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ORIGINAL ARTICLE

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Novel mechanism of cellular DNA topoisomerase II inhibition by the pyranonaphthoquinone derivatives α-lapachone and β-lapachone

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Abstract *Purpose*: The mechanisms of intracellular topoisomerase II inhibition by the pyranonaphthoquinone derivatives α-lapachone and β-lapachone were studied. *Methods*: Cell-based mechanistic studies were designed based on the in vitro mechanisms [17] and primarily involved the use of cultured KB (nasopharyngeal tumor cells) cells and the etoposide-resistant sub-line KB-7d. *Results*: The KB-7d cells exhibited collateral sensitivity to α-lapachone; this supports the possibility of catalytic inhibition of topoisomerase II in the cells. Interestingly, both compounds induced an increase (two- to threefold) in reversible double-stranded DNA breaks in cell lines with a reduced expression of topoisomerase II. However, these drug-induced DNA breaks became irreversible at treatment times greater than 1 h. Studies showed that DNA breaks in KB-7d cells were not caused by endonucleases. Use of antioxidants abolished the appearance of cellular DNA breaks; this suggests involvement of the oxidation–reduction cycle of pyranonaphthoquinones in topoisomerase II inhibition; however, irreversible DNA breaks were not a result of drug-induced oxidative stress. *Conclusions*: On the basis of the findings, it is proposed that the compounds, on longer incubation with cells, induce abortive dissociation of topoisomerase II from the DNA, leading to an irreversible accumulation of high molecular weight DNA fragments. In addition to establishing topoisomerase II as an intracellular target of α-lapachone, the results suggest that both compounds can be classified as neither typical poisons nor as typical catalytic inhibitors of the enzyme. In summary, both compounds are members of a new inhibitor class, and α-lapachone, in particular, can be considered a potential lead for the development of drugs to treat multidrug-resistant cell lines with lower expression of topoisomerase II.

Key words Catalytic inhibitors · DNA topoisomerase II · Naphthoquinones

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Introduction

Type II DNA topoisomerases are essential enzymes that resolve topological problems of DNA during replication, recombination, transcription and chromosome segregation [31]. Inhibitors of the enzyme are classified as ''poisons'' and ''catalytic inhibitors''. In cells, formation of reversible protein-linked DNA complexes is a marker for stabilization of topoisomerase II–DNA covalent complexes by enzyme poisons [2]. Study of catalytic inhibition in mammalian cells is more complicated and the only widely applicable method is limited since it involves an induced enzyme in a virus-infected cell system [26]. Knowledge of in vitro mechanisms of inhibition can therefore help the design and interpretation of cell-based experiments.

The in vitro mechanisms of irreversible catalytic inhibition of topoisomerase II by β-lapachone and α-lapachone (Fig. 1) were recently reported [17]. β-Lapachone induced the enzyme to re-ligate the DNA breaks in the presence of ATP, and dissociated the enzyme from the DNA. α-Lapachone inhibited initial non-covalent binding of topoisomerase II to DNA and, in addition, induced re-ligation of DNA breaks before dissociating the enzyme from the DNA. This report is on a continuation of the mechanistic studies but with a focus on cell-based experiments.

The most common form of drug resistance is due to decreased accumulation of cytotoxic compounds in the cells and is mediated by alterations in the expression of drug efflux pumps including P-glycoprotein and the multidrug-resistance-related protein, MRP [27]. Alterations other than membrane-associated drug transport can also confer a multidrug-resistant (MDR) phenotype.

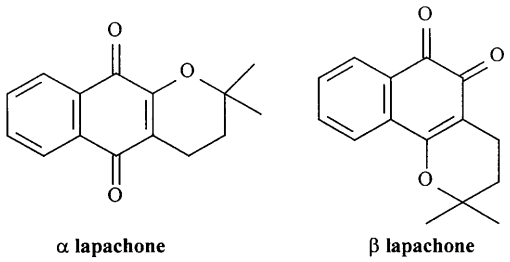


Fig. 1 Structures of the pyranonaphthoquinone derivatives α-lapachone and β-lapachone

Another form of MDR is associated with reduced expression or activity of topoisomerase II, and is termed atypical MDR (at-MDR). Reduction in nuclear topoisomerase II levels confers resistance to enzyme poisons due to a decreased amount of enzyme that can be trapped in ternary complexes. However, such cell lines are expected to exhibit collateral sensitivity to catalytic inhibitors [7, 32]. For example, at-MDR cell lines were not cross-resistant to the bis-dioxopiperazine derivative, ICRF-159, but they displayed hypersensitivity [4]. This concept is utilized in the present work and the majority of intracellular mechanistic studies reported here utilize KB-7d, an MDR sub-line of KB (nasopharyngeal tumor cells) with reduced levels of topoisomerase II. Interestingly, a recent study demonstrated that β-lapachone and related 1,2-naphthoquinones were active against fourteen diverse tumor-drug-resistant cell lines; however, none exhibited collateral sensitivity to the agents [6].

Due to the presence of a quinone moiety, the intracellular effects of β-lapachone have been attributed to hydrogen peroxide and free radical production [3, 5, 11]. α-Lapachone being a para-quinone was reported to have lower activities as either an anti-tumor or an anti-parasitic agent presumably due to a decreased production of reactive oxygen species [25]. The redox-cycling properties of pyranonaphthoquinones and the resulting oxidation of thiol enzymes is also one of the proposed mechanisms of cellular toxicity [21, 25]. This increased redox cycling by ortho-quinones as compared to para-quinones was clearly differentiated by Neder et al. in experiments demonstrating reactivity of pyranonaphthoquinones with 2-mercaptoethanol (a biomimetic model of thiol enzymes) [22].

In addition, β-lapachone can also induce apoptosis in prostrate cancer cell lines, breast cancer cells and human leukemia cell lines [19, 24, 33]. An effect of the drug on topoisomerase I/II or any other enzyme as a cause for apoptosis has not yet been demonstrated. So, the molecular mechanism(s) leading to induction of apoptosis remains unknown although Wuerzberger et al. speculated that a direct activation of proteases in MCF-7 cells was involved, through a non-nuclear signaling mechanism [33].

In this report, the intracellular effects of α-lapachone and β-lapachone have been studied with respect to inhibition of topoisomerase II. In the course of the work, some aspects of free radical formation, oxidative inactivation of thiol-enzymes and induction of apoptosis by pyranonaphthoquinones have also been addressed. Based on current findings, α-lapachone acts as a novel catalytic inhibitor of cellular topoisomerase II and is a potential lead molecule for developing drugs against MDR tumors with lower levels of the enzyme.

Materials and methods

The cell lines used in this study include KB cells (nasopharyngeal tumor cells) and the KB-7d sub-clone (a pleiotropic multidrug-resistant cell line resistant to topoisomerase II poisons) [8]. Some experiments used SV28, baby hamster kidney cells transformed with the SV40 virus, and two sub-lines SV-20ER and SV-V5ER, developed for resistance to etoposide, with twofold reduction and undetectable levels of topoisomerase II α-isoform, respectively [12]. All cell lines were propagated in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 100 μg/ml kanamycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. α-Lapachone and β-lapachone were synthesized from lapachol according to the methods published by Hooker [14]. Etoposide was generously provided by the Natural Products Laboratory, UNC-CH. 9-Ethoxycarbonyl berberine was a generous gift of Dr. K. Iwasa (Kobe Pharmaceutical University, Japan) [15]. The calcium ionophore (A23187) and α-tocopherol were purchased from Sigma (St. Louis, Mo.). The pBR322 DNA was prepared by standard techniques. All other chemicals were reagent grade.

