

Induction of CDK Inhibitors (p21^{WAF1} and p27^{Kip1}) and Bak in the β -Lapachone-Induced Apoptosis of Human Prostate Cancer Cells

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ABSTRACT

β -Lapachone, a novel anti-neoplastic drug, induces various cancer cells to undergo apoptosis. In a previous report, we showed that β -lapachone-induced apoptosis of HL-60 cells is mediated by oxidative stress. However, in the present study, we found that β -lapachone-induced apoptosis of human prostate cancer (HPC) cells may be independent of oxidative stress. In contrast to the 10-fold β -lapachone-induced increase in H₂O₂ production seen in HL-60 cells, only a 2- to 4-fold increase was observed in HPC cells. *N*-acetyl-L-cysteine (NAC), a thiol antioxidant, inhibited the apoptosis in DU145 cells after 12 h exposure to β -lapachone. Nonetheless, NAC, along with other antioxidants, failed to exert similar effect in HPC cells subjected to β -lapachone treatment for 24 h. Under this premise, we suggest that the oxidative stress may not play a

crucial role in β -lapachone-mediated HPC cell apoptosis. Here we demonstrate that damage to genomic DNA is the trigger for the apoptosis of HPC cells induced by β -lapachone. According to our results, β -lapachone stimulates DNA dependent kinase expression and poly(ADP-ribose) polymerase cleavage in advance of significant morphological changes. β -Lapachone promotes the expression of cyclin-dependent kinase (cdk) inhibitors (p21^{WAF1} and p27^{Kip1}), induces bak expression, and subsequently stimulates the activation of caspase-7 but not of caspase-3 or caspase-8 during the apoptosis of HPC cells. Taken together, these results suggest that the signaling pathway involving the β -lapachone-induced apoptosis of HPC cell may be by DNA damage, induction of cdk inhibitors (p21 and p27), and then subsequent stimulation of caspase-7 activation.

β -Lapachone (3,4 dihydro-2,2-dimethyl-2H-naphtho[1,2-*b*]pyran-5,6-dione), a natural plant product extracted from the lapacho tree (*Tabebuia avellanedae*), has been shown to have a variety of pharmacological effects, including antiviral, antibacterial, antifungal, and antiparasitic activities (Docampo et al., 1979; Goncalves et al., 1980; Schaffner-Sabba et al., 1984). Recently, it was also shown to have a significant antineoplastic effect on various cancer cells (Li et al., 1995; Chau et al., 1998; Lai et al., 1998). β -Lapachone is a DNA Topo I inhibitor and produces its cytotoxicity by directly interacting with the Topo I enzyme, rather than by stabilizing the DNA-cleavable complex (Li et al., 1993). Furthermore, it increases the sensitivity of tumor cells to DNA-

damaging agents, such as methylmethanesulfonate or ionizing radiation (Boorstein and Pardee, 1983), and interrupts the cell cycle (Boothman and Pardee, 1989). It is also suggested to be a DNA Topo II α inhibitor, because it suppresses the growth of yeast and blocks the DNA-unwinding enzyme Topo II α in vitro (Frydman et al., 1997; Neder et al., 1998). However, in contrast to other Topo-inhibitors, such as camptothecin or etoposide, as shown by alkaline or neutral filter elution studies, β -lapachone does not bind covalently to the Topo I-DNA complex or other protein-DNA complexes (Li et al., 1993; Frydman et al., 1997; Wuerzberger et al., 1998). In terms of its chemical structure, β -lapachone is a lipophilic *O*-naphthoquinonic compound. Many quinonic compounds are widely used as chemotherapeutic agents and exert their cellular toxicity by stimulating intracellular free radical production in mitochondrial and microsomal fractions (Dubin et al., 1990; Fry and Pudney, 1992; Henry and Wallace, 1995).

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ABBREVIATIONS: β -lapachone, 3,4 dihydro-2,2-dimethyl-2H-naphtho[1,2-*b*]pyran-5,6-dione; Topo, topoisomerase; ROS, reactive oxygen species; cdk, cyclin-dependent kinase; HPC, human prostate cancer; DCFH-DA, 2',7'-dichlorofluorescein diacetate; HE, hydroethidine bromide; NAC, *N*-acetyl-L-cysteine; AO, acridine orange; TUNEL, terminal deoxynucleotidyl transferase dUTP-biotin nick-end labeling; PI, propidium iodide; DNA-PK, DNA-dependent kinase; DAB, diaminobenzidine; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase.

Moreover, the β-lapachone-mediated cell death of human leukemic cells involves the up-regulation of intracellular ROS generation and the subsequent initiation of c-Jun amino terminal kinase and caspase-3 activation (Chau et al., 1998; Planchon et al., 1999; Shiah et al., 1999). Currently, it is still unclear whether β-lapachone mediates apoptosis in cancer cell lines by acting as a DNA Topo inhibitor or a free radical generator.

The progression of the cell cycle is regulated by a number of cyclin-dependent kinases (cdks). Binding of cdks to cyclins results in the activation of the cdks and passage through specific cell cycle checkpoints. The proteins p21 and p27 act primarily by inhibiting the kinase activities of cdk, thus blocking cell cycle progression (Zi et al., 1998; Robson et al., 1999). Prostate cancer is an epidemic disease in the world and is also the second leading cause of cancer deaths in American men. The number of patients who died from prostate cancer in 1997 accounts for 14% of all cancer deaths in American men (Parker et al., 1997). The main curative treatment for prostate cancer at early stage includes surgery, radiation therapy, and hormone ablation. However, the results of hormone ablation and prostatectomy for patients with metastatic prostate cancer have been disappointing because most metastatic prostate cancer cells become androgen-independent and escape apoptosis induced by androgen ablation and by many cytotoxic drugs (Marcelli et al., 1999). The aims of the present study, therefore, were to study 1) whether the cytotoxic effect of β-lapachone on androgen-independent human prostate cells is mediated by ROS production and 2) whether β-lapachone interrupts the cell cycle by the induction of cdk inhibitors. In addition, changes in expression of members of the bcl-2 protein family and caspase activity were also examined, because their involvement in the apoptotic pathway is well known.

In the present study, we demonstrate that the β-lapachone-induced cell death of HPC cells may not be mediated by ROS production. Results using DCFH-DA and HE probes showed that β-lapachone does not induce a large amount of ROS production in these cells. Furthermore, β-lapachone-induced cell death is not prevented by antioxidant treatment. Western blotting and immunocytochemical studies showed that β-lapachone induces DNA-PK expression and PARP cleavage, these events happening in advance of any morphological changes. In addition, β-lapachone up-regulates cdk inhibitors (p21 and p27), promotes bak expression, and induces activation of caspase-7, but not of caspase-3 or caspase-8.

Materials and Methods

Chemicals. β-Lapachone, prepared according to the procedures described by Schaffner-Sabba et al. (1984), was dissolved as a 20 mM stock solution in ice-cold absolute alcohol and stored in aliquots at −20°C. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and hydroethidine bromide (HE), obtained from Molecular Probes (Fremont, CA), were dissolved in DMF at a concentration of 2 mM. Other chemicals were obtained from Sigma (St. Louis, MO).

Cell Cultures. The human prostate cancer cell lines (DU145 and PC-3) used in this study were obtained from the American Type Culture Collection (Manassas, VA). Cells (2×10^6) were grown for 24 h at 37°C in a humidified 5% CO₂ atmosphere in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin. They were then washed twice with

prewarmed PBS and cultured in serum-free medium for an additional 24 h.

