*O*Experimental
*O*NCOLOGY

β-LAPACHONE INDUCES GROWTH INHIBITION AND APOPTOSIS IN BLADDER CANCER CELLS BY MODULATION OF BCL-2 FAMILY AND ACTIVATION OF CASPASES

*J.I. Lee[1], D.Y. Choi[1], H.S. Chung[1], H.G. Seo[1], H.J. Woo[2], B.T. Choi[2], Y. H. Choi[2, 3, *]*
[1]R&E Program, Korea Science Academy, Busan, South Korea
[2]Department of Oriental Medicine Dongeui University, Busan, South Korea
[3]Department of Biomaterial Control, Dongeui University, Busan, South Korea

Aim: To study *in vitro* the molecular mechanism of apoptosis caused by β-lapachone, a quinone obtained from the bark of the lapacho tree (*Tabebuia avellanedae*). *Materials and Methods:* The study was carried out on human bladder carcinoma T24 cell line. Determination of cell viability was done using trypan blue exclusion method, apoptosis quantitative estimation — by DAPI staining and agarose gel electrophoresis for DNA fragmentation. Flow cytometry analysis, RT-PCR and Western blot analysis, colorimetric assay of caspase activity were applied as well. *Results:* It was found that in micromolar range of concentrations β-lapachone inhibited the viability of T24 cells by inducing apoptosis, which could be proved by formation of apoptotic bodies and DNA fragmentation. Treatment of T24 cells with β-lapachone resulted in a down-regulation of Bcl-2 expression and up-regulation of Bax expression. β-lapachone-induced apoptosis was also associated with activation of caspase-3 and caspase-9, inhibition of IAP expression, and degradation of poly (ADP-ribose) polymerase, phospholipase C-γ1 and β-catenin proteins. At the same time Fas and FasL levels were inhibited upon treatment with β-lapachone in a concentration-dependent manner. *Conclusion*: β-lapachone-induced apoptosis in T24 cells is mediated, at least in part, by the mitochondrial-signaling pathway.
Key Words: β-lapachone, T24 bladder cancer cells, apoptosis, Bcl-2 family, caspases.

Apoptosis, programmed cell death, plays important role in controlling cells fate in normal physiological state and upon carcinogenesis [4, 5, 12]. Several genes' products have been demonstrated to be critical in the regulation of apoptosis. Caspases are essential for the execution of cell death upon various apoptotic stimuli [20].

Caspase activation is often regulated by various cellular proteins, including members of the inhibitor of apoptosis protein (IAP), the Bcl-2 family and/or the Fas/FasL system [4, 5, 13, 18]. Emerging evidence has demonstrated that anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer [4, 12].

β-lapachone (3,4-dihydro-2,2-dimenthyl-2H-napthol [1,2-b]pyran-5,6-dione) is natural plant quinone obtained from the bark of the lapacho tree *(Tabebuia avellanedae)* which is native to South America [21]. This compound inhibits reverse transcriptase and DNA polymerase-α, and blocks DNA repair thus sensitizing tumor cells to DNA — damaging agents [2, 21]. Several studies suggested that β-lapachone could directly target DNA topoisomerases and inhibit their activity [8, 15, 16].

However, its inhibitory mode is distinct from that of other typical topoisomerase inhibitors, such as camptothecin and related compounds [15]. β-lapachone exhibits anti-bacterial, anti-fungal and anti-trypanocidal action [10, 11, 17], which are linked to the formation of reactive oxygen species [7, 19]. In addition, it has been known that β-lapachone induces apoptotic cell death in human cancer cells, however, the molecular mechanisms of its anti-proliferative action are not known completely.

In this study we determined the antiproliferative activity of β-lapachone, and examined its effect on apoptosis in the human bladder carcinoma cell line T24. Furthermore, to establish the anticancer mechanism of β-lapachone, we assayed the levels of several important genes, which are strongly associated with apoptosis and influence the sensitivity of tumor cells to anticancer agents.

MATERIALS AND METHODS

Cell culturing. Human bladder carcinoma cell line T24 was purchased from the American Type Culture Collection (Rockville, USA), and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 µg/mL penicillin (Gibco BRL, USA) at 37 ˚C and 5% CO_2. β-lapachone was purchased from Biomol (Plymouth Meeting, PA, USA) and dissolved in ice-cold absolute alcohol as a stock solution at 10 mM concentration, and stored in aliquots at −20 ˚C. Cells were cultured in the absence and presence of variable concentrations of β-lapachone for 48 h, then trypsinized, washed with phosphate-buffered saline (PBS), and the viable cells were scored using hemocytometer and the exclusion of trypan blue.