Clonogenic assay

Clonogenic survival assays are based on the colony-forming ability of cells after short treatment with the test compounds α-lapachone, β-lapachone and etoposide [1]. Ten thousand cells were treated with the compounds for 90 min. The samples were then diluted to concentrations below inhibitory levels. A fraction of the cells were plated in 12-well plates and incubated for 10 days, at the end of which colonies were fixed and stained with 0.8% (w/v) crystal violet in 50% (v/v) ethanol, and counted by eye. Over the course of the experiments, plating efficiency of untreated KB or KB-7d cells was similar at $40 \pm 4\%$ ($n = 3$). The concentration of compounds that inhibited colony formation by 25 and 50%, the LD_{25} and LD_{50} values, respectively, were interpolated from the dose–response graphs.

Quantitative estimation of intracellular covalent protein/DNA cross-links

KB cells were seeded at 10^4 cells/cm^2 and labeled with [^{3}H] thymidine (1 μCi/ml; 60 Ci/mmol) for 24 h. At 1 h prior to drug treatment, the label was removed, and the cells were then washed with PBS (phosphate-buffered saline) and incubated in fresh medium. The cells were treated with compounds, alone, or in combination with etoposide for 3 h. Treatment schedules included (a) co-treatment of cells with α-lapachone/β-lapachone and etoposide; (b) pretreatment of cells with α-lapachone/β-lapachone for 1 h followed by etoposide; (c) post-treatment of etoposide-treated cells with α-lapachone/β-lapachone after 1 h. After the drug treatments, the protein–DNA complexes were collected by the method described by Caldecott et al. [2]. The protein–DNA complexes were dissolved in 200 μl water and added to 2 ml of scintillant (Ecoscint H, National Diagnostics). Radioactivity was measured with a TRI-CARB 2100 liquid scintillation analyzer with 64% counting efficiency. Results were analyzed by a two-tailed Student's t test with a software package from GraphPad Prism (San Diego, Calif.).

Gel lysis assay for detection of cellular DNA breaks

The gel lysis method detects genomic DNA breaks on the basis of the mobility of fragmented DNA compared to that of the chromosomal DNA that remains in the plug at the origin of electrophoresis. KB cells were plated at 10^4 cells/cm^2 overnight and treated with the test compounds for the indicated periods of time. In experiments that used an antioxidant or an endonuclease inhibitor, 0.1 mM of α-tocopherol or zinc chloride, respectively, the cells were pretreated for 1 h prior to the addition of test compounds. Reversibility studies were carried out by incubating the treated cells in pre-warmed drug-free medium for 30 min before harvesting the cells for plug preparation and analysis. The effect of treatment on genomic DNA was analyzed according to methods described by Bastow et al. [1]. Briefly, cells were scraped into ice-cold PBS and harvested by centrifugation. Washed cells were re-suspended in 80 μl of a 37 °C gel solution [1% (w/v) low melting point agarose in PBS], and were cast in a mold and refrigerated to form agarose plugs. Plugs of cells were incubated in lysis-digestion buffer (0.4 M EDTA, 0.01 M Tris-HCl, pH 8.0, 1% (w/v) *N*-lauryl-sarcosine and 100 μg/ml proteinase K) at 50 °C overnight. The treated plugs were equilibrated in TBE buffer and placed against a gel comb in a horizontal gel casting tray. Electrophoresis was at 3.5 V/cm (measured between electrodes) for 16 h at 4 °C. The gel was stained with 1 μg/ml ethidium bromide, treated with RNase (1 μg/ml) and photographed with Polaroid film (Type 667) under UV illumination. Intensities of DNA breaks were also analyzed with a STORM phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) operating in the blue fluorescence mode and the supplied ImageQuant software according to the manufacturer's instructions.

Statistical analysis

The relative amounts of double-stranded DNA were compared by measuring fluorescence intensity in each sample lane around the region marked as "DS DNA fragments" (DS, double-stranded) in representative results shown in Figs. 4, 5, and 7. Since the HMW (high molecular weight) smear above these DNA fragments was not included in the analyses, the level of double-stranded DNA breaks in drug-treated cells was underestimated in graphed data summarizing the overall results. Within each experiment the fluorescence intensity of double-stranded DNA breaks from the untreated control was subtracted from the values obtained from the drug-treated samples. The results were plotted and analyzed by a two-tailed Student's *t* test with a software package from GraphPad Prism (San Diego, Calif.).

Detection of endonuclease activity in cell-free extracts

The assay is based on the recent work of Walker et al. [30]. Briefly, KB-7d cells were treated with the test compounds in the presence or absence of a 0.1-mM zinc chloride pretreatment. One hour later, cells were harvested and resuspended in a buffer containing 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl. After microcentrifugation for 15 min, the low salt concentration cell extract (the supernatant) was collected for a plasmid DNA digestion assay. A portion of the supernatant (5–10 μg of protein) was resuspended in 25 μl of 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 0.2 mM $CaCl_2$ and 2 μg plasmid pBR322 for 20 min at 37 °C. The reaction was stopped by the addition of 4 μl of 10× DNA loading buffer (40% w/v sucrose, 0.01% bromophenol blue, 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA). The integrity of the plasmid DNA was analyzed by loading of the reaction mixture on a 0.8% agarose gel, followed by electrophoresis at 1.0 V/cm (measured between electrodes) for 16 h at room temperature. The gel was stained with 0.5 μg/ml ethidium bromide and photographed with Polaroid Type 667 film under UV illumination.

Results

Clonogenic assay

KB cells and the multidrug-resistant sub-line KB-7d were treated with α-lapachone and β-lapachone for 90 min and the surviving cells were measured as macroscopic colonies after 10 days in culture. The calculated LD_{50} and LD_{25} values are shown in Table 1. Figure 2 is a pictorial result clearly showing the differential cytotoxic activity of α-lapachone against KB cells versus KB-7d. Both pyranonaphthoquinones were cytotoxic over a very narrow dose range (data not shown), in agreement with the report of Dolan et al. [6]. As expected, KB-7d cells were more than 40-fold resistant to etoposide. β-Lapachone was equally active against both cell lines but the LD_{50} values were more than tenfold lower than the concentration at which topoisomerase II was inhibited with in vitro enzyme assays (87–700 μM range [17]). Interestingly, α-lapachone exhibited a twofold greater cytotoxic activity against KB-7d as compared to the parental cell line (based on comparison of LD_{50} values). Moreover, the LD_{50} value of α-lapachone against KB cells (80.1 ± 5.7 μM) was comparable to the concentrations at which the enzyme was inhibited by in vitro catalytic and non-catalytic assays (25–100 μM range [17]). Based on the collateral sensitivity of KB-7d cells to α-lapachone, specific inhibition of cellular topoisomerase II was considered to be a possible mechanism of drug action. In contrast, the structural isomer, β-lapachone appeared to have a more complex mechanism of action, possibly involving multiple targets.