Cell Death Assay. After a 24-h period in serum-free culture, cells were washed twice with prewarmed PBS, then treated with various concentration of β-lapachone for 12 to 48 h, after which they were trypsinized and cell viability was evaluated by Trypan Blue exclusion using phase contrast microscopy. For antioxidant studies, cells were pretreated for 3 h with various antioxidants [*N*-acetyl-L-cysteine (NAC; 30 mM), ascorbic acid (vitamin C, 25 μM), α-tocopherol (vitamin E, 10 mM), or propyl gallate (5 μM)], then coincubated with the antioxidant and β-lapachone for a further 6, 12, or 24 h before cell death was evaluated by Trypan Blue exclusion. Cells counts were performed on a hemocytometer using equal volumes of cell suspension and Trypan Blue solution (0.3% in PBS). Cells taking up Trypan Blue were classed as nonviable and expressed as a percentage of the total number.

Acridine Orange (AO) Staining and Terminal Deoxynucleotidyl Transferase dUTP-Biotin Nick-End Labeling (TUNEL). Cells cultured on coverslips in 24-well plates were used. For AO staining, cells exposed for 6, 12, or 24 h to β-lapachone were washed twice with cold PBS, then fixed with methanol/glacial acetic acid (3:1, v/v). After several PBS washes, they were then stained for 5 min with 0.5 ml of AO solution (10 μg/ml in PBS), washed three times with PBS, and examined using an Olympus BH-2 microscope with fluorescence attachment. For TUNEL labeling, a TACS In Situ Apoptosis Detection kit (R & D Systems, Minneapolis, MN) was used to detect DNA fragmentation in apoptotic cells. Briefly, after fixation with 3.7% paraformaldehyde in PBS and digestion with proteinase K, the cells were treated for 5 min with 0.3% hydrogen peroxide, then washed with distilled water. The slides were then immersed in TdT labeling solution, incubated for 1 h in a humidity chamber at 37°C, washed with distilled water, and incubated for 10 min with streptavidin-horseradish peroxidase complex. A positive TUNEL signal in apoptotic cells was detected as blue TACS labeling using an Olympus microscope.

Cell Cycle Analysis. β-Lapachone-treated or control cells were washed with ice-cold PBS, then fixed for 1 h at −20°C in 70% ethanol. They were then washed twice, incubated for 30 min at 37°C with 0.5 ml of 0.5% Triton X-100/PBS containing 1 mg/ml of RNase A, and stained for 10 min with 0.5 ml of 50 μg/ml of propidium iodide

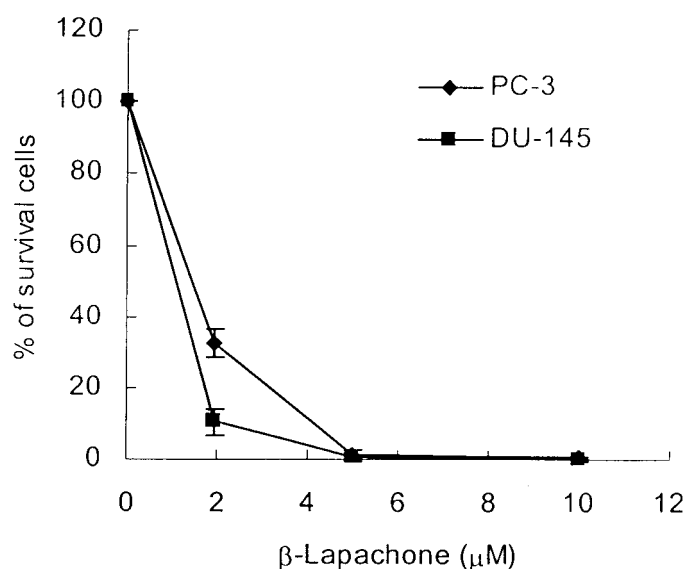


Fig. 1. Survival analysis of HPC cells after a 24-h exposure to various concentrations of β-lapachone. DU145 or PC-3 cells (5×10^5) were cultured in 6-well plates with serum-free medium for 24 h. Cells were then treated with 0 μM, 2 μM, 5 μM, and 10 μM β-lapachone, respectively, for an additional 24 h. Cell survival was determined by the Trypan Blue exclusion assay. The points are the mean ± S.D. of three measurements.

(PI). The intensity of the fluorescence emitted by the PI-DNA complex was quantified after laser excitation of the fluorescent dye using a FACScan flow cytometry (Becton Dickinson, Mountain View, CA).

Determination of Intracellular ROS Production. The production of hydrogen peroxide and superoxide anion, respectively, was monitored by flow cytometry using DCFH-DA and HE dyes. Briefly, cells (1×10^6) were incubated for 10 min at 37°C with 50 μ M DCFH-DA or 2 μ M HE in the presence or absence of β -lapachone, then were centrifuged, resuspended in ice-cold PBS, and subjected to FACScan flow cytometry (Becton Dickinson).

Immunocytochemical Studies of DNA-PK, cdk Inhibitors, and bcl-2-Related Proteins. Cells (1×10^5) plated on coverslips in 24-well plates were used to study the expression of DNA-PK, cdk inhibitors (p21 and p27), and bcl-2-related proteins (bcl-2, bcl-xs, bak, and bax,). After 6 h treatment with β -lapachone, cells were fixed for 10 min with 4% paraformaldehyde in PBS, pH 7.4, washed several times with PBS, then incubated for 2 h at 37°C with various primary antibodies obtained from Oncogene Research Products (Cambridge, MA) and Neo Marker (Fremont, CA). The antibodies were diluted 1:500 for bcl-2 family-related proteins and 1:200 for cdk

