Beta-Lapachone Suppresses Non-small Cell Lung Cancer Proliferation through the Regulation of Specificity Protein 1

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Received February 23, 2015; accepted May 11, 2015

Lung cancer is the leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) is the most common pathological type with a reported frequency of about 85% of all cases. Despite recent advances in therapeutic agents and targeted therapies, the prognosis for NSCLC remains poor, and therefore it is important to identify the biological targets of this complex disease since a blockade of such targets would affect multiple downstream signaling cascades. β-Lapachone (β-Lap) is an antiproliferative agent that selectively induces apoptosis-related cell death in a variety of human cancer cells. However, the mechanisms of its action require further investigation. In this study, we show that treatment with β-lap triggers apoptosis and cell-cycle arrest in two NSCLC cell lines: H1299 and NCI-H358. The transcription factor specificity protein 1 (Sp1) was markedly inhibited by β-lap in a dose- and time-dependent manner. Furthermore, β-lap modulated the protein expression levels of the Sp1 regulatory genes, including cell-cycle regulatory proteins and antiapoptotic proteins, resulting in apoptosis. Taken together, our results indicate that β-lap may be a potential antiproliferative agent candidate by inducing apoptotic cell death in NSCLC through downregulation of Sp1.

Key words β-lapachone; non-small cell lung cancer; specificity protein 1; apoptosis

In recent years, lung cancer has gained more attention due to the rapid increase in incidence and mortality, which is estimated at >100,000 deaths each year.1,2 Lung cancer can be divided into two histological groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for almost 80% of all lung cancer cases and mainly consists of adenocarcinoma, squamous cell carcinoma and large cell carcinoma.3 Although many factors, such as exposure to tobacco smoke, ionizing radiation and a viral infection, are known to increase the risk of this cancer type, the mechanisms involved in NSCLC formation remain largely unknown to date.3

The major treatment modality for cancer still involves a combination of surgery, radiation and chemotherapy, but some patients have conditions that are not suitable for surgery.4 Furthermore, the overall survival rate for lung cancer patients in advanced or late stages of the disease is still low, despite improvements in tumor response to chemotherapy, and has not markedly improved in the past recent decades.3 Since the mutation heterogeneity of lung cancer cells is associated with an acquired drug resistance, the search for novel anticancer drugs with an enhanced specificity remains urgent in order to increase the potency of chemotherapy.5

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]-pyran-5,6-dione) (β-lap) is a naturally occurring quinone derived from the bark of the lapacho tree (Tabebuia avellanedae) native to South America, and it is also being used as a novel bio-reductive anti-cancer drug5 (Fig. 1). This agent has been reported to have anti-cancer,3 anti-Trypanosoma cruzi,8 anti-inflammatory,9,10 antibacterial,11 antifungal,11 and antiviral12 and healing properties.13 β-Lap also promotes apoptosis-related cell death by sensitizing human tumor cells to ionizing radiation and DNA-damaging agents.14 Previous studies have shown that β-lap treatment can induce cell death in a variety of cancer cell lines.15,16 However, the mechanisms through which β-lap has an effect on NSCLC cell proliferation and apoptosis is not yet well understood.

Specificity protein 1 (Sp1) is a constitutive transcription factor that plays an important role and regulates a variety of cellular processes, such as cellular growth, angiogenesis, and apoptosis.17,18 In addition, Sp1 is highly expressed in many cancer types by modulating the expression of its target genes, which include oncogenes and tumor suppressor genes,19 and many studies have suggested that Sp1-mediated functions are potential targets for cancer therapy.

We examined the effects of administering β-lap in two NSCLC cell lines, H1299 and NCI-H358. The results indicated that β-lap suppresses cell growth and induces apoptosis in H1299 and NCI-H358 cells. Our results provide experimental evidence to support the hypothesis that β-lap decreases Sp1 expression and inhibits NSCLC cell viability by inducing cell cycle arrest and activating apoptosis-related pathways. The suppression of the Sp1 protein in NSCLC therefore suggests that β-lap is of potential pharmacological interest.

MATERIALS AND METHODS

Agents β-Lap (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in a 20 mM dimethyl sulfoxide (DMSO) stock solution and stored in aliquots at −20°C.