Interference of etoposide-stabilized protein-linked DNA breaks

α-Lapachone inhibits stabilization of in vitro ternary complexes induced by etoposide by inhibiting initial non-covalent binding of topoisomerase II to DNA, and by induction of re-ligation and dissociation of enzyme from enzyme–DNA binary complexes. β-Lapachone re-ligates DNA breaks in ternary complexes without

Table 1 Cytotoxic activities of α-lapachone and β-lapachone in KB and KB-7d cell lines

	KB		KB-7d	
	LD_{50} (μM)	LD_{25} (μM)	LD_{50} (μM)	LD_{25} (μM)
α-Lapachone	80.1 ± 5.7	52.8 ± 0.8	39.6 ± 4.9	25.2 ± 2.9
β-Lapachone	6.6 ± 0.08	5.3 ± 0.0	5.7 ± 0.4	4.5 ± 0.8
Etoposide	1.2 ± 0.05	ND	–	>40.0

Cells were treated with the test compounds for 90 min and were plated as described in the methods. LD_{50} and LD_{25} values were interpolated from the concentration response graphs and values are mean and standard deviations from three independent experiments. *ND* not determined

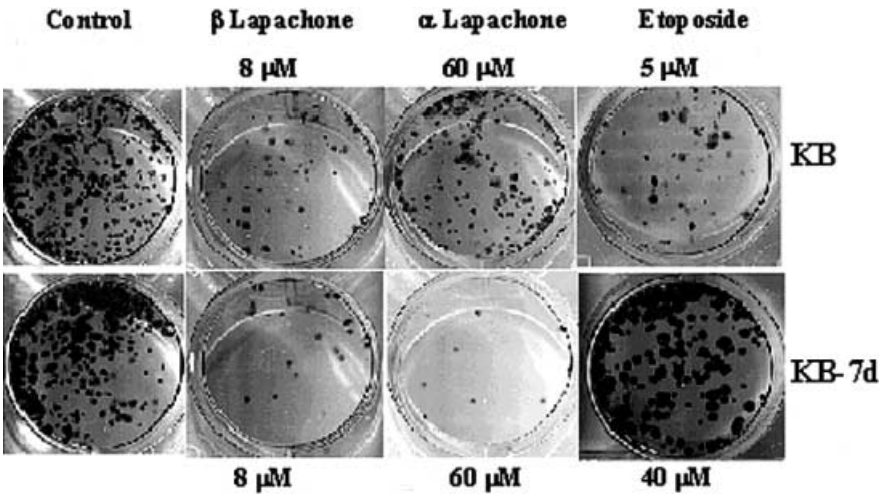


Fig. 2 Comparison of the cytotoxic activities of α-lapachone, β-lapachone, and etoposide in KB cells and in the KB-7d sub-line. The figure is a representative result of three independent experiments, showing colonies of cells surviving treatments with drug concentrations around the LD_{50} values determined with the use of KB cells

dissociating enzyme from DNA but induces dissociation of enzyme from DNA in the absence of etoposide and in the presence of ATP [17]. If the in vitro and intracellular mechanism of enzyme inhibition by both compounds were similar, then etoposide-stabilized covalent protein–DNA complexes would decrease in the presence of the pyranonaphthoquinones, irrespective of the treatment schedule.

To test the hypothesis, [^{3}H] thymidine-labeled KB cells were treated with etoposide, α-lapachone, and β-lapachone alone or under one of the following three treatment conditions: (a) Pre-incubation, that is, treatment of the compounds with cells for 1 h before the addition of etoposide; (b) Co-treatment, that is, the cells were incubated in the presence of the compounds and etoposide for 3 h. (c) Post-treatment, that is, etoposide was added to the medium for 1 h to allow protein–DNA complexes to form before the addition of the test compounds. Figure 3 shows cellular protein–DNA complexes as a fold increase over (multiple of) the control on treatment with the compounds or a combination of compounds. Etoposide increased protein–DNA complexes more than 40-fold over those detected in the control untreated cells. α-Lapachone and β-lapachone did not stabilize detectable protein–DNA complexes. α-Lapachone did not interfere with etoposide-stabilized complexes under any treatment schedule. β-Lapachone also did not significantly interfere ($P > 0.1$) with etoposide, and the slight fluctuations in observed interference could be simply explained as a change in cellular accumulation of etoposide in the presence of β-lapachone.

Overall, the intracellular effects of pyranonaphthoquinones as topoisomerase II inhibitors seemed to be different from their in vitro actions; however, the possibility that the protein-linked DNA break assay lacked sensitivity to detect changes arising from compound-induced re-ligation of double-stranded DNA breaks could not be ruled out. So, an alternate cell-based method that directly detects double-stranded DNA breaks was utilized to further explore enzyme inhibition.

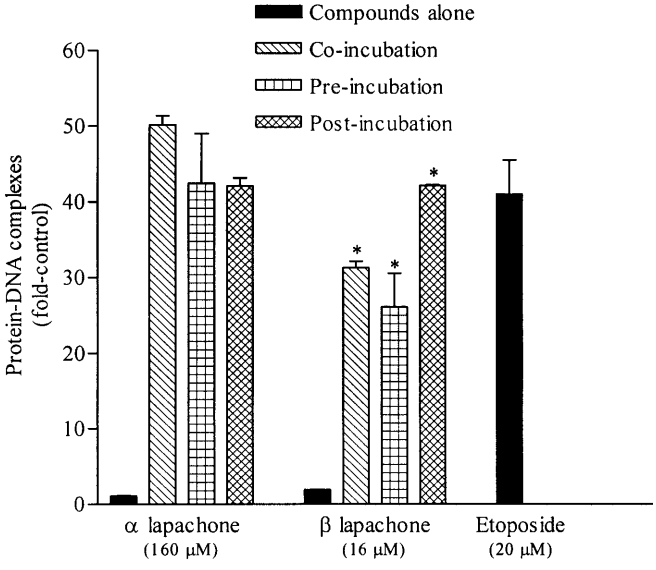


Fig. 3 Protein-linked DNA breaks in KB cells. Protein–DNA complexes stabilized by α-lapachone, β-lapachone, and etoposide, alone or in combination, are plotted as a fold increase over (multiple of) control levels. *Error bars* represent the standard deviations of mean values obtained from three independent experiments. *$P > 0.1$