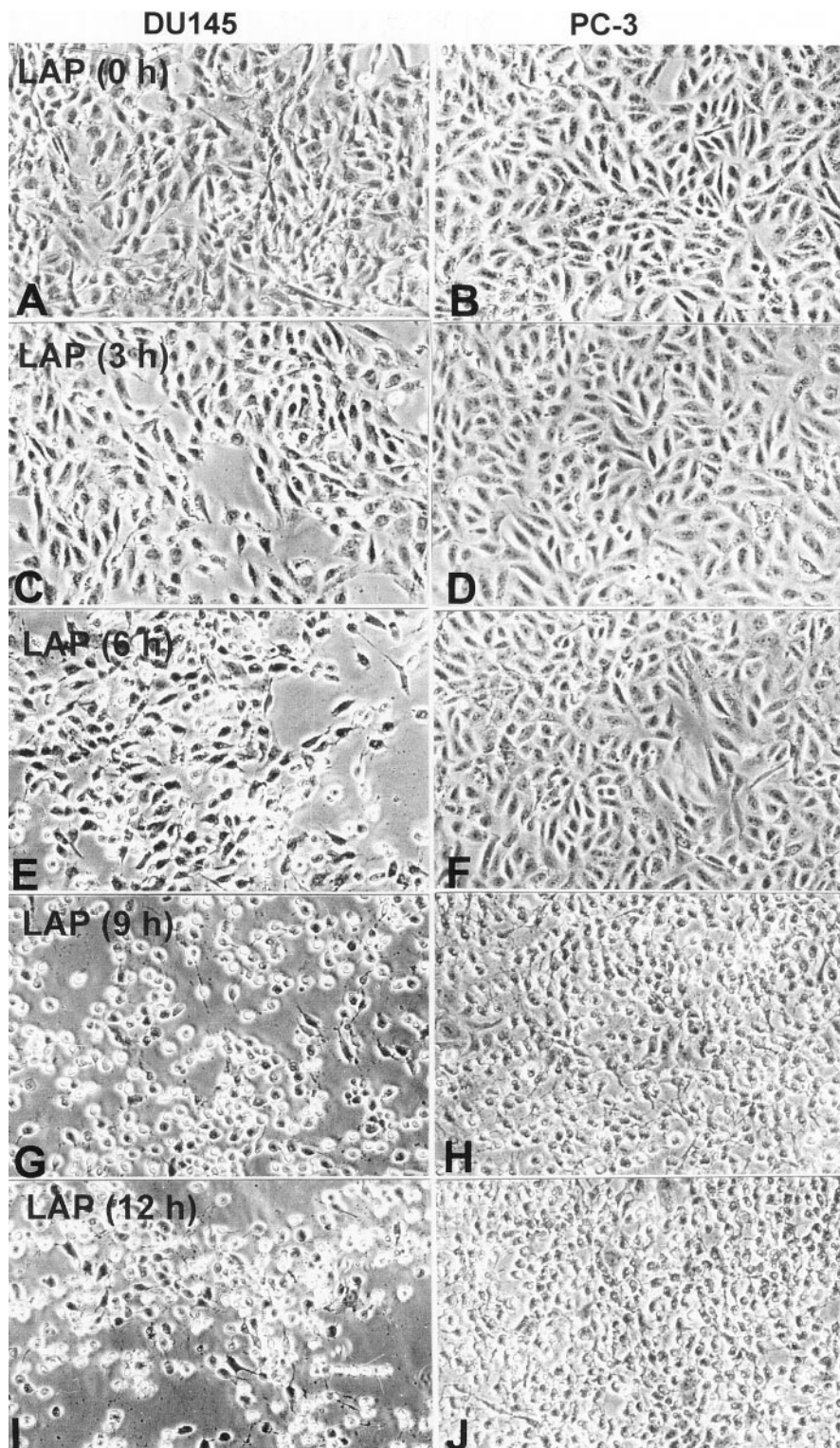


Fig. 2. Light microscopic photographs of HPC cells with β -lapachone treatment. DU145 cells treated with 2 μ M β -lapachone (left) and PC-3 cells treated with 5 μ M β -lapachone (right) for 0 h (A, B); 3 h (C, D); 6 h (E, F); 9 h (G, H), and 12 h (I, J) were observed under a light microscope. Note that cells shrink in size and round up after 6 h of β -lapachone treatment. Magnification, 75 \times .

inhibitors and DNA-PK. After washing for 30 min with PBS, the cells were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted 1:200, then bound antibody was visualized using diaminobenzidine (DAB) and 0.003% H₂O₂. For light microscopy, the stained cells were dehydrated using a graded alcohol series, then mounted on glass slides with Entellan (Merck, Germany).

Western Blot Analysis. Whole-cell or nuclear extracts from control and drug-treated cells were prepared at various times. Briefly, cells in 10-cm² tissue dishes were washed twice with ice-cold PBS, scraped off, and collected by centrifugation (800g for 10 min), then lysed in a lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml of aprotinin, and 10 μg/ml of leupeptin) at 4°C for 30 min with gentle agitation. After centrifugation (15,000g for 10 min), the supernatants were collected and stored at -80°C as whole-cell extracts. For nuclear extracts, the cell pellets were washed once with ice-cold PBS,

resuspended for 15 min in ice-cold hypotonic solution (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 2 mM KCl, 2 mM Mg acetate, 1 mM dithiothreitol, and 1 mM PMSF), pelleted at 4°C for 10 min at 500g, resuspended in hypertonic solution (10 mM Tris-HCl, pH 7.5, 400 mM KCl, 2 mM KCl, 2 mM Mg acetate, 20% glycerol, 1 mM dithiothreitol, and 1 mM PMSF), and incubated on ice for 10 min. Insoluble nuclear debris was removed by centrifugation at 4°C for 10 min at 15,000g, and the supernatants were aliquoted and stored at -80°C. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA) and 100- to 200-μg samples of protein separated by 12% SDS-PAGE. The separated proteins were transferred for 2 h at 200 V to Immobilon-P membranes (Millipore, Bedford, MA) in a Trans-Blot Electrophoretic Transfer cell, then the membranes were blocked with 5% skim milk in PBS-0.2% Tween 20, incubated for 2 h with various primary antibodies (using 1:500 to 1:2000 dilutions), washed for 1 h in PBS-0.2% Tween 20, then incubated with secondary antibody (using 1:2000 dilutions). Bound antibody was

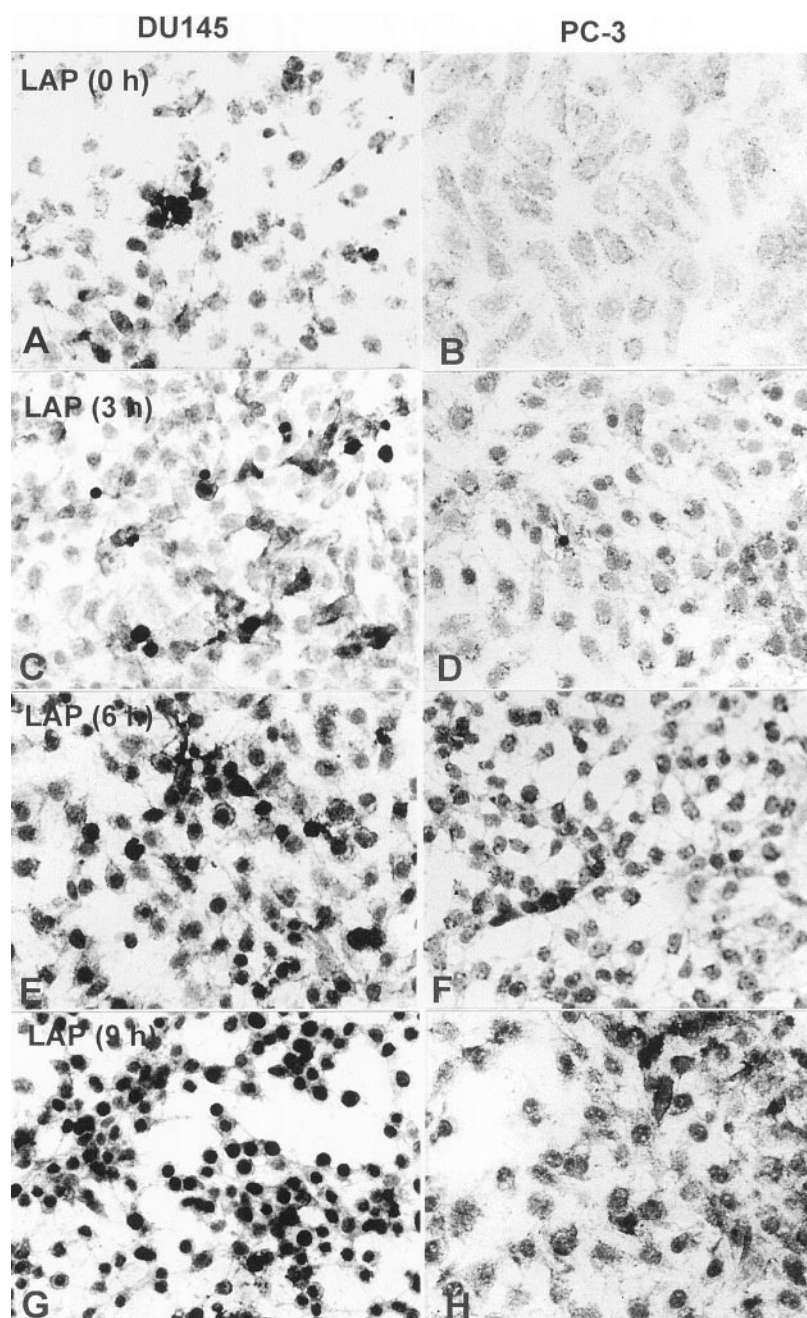


Fig. 3. TUNEL labeling for detection of DNA fragmentation in DU145 or PC-3 cells. DU145 or PC-3 cells grown on coverslips were incubated with serum-free medium for 24 h and then treated with 2 or 5 μM β-lapachone, respectively, for 0 to 9 h. Cells were fixed with 3.7% paraformaldehyde for 10 min. DNA damage was then detected with TUNEL labeling as described under *Materials and Methods*. The positive TUNEL signal in DU145 cells (A, C, E, G) and PC-3 cells (B, D, F, and H) for 0 h (A, B); 3 h (C, D); 6 h (E, F), and 9 h (G, H) were monitored after the blue TACS labeling and then observed with a light microscope. Magnification, 150×.

detected using the ECL Western blotting reagent (Amersham Pharmacia Biotech, Piscataway, NJ). The chemiluminescence was detected using Fuji Medical X-ray film (Tokyo, Japan). Induction levels were quantified using either densitometry or gel image analyses on a Bio-Rad Gel Doc 1000 system.