Cell Culture H1299 (CRL-5803) and NCI-H358 (CRL-5807) is human non-small cell lung cancer which can be...
obtained from the American type culture collection (ATCC). These cells were grown in cell culture dishes with RPMI 1640 containing 10% heat-inactivated fetal bovine serum and 100 U/mL each of penicillin and streptomycin (Thermo Scientific) at 37°C in a humidified atmosphere with 5% CO₂.

**Cell Viability Assay** H1299 and NCI-H358 cells were seeded in 96-well plates for 24 h and were treated with various concentrations of β-lap for 24 and 48 h. The percentage of viable cells was determined by using a CellTiter96® Aqueous One Cell Proliferation Assay Kit (Promega, Madison, WI, U.S.A.) to measure the solubility of formazan product on addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), an inner salt, for 2 h at 490 nm, as described by the manufacturer. All experiments were carried out in triplicate, and the percentages of the cell viability of β-lap treated cells were normalized to that of untreated cells.

**Nuclear Staining with DAPI Staining** H1299 and NCI-H358 cells treated with β-lap were harvested via trypsinization, washed with cold phosphate-buffered saline (PBS), and fixed in 100% methanol at room temperature for 20 min. The cells were then spread on a slide, stained with DAPI solution (2 µg/mL), and observed using a Fluoview confocal laser microscope (Fluoview FV10i, Olympus Corp., Tokyo, Japan).

**Cell Cycle Analysis** The cell cycle was analyzed using a Muse™ Cell Analyzer (Millipore, Billerica, MA, U.S.A.) following the manufacturer’s instructions. Briefly, after the H1299 and NCI-H358 cells were treated with β-lap, the detached cells were collected, washed with cold PBS, fixed with 70% ethanol overnight at −20°C, and treated with 150 µg/mL RNase A and 20 µg/mL propidium iodide (PI; Sigma-Aldrich, Inc.). The cells were then processed for cell cycle analysis post-staining.

**Annexin V and Dead Cell Assay** H1299 and NCI-H358 cells were seeded into 12-well plates for 24 h and were treated with various concentrations of β-lap for 48 h. The floating and attached cells were harvested to assess apoptosis. The apoptotic and necrotic cells were analyzed via flow cytometry (Muse cell analyzer, Merck Milipore, Billerica, MA, U.S.A.) by using the Muse Annexin V & Dead Cell Kit (Muse Annexin V and Dead Cell Reagent) according to the manufacturer’s instructions.

**Western Blot Analysis** H1299 and NCI-H358 cells treated with β-lap were washed with ice-cold PBS and were harvested in M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, U.S.A.) containing a protease inhibitor cocktail (Roche, Switzerland). The protein concentrations were measured using a BCA Protein Assay Kit (Thermo Scientific). The protein samples were separated in 10% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gel, and standard procedures were then used to transfer the samples to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% (v/v) skim milk in TBS-T buffer [20 mM Tris–HCl (pH 7.6), 0.1369 M NaCl, and 0.1% Tween 20] and then incubated with the primary antibody overnight on a rocking platform at 4°C.
The membrane was then washed 4 times for 10 min with TBS-T buffer and was incubated with horseradish peroxidase tagged anti-mouse or anti-rabbit immunoglobulin G (IgG) antibodies. Finally, the hybridized membrane was washed in TBS-T buffer and was visualized using an enhanced chemiluminescence (ECL) detection kit (Thermo Scientific, Rockford, IL, U.S.A.). The primary antibodies used in this study were as followings: α-Sp1, α-cyclin D1, α-survivin, α-Bcl-xl, α-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology), α-p27, α-p21, α-Bax, α-poly(ADP-ribose)polymerase (PARP) and α-caspase-3 (Cell Signaling Technology, Denver, MA, U.S.A.) antibodies.

**Statistical Analysis** Student’s t-test was used to determine the significance in the differences observed between the control and treatment groups, and values of p<0.05 were considered to be significant.