Nuclear staining with DAPI. Cells were washed with cold PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4, 6-diamidino-2-phenylindile (DAPI, Sigma Chemical Co., USA) solution for 10 min at room temperature. The cells were washed two more times with PBS and analyzed by fluorescent microscopy.

DNA fragmentation assay. After treatment with β-lapachone, cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10.000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol : chloroform : isoamylalcohol (25 : 24 : 1, v/v/v) and analyzed electrophoretically on 1% agarose gel containing ethidium bromide (EtBr, Sigma).

Flow cytometry assay. After treatment with β-lapachone, cells were collected, washed with cold PBS, and resuspended in PBS. DNA contents of cells were measured using a DNA staining kit (CycleTESTTM PLUS Kit, Becton Dickinson, USA). Propidium iodide (PI) — stained nuclear fractions were obtained by following the kit protocol. Fluorescence intensity was determined using a FACScan flow cytometer and analyzed by CellQuest software (Becton Dickinson).

RNA extraction and RT-PCR. Total RNA was isolated according to the method [3]. Single-stranded cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco BRL). The mRNAs were amplified by polymerase chain reaction (PCR) with desired primers (see Table). Conditions for PCR reaction were 1 x (94 °C, 3 min); 35 x (94 °C, 45 s; 58 °C, 45 s; and 72 °C, 1 min) and 1 x (72 °C, 10 min). Amplification products obtained by PCR were electrophoretically separated on 1% agarose gel.

Table 1. Gene-specific primers for RT-PCR

Name		Sequence of primers
Bax	sence	5´-ATG-GAC-GGG-TCC-GGG-GAG-3´
	antisence	5´-TGG-AAG-AAG-ATG-GGC-TGA-3´
Bcl-2	sence	5´-CAG-CTG-CAC-CTG-ACG-3´
	antisence	5´-GCT-GGG-TAG-GTG-CAT-3´
Bcl-X$_L$	sence	5´-CAG CTG CAC CTG ACG-3´
	antisence	5´-GCT GGG TAG GTG CAT-3´
XIAP	sence	5´-GAA-GAC-CCT-TGG-GAA-CAA-CA-3´
	antisence	5-CGC-CTT-AGC-TGC-TCT-CTT-CAG-T-3´
cIAP-1	sence	5´-TGA-GCA-TGC-AGA-CAC-ATG-C-3´
	antisence	5´-TGA-CGG-ATG-AAC-TCC-TGT-CC-3´
cIAP-2	sence	5´-CAG-AAT-TGG-CAA-GAG-CTG-G-3´
	antisence	5´-CAC-TTG-CAA-GCT-GCT-CAG-G-3´
Fas	sence	5´-TCT-AAC-TTG-GGG-TGG-CTT-TGT-CTT-C-3´
	antisence	5´-GTG-TCA-TAC-GCT-TTC-TTT-CCA-T-3´
Fas	sence	5´-GGA-TTG-GGC-CTG-GGG-ATG-TTT-CA-3´
	antisence	5´AGC-CCA-GTT-TCA-TTG-ATC-ACA-AGG-3´
GAPDH	sence	5´-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3´
	antisence	5´-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3´

Gel electrophoresis and Western blot analysis. The cells were harvested, lyzed, and protein concentrations were measured using the Bio Rad protein assay (BioRad Lab., USA), according to protocol of manufacturer. An equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, USA) by electroblotting. Blots were incubated with the desired antibodies for 1 h, incubated with diluted enzyme-linked secondary antibodies, and then visualized by the enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp., USA). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (USA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

In vitro caspase activity assay. Caspases activity was determined by colorimetric assay using the caspase-3 and caspase-9 activation kits from Clontech Lab. (USA) and R&D Systems (USA), respectively, following the manufacturer's protocol. The kits used in the present study utilize synthetic tetrapeptides, DEVD for caspase-3 and LEHD for caspase-9, labeled with p-nitroaniline (pNA). Briefly, β-lapachone-treated and untreated cells were lyzed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37 °C. The reaction was measured by changes in absorbance at 405 nm using the VERSAmax tunable microplate reader (Molecular devices, USA).