Results

β -Lapachone Induces Apoptosis of HPC cells. The cytotoxic effect of β -lapachone on HPC cells was evaluated using Trypan Blue exclusion. The results showed that more than 90% of DU 145 cells or PC-3 cells were killed after 24 h treatment with 2 μ M or 5 μ M β -lapachone, respectively (Fig. 1). In contrast to untreated cells, β -lapachone-treated cells became rounded up, shrank, then became detached from the plate (Fig. 2). We then used light and fluorescence microscopy to examine whether β -lapachone induced apoptosis of HPC cells and found that only β -lapachone-treated cells exhibited TUNEL-positive staining and showed intensive fluorescence with AO staining (Figs. 3 and 4). These results confirmed that β -lapachone induced cellular DNA damage, then triggered cell death of HPC cells.

Measurement of ROS Generation in HPC Cells. Intracellular H_2O_2 or ROS production, respectively, was determined using DCFH-DA or HE and flow cytometry. In positive controls, in which HPC cells were treated with 0.015% H_2O_2 for 15 min, then washed, and the endogenous H_2O_2 mea-

sured, an increase of ≤ 10 -fold in DCFH-DA fluorescence intensity was seen. However, only a 2- to 4-fold increase in H_2O_2 production was detected in β -lapachone-treated cells. Moreover, the HE probe showed no marked increase in the level of ROS generation in β -lapachone-treated cells (Fig. 5).

Antioxidants Fail to Block β -Lapachone-Induced HPC Cell Death. To determine whether antioxidants could protect these cells against β -lapachone-induced cell death, HPC cells were pretreated for 3 h with several potent antioxidants (NAC, vitamin C, vitamin E, and propyl gallate) before exposure for 24 h to 2 or 5 μ M β -lapachone in the continued presence of the antioxidant. Although the glutathione precursor, NAC, provided partial protection and delayed β -lapachone-induced cell death, all the antioxidants tested failed to prevent cell death (Fig. 6, A and B). Moreover, the positive TUNEL signals were also observed in HPC cells treated with both antioxidant and β -lapachone (Fig. 6C). These results and the ROS result strongly suggest that oxidative stress may not be a causal factor in the pathway of β -lapachone-mediated HPC cell apoptosis.

DNA-PK Expression and PARP Cleavage in β -Lapachone-Treated Cells. Because of the essential roles of DNA-PK and PARP in DNA repair, DNA damage is always associated with increased DNA-PK expression and PARP cleavage. As shown in Fig. 7, increased DNA-PK expression was seen in β -lapachone-treated cells compared with un-

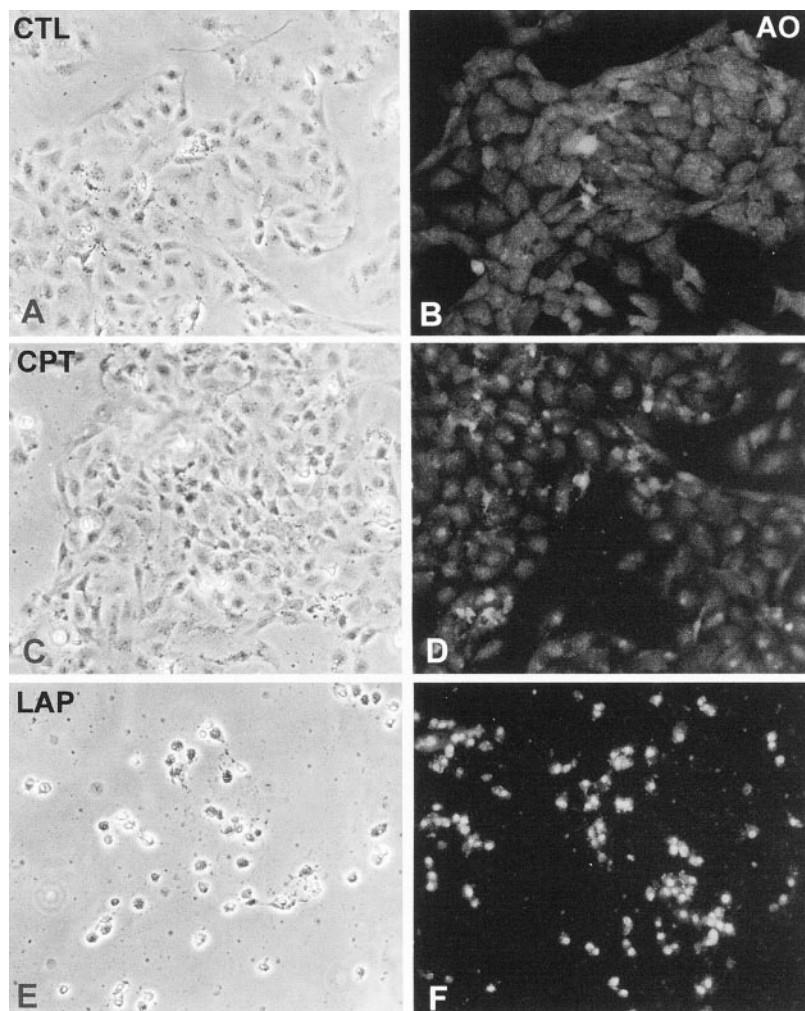


Fig. 4. Visualization of apoptotic response of DU145 cells after camptothecin or β -lapachone treatments. DU145 cells grown on coverslips were incubated with serum-free medium for 24 h and then treated with vehicle (A, B), 10 μ M camptothecin (C, D), or 2 μ M β -lapachone (E, F), respectively, for an additional 24 h. Cells were fixed with methanol: glacial acid (3:1) for 10 min. After several PBS washes, cells were observed by a phase-contrast microscope (A, C, E) or by an Olympus fluorescence microscope (B, D, F) after AO staining. Magnification, 150 \times .

treated cells; this effect was seen after 2- and 4-h exposure to β -lapachone, but lost after 12 h (data not shown). Immunoblot studies showed that the PARP cleavage product was seen after 1 h of β -lapachone treatment. Taken together, these results suggest that the induction of DNA-PK expression and PARP cleavage is an early event that occurs before the morphological changes and DNA fragmentation in the apoptotic pathway.

β -Lapachone Halts the Cell Cycle by Inducing Expression of the Cyclin Inhibitors p21 and p27. Cell cycle analysis was performed on β -lapachone-treated cells using PI staining and flow cytometry. Although the result showed no apparent change in the cell cycle after β -lapachone treatment, the appearance of apoptotic cells ($<2N$) was accompanied by the disappearance of G_0/G_1 cells (Fig. 8). The checkpoints of the cell cycle are regulated by cyclin kinases and their inhibitors, the proteins p21 and p27. To investigate whether β -lapachone interrupts the progression of the cell cycle, we next examined the expression of p21 and p27 in

β -lapachone-treated cells. As shown in Fig. 9, the expression of both p21 and p27 was significantly increased in HPC cells exposed to β -lapachone for 6 h. These results suggest that β -lapachone may be a potent topoisomerase inhibitor that halts the progression of the cell cycle by inducing the expression of cdk inhibitors.