Fig. 2. The Apoptotic Effect Induced by β-Lap in H1299 and NCI-H358 Cells

(A) H1299 and NCI-H358 cells were treated with β-lap or DMSO for 48 h at the indicated doses, and DAPI staining was performed as described in Materials and Methods. DNA fragmentation and nuclear condensation were quantified, and the data represent the mean of 3 experiments with error bars representing the standard deviation. (B) H1299 and NCI-H358 cell cultures were treated with 1, 2 and 3 µM β-lap or PBS (vehicle). The cells were then washed, fixed and stained with PI and analyzed for DNA content via flow cytometry. (C) H1299 and NCI-H358 cells were incubated with β-lap for 48 h, and apoptosis was analyzed via Annexin V-FITC. Annexin V-FITC staining is represented on the x-axis, and PI staining is represented on the y-axis. The data are shown as the average of triplicate samples from independent experiments.
RESULTS

**β-Lap Suppresses the Viability of NSCLC Cells**

β-Lap reportedly inhibits cell proliferation and possesses anti-tumor properties for various cell lines derived from different cancers.15) Figure 1A shows the structure of β-lap. The efficacy of β-lap as an anticancer drug was first investigated by treating H1299 and NCI-H358 cells with β-lap and using an MTS assay to determine cell viability. Figure 1B shows that the viability of H1299 and NCI-H358 cells decreased in a dose- and time-dependent manner (p<0.05) after treatment with β-lap. A maximal decrease was observed at 48 h relative to the levels at 24 h. The morphological changes were observed under an optical microscope after 48 h, and the apoptotic phenotype was present as a rounded cell with cytoplasmic blebbing and irregularities in shape (Fig. 1C). These results therefore indicate that β-lap inhibits the growth of NSCLC cells.

**β-Lap Treatment Induced Apoptosis in NSCLC Cells**

The suppression of cancer cell proliferation is associated with cell cycle arrest and apoptosis.20) We carried out DAPI staining under a confocal laser microscope for β-lap-treated H1299 and NCI-H358 cells to investigate the morphological changes related to apoptosis. The results revealed the presence of nuclear fragmentation upon treatment with β-lap at a concentration of 1, 2, and 3 µM for 48 h, and the percentage of cells with nuclear fragmentation in the β-lap-treated group, relative to control, is shown in Fig. 2A. Next, the cell cycle distribution was analyzed via FACS to determine whether β-lap-mediated apoptosis of H1299 and NCI-H358 cells could be attributed to cell cycle arrest. As shown in Fig. 2B, the H1299 and NCI-H358 cells showed a significant dose-dependent increase in the number of sub-G1 cells after β-lap treatment relative to untreated control cells. Finally, we verified β-lap-mediated apoptosis using an annexin V assay and found that the ratio of early and late apoptotic cells significantly increased in β-lap treated H1299 and NCI-H358 cells relative to the control group (Fig. 2C). These data therefore indicate that β-lap treatment effectively inhibits cell proliferation and leads to apoptotic cell death in OSCC cells.

**β-Lap Suppresses Sp1 Expression in NSCLC Cells**

Sp1 plays an important role in oncogenesis, and hence, effective modulation of its expression level through the use of a chemotherapeutic agent is a potent approach to suppress tumor progression. H1299 and NCI-H358 cells were treated with 3 µM β-lap for different time periods (0, 6, 12, 24 and 48 h) in order to determine whether Sp1 protein expression was reduced by β-lap. Figures 3A and B show that β-lap treatment induced a significant time-dependent decrease in Sp1 protein expression in H1299 and NCI-H358 cells. To further investigate the apoptotic effects of Sp1 downregulation, both cell lines were treated with various concentrations of β-lap at 0, 1, 2 and 3 µM for 48 h. We further showed a decrease in caspase-3 levels, which indicated apoptosis had been induced in H1299 and NCI-H358 (Figs. 3C, D). Our data suggested that Sp1 downregulation due to β-lap treatment may lead to apoptotic cell death.

![Fig. 3. The Effects of β-Lap on Sp1 Protein Expression in H1299 and NCI-H358 Cells](image-url)
β-Lap Regulates Cell Cycle Arrest and Apoptosis in NSCLC Cells

Sp1 regulates the expression of various proteins involved in cell cycle arrest, growth and apoptosis. To further support the association between β-lap and Sp1-mediated apoptosis, we examined Sp1 target proteins and apoptotic proteins and found that proteins involved in cell cycle arrest, such as p27 and p21, had been markedly increased by β-lap whereas proteins related to cell proliferation and survival, such as cyclin D1, Myeloid cell leukemia-1 (Mcl-1) and survivin, were significantly attenuated in a dose-dependent manner by β-lap treatment (Figs. 4A, B). Furthermore, various pro-apoptotic and anti-apoptotic proteins were also expressed in H1299 and NCI-H358 cell lines (Figs. 5A, B). Western blot analysis revealed that the upregulation of Bax and downregulation of Bid were involved in β-lap-induced apoptotic cell death, and in addition, PARP and caspase-3 decreased in a dose-dependent manner as a result of β-lap treatment. Our results clearly indicate that cell cycle arrest and apoptotic cell death are induced in NSCLC cells treated with β-lap through the Sp1-mediated pathway.

