Induction of Apoptosis by β-Lapachone in Human Prostate Cancer Cells

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Abstract

β-Lapachone, a plant product, has been shown to be a novel inhibitor of DNA topoisomerase I, with a mode of action different from camptothecin and a chemical structure distinct from those of current anti-cancer drugs. We observed that β-lapachone, at concentrations of less than 8 μM, induces cell death with characteristics of apoptosis in human prostate cancer cell lines. This effect of β-lapachone was also observed in a human promyelocytic leukemia cell line (HL-60). β-Lapachone-induced apoptosis is independent of p53 expression, and ectopic overexpression of bcl-2 did not confer significant resistance to β-lapachone. Among other human carcinoma and adenoma cell lines tested, human breast and ovary carcinoma showed sensitivity to the cytotoxic effect of β-lapachone without manifesting signs of apoptosis. These results suggest that β-lapachone is a potential compound to be added to cancer chemotherapy, particularly for prostate cancer.

Introduction

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) is a simple plant product with a chemical structure different from currently used anti-cancer drugs. It is obtained by sulfuric acid treatment of the naturally occurring lapachol, which is readily isolated from Tabebuia avellanedae growing mainly in Brazil, or is easily synthesized from lomatol, isolated from seeds of lomatia growing in Australia (1, 2).

β-Lapachone has been shown to have a variety of pharmacological effects. Numerous derivatives have been synthesized and tested as anti-viral and anti-parasitic agents (3–5). β-Lapachone and its derivatives, e.g., 3-allyl-β-lapachone, show anti-trypanosomal effects (3), the mechanism of which is unclear. It significantly prolongs the survival of mice infected with Rauscher leukemia virus, probably through inhibition of reverse transcriptase (4, 6). We have demonstrated that β-lapachone inhibits gene expression directed by the long terminal repeat of the human immunodeficiency virus type 1 and viral replication (5). It has also been shown to be a DNA repair inhibitor that sensitizes cells to DNA-damaging agents (7, 8). β-Lapachone is well tolerated in dogs, rats, mice, and chickens. The maximum tolerated dose, when given p.o. daily for 1 month, is 200 mg/kg in rats and 500 mg/kg in dogs. Higher doses cause gastric ulceration and loss of erythrocytes but not signs of bone marrow suppression.3 The experience in humans has been extremely limited.

We have reported eukaryotic Topo I to be a molecular target for β-lapachone and its derivatives (9). β-Lapachone inhibits the catalytic activity of DNA Topo I through a different mechanism than camptothecin (9). We have suggested that this inhibition may be mediated by a direct interaction of β-lapachone with Topo I rather than stabilization of the cleavable complex (9).

This unique mode of action and chemical structure of β-lapachone, in comparison with the currently used anti-cancer drugs (4, 9), motivated us to test the sensitivity of a variety of human cancer cell lines to this compound. In this study, we observed that β-lapachone induces suppression of survival in several human cancer cell lines and selectively triggers programmed cell death in human prostate cancer cells, including hormone refractory cells. Similar findings made by Planchnon et al. (10) are reported in the preceding paper.

Materials and Methods

Chemicals. β-Lapachone was kindly provided by Dr. A. Matter (Ciba-Geigy, Ltd., Basel, Switzerland). It was dissolved in DMSO at 20 mM concentration, aliquoted, and kept at −20°C.

Cell Cultures. All cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD) unless specified otherwise. Cells were maintained at 37°C in 5% CO2 in complete humidity. Human prostate tumor cells PC-3, DU145, and LNCaP were grown in DMEM (Life Technologies, Inc.) supplemented with 10% FCS and 2 mM l-glutamine. HL-60 (human promyelocytic leukemia cell line) was cultured in RPMI with 10% heat-inactivated FCS. MCF-7 and 21 MT (human breast epithelial cell line), kindly provided by Dr. R. Sager (Dana-Farber Cancer Institute, Boston, MA), were cultured in α-MEM (Life Technologies, Inc.) supplemented with 10% FCS, 2 mM l-glutamine, and 1 mg/ml insulin. AD 2780 s (human ovary carcinoma), a generous gift from Dr. K. J. Scanlon (City of Hope Medical Center, Duarte, CA), 293 (human kidney epithelial cell line), SW1116 (human colon adenocarcinoma), and human lung carcinoma cell lines (HS96 and HS520) were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% FCS and 2 mM l-glutamine. HeLa and HeLa-bcl-2 cells (11), kindly provided by Drs. W. Meikrantz and R. Schlegel (Harvard School of Public Health, Boston, MA), were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% FCS, 2 mM l-glutamine, and 800 μg/ml of G418.