RESULTS AND DISCUSSION

Inhibition of viability and induction of apoptosis by β-lapachone. Cells were cultured in 10% FBS-containing medium, with or without β-lapachone for 48 h, and cell viability was evaluated by hemocytometer counts. As shown in Fig. 1, *a*, β-lapachone had a marked dose — dependent inhibitory effect on T24 cell viability. Then we examined whether β-lapachone may influence apoptosis of T24 cells. By morphological analysis with DAPI staining, the untreated control cells displayed intact nuclear structures, while in cells treated with β-lapachone chromosomal condensation and the formation of apoptotic bodies were observed, indicating that β-lapachone induced apoptotic cell death in a concentration-dependent manner (Fig. 1, *b*).
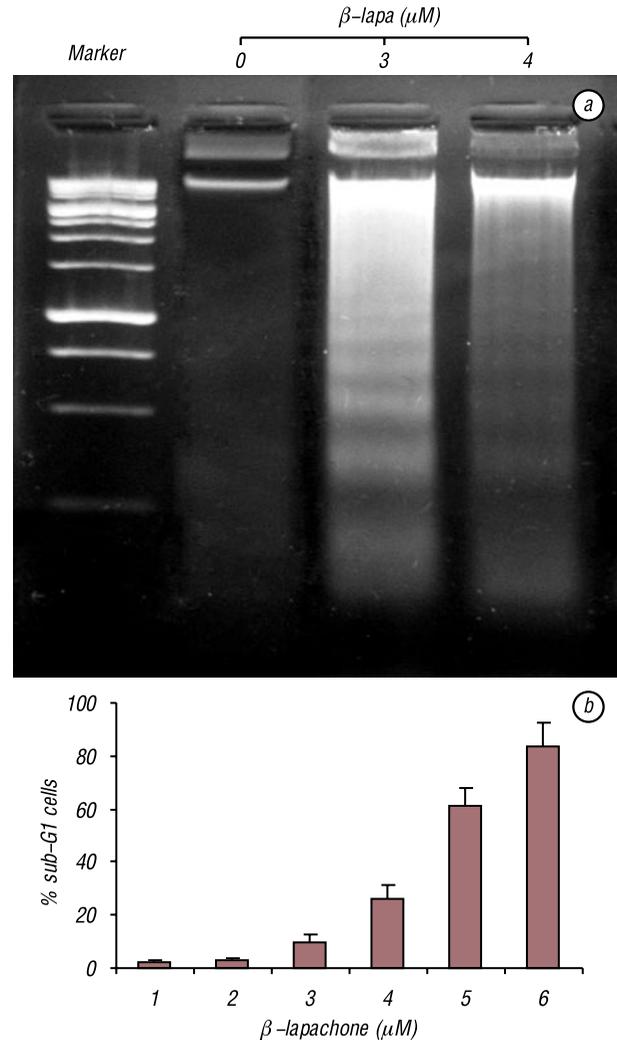
Another hallmark of apoptosis is degradation of chromosomal DNA at internucleosomal linkages [22]. So, we analyzed whether DNA fragmentation was induced by β-lapachone in T24 cells. After treatment of T24 cells with β-lapachone, a typical ladder pattern of internucleosomal fragmentation was observed (Fig. 2, *a*). To evaluate an apoptosis rate, we analyzed the amount of sub-G1 population by the flow cytometry of fixed nuclei. As shown in Fig. 2, *b*, the addition of β-lapachone to T24 cells resulted in a markedly increased accumulation of cells on the sub-G1 phase in a dose-dependent manner. These results suggest that upon treatment with β-lapachone, T24 cells underwent apoptosis, and that there is a good correspondence between apoptosis rate and inhibition of cell viability.
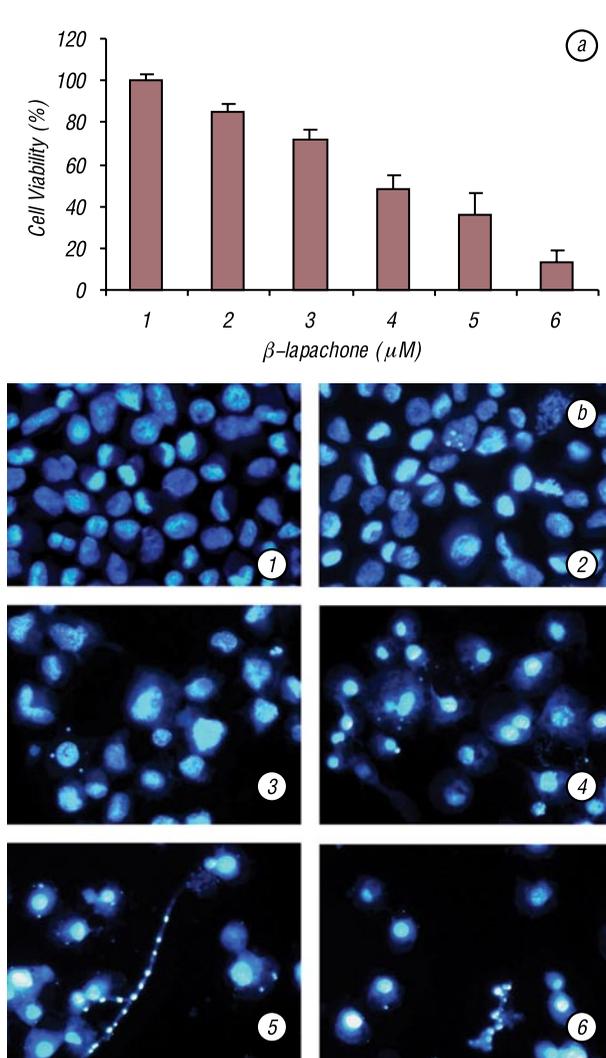
Fig. 1. Viability of T24 cells treated with β-lapachone (1–5 μM) for 48 h (*a*) and induction of apoptotic bodies (*b*). (*a*) Results are expressed as the means ± S.E. of three independent experiments. (*b*) Cells were incubated with β-lapachone (1–5 μM) for 48 h. A number in the right corner responds to concentration of β-lapachone. Cells were stained with DAPI, stained nuclei were observed in fluorescent microscope using a blue filter. Magnification, X 400