DISCUSSION

For the past 30 years, natural compounds have been rich agents of value to medicine and have been the mainstay for cancer therapy. Natural compounds that have anticancer abilities must be evaluated in order to continue development of anticancer drugs, and to this end, the present study investi-
gated the anticancer properties of β-lap, a naturally occurring quinone obtained from the bark of the lapacho tree (*Tabebuia avellanedae*) native to South America. Several mechanisms for the anticancer properties of β-lap had been previously investigated, and β-lap was found to induce sub-G1 arrest in the cell cycle and apoptosis in various cancer cells, such as breast carcinoma, prostate carcinoma, and human leukemia.\(^{5,23–25}\) suggesting that β-lap could interfere with proliferation and could induce apoptosis in close association with sub-G1 arrest. Although numerous studies have been carried out on β-lap-induced apoptosis for many cancer cell lines, the cytotoxic mechanisms of β-lap in human NSCLC cells had not yet been determined. We therefore investigated the molecular mechanisms of apoptosis resulting from administering β-lap in NSCLC cell lines, H1299 and NCI-H358. The induction of apoptotic cell death by β-lap was associated with characteristic morphological changes, such as cell rounding, membrane blebbing and chromatin condensation/fragmentation.

The ubiquitous transcription factor Sp1 regulates various aspects of cancer biology, including survival, invasion, metastasis, and angiogenesis.\(^{29}\) Since Sp1 accumulates in several types of cancers,\(^{27}\) an understanding of the Sp1 regulatory network may provide novel insight into cancer treatment.

Several studies have investigated whether or not Sp1 down-regulation by anti-cancer agents could regulate cell cycle- and apoptosis-related proteins to inhibit growth and induce apoptosis.\(^{28,29}\) In particular, Sp1 is known to mediate prostaglandin E2- and nicotine-induced growth of NSCLC cells,\(^{30,31}\) and we show that Sp1 is significantly reduced in cells treated with β-lap and that cell cycle- and apoptosis-related proteins are regulated by Sp1.

To further characterize the effects of β-lap on Sp1, we analyzed the effects that β-lap had on p27, p21, cyclin D1 and survivin, which are Sp1 target proteins.\(^{32–34}\) p21 expression produces an accumulation of cells in G0/G1, altering the cell morphology and differentiation, but not triggering apoptosis, and in addition, p21 regulates G1 cell cycle arrest resulting from DNA damage.\(^{35}\) p27 modulates cell progression from the G1- to the S-phase by mediating G1 arrest through the inhibition of cyclin/CDK-complex activities in response to a variety of growth inhibitory signals, which in turn leads to apoptotic cell death in all cell types.\(^{36}\) Cyclin D1 is indispensable for cell cycle progression and is related to tumorigenesis and cell maintenance because it promotes G1/S phase transition,\(^{37}\) and it was also regulated by β-lap treatment. In addition, Sp1 was reported to be related to a variety of tumor related genes, including survivin, that contains Sp1 sites in their promoter regions.\(^{38}\) In addition, β-lap induced Bax and reduced Bcl-xL expression, and it also activated caspase-3 and PARP, suggesting that β-lap regulated Sp1 and ultimately led to apoptotic cell death.

To the best of our knowledge, this was the first report to demonstrate the cancer chemoprevention effect of β-lap on NSCLC cells. The results of our study indicate that β-lap may inhibit cell proliferation and induce apoptosis by Sp1 via Sp1 target proteins and apoptosis associated proteins. Altogether, β-lap might be a promising therapeutic agent in the treatment of NSCLC.

**Acknowledgment** This work was supported by the Agen-da Program (PJ00932102) from Rural Development Adminis-

**Conflict of Interest** The authors declare no conflict of interest.