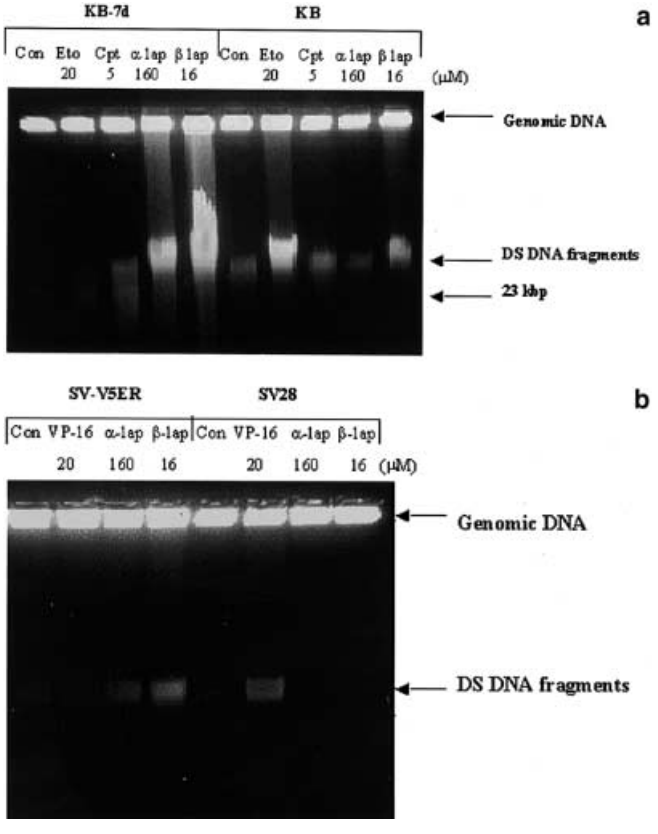
Double-stranded DNA breaks

Resistant cell lines with lower levels of topoisomerase II

Genomic DNA has a nucleosome structure, arranged as solenoids that form individual loop domains. These loops are further ordered as rosettes, which are in turn stacked to form the chromatids. Scaffold attachment regions (SAR), containing enzymes necessary for DNA metabolism including topoisomerase II, are located at the base of the DNA loop domains [9, 10, 23]. On treatment of cells with topoisomerase II poisons, DNA breaks occur at the base of loop domains; upon gel electrophoresis the released DNA fragments migrate

faster than the genomic/intact DNA, which remains at the origin of the gel [29]. Topoisomerase-II-induced double-stranded DNA breaks are generally reversible on removal of the drug and upon replacement with drug-free medium. This characteristic is considered to be a marker of enzyme inhibition by poisons such as etoposide [20].

KB cells were treated with α-lapachone and β-lapachone in combination with etoposide; however, neither interference nor re-ligation of DNA breaks was observed (data not shown). These results are consistent with results of experiments using the protein-linked DNA-break assay (Fig. 3). Surprisingly, treatment of KB cells with β-lapachone alone induced double-stranded DNA breaks. Further studies using KB-7d showed an increased occurrence of double-stranded breaks on treatment with either α-lapachone or β-lapachone, as shown in Fig. 4a, c. KB and KB-7d cells were also treated with camptothecin, a topoisomerase I poison. Camptothecin induces irreversible double-stranded DNA breaks at the replication forks during the S phase of the cell cycle. Since double-stranded DNA breaks were not detected in either KB or KB-7d cells treated with camptothecin (Fig. 4a), the results indicate that a minor fraction of cells were cycling through the S phase during the time course of treatment employed in the experiments. Therefore, α-lapachone- and β-lapachone-induced double-stranded DNA breaks in KB-7d cells were not likely a consequence of topoisomerase I inhibition. That the relative increase in double-stranded DNA breaks was associated with lower intracellular levels of topoisomerase II was further investigated with a baby hamster kidney cell line, SV28, and two sub-lines SV28-V5ER (Fig. 4b) and SV28-20ER (pictorial representation is not shown), which were selected for resistance to etoposide, and which have reduced levels of the topoisomerase II α-isoform [12]. Upon treatment



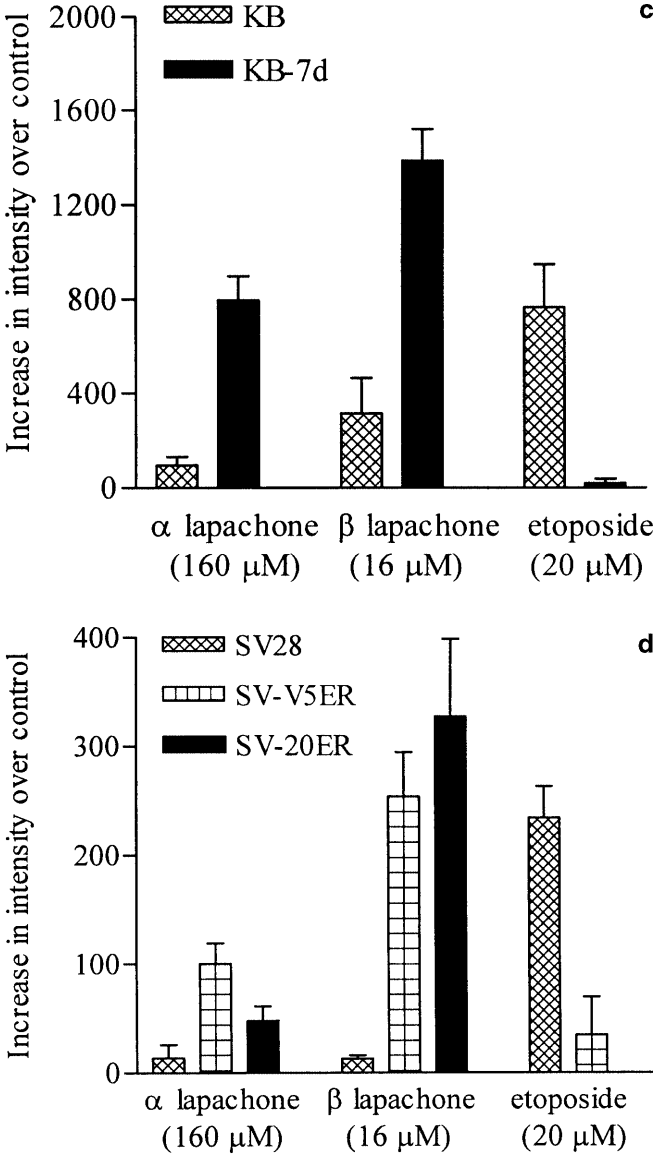


Fig. 4a–d Double-stranded DNA breaks. **a** KB and KB-7d cells were mock treated (medium change only) or treated with etoposide (20 μM), camptothecin (5 μM), α-lapachone (160 μM), or β-lapachone (16 μM). The increases in intensities of drug-induced double-stranded DNA breaks in KB-7d cells as compared to KB cells are summarized in **c**. *Error bars* represent the standard deviations of mean values obtained from three independent experiments. **b** SV28 and SV-V5ER cells were treated with etoposide (20 μM), α-lapachone (160 μM) or β-lapachone (16 μM). The increases in intensities of drug-induced double-stranded DNA breaks in SV-V5ER and SV-20ER cells as compared to SV28 cells are summarized in **d**. *Error bars* represent the standard deviations of mean values obtained from three independent experiments

