ± 0.10	0.8 ± 0.10	6.23 ± 1.15
H_2O_2[b]	193.08 ± 0.10[e]	9.91 ± 2.05[e]	2.17 ± 0.25[e]
H_2O_2 plus NAC[c]	13.45 ± 0.75	1.14 ± 0.30	6.82 ± 0.10
H_2O_2 plus BH[d]	273.27 ± 4.15[e]	15.61 ± 2.25[e]	1.10 ± 0.20[e]
nor-β-lapachone	105.73 ± 2.45[e]	19.60 ± 1.08[e]	2.15 ± 0.11
nor-β-lapachone plus NAC	15.35 ± 0.17	1.25 ± 0.11	6.82 ± 0.22
nor-β-lapachone plus BH	147.18 ± 2.56[e]	34.05 ± 1.15[e]	1.08 ± 0.56[e]

[a] Negative control was treated with the vehicle (0.1% DMSO) used for diluting the test substances. [b] Positive control (10 μM). [c] NAC (5 mM). [d] BH (50 μM). [e] Data significant in relation to control group (vehicle) at $p < 0.05$/ANOVA followed by Student's t test.

their hydroquinone derivatives, which can then be conjugated and excreted.[62] Two-electron reduction directly to the hydroquinone bypasses the formation of redox-cycling semiquinones and the generation of ROS.[63] On the other hand, reoxidation of either the semiquinone or the hydroquinone can lead to the production of ROS and ROS-mediated DNA fragmentation.[64,65]

High levels of NQO1 gene expression have been observed in liver, lung, colon, and breast tumors as compared to normal tissues of the same origin.[66] Fitzsimmons et al.[67] reported that reductase enzyme expression is heterogeneous across human tumor cell lines (NCI tumor cell line panel), and tissue-specific patterns of expression are apparent. According to them, cancer cell lines originating from lung, colon, central nervous system, breast, prostate, melanoma, and renal groups have an elevated NQO1 activity. While the overall level of NADPH:cytochrome P-450-reductase activity was lower than that of NQO1. Regarding leukemia cell lines, NQO1 activity was not detected in some, namely, HL-60 and MOLT-4.[67,68]

Interestingly, as shown in our recent studies,[14,16] human cancer cell lines with no expression of NQO1 or those expressing different levels of this enzyme were greatly sensitized by nor-β-lapachone. Also, the nontumor cells used in our present work have different histopathological origins and different levels of metabolizing enzymes. In relation to human cells, polymorphisms in the NQO1 gene were detected in human populations, correlating with low NQO1 activity,[69,70] and showed that human bone marrow mononuclear and progenitor cells fail to express NQO1 protein. Moreover, Chinese hamster lung V79 cells lack endogenous cytochrome P450 activity[71,72] but have normal levels of NQO1 expression.[73] These data suggest that more than one enzyme may be important in reductive activation of nor-β-lapachone.

The low selectivity of most current anticancer agents makes the search for new molecules with more selective action and few side effects indispensable and challenging. Doxorubicin, used here as a positive control, is a well-known chemotherapeutic agent, which is cytotoxic to several cancer cell lines[14,16] and also to human lymphocytes and V79 cells (Table 1). As expected in the cancer chemotherapy regimens, cells with high proliferative rates are often more susceptible to antineoplastic drugs. Data from our current study showed that nor-β-lapachone elicited a significant antiproliferative effect in human promyelocytic HL-60 leukemia cells with an IC_{50} value below 2 μM after 24 h of exposure. HL-60 cells and V79 fibroblasts have similar doubling times ($\sim$15 h);[74] on the other hand, these leukemia cells were almost 8-fold more sensitive to nor-β-lapachone than were V79 cells. Thus, we suggest that the ability of nor-β-lapachone to kill rapidly proliferating cancer cells without effects on lymphocyte proliferation makes this semisynthetic NQ, in general, a promising candidate as a prototype for drug development for the treatment of pathologically activated lymphocytes such as those in acute lymphoid leukemia or other cancer types.

Many cancer chemotherapeutic agents, such as ionizing radiation, and DNA-damaging chemotherapeutic compounds (e.g., doxorubicin) cause cell death by causing the formation of DNA double-strand breaks (DSBs).[75,76] DSBs can occur from endogenously produced ROS or conversion of single-strand breaks (SSBs) to DSBs by advancing replication forks.[77] Although cells maintain the capability to survive low levels of DNA damage, as little as one nonrepaired DSB can be lethal.[78] The response to stress turns on a complex network of molecular interactions including alteration of mRNA levels of various genes leading to activation or repression of pathways involved in repair, survival, and/or cell death.[79]

The alkaline version of the comet assay (standard protocol) is a sensitive procedure to quantify different types of DNA damage in cells, which include alkali-labile sites, SSBs, and DSBs.[80] Our data show that the exposure of lymphocytes or V79 cells to nor-β-lapachone concentrations up to 5 μM did not induce any significant increase in the pattern of DNA migration (DNA breaks). The standard comet assay showed a significant increase in DNA strand breaks in lymphocytes and V79 cells only at the highest concentrations (10 or 20 μM) of nor-β-lapachone. Moreover, our electrochemical experiments revealed that nor-β-lapachone does not damage DNA directly (Figure 5) at concentrations similar to those used in the comet and CAs tests. Nor-β-lapachone displayed very weak direct DNA interaction only at the highest concentration (200 μM), while some DNA lesions were detected as DSBs, as measured by the neutral comet assay protocol (Table 3). Interestingly, the levels of DNA breakage after nor-β-lapachone exposure were more pronounced in V79 cells than lymphocytes and were observed in cultures treated with the half genotoxic concentration in lymphocytes. By employing FPG and ENDOIII proteins, we found that nor-β-lapachone promoted an important additional increase in DNA strand breaks, suggesting that these breaks are caused by ROS (Figure 5). Indeed, it has been shown that NQs are able to produce adducts with DNA, which may lead to abasic sites, SSBs, or DSBs.[81,82] As recently published by our group, ROS generated by nor-β-lapachone (5 μM) were able to oxidize purine and pyrimidine bases such as 8-oxo-7,8-dihydroguanine and thymine glycol, respectively, and to damage DNA indirectly, triggering apoptosis in several human cancer cell lines at a concentration several times lower than that used in the present study.[14]