β -Lapachone Promotes the Expression of the Proapoptotic Protein, bak, in HPC Cells. To determine whether changes in bcl-2-related proteins occurred during the β -lapachone-induced apoptosis of HPC cells, the expression of the antiapoptotic bcl-proteins (bcl-2 and bcl-xs) and the proapoptotic bcl-2 family proteins (bak and bax) was examined. As shown in Fig. 10, both immunostaining and immunoblot analysis showed that only the expression of bak was increased in cells after 6-h exposure to β -lapachone.

β -Lapachone Induces Activation of Caspase-7 in the Apoptosis of HPC Cells. Because cleavage of caspases is recognized to be a common event in the apoptotic pathway, caspase activation was studied by Western blotting. Immu-

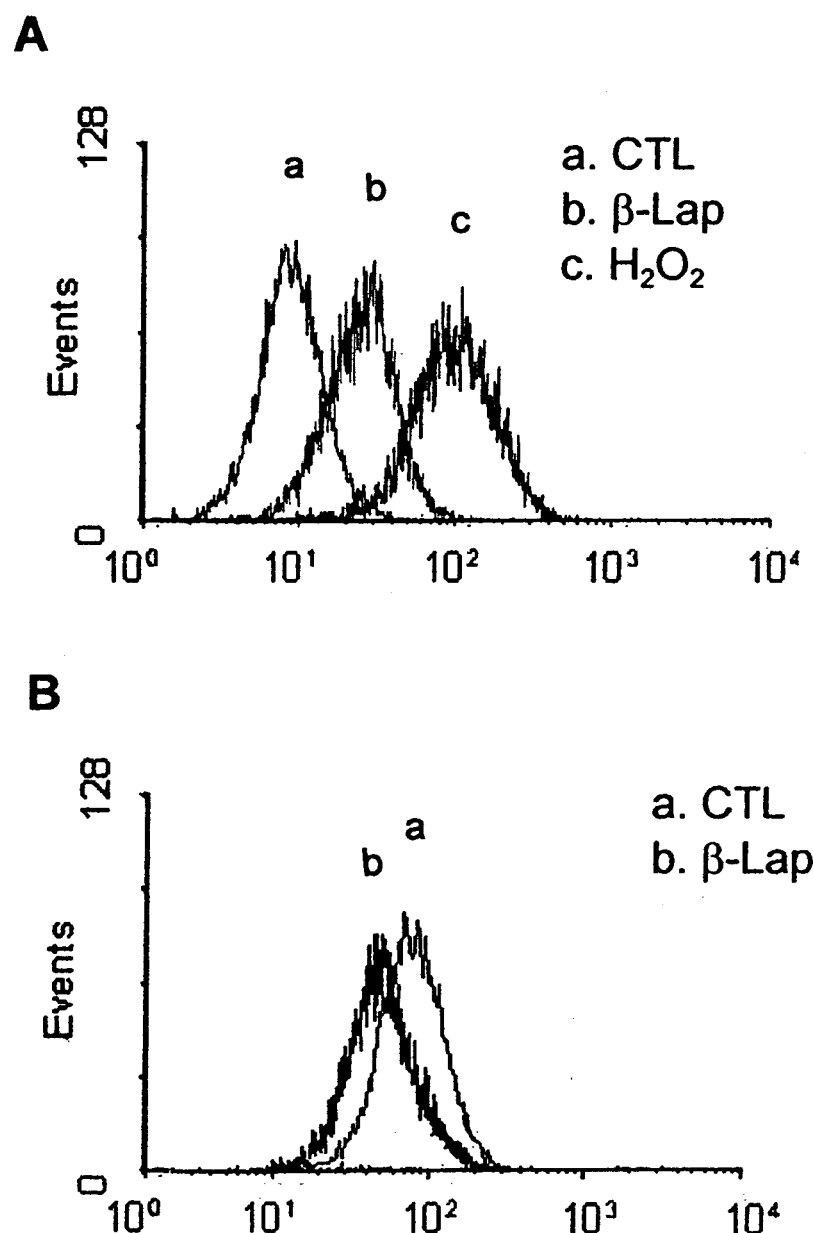


Fig. 5. β -Lapachone-induced ROS generation in PC-3 cells. A, intracellular hydrogen peroxide generation in β -lapachone-treated PC-3 cells was detected by flow cytometry using a peroxide-sensitive dye, DCFH-DA. Note that only a small increase in the level of hydrogen peroxide was observed in PC-3 cells after treatment with β -lapachone (peak b) compared with control cells (peak a). PC-3 cells were treated with 0.015% H_2O_2 for 15 min as a positive control (peak c). B, superoxide generation was also quantified by flow cytometry using the dye HE. Note that β -lapachone failed to enhance the production of superoxide (peak b) compared with cells treated with solvent control (peak a).

Discussion

β -Lapachone Causes Apoptosis of HPC Cells through an ROS-Independent Pathway.

Our previous study demonstrated that the main cause of β -lapachone-mediated apoptosis of HL-60 cells is the increased production of intracellular ROS (Chau et al., 1998). However, in this study, we demonstrated that the induction of oxidative stress by β -lapachone may not a crucial factor in the apoptosis of HPC cells. It is generally accepted that the susceptibility of oxidative stress in various cell lines is different and dependent on the cellular thiol content and activities of SOD and catalase. Compared with human leukemic cell lines, androgen-independent human prostate cancer cell lines such as DU145 and PC-3 cells are less susceptible to oxidative damage (Sun et al., 1999a,b). In the present study, we demonstrated that there was only a small increase (2- to 4-fold) in hydrogen peroxide production in β -lapachone-treated HPC cells (Fig. 5), whereas under the same experimental conditions, an increase of ≤ 10 -fold in fluorescence intensity was seen in the positive control. Furthermore, although our result showed that NAC, the thiol antioxidant, provided a partial protection on DU145 cells after treatment with β -lapachone for 12 h, other antioxidants such as ascorbic acid (vitamin C), α -tocopherol (vitamin E), and propyl gallate, had no significant protective effect on β -lapachone-induced cytotoxicity in HPC cells. Subsequently, NAC and other antioxidants (vitamin C, vitamin E and propyl gallate) did not block the β -lapachone-induced apoptosis on HPC cells. Positive TUNEL labeling was also found on HPC cells treated with both antioxidant and β -lapachone (Fig. 6C). Together, these results suggest that the ROS generation induced by β -lapachone may not be the key component to trigger the apoptosis in HPC cells. The mechanism by which NAC might promote cell survival is still unclear. In addition to its effect on reducing the generation of ROS, NAC might lead to alteration of the cellular redox state, which in turn might modulate the activity of specific transcription factors (Moynagh et al., 1994; Lin et al., 1995; Yan et al., 1995). Recently, other studies have shown that β -lapachone induced cytotoxicity in cell lines such as MCF-7 and SW620 cells without stimulating hydrogen peroxide production (Chau et al., 1998; Wuerzberger et al., 1998; Huang and Pardee, 1999).

β -Lapachone Induces DNA Damage and PARP Cleavage.