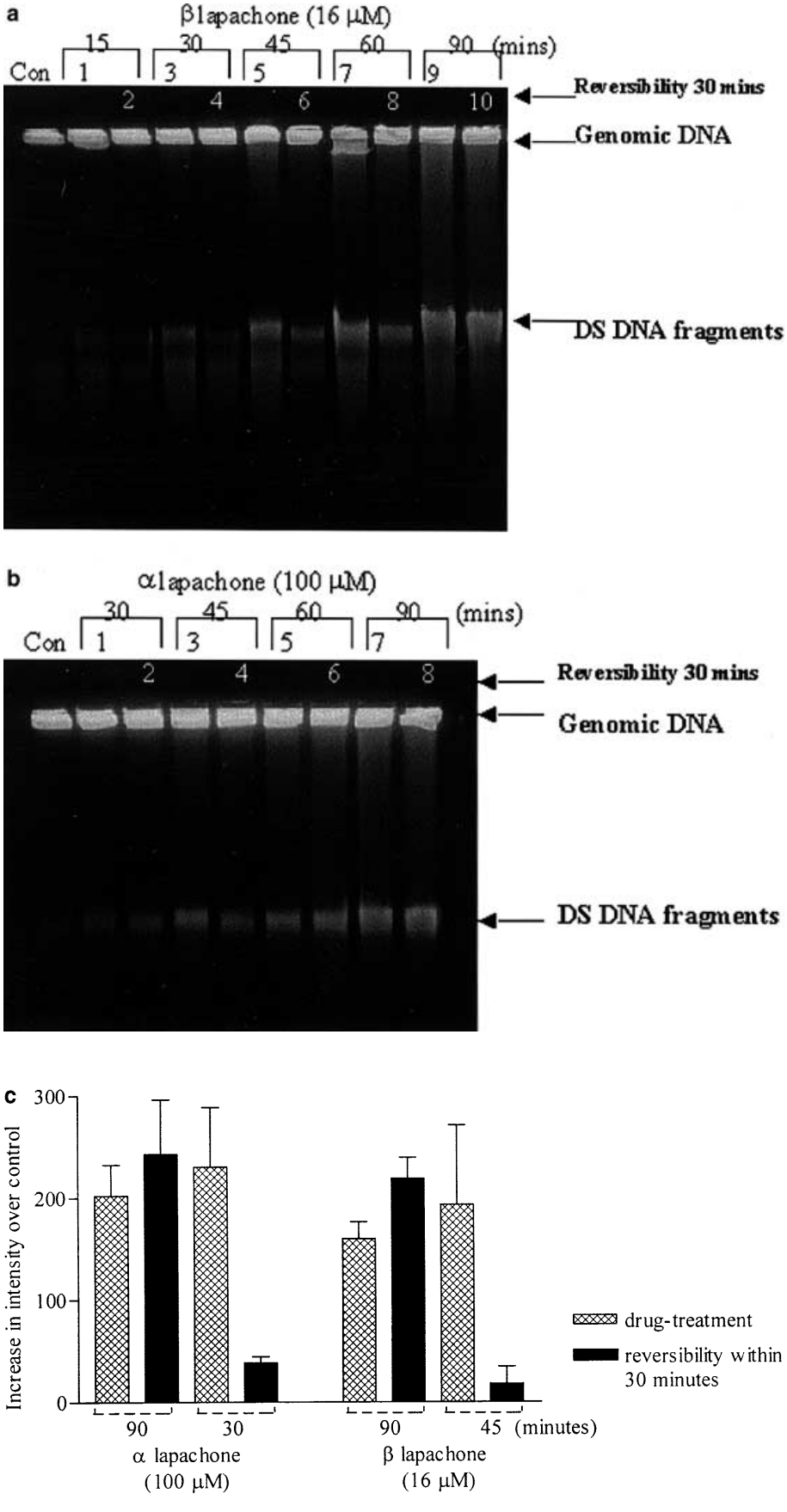


Fig. 5a–c Reversibility of double-stranded DNA breaks in KB-7d cells. KB-7d cells were treated with β-lapachone (16 μM) (**a**) or α-lapachone (100 μM) (**b**) for the indicated times. One of each duplicate culture shown in alternate lanes were re-fed with drug-free medium, followed by additional incubation for 30 min. **c** Summary of the intensities of double-stranded DNA breaks upon treatment reversal after a 45- and 90-min exposure to β-lapachone and after a 30- and 90-min treatment with α-lapachone. *Error bars* show standard deviations of mean values, obtained from three independent experiments

of SV28-derived resistant sub-lines, there was an increased induction of double-stranded DNA breaks, as shown in Fig. 4d, again suggesting involvement of topoisomerase II as an intracellular target.

Subsequent cell-based experiments were mainly done with KB-7d cells, because of the relative ease of detecting and quantifying the α-lapachone- and β-lapachone-induced double-stranded DNA breaks in this drug-resistant sub-line.

Reversibility of double-stranded DNA breaks

The DNA breaks that occur at the base of DNA loop domains can also be due to induction of apoptosis. During programmed cell death, endonucleases are activated, high molecular weight DNA fragments are produced that are eventually processed further via internucleosomal breaks [16]. By conventional agarose gel electrophoresis, the initial DNA fragments can be detected, but they are not resolved on the basis of size. Endonucleolytic DNA breaks are irreversible unlike those induced by topoisomerase II. Therefore, reversibility or re-ligation of double-stranded breaks subsequent to removal of the drug and its replacement with drug-free medium can serve as a marker for topoisomerase II inhibition.

Cells were treated with α-lapachone and β-lapachone various times, and were then placed in drug-free medium in a replicate at each time point, after which incubation was continued for 30 min. Figure 5a,b shows the results obtained with KB-7d cells (similar observations were made with KB cells treated with β-lapachone; data not shown). Double-stranded breaks were reversible up to 45 min of treatment with α-lapachone and up to 60 min with β-lapachone. After longer treatments with either drug, the breaks became irreversible.

These results strongly support involvement of topoisomerase II as a biochemical target. At earlier incubation periods, HMW DNA fragments were bound to topoisomerase II and were reversible, but after additional treatment for 15–30 min, the irreversible nature of the DNA breaks could not be so easily accounted for and required explanation. Two hypothetical mechanisms of action to account for reversible and irreversible breaks were considered and they are illustrated in Fig. 6. Li et al. recently reported interesting observations from a study of cells treated with hydrogen peroxide [20]. Formation of high molecular weight reversible DNA breaks in cells along with some in vitro correlations led to the conclusion that topoisomerase II was targeted by hydrogen peroxide during oxidative stress of human pro-monocytic U937 cells. Since quinone derivatives are known to generate hydrogen peroxide, the first mechanism shown in Fig. 6 to account for double-stranded DNA breaks was proposed. According to this model, pyranonaphthoquinones could induce intracellular oxidative stress and the resulting generation of hydrogen peroxide could inhibit topoisomerase II and form reversible HMW DNA breaks. Alternatively, the compounds could induce apoptosis resulting in activation of endonucleases, thus explaining the irreversible nature of DNA breaks upon prolonged treatment. This is the second model shown in Fig. 6.