**Fig. 2.** Induction of DNA fragmentation (*a*) and flow cytometry analysis of T24 cells treated by β-lapachone (1–5 μM). (*b*) Each point represents the mean of two independent experiments

Modulation of Bcl-2 family and activation of caspases by β-lapachone. Apoptotic cell death is known to be regulated by pro-apoptotic and anti-apoptotic modulators, including these of Bcl-2 family [5, 20]. To analyze whether β-lapachone induces cell death by modulating the expression of Bcl-2 family proteins, after exposure to different concentrations of β-lapachone, total RNAs and cellular proteins were isolated, and RT-PCR and immunoblotting were performed. The results indicated that treatment of cells with β-lapachone resulted in a down-regulation of expression of anti-apoptotic Bcl-2 protein and up-regulation of pro-apoptotic Bax (Fig. 3, *a* and *b*).

Recent studies have identified caspases as important mediators of apoptosis induced by various apoptotic stimuli [5, 20]. To determine the role of caspases in β-lapachone-induced apoptosis, we determined the levels of caspase-3 and caspase-9 expression in T24 cells treated with β-lapachone. As shown in Fig. 4, *a*, β-lapachone-induced apoptosis was associated with a decreased expression of the pro-caspase-3 and pro-caspase-9 protein; however, we did not observed the active subunits of both caspases. To further quantify the proteolytic activities of caspase-3 and caspase-9, lyzates equalized for protein from cells treated with β-lapachone were assayed for *in vitro* caspase-3 and caspase-9 activity using as substrates DEVD-pNA and LEHD-pNA, respectively. The results showed that exposure of cells β-lapachone increased both caspase-3 and caspas-9 activity in a concentration-dependent manner.