It is still unclear whether β -lapachone is a DNA topoisomerase inhibitor, because it has been reported not to form an SDS- K^+ precipitable protein-DNA complex or to arrest the cell cycle (Li et al., 1993). In the present study, β -lapachone-induced DNA damage was studied using the TUNEL method (Fig. 3). Genomic DNA is an important subcellular target for most chemotherapeutic drugs and, in response to DNA damage, cells activate DNA repair enzymes, such as DNA-PK and PARP (Szumiel, 1998; Teyssier et al., 1999). Thus, to demonstrate that β -lapachone was a DNA-damaging agent, it was necessary to demonstrate that DNA-PK expression and PARP activity were increased. The present study shows that this is indeed the case and that both are early events occurring before the morphological changes and the induction of cdk inhibitors.

β -Lapachone Halts the Cell Cycle by Increasing Levels of cdk Inhibitors. β -Lapachone has been reported to induce apoptosis in HPC cells and breast cancer cells without

noblot analysis of lysates of HPC cells treated for various times with β -lapachone showed that caspase-7, but not caspase-3 and caspase-8, was activated and proteolytically cleaved after 6-h exposure to β -lapachone (Fig. 11A). In addition, the activation of caspase-7 was also detected on the antioxidant-pretreated cells after 24-h exposure to β -lapachone in the presence of antioxidant (NAC or vitamin C) (Fig. 11B).

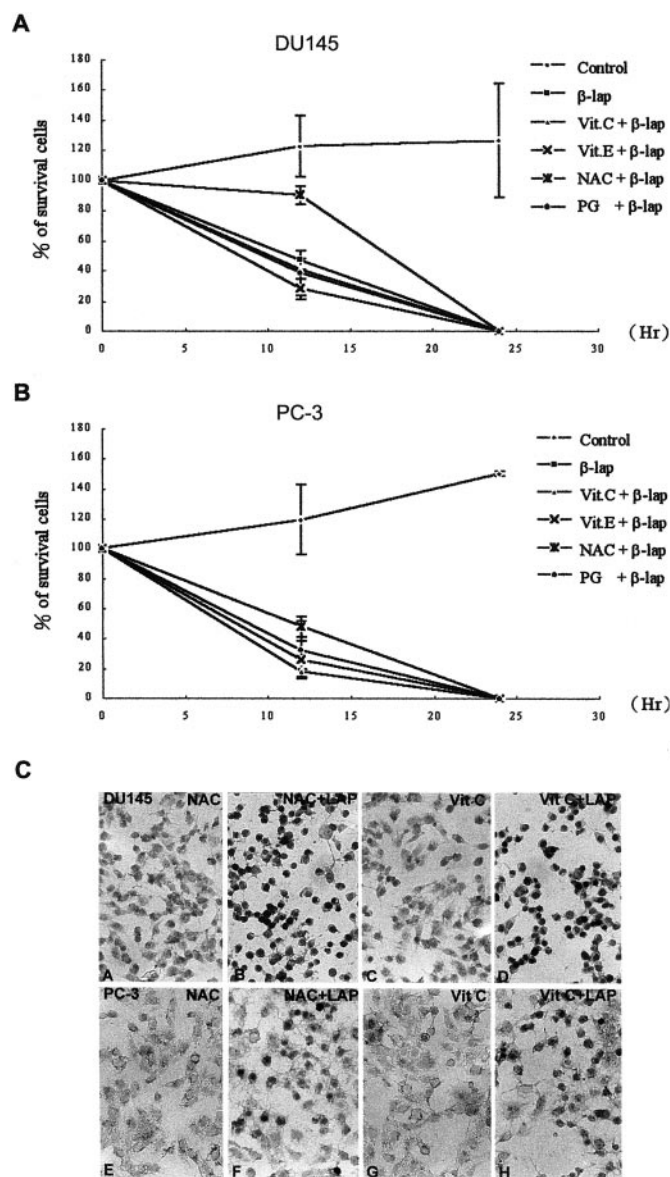


Fig. 6. Effects of various antioxidants on β -lapachone-induced apoptosis in HPC cells. After a 3-h pretreatment with various antioxidants (NAC, vitamin C, vitamin E, or propyl gallate), DU145 (A) and PC-3 cells (B) were exposed to 2 or 5 μ M β -lapachone for 0, 12, or 24 h, respectively. Cells viability was then determined by Trypan Blue exclusion assay. Data represent the means \pm S.D. from at least three measurements. C, the TUNEL labeling assay for detection of DNA fragmentation in HPC cells pretreated with various antioxidants. DU145 or PC-3 cells grown on coverslides were incubated with serum-free medium for 24 h and then pretreated with NAC or vitamin C for 3 h. After exposure to 2 or 5 μ M β -lapachone for 24 h in the presence of antioxidants, cells were fixed with 3.7% paraformaldehyde for 10 min. DNA damage were then detected with TUNEL labeling as described under *Materials and Methods*. Experimental results showed that the positive TUNEL labeling was only detected in DU145 cells (B, D) and PC-3 cells (G, H) treated with both antioxidant (NAC or vitamin C) and β -lapachone but not in cells treated with antioxidant (NAC or vitamin C) alone (A, C, E, and G). Magnification, 75 \times .

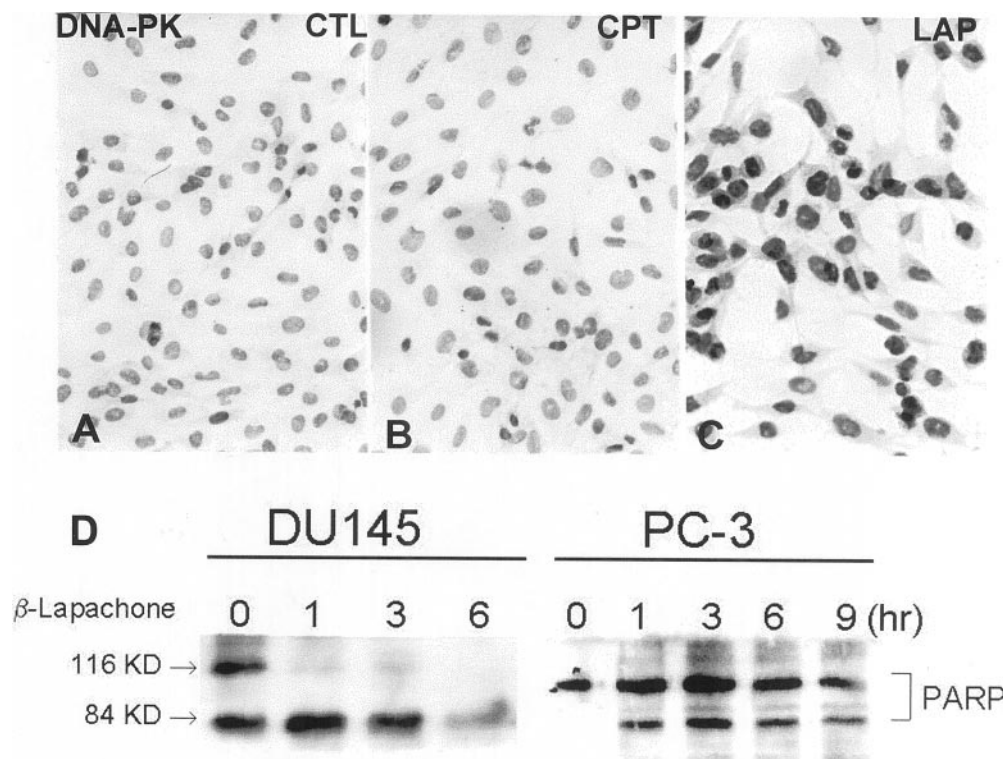


Fig. 7. β-Lapachone increased the expression of DNA-PK and the cleavage of PARP in PC-3 cells. PC-3 cells grown on coverslips were treated for 3 h with vehicle (A), 10 μM camptothecin (B) or 5 μM β-lapachone (C). Afterward, cells were fixed and immunostained with DNA-PK antibody. The immunoreactivity was visualized by DAB reaction. Note that the intense DNA-PK immunoreactivity was seen in the nuclei of β-lapachone treated cells. Magnification, 150×. D, immunoblot analysis of proteolytic cleavage of PARP in β-lapachone treated HPC cells. DU145 or PC-3 cells were treated with 2 or 5 μM β-lapachone, respectively, for 0 to 9 h. Nuclear protein (100 μg/lane) prepared as detailed in *Materials and Methods*, were subjected to SDS-PAGE followed by Western blot analysis using a PARP antibody. Both native (115-kDa) and cleaved (84-kDa) forms of PARP were monitored in cells after β-lapachone treatment.