Colony Formation Assay. Exponentially growing cells were seeded (2000 cells/dish) in 60-mm culture dishes and allowed to attach for 48 h. β-Lapachone was added in less than 5 μl volume (corresponding to a final DMSO concentration of less than 0.1%) directly to dishes from concentrated working stocks. Cultures were observed daily for 10 to 20 days, and cells were fixed and stained with modified Wright-Giemsa Stain (Sigma Chemical Co.). Colonies of greater than 30 cells were scored as survivors.3

Agarose Gel Electrophoresis of Apoptotic DNA (12). Cells were treated with β-lapachone and then incubated in drug-free media. They were harvested and lysed in 50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K, and 0.15 mg/ml RNase. The supernatant of the cell lysate was loaded onto a 2% agarose gel. The electrophoresis was carried out at 24 V for 16 h. The gel was stained with ethidium bromide. A Polaroid picture was taken after destaining the gel for 1 h.

Flow Cytometry Analysis. Cytofluorometric analysis of apoptosis and cell cycle analysis was performed by propidium iodide staining of nuclei as reported previously (13).

Western Blot Analysis. Nuclear extract was prepared from exponentially growing cells (14). The ECL assay system was used to detect p53 and bcl-2 levels. Briefly, nuclear protein samples (10 μg/sample) were electrophoresed in a SDS-polyacrylamide gel and then electrophotochemically transferred to a
Results

Effect of β-Lapachone on Survival of Human Cancer Cells.

Human carcinoma cell lines of different histotypes were used to test the anti-survival effect of β-lapachone. Androgen-independent human prostate tumor cells PC-3 and DU145 were treated with β-lapachone in vitro. Cell survival was determined by colony formation assay. β-Lapachone inhibits proliferation of both cell lines with an IC50 of 4 to 8 μM (Fig. 1). LNCaP cells were equally sensitive to β-lapachone. 21 MT (a human breast carcinoma cell line) and AD2780 s (a human ovary carcinoma cell line) were also relatively sensitive to the anti-proliferative effect of β-lapachone (IC50 > 16 μM). Proliferation of SW116, a human colon adenocarcinoma cell line, was not significantly inhibited by β-lapachone up to 128 μM, the maximum concentration used. Other cell lines tested, which included H596, H520 (human lung carcinoma cell lines), and 293 (a human kidney epithelial cell line) were relatively resistant to β-lapachone (IC50 > 32 μM).

Seventeen derivatives of β-lapachone were tested in PC-3 and DU145 cells. 3-Allyl-β-lapachone and 3-bromo-β-lapachone were found to be more active than β-lapachone against human prostate cells (data not shown).

Induction of Apoptosis by β-Lapachone in Human Prostate Cancer Cells.

Extensive cell death was observed in proliferating human prostate cancer cells after treatment with β-lapachone. To determine whether this cell death occurs through necrosis or apoptosis, cells were harvested by trypsinization, and their genomic DNA was subjected to gel electrophoresis. As shown in Fig. 2, A and B, β-lapachone induced a typical DNA laddering in human prostate cancer cells, consistent with cell death by apoptosis, a process that is specifically activated in prostate cells after they are deprived of testosterone (15, 16). This β-lapachone-induced apoptosis was observed in every prostate cell line we used, including PC-3, DU145, and LNCaP. To test whether quiescent cells are similarly sensitive to β-lapachone, both PC-3 and DU145 cells were serum starved for 48 h before drug treatment. Apoptosis was similarly induced in nonproliferating cells (data not shown). Apoptotic cells were quantitated with flow cytometry analysis. As shown in Fig. 2D, β-lapachone induced apoptosis in 68% of LNCaP cells by 24 h after initial treatment with β-lapachone. In PC-3 cells, apoptosis occurs in 62% by 24 h (data not shown). Signs of apoptosis, as determined by DNA laddering and chromosomal condensation, were not detected in β-lapachone-treated 21-MT, H520, SW1116, and A2780 s cells.


