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# Involvement of endoplasmic reticulum stress and activation of MAP kinases in ß-lapachone-induced human prostate cancer cell apoptosis

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**Summary.** ß-Lapachone, an *o*-naphthoquinone, induces various carcinoma cells to undergo apoptosis, but the mechanism is poorly understood. In the present study, we found that the ß-lapachone-induced apoptosis of DU145 human prostate carcinoma cells was associated with endoplasmic reticulum (ER) stress, as shown by increased intracellular calcium levels and induction of GRP-78 and GADD-153 proteins, suggesting that the endoplasmic reticulum is a target of ß-lapachone. ß-Lapachone-induced DU145 cell apoptosis was dose-dependent and accompanied by cleavage of procaspase-12 and phosphorylation of p38, ERK, and JNK, followed by activation of the executioner caspases, caspase-7 and calpain. However, pretreatment with the general caspase inhibitor, z-VAD-FMK, or calpain inhibitors, including ALLM or ALLN, failed to prevent ß-lapachone-induced apoptotic cell death. Blocking the enzyme activity of NQO1 with dicoumarol, a known NQO1 inhibitor, or preventing an increase in intracellular calcium levels using BAPTA-AM, an intracellular calcium chelator, substantially inhibited MAPK phosphorylation, abolished the activation of calpain, caspase-12 and caspase-7, and provided significant protection of ß-lapachone-treated cells. These findings show that ß-lapachone-induced ER stress and MAP kinase phosphorylation is a novel signaling pathway underlying the molecular mechanism of the anticancer effect of ß-lapachone.

**Key words:** ß-Lapachone, Endoplasmic reticulum (ER) stress, MAPK phosphorylation

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## Introduction

ß-Lapachone, an ortho-naphthoquinone originally extracted from the lapacho tree (*Tabebuia avellaneda*), has anticancer activity against various human carcinoma cells, including human bladder (Lee et al., 2006), breast (Pink et al., 2000b), lung (Bey et al., 2007) and prostate cancer cells (Choi et al., 2002). It acts as an inhibitor of DNA topoisomerases, including DNA Topo I and II, and interrupts the cell cycle by blocking DNA transcription and translation by interacting with the DNA topo enzymes (Frydman et al., 1997; Li et al., 1999). More recent studies have shown that ß-lapachone undergoes a redox cycle in the presence of NAD(P)H:quinone oxidoreductase (NQO1), which reduces ß-lapachone to an unstable semiquinone, which rapidly undergoes a two-step oxidation back to the parent compound, perpetuating a futile redox cycle and resulting in the generation of reactive oxygen species (ROS) and superoxide (Pink et al., 2000a; Planchon et al., 2001; Choi et al., 2007). These reactive species can oxidize thiol groups of the mitochondrial potential transition pore complex, leading to an increase in permeability of the mitochondrial inner membrane and a reduction in mitochondrial membrane depolarization and the release of cytochrome c, subsequently leading to cell death (Lemasters et al., 1998; Smaili et al., 2000; Rivers et al., 2005).

Apoptosis is a tightly regulated process. Previous studies have focused on mitochondrial function, and energetic perturbations have been recognized as an initial trigger for cellular dysfunction (Smaili et al., 2000; Shoshan-Barmatz et al., 2006). Recently, it was shown that many factors, such as oxidative stress, excitotoxicity, and apoptotic-like mechanisms that cause cell death, may be associated with endoplasmic reticulum (ER) stress (Hitomi et al., 2004; Pattacini et

al., 2004). ER stress triggers many pathological responses in cells, including imbalance of $Ca^{2+}$ homeostasis, induction of the unfolded protein response (UPR), and activation of the caspase cascade (Mandic et al., 2003; Sharma and Rohrer, 2004; Bentle et al., 2006). Importantly, pro-apoptotic organelle crosstalk has been demonstrated between the ER and mitochondria, with ER stress being upstream of mitochondrial dysfunction, cytochrome c release, and apoptosome assembly for caspase activation (Reeve et al., 2007; Zhang and Armstrong, 2007; Ferreiro et al., 2008). Whether ER stress is involved in ß-lapachone-mediated cell death and, if so, the pathway involved, are not known.

In this study, we used an androgen-independent human prostate carcinoma cell line, DU145, as a model to examine whether ER stress and its associated signals were involved in ß-lapachone-induced apoptosis. ß-Lapachone induction of ER stress was supported by alterations in $Ca^{2+}$ homeostasis, activation of procaspase-12, and up-regulated expression of glucose-regulated protein-78 (GRP-78) and pro-apoptotic growth arrest and DNA gene product 153 (GADD-153) proteins. Moreover, the ß-lapachone response was accompanied by MAP kinase activation (phosphorylation of p38, ERK, and JNK) and the subsequent cleavage of executioner caspases (caspase-7 and calpain). Reduction of NQO1 enzyme activity using the specific NQO1 inhibitor, dicoumarol, or prevention of an increase in intracellular calcium levels using a calcium chelator, BAPTA-AM/AM, significantly abolished the ß-lapachone-induced imbalance in $Ca^{2+}$ homeostasis, inhibited the activation of executioner caspases and prevented apoptosis.

## Materials and methods

### *Chemicals*

ß-Lapachone, prepared as 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. Propidium iodide (PI), Annexin-V-FITC and Fluo-4/AM were obtained from Molecular Probe (Fremont, CA), and z-VAD-FMK, EGTA and BAPTA-AM/AM were obtained from Calbiochem (Gibbstown, NJ). Other chemicals were obtained from Sigma Chemical Co (St. Louis, MO).