Proliferating lymphocytes repaired DNA lesions induced by oxygen radicals more rapidly than quiescent lymphocytes, which are consistent with the evidence that a number of DNA repair enzymes have greater activity in proliferating cells than in resting ones.[83] The statistically significant elevation in structural chromosomal changes appeared in lymphocytes at twice the nor-β-lapachone concentration than in V79 fibroblast cells (Tables 4 and 5). Despite the different sensitivity of the cells used in our experiments, this discrepancy may be related to the different repair systems of these two cell types used in the present study (Figure 7). Additionally, it has been reported that differences in cell cycle effects with regard to cytotoxicity may play a role in sensitivity to micronucleus (MN) formation in some cell types.[84] Therefore, the likelihood of cell cycle kinetic differences in the two cell lines contributing to the differential sensitivity of DNA strand breaks and CAs formation in lymphocytes and V79 cells can be supported by the present study.

Recently, Honma and Hayashi[85] reported that p53 status did not seriously affect the test outcome of several known chemicals (mutagens, clastogens, and spindle poisons) in the MN test, although p53-deficient cells had higher MN frequencies than p53-competent cells. The high frequency of positive results in the nor-β-lapachone may be due to the fact that V79 cells carry a p53 mutation.[86] After genotoxic insult, cells deficient in functional p53 protein fail to repair the DNA damage, resulting in the accumulation of CAs and gene mutations,[87] some of which may not be biologically relevant considering human hazard or risk. Corroborating with p53 mutation status, nor-β-lapachone (5 μM) induces DNA damage and apoptosis in human leukemia HL-60 cells,[14] which are p53 null,[88] as well as in other human cancer cell lines,[14] which carry a p53 mutation,[89] showing that nor-β-lapachone-induced cytotoxicity and genetic toxicity are p53-independent.

In agreement with our comet assay data, nor-β-lapachone at the lowest concentrations (2.5 and 5 μM) failed to induce CAs in human lymphocytes and V79 fibroblasts. Cytogenetic

abnormalities were observed only at the highest concentrations (10 or 20 μM). A detailed analysis of structural CAs showed that chromatid type aberrations prevailed. This result suggests S-phase dependence of nor-β-lapachone. It is well-known that the type of aberrations induced by genotoxic agents is cell cycle dependent. Most of the chemically induced aberrations are formed only during the DNA synthesis phase (probably due to misreplication). Such chemical agents induce mainly chromatid type aberrations and are also very efficient in inducing sister chromatid exchanges.[90]

If GSH is involved in the pro-oxidant properties of nor-β-lapachone, pretreatment with compounds that stimulate an increase or a decrease in cellular GSH may modulate its effects. Therefore, insights into the ROS contribution to the genotoxicity and mutagenicity of nor-β-lapachone against lymphocytes and V79 cells were obtained by the addition of a nontoxic concentration of BH (50 μM), which depletes GSH content,[40] and by using NAC (5 mM), which protects cells by promoting GSH synthesis, and by its ROS scavenger ability.[39] As shown in Table 6, in both nontumor cell lines, NAC had a strong protective effect against nor-β-lapachone-induced oxidative DNA breakage, as measured by the alkaline comet assay and prevented induced mutations, as verified by the lower rate of CAs. In contrast, in GSH-depleted cell cultures (lymphocytes and V79 cells), an increase in the levels of DNA strand breaks and in the frequency of aberrant cells was noted. The participation of ROS in the genetic toxicity of nor-β-lapachone is in agreement with a recent study[14] in which we reported that nor-β-lapachone cytotoxicity against human cancer cell lines is mediated by ROS production.

In summary, the biological effects of nor-β-lapachone can be ascribed to its ability to deplete GSH, which leads to a pro-oxidant cellular status contributing to its antiproliferative properties. By increasing intracellular GSH content, NAC pretreatment can prevent these effects. It is important to emphasize that nor-β-lapachone showed a weak in vitro cytotoxic activity in nontumor cells with genotoxic and mutagenic effects appearing only at concentrations several times higher than that needed to exert antiproliferative effects on cancer cells.

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ABBREVIATIONS

BH, 1-bromoheptane; CAs, chromosomal aberrations; DMEM, Dulbecco's modified Eagle's medium; DSBs, DNA double-strand breaks; DPV, differential pulse voltammetry; DTNB, 5,5′-dithionitrobenzoic acid; ENDOIII, endonuclease III; FGP, formamidopyrimidine DNA-glycosylase; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; NO, nitric oxide; NQs, naphthoquinones; PHA, phytohemagglutinin; ROS, reactive oxygen species; SSBs, single-strand breaks; TBA, thiobarbituric acid; TBARS, thiobarbituric reactive species; TNB, 5-thio-2-nitrobenzoate.

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