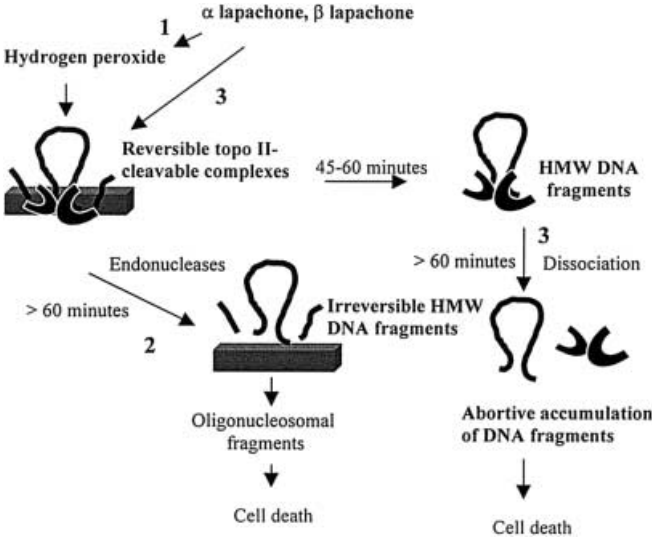


Fig. 6 Schematic representation of the three possible mechanisms for induction of double-stranded DNA breaks by pyranonaphthoquinones. *Numbered arrows* identify the three models discussed in the text. The scheme has been modified from the model published by Li et al. [20]

Double-stranded breaks induced by oxidative stress

Since pyranonaphthoquinones, especially β-lapachone, could induce hydrogen peroxide formation [3, 5, 11], the pattern of hydrogen peroxide induced double-stranded DNA breaks was evaluated. Figure 7a shows DNA breaks at 45- and 90-min treatments of KB cells and the KB-7d sub-line with 20 and 40 mM hydrogen peroxide. The levels of double-stranded DNA breaks induced by hydrogen peroxide in the KB-7d sub-line and KB cells were similar. Moreover, the breaks were irreversible at incubation times between 30 (data not shown) and 90 min. Taken together, the results suggest that the mechanism(s) of hydrogen peroxide induced DNA breaks in KB cell lines was entirely different from the action of the pyranonaphthoquinones.

A common method of evaluating oxidative stress is to use antioxidants to negate cellular damage. Inhibition of hydrogen peroxide induced DNA breaks by α-tocopherol served as a positive control. KB and KB-7d cells were pretreated with α-tocopherol and were then treated with hydrogen peroxide, α-lapachone or β-lapachone for 90 min. Figure 7b,c shows the effect of the antioxidant on induction of double-stranded breaks when the KB-7d sub-line is used. The DNA breaks by both

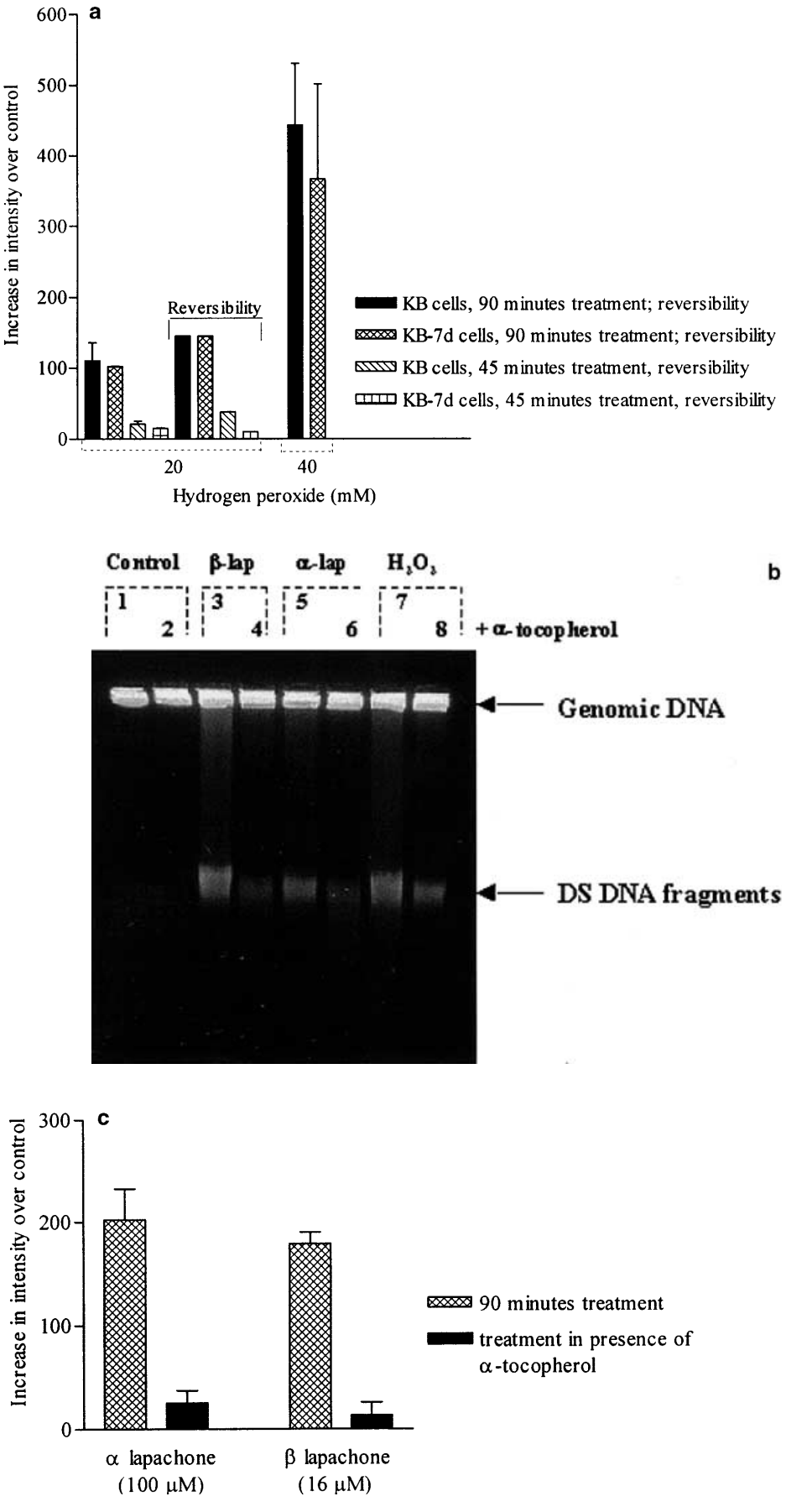


Fig. 7a–c Effects of antioxidants on induction of double-stranded DNA breaks. **a** A graph summarizing double-stranded DNA breaks in KB cells and the KB-7d sub-line in the presence of 20 mM and 40 mM hydrogen peroxide upon treatment for 45 and 90 min, followed by reversibility in drug-free medium for 30 min. *Error bars* show standard deviations of mean values obtained from three independent experiments. The reversibility result is obtained from a single experiment. **b** A representative result showing induction of double-stranded DNA breaks in KB-7d cells treated in the presence or absence of 0.1 mM α-tocopherol with α-lapachone (100 μM), β-lapachone (16 μM) and hydrogen peroxide (10 mM). **c** A graph summarizing DNA breaks induced by the test compounds in the presence or absence of α-tocopherol. *Error bars* show standard deviations of mean values obtained from three independent experiments

pyranonaphthoquinone derivatives dropped to basal levels in the presence of α-tocopherol. This implied that use of antioxidants could directly reduce free radical formation and could therefore also inhibit oxidation of a potential target enzyme (topoisomerase II). In addition, induction of irreversible DNA breaks by α-lapachone and β-lapachone may have resulted from the drug-induced oxidative stress of the cells.