### *Cell culture*

The human prostate cancer cell line, DU145, was obtained from the American Type Culture Collection (Rockville, MD). Cells ($2x10^6$) were grown for 24 h at 37°C in a humidified 5% $CO_2$ atmosphere in RPMI medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 unit/ml each of penicillin and streptomycin. They were then washed twice with prewarmed phosphate-buffered saline (PBS) and cultured in serum-free medium for an additional 12 h.

### *Cell death assay*

For the MTT assay, DU145 cells ($1x10^4$) in 100 µl of medium were seeded for 24 h at 37°C in a 96-well culture plate in a humidified 5% $CO_2$ atmosphere, washed twice with prewarmed PBS, and cultured for 12 h in serum-free medium before being treated with various concentrations of ß-lapachone for 24 h. Ten microliters of a 5 mg/ml stock solution of MTT was added to each well and incubation continued for another 4 h at 37°C, then the insoluble formazan product was dissolved for 30 min at 37°C in 100 ml of DMSO and the absorbance at 570 nm measured using a microplate reader (Tecan, Austria). For protease inhibitor and MAP kinase inhibitor studies the cells were pretreated for 3 h with various drugs, then cotreated with ß-lapachone for a further 6-24 h before cell viability was evaluated using the MTT assay. The data are presented as the mean ± SD for at least three sets of independent experiments, each carried out in triplicate. Differences among groups were examined using one-way ANOVA with the *Scheffe* test and a $p$ value less than 0.05 was considered statistically significant. For the Trypan blue exclusion assay, after a 12 h period in serum-free culture, the cells ($3x10^5$) in each well of a 6-well culture plate were washed twice with prewarmed PBS, then treated with various concentrations of ß-lapachone for 24 h, after which they were trypsinized and cell viability was evaluated by Trypan blue exclusion using phase contrast microscopy. Cells taking up Trypan blue were classed as nonviable and expressed as a percentage of the total number.

### *Acridine orange (AO) staining*

DU145 cells ($5x10^4$) cultured on 12 mm coverslipes in 24-well plates were used. For AO staining, the cells were incubated with 2 µM ß-lapachone for 0, 3, 6, or 12 hours, then were fixed with methanol : glacial acetic acid (3:1, v/v), stained for 5 min with 0.5 µl of AO solution (10 mg/ml in PBS), and examined using an Olympus BH-2 microscope with a fluorescence attachment.

### *Detection of apoptosis and measurement of intracellular calcium levels*

To detect apoptosis, ß-lapachone-treated or untreated cells ($1x10^6$) were washed with ice-cold PBS and trypsinized with 0.05% trypsin-0.02% EDTA. After several washes with cold PBS, the cells were stained for 15 min at 37°C with annexin V-FITC (10 µg/ml) and propidium iodide (50 mg/ml) in 100 ml of binding buffer (10 µM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After dilution with 400 ml of binding buffer, the cells were analyzed by flow cytometry using FACScan flow cytometry (Becton Dickinson). For measurement of intracellular calcium levels, cells were incubated for 10

min at 37°C with 2 mM Fluo-4/AM in medium, washed once with PBS, trypsinized, and immediately analyzed by FACSan flow cytometry.

### *Western blot analysis*

Whole cell extracts from control and drug-treated cells were prepared. Briefly, $3x10^5$ DU145 cells cultured in 6-well plates were washed twice with ice-cold PBS, scraped off, and collected by centrifugation (800xg for 10 min, 4°C), then lysed for 30 min at 4°C with gentle agitation in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% NP-40, 0.1% SDS, 1 mM PMSF, 10 mg/ml of aprotonin, and 10 mg/ml of leupeptin). After centrifugation (15000xg for 10 min, 4°C), the supernatants were collected and stored at -80°C as whole cell extracts. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA), then 40-80 µg samples of protein were separated by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) in a Trans-Blot Electrophoretic Transfer cell (2 h at 200 V). The membranes were then blocked with 5% skim milk in PBS-0.2% Tween 20, incubated for 2 h at room temperature with various primary antibodies (using 1:500 to 1:2000 dilutions), washed for 2 h at room temperature in PBS-0.2% Tween 20, then incubated for 1 h at room temperature with secondary antibodies (1:2000 dilution). Bound antibody was detected using the ECL Western blotting reagent (Amersham, Piscataway, NJ), the chemiluminescence being 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.

### *Statistical analysis*

Results are presented as mean ± SEM for at least three replicate experiments. Differences among groups were examined using one-way ANOVA with the *Scheffe* test and a $p$ value less than 0.05 was considered statistically significant.

## Results

### *ß-Lapachone induces apoptosis of DU145 cells*

The cytotoxic effect of ß-lapachone (Fig. 1A) on DU145 cells was evaluated using the MTT assay and Trypan blue exclusion. As shown in Fig. 1B, the cytotoxic effect seen after 24 h of treatment was concentration-dependent, with more than 70% of the cells being killed using 1.25 mM ß-lapachone. In all subsequent studies, 2 mM ß-lapachone was used. Fig. 1C shows the time course of killing. When the ß-lapachone-treated cells were examined by AO staining and fluorescent microscopy, or annexin-V labeling and flow cytometry, time-dependent cytotoxicity was seen (Fig. 2).