any apparent arrest of the cell cycle or induction of the cdk inhibitor p21 (Li et al., 1995), but this is still controversial (Li et al., 1999). In this study, we demonstrated a significant increase in the expression of cdk inhibitors p21 and p27 after a 6-h exposure of the HPC cells to β-lapachone. Both p21 and p27 have been suggested to be negative regulators of the cell cycle. Overexpression of p21 or p27 results in accumulation of cells in G₁ arrest (Kwon and Nordin, 1997; Ladha et al., 1998). However, increased levels of p27 and G₂/M blockage of the cell cycle were seen in androgen-independent prostate cancer cells after camptothecin or etoposide treatment (Frydman et al., 1997; Furuya et al., 1997). The increase of cdk inhibitors (p21 and p27 proteins) is not a common event in β-lapachone-mediated apoptotic pathway because no elevation of p21 and p27 proteins was detected in the apoptotic pathway of human leukemic cell lines by β-lapachone (data not shown). Consistent with this result, the increase of p21 by β-lapachone was not found in colon cancer cell lines (SW620 and DLD1) (Huang and Pardee, 1999). However, the elevated expressions of p21 and p27 have been confirmed in DU145 cells and SW480 cells, respectively, after β-lapachone treatment (Huang and Pardee, 1999; Li et al., 1999). Taken together, these results suggest that the different mechanisms of β-lapachone-mediated cytotoxicity may be involved in different cell lines. Moreover, the induction of p21 and p27 in HPC cells by β-lapachone occurred before the induction of cell death, indicating that it may be an important signal in the β-lapachone-mediated apoptotic pathway. The induction of both p21 and p27, seen in the present study, was independent to p53 expression, because the HPC cell lines (DU145 and PC-3 cells) used are p53-mutant. Based on our data, β-lapachone seems to be a potent DNA topoisomerase inhibitor and DNA-damaging agent that induces the expression of the cdk inhibitors p21 and p27 and halts the progression of the cell cycle.

β-Lapachone Enhances bak Expression during HPC Cell Apoptosis. Several studies have suggested that cell death or survival is determined by the relative amounts of pro- and

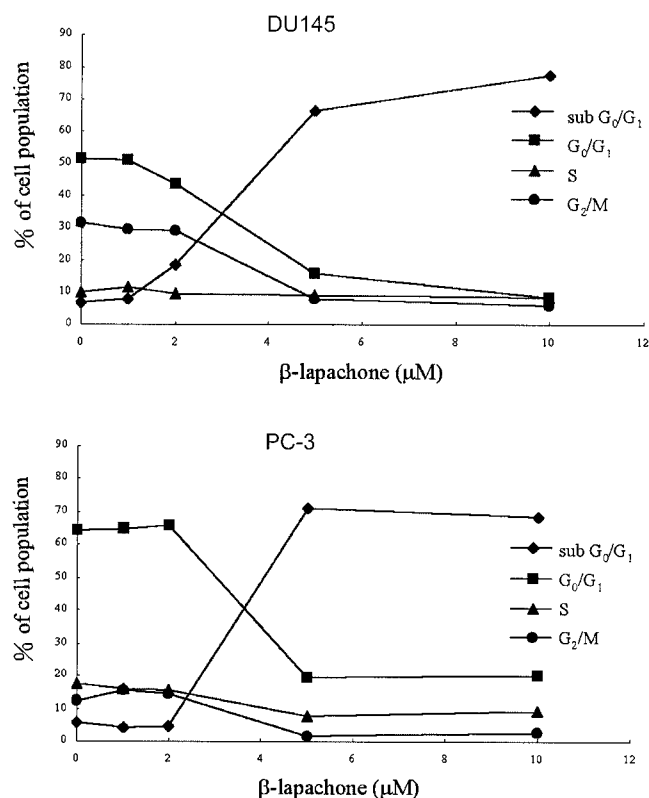


Fig. 8. Cell cycle analysis of β-lapachone-treated HPC cells. DU145 or PC-3 cells were cultured in serum-free medium for 24 h and then treated with various concentrations of β-lapachone for an additional 24 h. Cells were fixed in cold 70% ethanol, stained with PI, and then analyzed for DNA content by flow cytometry as described under *Materials and Methods*.

antiapoptotic members of the bcl-2 family (Adams and Cory, 1998). An increase in the proapoptotic proteins bax and bak and a decrease in bcl-2 protein is always accompanied by apoptosis or differentiation in various cell lines. To investigate the roles of bcl-2-related proteins in the pathway involved in β -lapachone-

induced HPC cell death, the expression of a pro-apoptotic protein (bak and bax) and of two antiapoptotic proteins (bcl-2 and bcl-xs) was studied by immunocytochemistry and Western blotting. The results showed that β -lapachone induced the expression of bak, but not of bax, bcl-2, or bcl-x (Fig. 10).

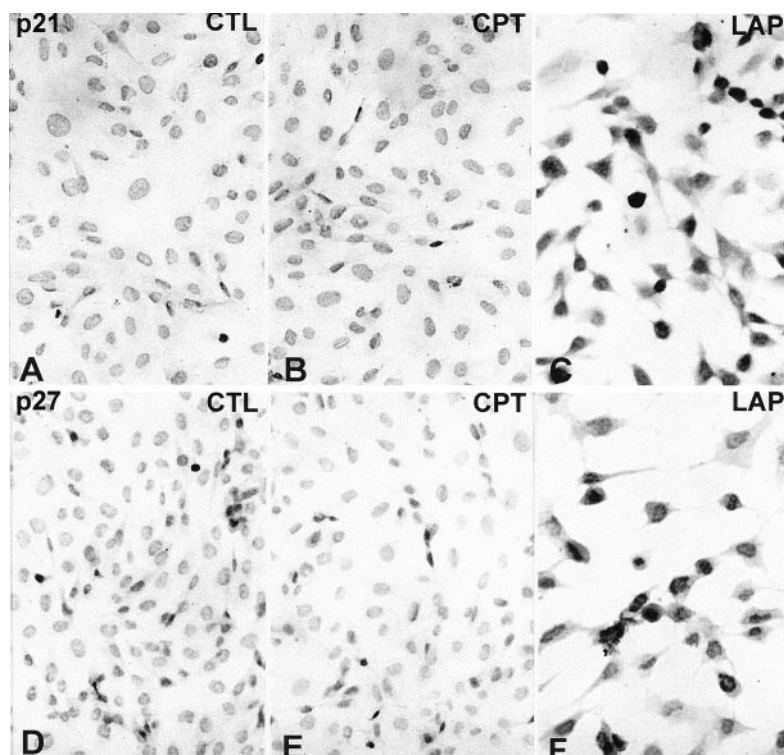


Fig. 9. Immunostaining and Western blotting of cyclin inhibitors p21 and p27 in PC-3 cells after 5 μ M β -lapachone treatment. PC-3 cells were incubated with serum-free medium for 24 h. Afterward, cells were treated with vehicle (A, D), camptothecin (B, E), and β -lapachone (C, F). Cells were then fixed with 3.7% paraformaldehyde and immunostained with p21 (A, B, and C) or p27 antibody (D, E, and F), respectively. The immunoreactivity was lastly visualized by DAB reaction. Magnification, 150 \times . G, the expression of p21 and p27 proteins in DU145 or PC-3 cells after β -lapachone treatment. Nuclear protein (100 μ g/lane), prepared as detailed under *Materials and Methods*, was subjected to SDS-PAGE followed by Western blot analysis by p21 or p27 antibody. Immunoblotting with α -tubulin antibody in the same blotting membrane was performed to monitor for protein loading.