Expression of bcl-2 has been implicated in the resistance of cancer cells to chemotherapeutic drugs including prostate cells (17). To determine whether apoptosis in prostate cancer cells is due to lack of bcl-2 expression, we measured bcl-2 expression by Western blot assay. As shown in Fig. 3, bcl-2 expression is high in LNCaP cells (B). In A, cells were treated with 4 μM β-lapachone for 4 h, followed by incubation in drug-free medium for 4 h (Lanes 2 and 7), 12 h (Lanes 3 and 8), 20 h (Lanes 4 and 9), and 44 h (Lanes 5 and 10). As controls, cells were treated with an equal volume of DMSO (Lanes 1 and 6). DNA was extracted and subjected to electrophoresis. In B, LNCaP cells were treated with β-lapachone for 4 h at concentrations of 0 μM (Lane 1), 0.5 μM (Lane 2), 2 μM (Lane 3), and 4 μM (Lane 4), followed by drug-free incubation for 20 h. To quantify apoptotic fraction, LNCaP cells were treated with DMSO (C) or 8 μM β-lapachone (D) for 1 h, followed by incubation in drug-free media for 23 h before they were subjected to flow cytometric analysis.

Fig. 2. Induction of apoptosis by β-lapachone in human prostate cancer cells. DNA laddering, a typical feature of apoptosis, was induced in PC-3, DU145 (A), and LNCaP cells (B). In A, cells were treated with 4 μM β-lapachone for 4 h, followed by incubation in drug-free medium for 4 h (Lanes 2 and 7), 12 h (Lanes 3 and 8), 20 h (Lanes 4 and 9), and 44 h (Lanes 5 and 10). As controls, cells were treated with an equal volume of DMSO (Lanes 1 and 6). DNA was extracted and subjected to electrophoresis. In B, LNCaP cells were treated with β-lapachone for 4 h at concentrations of 0 μM (Lane 1), 0.5 μM (Lane 2), 2 μM (Lane 3), and 4 μM (Lane 4), followed by drug-free incubation for 20 h. To quantify apoptotic fraction, LNCaP cells were treated with DMSO (C) or 8 μM β-lapachone (D) for 1 h, followed by incubation in drug-free media for 24 h before they were subjected to flow cytometric analysis.
overexpression of bcl-2 (HeLa-bcl-2; Ref. 11) were not significantly resistant to ß-lapachone in comparison with its parental cell line (IC50; parental cells, 16 μM; HeLa-bcl-2 cells, 32 μM; data not shown).

p53 status has been shown to be important for apoptosis in cancer cells provoked by ionizing radiation and chemotherapeutic drugs (18). p53 was expressed in DU145 cells but was not detectable in PC-3 cells (Fig. 3A), although apoptosis was induced in both cell lines (Fig. 2A). ß-Lapachone treatment did not significantly induce expression of p53 (Fig. 3B). These results suggest that apoptosis induced by ß-lapachone is not dependent on p53 expression. Expression of p21 is not up-regulated in prostate cancer cells undergoing apoptosis (Fig. 3B).

**ß-Lapachone-induced Apoptosis and Cell Differentiation in p53 −/− HL-60 Cells.** The effect of ß-lapachone was also tested in hematopoietic cancer cells. Treatment of HL-60, a human leukemia cell line, with ß-lapachone also induced apoptosis. Chromosomal laddering was detectable within 4 h after ß-lapachone treatment (Fig. 4A). At subapoptotic doses, ß-lapachone induced an increase in the G2 fraction (data not shown) and morphological differentiation in HL-60 cells (Fig. 4, B and C).