Endonuclease activation versus topoisomerase II inhibition at longer incubation periods

To differentiate between the two proposed mechanisms, endonucleases were inhibited by pretreatment of cells with zinc chloride for 1 h. Double-stranded DNA breaks induced by the calcium ionophore, A23187 [28], and decreased induction of these breaks in the presence of zinc chloride was used to validate endonuclease activation/inhibition in the KB cell system. Since KB-7d is more than 40-fold resistant to etoposide, this drug could not be used as a control for intracellular topoisomerase II poisoning. 9-Ethoxycarbonyl berberine was chosen since the agent is a topoisomerase II poison but KB-7d is only sixfold cross-resistant to the agent [18]. Levels of double-stranded DNA breaks remained unaffected by zinc chloride, indicating normal functioning of topoisomerase II in its presence (data not shown). Figure 8a shows the effects of the compounds on double-stranded DNA breaks when endonucleases were inhibited. DNA fragmentation induced by α-lapachone and β-lapachone

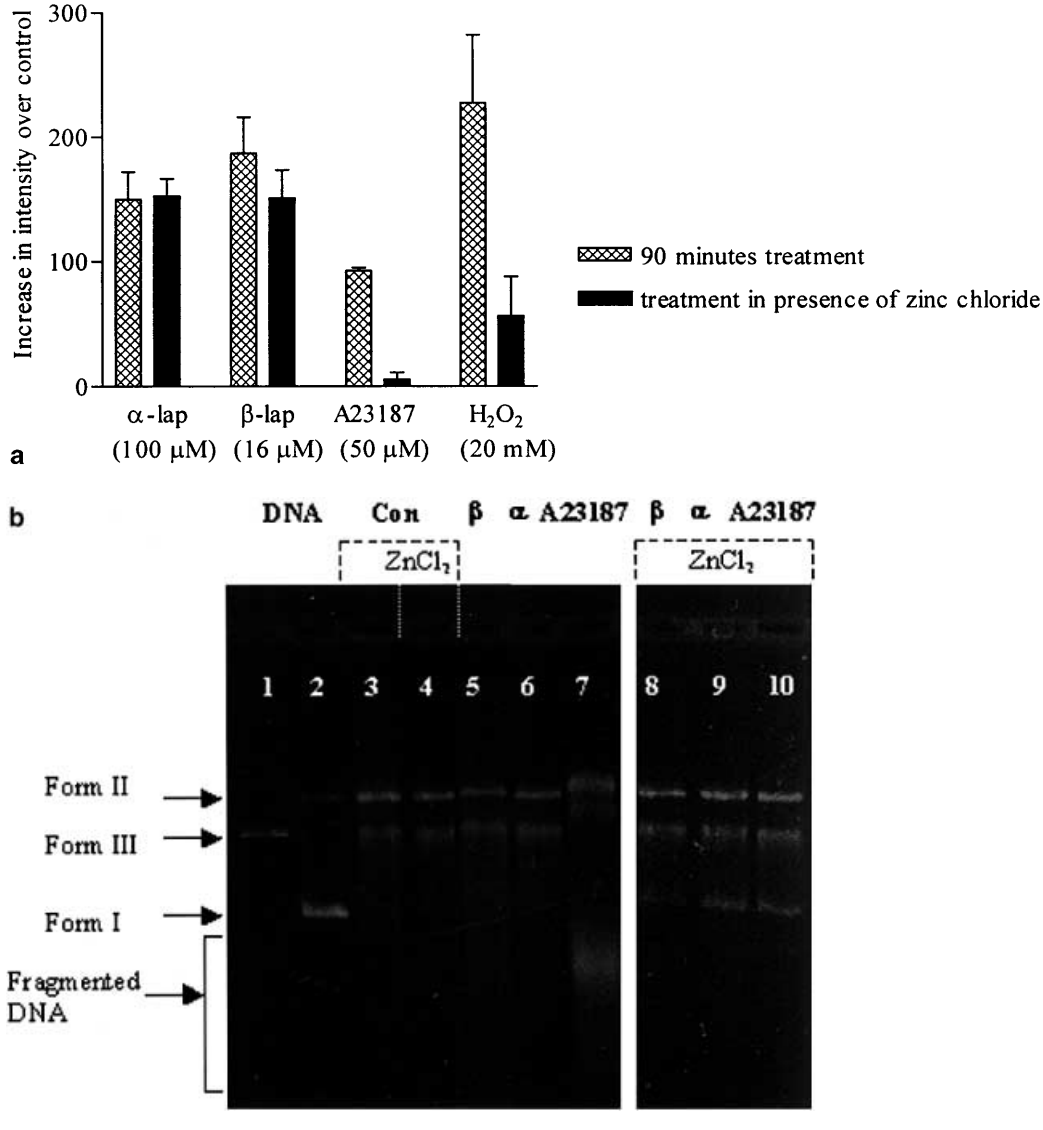


Fig. 8a, b Effects of endonuclease inhibitors on induction of double-stranded DNA breaks. **a** A graph summarizing DNA breaks induced by α-lapachone (100 μM), β-lapachone (16 μM), A23187 (50 μM), and hydrogen peroxide (20 mM), in the presence or absence of 0.1 mM zinc chloride. *Error bars* show standard deviations of mean values obtained from three independent experiments. **b** Endonuclease activity in cell-free extracts. KB-7d cells were treated with α-lapachone (100 μM), β-lapachone (16 μM) and A23187 (50 μM) in the presence or absence of 0.1 mM zinc chloride. The figure is a representative result from two independent experiments showing digestion of pBR322 DNA by salt extracts of treated cells. Non-adjacent lanes from a single gel were juxtaposed to create this figure

remained unaffected even at incubation times when DNA breaks became irreversible (90 min). In contrast, hydrogen peroxide induced DNA breaks decreased in the presence of zinc chloride.

Endonuclease involvement in DNA damage was checked by the independent use of digestion of plasmid DNA as an alternative method of detecting endonuclease activation, as shown in Fig. 8b. Low-concentration salt extracts from either treated or untreated KB-7d cells were added to plasmid pBR322 and incubated at 37 °C. Digestion of supercoiled DNA to form nicked and linear DNA by the untreated control sample reflects basal endonuclease activity of the extract. The pattern of cleavage by the ionophore-treated extract was quite unique and it was not detected if cells were pretreated with zinc chloride before A23187 exposure and subsequent extraction. α-Lapachone- and β-lapachone-treated cell extracts caused basal cleavage of plasmid DNA, similar to the untreated control. Plasmid digestion by hydrogen peroxide treated cell extracts varied over the course of the studies. In some experiments the endonuclease signature was detected and in others it was not. Hydrogen peroxide was the only agent that was irreproducible in the plasmid assay for reasons presently not understood, and representative results are therefore not included in Fig. 8b.

However, the overall results indicate that irreversibility of DNA fragmentation after longer treatments with α-lapachone and β-lapachone was not due to endonuclease activation.