### *Activation of caspases and calpain during ß-lapachone-induced DU145 cell apoptosis*

Our previous study (Don et al., 2001) showed that ß-lapachone induces human prostate cancer cell apoptosis through the activation of procaspase-7, rather than procaspase-3. In the present study, Western blots showed that a 3 h treatment of DU145 cells with ß-lapachone resulted in activation of calpain, procaspase-12 and procaspase-7 (Fig. 3A). However, ß-lapachone -induced DU145 cell apoptosis was not attenuated by cotreatment with either a general caspase inhibitor, z-VAD-FMK, or the calpain inactivators, ALLM and ALLN (Fig. 3B), suggesting that ß-lapachone did not induce DU145 cell apoptosis through a caspase- / calpain-dependent pathway.

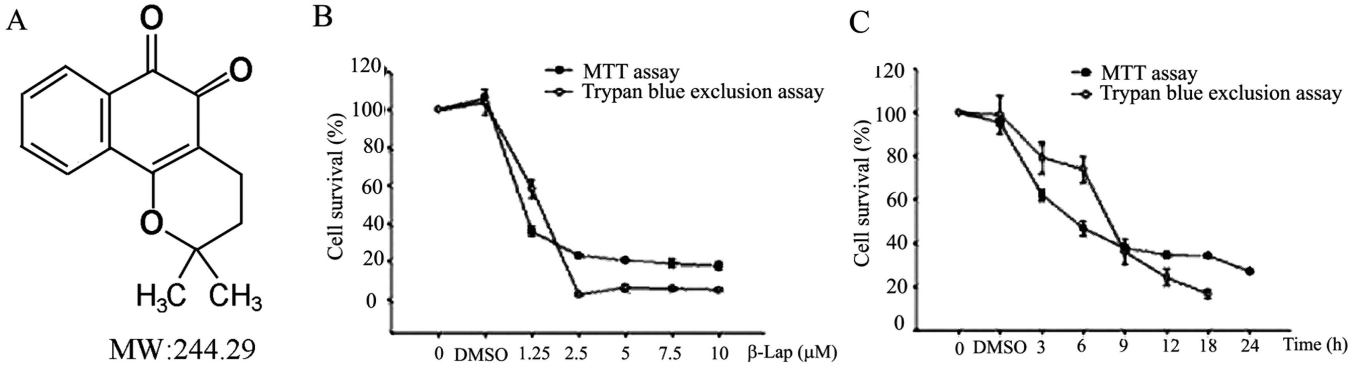


**Fig. 1.** Survival of DU145 cells following exposure to ß-lapachone. **A.** Chemical structure of ß-lapachone. For the MTT assay, DU145 cells ($1x10^4$) were cultured in 96-well plates in serum-free medium for 12 h. For the Trypan blue exclusion assay, DU145 cells ($3x10^5$) were cultured in 6-well plates in serum-free medium for 12 h. They were then treated with 0-10 mM of ß-lapachone for 24 h **(B)** or with 2 mM ß-lapachone for the indicated time **(C)** and cell survival measured. The points are the mean ± SD for three independent measurements, each in triplicate.

*ß-Lapachone induces endoplasmic reticulum (ER) stress, triggers disturbance of intracellular calcium homeostasis and activates MAP kinase phosphorylation*

It is known that the ER is exquisitely sensitive to alterations in homeostasis. It is also an important regulator of programmed cell death, cytotoxic insult and DNA damage, and alterations in $Ca^{2+}$ homeostasis initiate diverse molecular defense mechanisms referred to as "ER stress" (Shuda et al., 2003; Kim et al., 2006). To examine whether ß-lapachone caused ER stress, we analyzed the expression of glucose-regulated protein-78