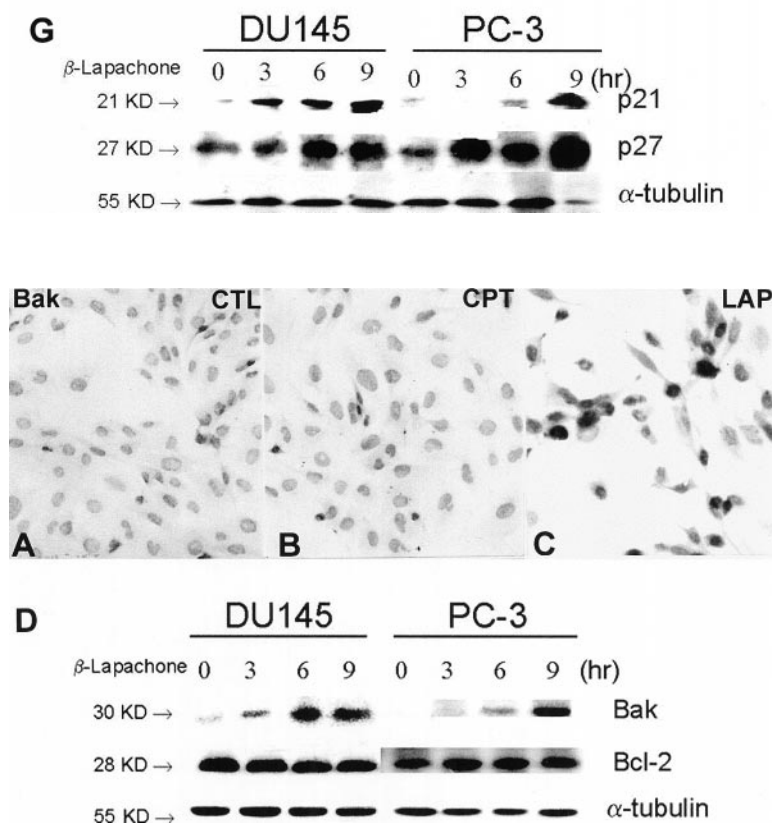


Fig. 10. Induction of bak expression in DU145 cells after 2 μ M β -lapachone treatment. DU145 cells were incubated with serum-free medium for 24 h. Afterward, cells were treated with vehicle (A), camptothecin (B), and β -lapachone (C). Cells were then fixed with paraformaldehyde and immunostained with bak antibody. The immunoreactivity was lastly visualized by DAB reaction. Magnification, 150 \times . D, immunoblot analysis of bak and bcl-2 levels in DU145 or PC-3 cells after exposure to 2 or 5 μ M β -lapachone, respectively, for 0 to 9 h. Total protein (100 μ g/lane) was subjected to 12% SDS-PAGE followed by Western blot analysis by Bak or Bcl-2 antibody. Reprobing the same blot with α -tubulin antibody was performed to monitor for protein loading.

β-Lapachone Induces Activation of Caspase-7 in the Pathway of HPC Cell Apoptosis. Because caspase activation is a common event in apoptosis, we determined whether it occurred during β-lapachone-induced HPC cell apoptosis. Western blots showed that caspase-7, but not caspase-3 or caspase-8, underwent proteolytic activation during this process. Both caspase-3 and caspase-7 are widely recognized to be downstream effectors of apoptotic pathways (Marcelli et al., 1999). Caspase-7 is a cytosolic protein that can be processed into its mature form by caspase-3, caspase-8, or caspase-10 (Marcelli et al., 1998). Caspase-3 activation is known to be induced by β-lapachone during apoptosis of HL-60 cells (Planchon et al., 1999; Shiah et al., 1999). However, in this study, no activation of caspase-3 or caspase-8 was seen during β-lapachone-induced HPC cell apoptosis, so it is likely that caspase-10 or an unknown protease is responsible for the proteolytic activation of caspase-7 in this pathway.

In summary, we have demonstrated that the oxidative stress may not be a crucial factor in the β-lapachone-induced apoptosis in HPC cells. The mechanism by which β-lapachone-induced apoptosis in human prostate cancer cells involves the DNA damage, induction of cdk inhibitors, in-

creased bak expression, and subsequent stimulation of caspase-7 activation.

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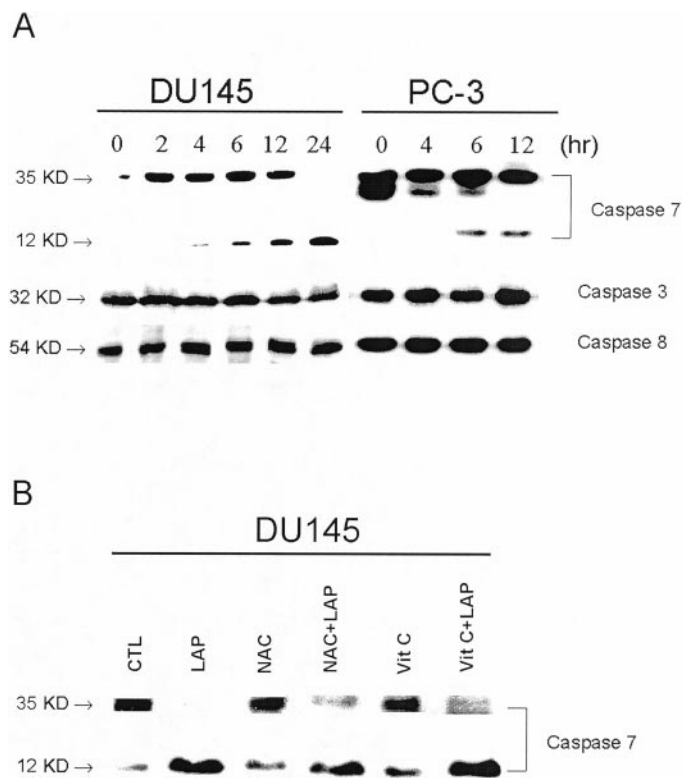


Fig. 11. Activation of caspases during β-lapachone-induced apoptosis of HPC cells (A). Immunoblot analysis of the activation of caspase-7, caspase-3, and caspase-8 after treatment of DU145 with 2 μM β-lapachone or PC-3 cells with 5 μM β-lapachone for 0–24 h. Total protein (100 μg/lane) was subjected to 12% SDS-PAGE followed by Western blot analysis with caspase 3, 7 or 8 antibody. Activation of caspase-7 was shown by the progressive appearance of its proteolytic fragment (12 kDa) in both DU145 or PC-3 cells after β-lapachone treatment (B). Immunoblot analysis of the activation of caspase-7 in DU145 cells treated with β-lapachone in the absence or presence of antioxidant (30 mM NAC or 25 μM vitamin C) for 24 h. Increased amounts of the caspase-7 proteolytic fragment (12 kDa) were found in cells treated with β-lapachone (lane 2) or treated with both antioxidant (NAC or vitamin C) and β-lapachone (lane 4 and lane 6).

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