Activated caspases induce a limited proteolysis in a number of cellular proteins, which are degraded and have been used as a marker of chemotherapy-induced apoptosis [1, 9, 14]. Here, we examined whether the substrate proteins such as poly (ADP-ribose) polymerase (PARP), phospholipase C-γ1 (PLC- γ1) and β-catenin were cleaved in cells treated with β-lapachone. A dose-dependent degradation of mentioned proteins was observed (Fig. 4, *b*), being in correlation with caspases activation upon treatment with β-lapachone. Because the anti-apoptotic IAP family proteins bind to caspases leading to their inactivation in eukaryotic cells [5, 6], we further examined the involvement of
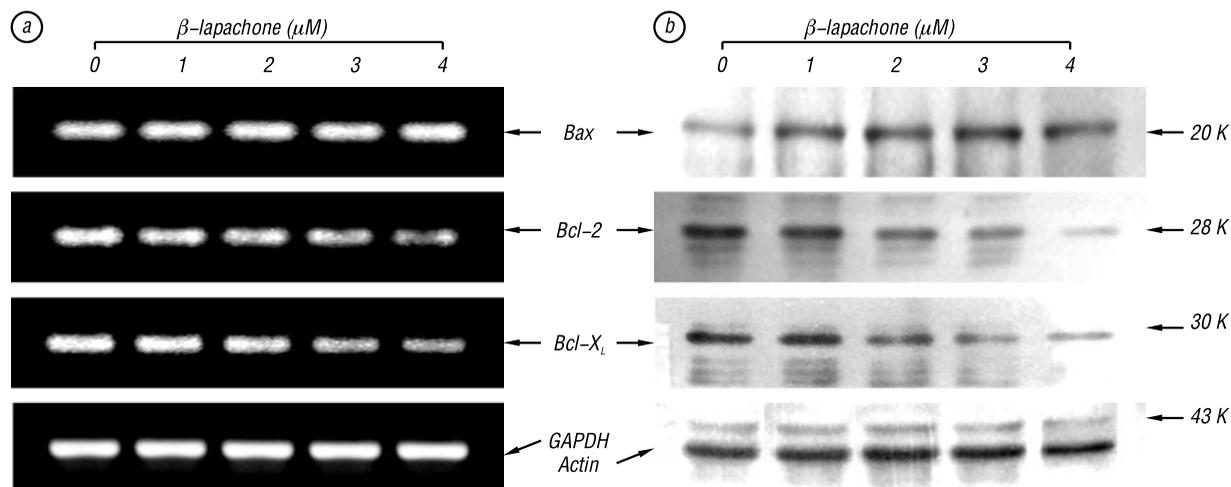
Fig. 3. Influence of β-lapachone (1–5 μM) on expression of Bcl-2 and Bax in T24 cells on mRNA (*a*) and protein (*b*) levels evaluated by RT-PCR and Western-blotting, respectively
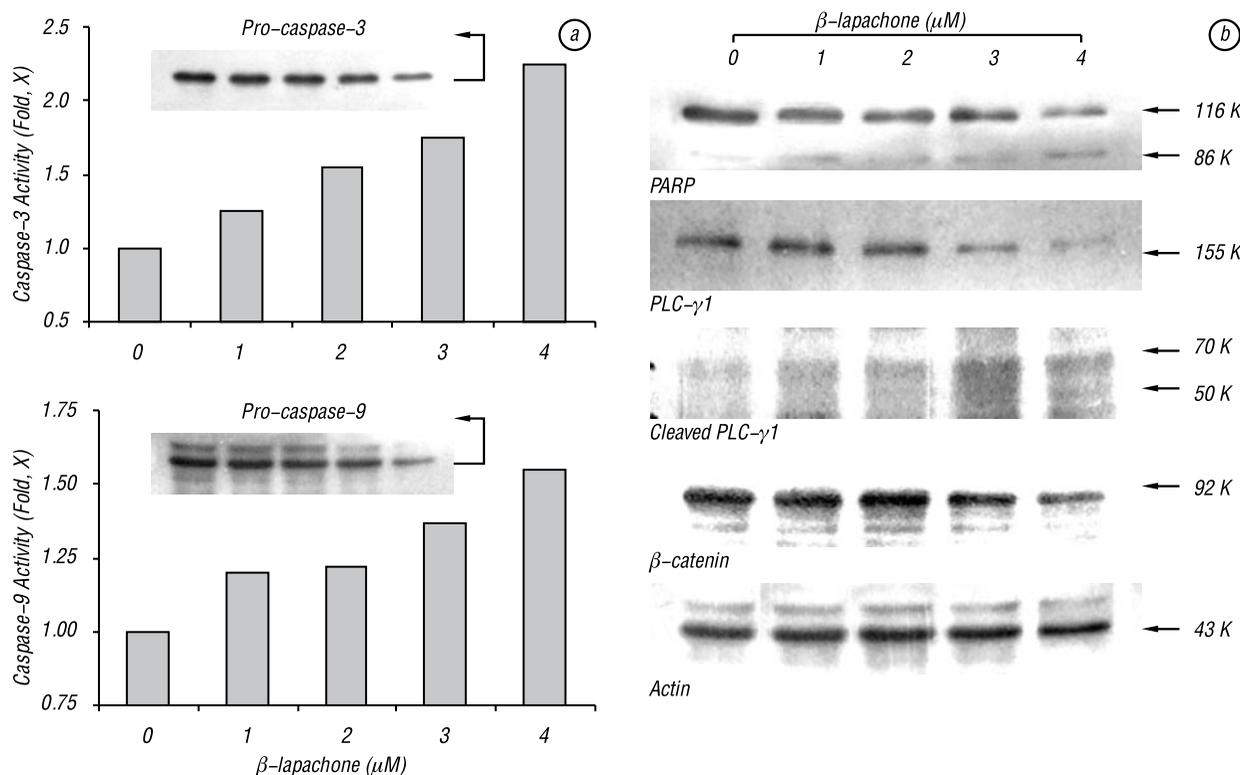