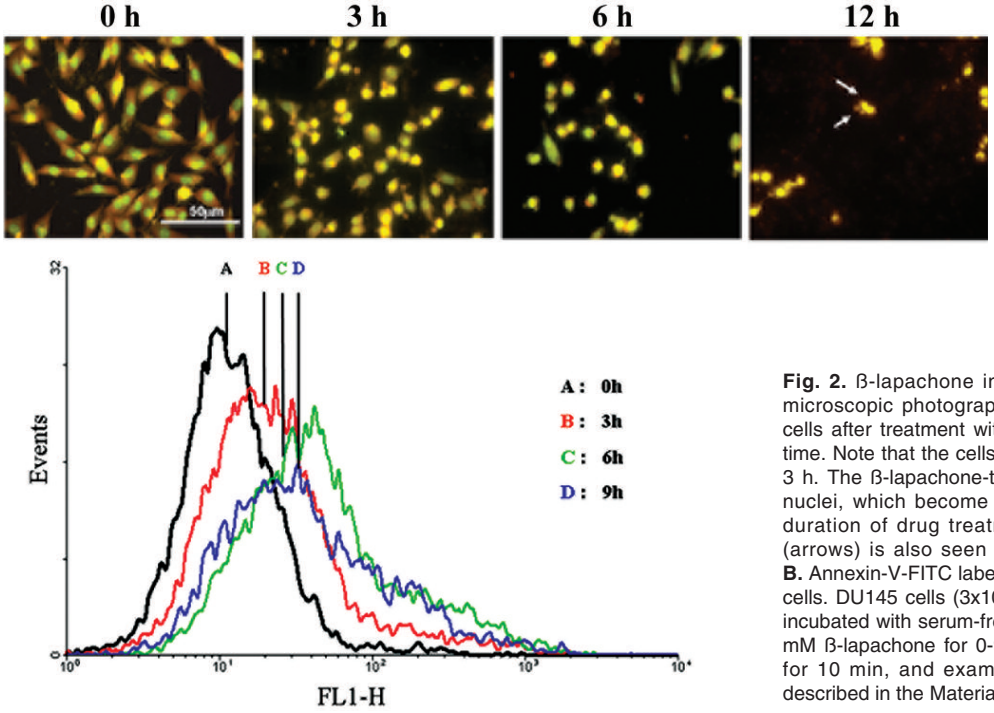


**Fig. 2.** ß-lapachone induces apoptosis of DU145. **A.** Light microscopic photographs of acridine orange-stained DU145 cells after treatment with 2 mM ß-lapachone for the indicated time. Note that the cells shrink in size and become round up at 3 h. The ß-lapachone-treated cells have intensely fluorescent nuclei, which become progressively more prominent as the duration of drug treatment is increased. Nuclear blebbing (arrows) is also seen after 12 h of ß-lapachone treatment. **B.** Annexin-V-FITC labeling for detection of apoptosis in DU145 cells. DU145 cells ($3x10^5$) grown on 6-well culture plates were incubated with serum-free medium for 12 h, then treated with 2 mM ß-lapachone for 0-9 h, fixed with 3.7% paraformaldehyde for 10 min, and examined for annexin-V-FITC labeling as described in the Materials and Methods.

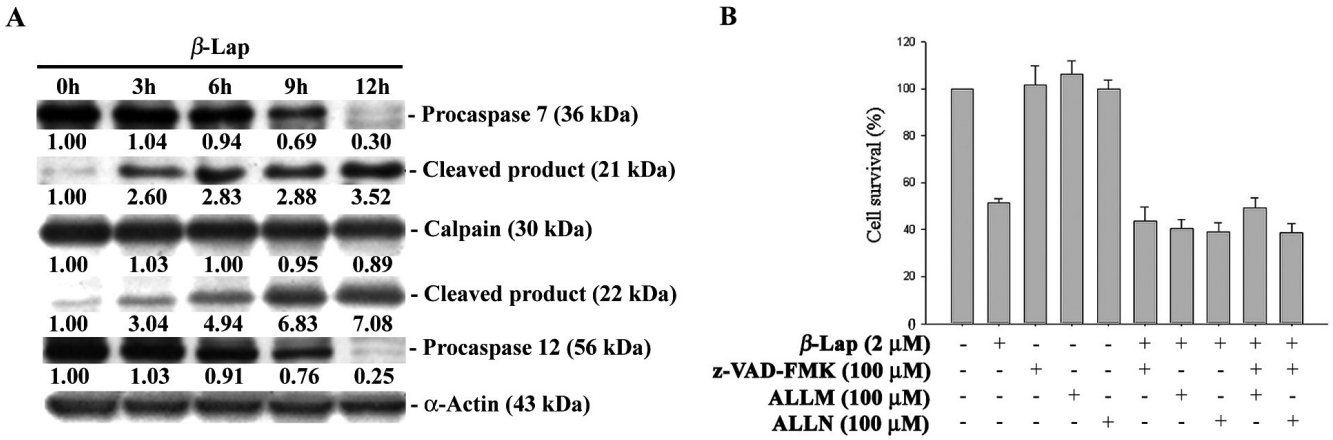


**Fig. 3.** Caspase/calpain activation during ß-lapachone-induced DU145 cell apoptosis. **A.** Immunoblot analysis of the activation of procaspase-7, calpain, and procaspase-12 in cells treated with 2 mM ß-lapachone for 0-12 h. Cleavage of procaspase-7 and calpain is shown by the progressive appearance of their proteolytic fragments (21 kDa and 22 kDa), while cleavage of procaspase-12 is shown by the loss of the procaspase-12 band. **B.** Lack of effect of caspase/calpain inhibitors on the survival of ß-lapachone-treated cells measured using the MTT assay. DU145 cells were incubated for 3 h in the presence or absence of a general caspase inhibitor (z-VAD-FMK) or calpain inhibitors (ALLM or ALLN), then with the inhibitor and 2 mM ß-lapachone for 6 h, and evaluated for survival, as described in the Methods.