Discussion

α-Lapachone and β-lapachone are two pyranonaphthoquinone derivatives that were recently identified as catalytic inhibitors of human DNA topoisomerase II α-isoform in vitro [17]. Consistent with this proposed action, cell-based studies established increased cytotoxic activity of α-lapachone against an etoposide-resistant cell line, KB-7d. However, unlike the novel mechanism of inhibition in vitro, neither derivative interfered with the formation of etoposide-stabilized protein–DNA complexes in cells. Studies were conducted to directly visualize double-stranded DNA breaks in order to measure potential drug effects on enzyme re-ligation. Interestingly, the pyranonaphthoquinones themselves were found to induce HMW double-stranded DNA breaks. Moreover, both agents caused a dramatic increase in double-stranded DNA breaks in KB-7d, SV-V5ER and SV-20ER, drug-resistant sub-lines with a lower expression of topoisomerase II as compared to the respective parental cells. These results strongly supported topoisomerase II as an intracellular target of the pyranonaphthoquinone derivatives.

HMW DNA fragments stabilized by topoisomerase II are generally reversible and this serves as a good marker of enzyme poisoning. The α-lapachone- and β-lapachone-induced DNA breaks in KB-7d cells were reversible up to 30 and 45 min of treatment, respectively. However, the breaks rapidly became irreversible (within an additional 15 min of drug exposure). The results implied that the initial response to treatment was stabilization of topoisomerase II-DNA breaks, but the biochemical basis for the irreversible DNA damage in drug-treated cells was unclear.

Two possible modes of action were considered to explain irreversible double-stranded DNA breaks and they are summarized below, and in Fig. 6. (a) Quinones are capable of forming free radicals and undergo redox cycling which can lead to oxidative stress and/or oxidation of thiol enzymes. This mechanism is based on the recent report of Li et al. on topoisomerase II inhibition and induction of double-stranded DNA breaks in response to hydrogen peroxide treatment [20]. (b) Induction of apoptosis accompanied by endonuclease activation can result in HMW irreversible DNA fragments.

The irreversible nature of hydrogen peroxide induced double-stranded DNA breaks as well as the similar levels of DNA breaks in both KB and KB-7d cells indicated that pyranonaphthoquinone action could not be explained simply by hydrogen peroxide generation inside cells. Oxidative stress in cells treated with hydrogen peroxide is accompanied by several one- and two-electron transfers, and involves various enzymes, such as NADPH–cytochrome P450 reductase, xanthine oxidase, and xanthine dehydrogenase. These redox reactions in turn result in the production of reactive oxygen species including the superoxide radical, the hydroxy radical, and the oxygen anion. Different responses of cell lines to oxidative stress may depend on several variables [13]. Therefore, the dissimilar behavior of double-stranded DNA breaks induced by hydrogen peroxide in KB, KB-7d, and U937 cells is not unexpected. The biochemical basis of the different responses is, however, currently unknown.

The proposed mechanism involving oxidative stress was further evaluated by use of α-tocopherol to scavenge free radicals. Double-stranded DNA breaks induced by α-lapachone, β-lapachone, and hydrogen peroxide dropped to basal levels in the presence of α-tocopherol. Thus, an oxidative mechanism appeared to be involved. Moreover, since oxidative stress in response to the compounds could also account for the irreversible nature of DNA breaks at longer treatment times, this possibility required further investigation.

Two methods were used to detect endonuclease activation in response to the compounds. The first method involved inhibition of endonucleases with zinc chloride followed by treatment with the compounds. The calcium ionophore A23187 was used as a positive control for the induction of endonucleases [28]. Even after 90 min of drug exposure, DNA breaks induced by α-lapachone and β-lapachone remained unchanged in the presence of zinc chloride; this implies that the irreversible nature of the DNA breaks were not a result of endonuclease activation. In contrast, hydrogen peroxide induced DNA breaks decreased in the presence of the endonuc-

lease inhibitor; this suggests that generalized oxidative stress could not account for the irreversible DNA breaks in response to the pyranonapthoquinones. The second approach was to directly measure activated endonucleases in extracts of pretreated cells by a plasmid digestion assay. In complete agreement with the gel lysis assay results, untreated, the α-lapachone- and β-lapachone-treated cell extracts contained basal levels of endonuclease activity since supercoiled DNA was digested to nicked and linear DNA. In contrast, the calcium ionophore-treated sample caused massive fragmentation of the plasmid and this activity was inhibited by pretreatment of cells with zinc chloride. The assay results obtained when hydrogen peroxide was used varied over the course of the study for reasons that were not resolved.

The overall findings show that neither of the two models can account for the behavior of double-stranded DNA breaks induced by pyranonaphthoquinones. Oxidative stress or hydrogen peroxide formation was not responsible for the induction of reversible/irreversible DNA breaks in response to the pyranonaphthoquinones; endonucleases were also not activated. On the basis of the present findings, an alternate mechanism is proposed to explain the onset of irreversible DNA breaks. This third model, shown in Fig. 6, involves initial stabilization of topoisomerase-II-associated DNA breaks, followed by drug-induced abortive enzyme dissociation leading to accumulation of irreversible HMW double-stranded DNA. This model predicts that a fraction of chromatin-bound enzyme will redistribute in drug-treated cells; however, initial experiments to test this possibility proved inconclusive.

Under physiological conditions, both α-lapachone and β-lapachone are capable of oxidizing dithiols such as dithiothreitol but not monothiols such as 2-mercaptoethanol [17]. β-Lapachone was also previously reported to be a redox cycling agent that can oxidize cysteine residues on topoisomerase II, resulting in inactivation of the enzyme [22]. Based on current results, it is proposed that this oxidative mechanism is responsible for the induction of double-stranded DNA breaks. Since this report has established that topoisomerase II is a likely intracellular target, it is speculated that α-lapachone and β-lapachone induce oxidative damage to the enzyme, possibly via oxidation of cysteine residues on the enzyme.

In vitro assay conditions were used, and the concentrations required to inhibit (IC_{50}) and dissociate topoisomerase II from DNA were 50 μM and 175 μM for α-lapachone and β-lapachone, respectively [17]. For the induction of double-stranded DNA breaks in KB-7d cells, β-lapachone was about ten times (used at 16 μM) more active than α-lapachone as an enzyme inhibitor, whereas the corresponding activity of α-lapachone (used at 100 μM) was compatible with enzyme inhibition. Based on these observations and comparisons, β-lapachone seems to be less specific in its action, whereas α-lapachone may primarily target cellular topoisomerase II. The phenomenon of drug-induced re-ligation (observed in the in vitro assays) was not detected directly through cell-based assays. Since the present studies do not differentiate between inhibition of the α- and/or β isoforms of topoisomerase II, pharmacological differences in isoenzyme sensitivity could possibly account for the apparent discrepancies between the in vitro and cell-based assay results. This explanation is currently under investigation.

Due to the novel mechanism of action that involves initial stabilization of topoisomerase II–DNA covalent complexes, leading to possible abortive dissociation of the enzyme from the DNA, α-lapachone and β-lapachone cannot be classified as classical enzyme poisons (higher activity in cell lines with lower expression of topoisomerase II) or as classical catalytic inhibitors (initial stabilization of DNA breaks). This new class of topoisomerase II inhibitor, especially paraquinones such as α-lapachone, will be useful for generating candidate anticancer agents with hyperactivity against MDR tumors expressing lower levels of topoisomerase II.

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