Fig. 4. Activation of caspase-3 and caspase-9 (*a*) and degradation of PARP, PLC-γ1 and β–catenin protein (*b*) upon treatment of T24 cells β-lapachone (1–5 μM). Data represent the relative mean values of two independent experiments

the IAP family in β-lapachone-dependent apoptosis. As shown in Fig. 5, the levels of both X-linked inhibitor to apoptosis (XIAP) and cellular inhibitor of apoptosis protein (cIAP)-1 expression were decreased upon treatment with β-lapachone and cIAP-2 protein was cleaved in a concentration dependent fashion. These results suggest that β-lapachone-induced apoptosis in T24 cells is mediated, at least in part, by the mitochondrial-signaling pathway.

Effects of β-lapachone on the levels of Fas and FasL expression. Fas/FasL system is a key signaling transduction pathway of apoptosis in cells and tissues [13, 18]. Ligation of Fas by an agonistic antibody or its mature ligand induces receptor oligomerization and formation of death-inducing signaling complex, followed by activation of caspase-8, with further activation of caspase

cascade resulting in cell apoptotic death [5, 20]. However, the role of Fas/Fas ligand system in the control of apoptosis in bladder cancer is controversial. We further examined the involvement of the Fas/FasL system in T24 cells treated with β-lapachone. As shown in Fig. 6, Fas and Fas-L expression decreased upon the treatment with β-lapachone in a concentration dependent fashion.

In summary, we have shown here that β-lapachone induces apoptosis in T24 cell line, which appears to account for its anti-proliferating activity. These apoptotic mechanisms were mediated by an activation of caspases and an upregulating of the ratio Bax/Bcl-2, and associated with a down-regulation of IAP family. Our study has demonstrated that β-lapachone may be further studied as a promising agent for treatment of bladder cancer.
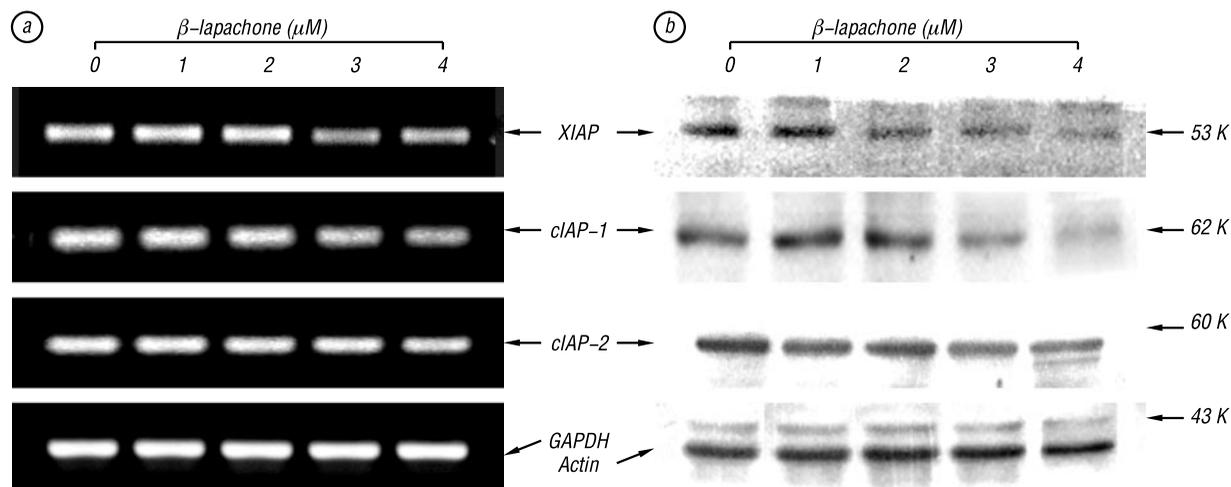
Fig. 5. Influence of β-lapachone (1–5 µM) on expression of XIAP, cIAP-1 and cIAP-2 in T24 cells on mRNA (*a*) and protein (*b*) levels evaluated by RT-PCR and Western-blotting, respectively
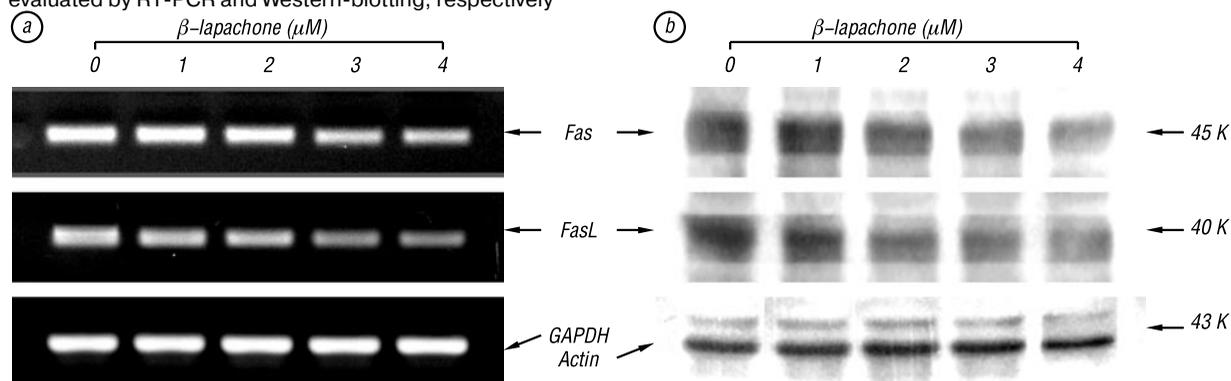
Fig. 6. Influence of β-lapachone (1–5 µM) on expression of Fas and FasL in T24 cells on mRNA (*a*) and protein (*b*) levels evaluated by RT-PCR and Western-blotting, respectively