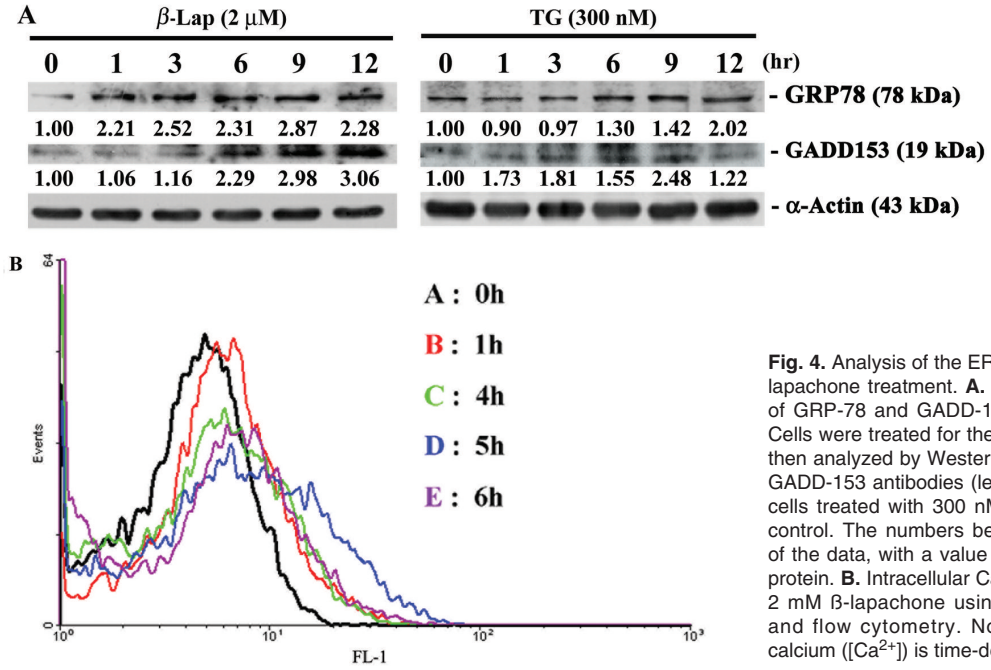


**Fig. 4.** Analysis of the ER stress response in DU145 cells after ß-lapachone treatment. **A.** Immunoblot analyses of the expression of GRP-78 and GADD-153 in ß-lapachone-treated DU145cells. Cells were treated for the indicated time with 2 mM ß-lapachone, then analyzed by Western blot analysis with anti-GRP-78 or anti-GADD-153 antibodies (left panel). The right panel shows DU145 cells treated with 300 nM thapsigargin for 0-12 h as a positive control. The numbers below represent densitometric evaluation of the data, with a value of 1.0 reflecting the basal level of each protein. **B.** Intracellular $Ca^{2+}$ levels measured in cells treated with 2 mM ß-lapachone using the $Ca^{2+}$-indicating dye, Fluo-4-AM, and flow cytometry. Note that the change in cytosolic free calcium ([$Ca^{2+}$]) is time-dependent.

(GRP-78), pro-apoptotic growth arrest and DNA gene product 153 (GADD-153), which are markers of the ER stress response. As shown in Fig. 4A, induction of GRP-78 or GADD-153 protein expression was significantly seen in cells treated with ß-lapachone for 1 or 3 h ($p<0.05$), respectively. Furthermore, flow cytometry showed a significant increase in Fluo-4/AM fluorescence intensity in cells following 1 h of ß-lapachone treatment (Fig. 4B), indicating that a disturbance of $Ca^{2+}$ homeostasis was associated with ER stress in the initial phase of ß-lapachone-mediated cell death. We then examined whether ß-lapachone caused activation of the stress-activated protein kinases p44/p42 MAP kinase, p38 MAP kinase, and JNK. As shown in Fig. 5, ß-lapachone induced marked activation of all three. Phosphorylation of p38 MAP and ERK kinases was seen at 6 h and reached a maximum at 9 h, while phosphorylation of JNK was seen at 3 h and also reached a maximum at 9 h. However, pretreatment of the cells with SB203580, a p38MAPK inhibitor, PD98059, a MEK inhibitor, or SP600125, a JNK inhibitor, did not protect the cells against ß-lapachone (data not shown), suggesting that the MAP kinase signaling pathway may be a downstream signal in ß-lapachone-mediated cell death.

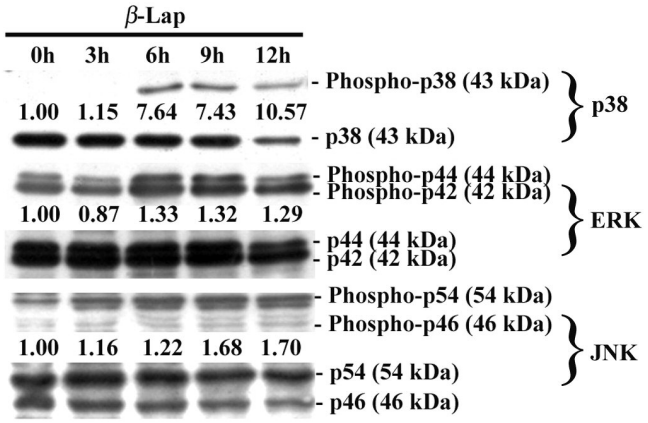


**Fig. 5.** Induction of MAP kinase activation in ß-lapachone -treated cells. Cells were incubated with 2 mM b ß-lapachone for 0-12 h, lysed with lysis buffer, and subjected to Western blot analysis using antibodies against phosphorylated p38 (anti-p38 antibody as control), phosphorylated ERK (anti-ERK antibody as control), or phosphorylated JNK (anti-JNK antibody as control). The numbers below represent densitometric evaluation of the data, with a value of 1.0 reflecting the basal level of phosphorylation of each signaling protein (p38, ERK and JNK, respectively). Each blot was re-hybridized with the control antibody; the results are representative of those for three blots.

### *Inhibition of NQO1 activity or the increase in intracellular $Ca^{2+}$ levels attenuates the cytotoxicity of ß-lapachone for DU145 cells*

Previous studies have shown that the toxicity of quinone-based anticancer drugs is caused, in part, by the bioreductive activity of NQO1. ß-lapachone is reduced to the unstable semiquinone intermediate form instead of the stable hydroquinone form by NQO1 (Pink et al., 2000a; Planchon et al., 2001). Furthermore, NQO1-deficient cell lines, such as human prostate carcinoma

LNCaP cells, are less sensitive to ß-lapachone treatment than NQO1-expressing cell lines, such as human prostate carcinoma DU-145 and PC-3 cells (Reinicke et al., 2005; Ross et al., 2006). To test whether NQO1 activity was an important factor in ß-lapachone-induced cell death, the effect of dicoumarol, a specific NQO1 inhibitor, on ß-