**Discussion**

In this study, we have tested a variety of human cancer cells for their sensitivity to ß-lapachone, a novel inhibitor of DNA Topo I (9). Human prostate cancer cells were most sensitive to ß-lapachone and were induced to undergo apoptosis, a process specifically activated in prostate cells after they are deprived of testosterone (15, 16). Our preliminary experiments in nude mice suggest that ß-lapachone is effective against human prostate tumor growth (data not shown). This compound also causes suppression of survival, albeit at slightly higher concentrations, in human ovary and breast cells. Typical apoptosis is not detected in any other human cancer cell of epithelial origins including the colon, kidney, lung, breast, or ovary exposed to the drug. Human hematopoietic leukemia cells (HL-60) are induced to undergo either apoptosis or differentiation, depending on the concentration used. These results suggest that ß-lapachone is a candidate for addition to cancer chemotherapy, particularly for human prostate cancer.

The prevalence of p53 inactivation and/or bcl-2 expression in human tumors is believed to be at least partially responsible for the general ineffectiveness of current chemo- and radiation therapy for cancer (17, 18). Thus, it is important to develop novel anticancer drugs that induce cell death in a p53-independent manner. One way such compounds could work is through activation of p53-targeting genes, e.g., p21 (SDII/WAF1/Cip1), by a p53-independent pathway (19). ß-Lapachone induced apoptosis in the absence of p53 expression (Fig. 3A). There was no significant induction of p53 and p21 (Fig. 3B) in human prostate cancer cells during apoptosis, suggesting that ß-lapachone-induced apoptosis occurs independent of the p53 pathway. ß-Lapachone-induced cell death did not correlate with bcl-2 expression and was not protected by ectopically overexpressed bcl-2. These results suggest the existence of a cell death program that is independent of both p53 and bcl-2, which can be activated by ß-lapachone.

The molecular mechanism of the observed apoptotic effect of ß-lapachone remains to be determined, although DNA Topo I is a potential candidate. Cytotoxic effect of DNA topoisomerase poisons and other DNA-damaging agents is always associated with G2-M arrest (20, 21). Induction of cell death by ß-lapachone was not accompanied by G2-M arrest (data not shown). Instead, ß-lapachone treatment was associated with increases in the fractions of G1, sub-G1, and cells with higher DNA ploidy (Fig. 2D). Further studies are needed to define the molecular target for ß-lapachone-induced cell death and the apparent selectivity for prostate cancer cells.

**Acknowledgments**

We thank Dr. Philip Kantoff for helpful discussion; David J. Friedman, Kara A. Zaccardi, and Victoria Wang for their technical assistance; and Scott Boyd for reading the manuscript. We are indebted to Dr. A. V. Pinto for supplying seventeen ß-lapachone derivatives.

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**Fig. 3.** Lack of correlation between ß-lapachone-induced apoptosis and the expression of p53 and bcl-2. A, expression pattern of p53 and bcl-2 in DU-145 cells (Lane 1) and PC-3 cells (Lane 2). ß-Lapachone did not induce expression of p53 and p21. Lanes 1-4, PC-3 cells; Lanes 5-8, DU-145 cells. Cells were treated with DMSO (Lanes 1 and 5) or ß-lapachone at 2 μM (Lanes 2 and 6), 4 μM (Lanes 3 and 7), and 8 μM (Lanes 4 and 8) for 1 h, followed by incubation in drug-free media for 23 h. Expressions of p53, p21, and bcl-2 were determined by Western blot assay as described in "Materials and Methods."

**Fig. 4.** Induction of apoptosis (A) and differentiation in HL-60 cells (B and C) by ß-lapachone. In A, HL-60 cells were treated with 8 μM ß-lapachone for 24 h (Lane 1), 16 h (Lane 2), 8 h (Lane 3), 4 h (Lane 4), 2 h (Lane 5), and 0 h (Lane 6). Cellular DNA was extracted and subjected to gel electrophoresis. To analyze morphological changes induced by ß-lapachone, HL-60 cells were treated with ethanol (1:1000, v/v; B) or 0.8 μM ß-lapachone dissolved in ethanol (C) for 6 days before harvest. A thin film of cells was spread on a slide and stained with modified Wright-Giemsa Stain (Sigma).
Note Added in Proof

β-Lapachone induced substantial necrosis in Dunning R3327 (AT-3) rat prostatic tumor at a dosage that caused considerable lethality in a preliminary experiment.

References


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