REFERENCES

1. **Bae SS, Perry DK, Oh YS, Choi JH, Galadari SH, Ghayur T, Ryu SH, Hannun YA, Suh PG.** Proteolytic cleavage of phospholipase C-γ1 during apoptosis in Molt-4 cells. FASEB J 2000; **14**: 1083–92.

2. **Boothman DA, Trask DK, Pardee AB.** Inhibition of potentially lethal DNA damage repair in human tumor cells by β-lapachone, an activator of topoisomerase I. Cancer Res 1989; **49**: 605–12.

3. **Chorna I, Fedorenko O, Datsyuk L, Stoika R.** Expression of mRNA coding for TGF-β and its receptors in irradiated human breast carcinoma MCF-7 cells differing in their sensitivity to doxorubicin. Exp Oncol 2005; **27**: 156–8.

4. **D'Agostini F, Izzotti A, Balansky RM, Bennicelli C, De Flora S.** Modulation of apoptosis by cancer chemopreventive agents. Mutat Res 2005; **591**: 173–86.

5. **Debatin KM.** Apoptosis pathways in cancer and cancer therapy. Cancer Immunol Immunother 2004; **53**: 153–9.

6. **Deveraux QL, Reed JC.** IAP family proteins-suppressors of apoptosis. Genes Dev 1999; **13**: 239–52.

7. **Fernandez VS, Stoppani AO, Dubin M.** Redox cycling of β-lapachone and structural analogues in microsomal and cytosol liver preparations. Methods Enzymol 2004; **378**: 67–87.

8. **Frydman B, Marton LJ, Sun JS, Neder K, Witiak DT, Liu AA, Wang HM, Mao Y, Wu HY, Sanders MM, Liu LF.** Induction of DNA topoisomerase II-mediated DNA cleavage by β-lapachone and related naphthoquinones. Cancer Res 1997; **57**: 620–27.

9. **Fukuda K.** Apoptosis-associated cleavage of β-catenin in human colon cancer and rat hepatoma cells. Int J Biochem Cell Biol 1999; **31**: 519–29.

10. **Goijman SG, Stoppani AO.** Effects of β-lapachone, a peroxide-generating quinone, on macromolecule synthesis and degradation in *Trypanosoma cruzi*. Arch Biochem Biophys 1985; **240**: 273–80.