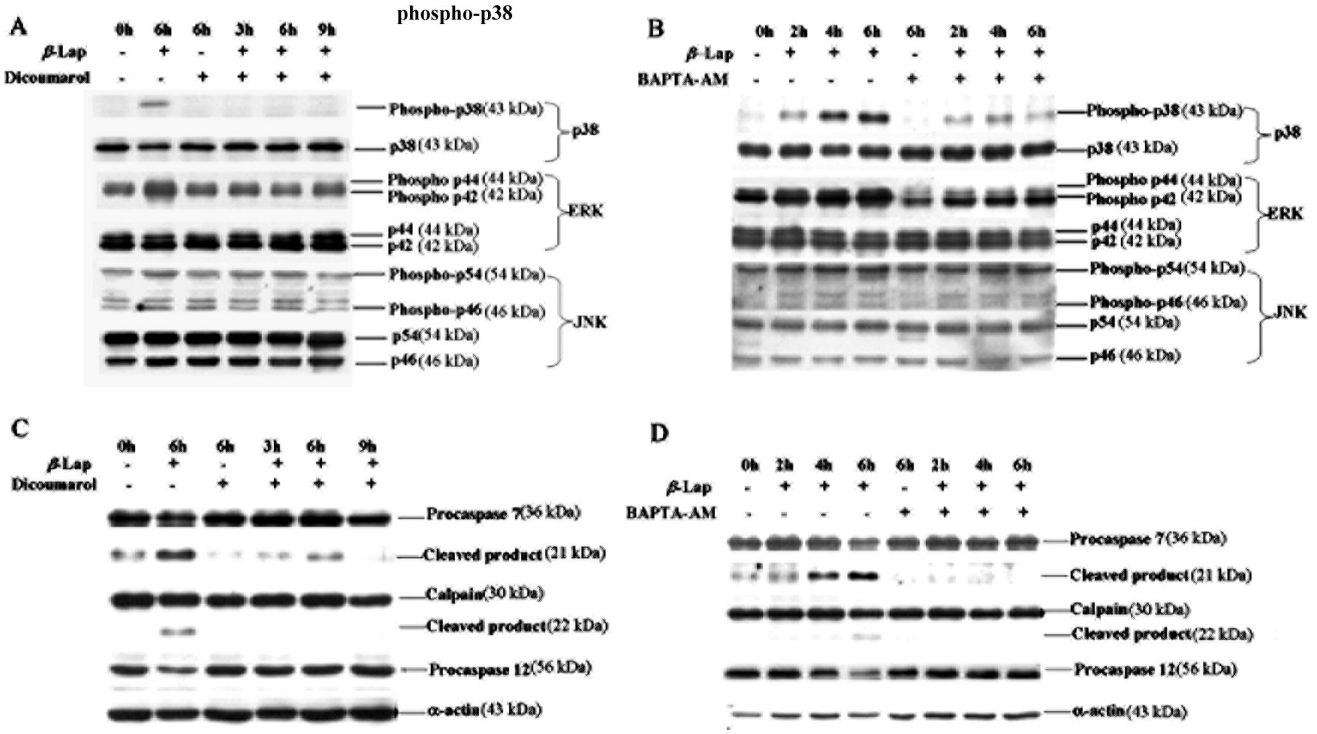


**Fig. 6.** Effects of the NQO1 inhibitor, dicoumarol, and the intracellular calcium chelator, BAPTA-AM, on ß-lapachone -mediated DU145 cell apoptosis. **A.** Dicoumarol inhibits the activation of MAP kinases by ß-lapachone. DU145 cells were cotreated with 10 mM dicoumarol and 2 mM ß-lapachone for the indicated time, and phospho-p38, phospho-ERK and phospho-JNK levels were measured by Western blotting as in Fig. 5. **B.** As in A, but using 5 mM BAPTA-AM instead of dicoumarol. **C.** Dicoumarol inhibits caspase/calpain activation during ß-lapachone-induced apoptosis. Cells were cotreated with 10 mM dicoumarol and 2 mM ß-lapachone for the indicated times, and activation of procaspase-7, calpain and procaspase-12 was examined by Western blotting. **D.** BAPTA-AM blocks ß-lapachone-induced caspase/calpain activation in DU145 cells. As in C, using 5 mM BAPTA-AM instead of dicoumarol.

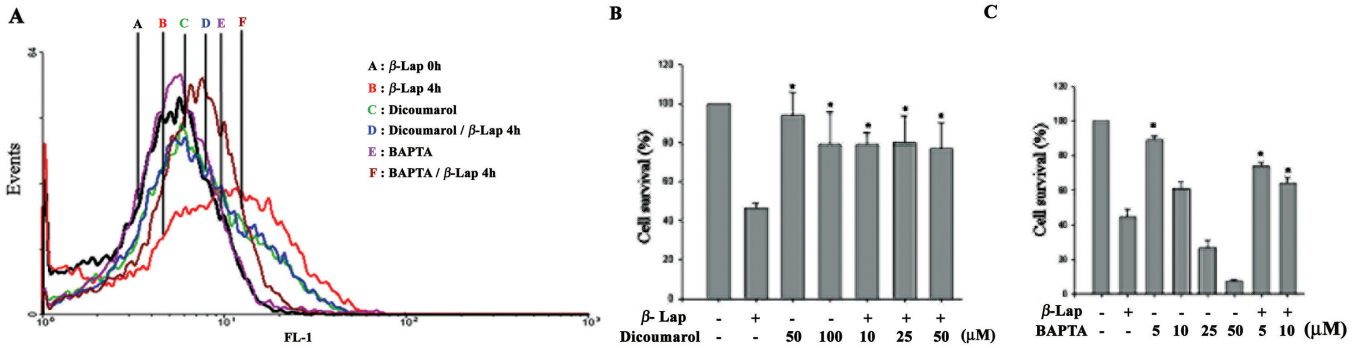


**Fig. 7.** Dicoumarol or BAPTA-AM attenuated the ß-lapachone–mediated $Ca^{2+}$ influx and provided protection against ß-lapachone. **A.** Intracellular $Ca^{2+}$ levels measured using the $Ca^{2+}$-indicating dye, Fluo-4-AM, and flow cytometry in untreated cells or cells cotreated for 4 h with 2 mM ß-lapachone and either 10 mM dicoumarol or 5 mM BAPTA-AM-AM. **B.** MTT assay for the survival of DU145 cells left untreated or treated with 2 mM ß-lapachone in the presence or absence of the indicated concentration of dicoumarol for 6 h. **C.** As in B, using BAPTA-AM instead of dicoumarol. The data are presented as the mean ± SD for at least three sets of independent experiments, each performed in triplicate. Differences between groups were examined using one-way ANOVA with the Scheffe test. *: denotes a statistically significant difference ($p<0.05$).