11. **Guiraud P, Steiman R, Campos-Takaki GM, Seigle-Murandi F, Simeon de Buochberg M.** Comparison of antibacterial and antifungal activities of lapachol and β-lapachone. Planta Med 1994; **60**: 373–4.

12. **Hengartner MO.** The biochemistry of apoptosis. Nature 2000; **407**: 770–6.

13. **Jiang C, Yang YF, Cheng SH.** Fas ligand gene therapy for vascular intimal hyperplasia. Curr Gene Ther 2004; **4**: 33–9.

14. **Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC.** Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 1994; **371**: 346–7.

15. **Li CJ, Averboukh L, Pardee AB.** β-lapachone, a novel DNA topoisomerase I inhibitor with a mode of action different from camptothecin. J Biol Chem 1993; **268**: 22463–8.

16. **Li CJ, Wang C, Pardee AB.** Induction of apoptosis by β-lapachone in human prostate cancer cells. Cancer Res 1995; **55**: 3712–5.

17. **Lopes JN, Cruz FS, Docampo R, Vasconcellos ME, Sampaio MC, Pinto AV, Gilbert B.** *In vitro* and *in vivo* evaluation of the toxicity of 1,4-naphthoquinone and 1,2-naphthoquinone derivatives against *Trypanosoma cruzi*. Ann Trop Med Parasitol 1978; **72**: 523–31.

18. **O'Brien DI, Nally K, Kelly RG, O'Connor TM, Shanahan F, O'Connell J.** Targeting the Fas/Fas ligand pathway in cancer. Expert Opin Ther Targets 2005; **9**: 1031–44.

19. **Pardee AB, Li YZ, Li CJ.** Cancer therapy with β-lapachone. Curr Cancer Drug Targets 2002; **2**: 227–42.

20. **Philchenkov A, Zavelevich M, Kroczak TJ, Los M.** Caspases and cancer: mechanisms of inactivation and new treatment modalities. Exp Oncol 2004; **26**: 82–97.

21. **Schaffner-Sabba K, Schmidt-Ruppin KH, Wehrli W, Schuerch AR, Wasley JW.** β-lapachone: synthesis of de-

rivatives and activities in tumor models. J Med Chem 1984; **27**: 990–4.

22. **Walker PR, Sikorska M.** New aspects of the mechanism of DNA fragmentation in apoptosis. Biochem Cell Biol 1997; **75**: 287–99.

β-ЛАПАКОН ИНДУЦИРУЕТ ПОДАВЛЕНИЕ РОСТА И АПОПТОЗ ОПУХОЛЕВЫХ КЛЕТОК МОЧЕВОГО ПУЗЫРЯ ПУТЕМ МОДУЛЯЦИИ СЕМЕЙСТВА BCL-2 И АКТИВАЦИИ КАСПАЗ

Цель: изучить механизмы апоптоза клеток карциномы мочевого пузыря человека Т24 при действии β-лапакона, хинона из коры дерева *Tabebuia avellanedae*. *Материалы и методы:* для определения жизнеспособности клеток использовали окраску трипановым синим; окрашивание DAPI и электрофоретический анализ фрагментации ДНК в агарозном геле, метод проточной цитометрии (для количественной оценки апоптоза); полимеразную цепную реакцию в режиме реального времени (РВ-ПЦР) и Вестерн блот-анализ (для оценки уровня экспрессии генов и белков), а также колориметрический анализ активности каспаз. *Результаты:* выявлено, что в микромолярных концентрациях β-лапакон понижает жизнеспособность клеток линии Т24 путем активации апоптоза, что подтверждается формированием апоптотических тел и фрагментацией ДНК. Результаты РВ-ПЦР и иммуноблоттинга указывают на то, что обработка клеток β-лапаконом приводит к снижению экспрессии Bcl-2 и к активации Bax. Апоптоз, индуцированный β-лапаконом, также сопровождается активацией каспаз -3 и -9, ингибированием экспрессии семейства IAP, а также деградацией поли-(ADP-рибозо) полимеразы, фосфатазы С-γ1 и β-катенина. Тем не менее, уровень экспрессии Fas и FasL снижался при увеличении концентрации β-лапакона. Выводы: апоптоз, индуцированный при действии β-лапакона в клетках Т24, может быть частично опосредован митохондриальным сигнальным каскадом.
Ключевые слова: β-лапакон, клетки рака мочевого пузыря линии Т24, апоптоз, семейство Bcl-2, каспазы.