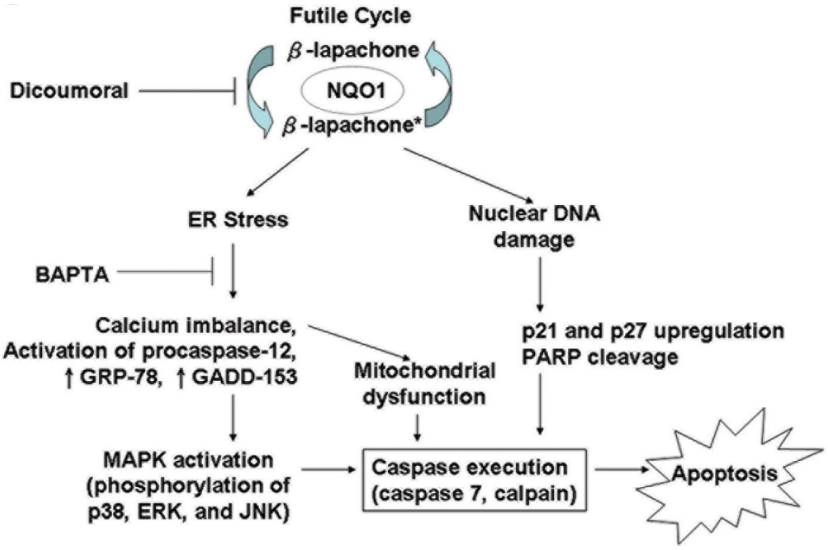


**Fig. 8.** A schematic representation of ß-lapachone-induced apoptosis in human prostate cancer DU145 cells. NQO1-activatedß-lapachone induces both ER stress response and nuclear DNA damage. The perturbation of cytosolic calcium leads to activation of MAP kinases, as well as mitochondrial dysfunction. These signal transduction pathways eventually converge on the activation of caspase executioners, leading to cell apoptosis.

lapachone -treated DU145 cells was studied. As shown in Fig. 6, 10 mM dicoumarol significantly blocked the ß-lapachone-induced phosphorylation of MAP kinases (Fig. 6A) and calpain/caspase activation (Fig. 6B). A similar effect was seen using 5 mM BAPTA-AM/AM, a calcium chelator (Fig. 6C,D). Consistent with this, co-administration of 10 mM dicoumoral or 5 mM BAPTA-AM/AM for 4 h prevented the ß-lapachone-induced increase in the $[Ca^{2+}]_i$ (Fig. 7A). In addition, co-treatment for 6 h with 10-50 mM dicoumarol (Fig. 7B) or 5-10 mM BAPTA-AM/AM (Fig. 7C) significantly protected DU145 cells against ß-lapachone cytotoxicity.

## Discussion

In this study, we examined the cytotoxic effect of ß-lapachone on human prostate carcinoma DU145 cells. Prostate cancer is an epidemic disease worldwide and is the second leading cause of cancer deaths in American men. Approximately 107 and 180 per 100,000 white and black American men die from prostate cancer every year (Parkin et al., 2002). The main curative treatments for early stage prostate cancer include surgeryand radiation therapy. If the disease advances to the seminal vesicle or pelvic lymph nodes surgery alone is not a cure. Hormone therapy is used for disease control. For metastasis status, hormone treatment becomes the major treatment. However, the results of hormone ablation for metastatic prostate cancer have been disappointing. After 12-18 months of hormone ablation prostate cancer cells in more than 50% of patients become androgen-independent and escape apoptosis induced by androgen ablation and be resistant to many anticancer drugs (Borden et al., 2006). In our previous study (Don et al., 2001), we reported that ß-lapachone is cytotoxic for various human prostate carcinoma cell lines, and suggested that ß-lapachone -mediated human cancer cell death may occur through activation of the calpain / caspase pathway. In the present study, we explored the underlying mechanism of ß-lapachone cytotoxicity on human prostate cancer DU145 cells. We found that exposure to ß-lapachone resulted in a concentration- and time-dependent decrease in cell viability (Figs. 1,2). Since activation of procaspase-7, procaspase-12, and calpain was also detected during ß-lapachone-induced DU145 cell apoptosis (Fig. 3A), we examined whether calpain or caspase inhibitors could protect the cells against ß-lapachone cytotoxicity, but found that this was not the case (Fig. 3B), demonstrating that calpain/caspase activation is not a critical factor in the mechanism of ß-lapachone-mediated DU145 cell death.

It is generally accepted that $Ca^{2+}$ is an important factor in regulating cellular homeostasis. A large body of evidence suggests that the ER may be the target of a variety of anti-tumor agents, including DNA damaging agents, calcium ionophores and inhibitors of protein glycosylation, which can induce ER stress and alter calcium homeostasis, leading to cell death (Mandic et al., 2003; Carew et al., 2006). These hypotheses suggest that ER stress responses can induce procaspase-12 activation and calcium ion efflux from the ER. Pro-caspase-12 is specifically localized to the ER and has been reported to be cleaved during disruption of ER function. Caspase-12 cleavage leads to activation of the $Ca^{2+}$-dependent cysteine protease, calpain. In several cell culture studies, the calpain-mediated cleavage of executioner caspases, such as caspase-3, -7, -8, and -9, has been shown to be involved in the process of apoptosis (Hitomi et al., 2004; Bentle et al., 2006) To determine whether ß-lapachone induced ER stress responses, one ER stress response, the UPR, characterized by upregulation of chaperons, such as GRP-78 and GADD-153, was examined (Wu et al., 2005; Zu et al., 2006). Our study showed that

upregulation of GRP-78 or GADD-153 was detectable after 1 h or 3 h of ß-lapachone treatment, respectively (Fig. 4A). In addition, we observed that exposure of DU145 cells to ß-lapachone for 1-6 h led to an increase in intracellular $Ca^{2+}$ levels (Fig. 4B).

The role of the MAPK signaling transduction pathway in response to external stimuli and ER stress has been extensively studied. However, the role of MAP kinases, including ERK, p38 MAPK and JNK in ß-lapachone-mediated cell death has only been investigated in few studies (Shiah et al., 1999; Dubin et al., 2001). In most cases, activation of the JNK and p38 kinase pathways are associated with induction of apoptosis, whereas activation of the ERK1/2 signal pathway usually functions to protect cells from a variety of cellular stresses (Johnson and Lapadat, 2002; Olson and Hallahan, 2004; Zhang and Dong, 2007). However, ERK1/2 has also been suggested to mediate cell apoptosis induced by substances such as asiatic acid, quercetin, and thapsigargin (Arai et al., 2004; Nguyen et al., 2004; Hsu et al., 2005). Our results showed that 2-6 h of ß-lapachone treatment resulted in phosphorylation of the MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and JNK (Fig. 5). However, co-treatment with the MAP kinase inhibitors, SB203580, a specific p38 MAP kinase inhibitor, PD98059, a MEK inhibitor, or SP600125, a specific JNK inhibitor, failed to prevent the cell death event caused by ß-lapachone, suggesting that the MAPK signal pathway only constitutes one of the multiple mechanisms by which ß-lapachone induces apoptosis. According to the model we propose below (see Fig. 8), ß-lapachone -induced cell apoptosis involves at least three distinct signal transduction pathways that eventually converge on the activation of caspase executioners. Therefore, inhibition of the MAPK signal pathway alone will not be expected to significantly affect the cytotoxic effect of ß-lapachone .

Several recent studies have shown that NQO1 (DT-diaphorase, quinine oxidoreductase, EC 1.6.99.2) regulates the cytotoxic effect of ß-lapachone (Pink et al., 2000a; Reinicke et al., 2005). NQO1, a cytosolic enzyme, protects against the toxicity of naturally occurring quinones and catalyzes a two-electron reduction of quinones, using either NADH or NAD(P)H as electron donor. The reducing activity of NQO1 detoxifies quinone directly to hydroquinone, thus bypassing the unstable and highly reactive semiquinone intermediates (Ross et al., 1993). However, NQO1 can also reduce certain quinone-containing antitumor agents, such as MMC, ß-lapachone and tanshinone IIA to more reactive forms, semiquinones, which are excellent free radical generators, initiating a redox cycle that results in the generation of superoxide or ROS. These highly reactive oxygen species may directly react with DNA or other cellular macromolecules, such as lipids and proteins, causing cell damage (Dubin et al., 2001; Yang et al., 2005). In addition, NQO1 uses NADH or NADPH as electron donors in the redox cycle, leading to a substantial loss of NADH and NADPH, which can cause dramatic cellular effects, including (i) an increase in cytosolic $Ca^{2+}$, (ii) inactivation of the electron transport chain, (iii) a decrease in the mitochondrial membrane potential, (iv) the loss of ATP, and (v) the initiation of apoptotic cell death (Pink et al., 2000a; Liu et al., 2002). High-level expression of NQO1 in human prostate cancer DU145 cells was confirmed in our previous study (Yang et al., 2005). To determine whether NQO1 activity was a critical determinant in ß-lapachone-induced DU145 cell death, the effect of an NQO1 inhibitor was tested. The results showed that inhibiting NQO1 activity with dicoumoral (a specific NQO1 inhibitor) or preventing the increase in intracellular $Ca^{2+}$ levels using the calcium chelator, BAPTA-AM, substantially prevented the ß-lapachone-induced $Ca^{2+}$ perturbation (Fig. 7A) and activation of MAP kinases (Fig. 6A,B), and subsequently abolished caspase activation (Fig. 6C,D), thus protecting DU145 cells against ß-lapachone-mediated cell death (Fig. 7B).

Taken together we propose a modified model of ß-lapachone-induced cell apoptosis (Fig. 8). In human prostate cancer DU145 cells, NQO1-activated ß-lapachone induces at least three distinct signal transduction pathways that eventually converge on the activation of caspase executioners: (1) an ER stress-mediated $Ca^{2+}$-dependent signaling pathway leads to the activation of the MAP kinases; (2) calcium perturbation results in mitochondrial dysfunction (Chan et al., 2002); and (3) bioactive ß-lapachone induces nuclear DNA damage, followed by up-regulation of p21 and p27, and the cleavage of PARP (Don et al., 2001 ). A further understanding of the multiple mechanisms of ß-lapachone effects may significantly enhance the agent's pharmaceutical potential on prostate cancer in future.

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