β-lapachone (β-lap) is a naturally occurring quinone obtained from the bark of lapacho tree (Tabebuia avellanedae) with anti-proliferative properties against various cancers. The present study investigated the cell proliferation and apoptosis effect of β-lap on two oral squamous cell carcinoma lines (OSCCs). We carried out a series of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium (MTS) assays, 4',6-diamidino-2-phenylindole (DAPI) staining, cell cycle analysis, and western blot analysis to characterize β-lap and its underlying signaling pathway. We demonstrated that β-lap-treated cells significantly reduced cell proliferation but increased DNA condensation and increased sub-G1 population in OSCCs. Particularly, β-lap suppresses activation of transcription factor specificity protein 1 (Sp1) followed by apoptosis in a concentration-dependent manner in OSCCs. Furthermore, β-lap modulated protein expression levels of cell cycle regulatory proteins and apoptosis-related proteins that are known as Sp1 target genes, resulting in apoptosis. Our results collectively indicated that β-lap was able to modulate Sp1 transactivation and induce apoptosis through the regulation of cell cycle and apoptosis-related proteins. Therefore, β-lap may be used in cancer prevention and therapies to improve clinical outcome as an anticancer drug candidate.

Introduction

The prognosis of patients with oral squamous cell carcinoma (OSCC) remains poor despite advances in diagnosis and conventional treatment (1). OSCC, the most common development in areas of carcinogen-exposed epithelium, likely results from the accumulation of cellular and genetic alteration, leading to aberrant expression of proteins involved in cell growth regulation (2). Blocking or modifying the function of these proteins may impede or delay the development of cancer. Chemotherapy is the mainstay treatment for patients with recurrent or metastatic OSCC, either alone or in combination with other agents (3). Although this conventional treatment of OSCC has been well advanced to date, five-year survival rate of OSCC remains <50% (4). Therefore, discovery and development of effective chemotherapeutic agents for OSCC might improve the survival rate for patients with OSCC.

β-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) (β-lap), a novel bio-reductive anticancer drug is a derivative of naturally occurring substance lapachol found in the South American lapacho tree (Tabebuia avellanedae) (5). β-lap is reported to have anticancer (5), anti-Trypanosoma cruzi (6), anti-inflammatory (7,8), antibacterial (9), antifungal (9), antiviral (10), and wound healing properties (11). Previous studies also have demonstrated that β-lap treatment induced cell death in a variety of different human cancer cells (12,13). In particular, β-lap is a topoisomerase I (14) and II (15) inhibitor that can repress telomerase

Contributed equally
activity in human prostate carcinoma and lung carcinoma cells in vitro, resulting in the induction of apoptosis (16).

Specificity protein 1 (Sp1), belonging to the specificity protein/Krüppel-like factor family of transcription factors that bind to GC-rich promoter element through three Cys2His2-type zinc-fingers, plays an essential role in a variety of cellular functions (17). Sp1 has been reported to contribute to tumorigenesis through regulating gene transcription related to growth and proliferation (18). In particular, Sp1 has been shown to increase during the process of transformation in fibrosarcoma transformation model. Inhibition of Sp1 by small interfering RNA was shown to reduce tumor proliferation in nude mice (19). In this regard, other recent studies suggest that Sp1-mediated functions are novel targets for cancer therapy (20,21).

Although the anticancer effect of β-lap has been demonstrated against several cancer cell lines and models, its effects on oral cancer or mechanisms of β-lap induced apoptosis remain poorly understood. Therefore, the objective of this study was to examine the regulatory effect of β-lap on the growth and apoptosis of OSCC and investigate its anticancer mechanism in relation to Sp1.

Materials and methods

Cell culture. Human oral squamous cell carcinoma cell lines HN22 and HSC4 were cultured in Hyclone Dulbecco's modified Eagle's medium (DMEM) (Wegene, Daegu, Korea) containing 10% heat-inactivated fetal bovine serum and 100 U/ml each of penicillin and streptomycin (Gibco, Grand Island, NY, USA) at 37°C with 5% CO2 in a humidified atmosphere.

Cell viability assay. The effect of β-lap on HN22 and HSC4 was estimated using CellTiter96® Aqueous One Cell Proliferation assay kit (Promega, Madison, WI, USA) according to the manual for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. HN22 (3×103) and HSC4 (2×103) cells were seeded into 96-well plates for 24 h and treated with various concentrations of β-lap for 24 and 48 h. MTS solution was added into 96-well plate and incubated for 2 h. The absorbance was measured at 490 nm using Absorbance Microplate Reader (Biotek, Winooski, VT, USA). Experiments were carried out in triplicates on different days. The percentage of viability was calculated as: (viable cells)% = (OD of β-lap-treated sample / OD of untreated sample) x 100.

DAPI staining. In order to determine apoptosis induction, HN22 and HSC4 treated by β-lap were detected by DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) staining techniques. Briefly, HN22 and HSC4 cells treated with 0-3 µM β-lap for 48 h were fixed with 100% methanol for 30 min at room temperature. After washing twice with PBS, fixed cells were incubated with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Finally, slides were mounted with a mounting solution containing 10% glycerol and observed under a Fluoview confocal laser microscope (Fluoview FV10i, Olympus Corp., Tokyo, Japan).

Cell cycle analysis. Cell cycle analysis was performed using Muse™ Cell Analyzer from Millipore (Billerica, MA, USA) following the manufacturer’s instructions. Briefly, after the treatment with β-lap 0, 1, 2 and 3 µM for 48 h, HN22 and HSC4 cells were collected, washed with cold PBS, fixed in 70% ethanol overnight at -20°C. Cells were then washed again with PBS before staining (150 µg/ml RNase A and 20 µg/ml propidium iodide (PI, Sigma-Aldrich) and incubated at 37°C for 30 min. After staining, cells were processed for cell cycle analysis.

Western blot analysis. After β-lap treatment, HN22 and HSC4 cells were washed with ice-cold PBS and lysed in M-PER® Mammalian Protein Extraction reagent (Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, Switzerland). Protein concentrations were measured using BCA protein assay kit (Thermo Scientific). Protein samples were separated by 8 or 12% SDS-poly-acrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) using standard procedures. After blocking with 5% (v/v) skim milk in TBS-T buffer, membranes were incubated with primary antibody overnight on a rocking platform at 4°C. The membrane was then washed 5 times with TBS-T buffer for 10 min and incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG antibodies. After washing in TBS-T buffer, membranes were visualized using a chemiluminescent ECL detection kit (Thermo Scientific). The primary antibodies used in this study were: Sp1, β-actin, PARP, cleaved PARP, p27, p21, cyclin D1, survivin, Bcl-2, Bcl-xl, Bax (Santa Cruz Biotechonology, Santa Cruz, CA, USA), caspase-3 and cleaved caspase-3 antibodies (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis. Data are reported as the average ± SD of triplicate independent experiment. Statistical significance was assessed using Student’s t-test. Compared to non-treated group, p<0.05 was considered statistically significant.

Results

Growth inhibition effects of β-lap on OSCC cells. β-lap was reported to have anti-proliferation with antitumor properties in different cancers (12). Initially, to investigate the efficacy of β-lap as an anticancer drug, HN22 and HSC4 cells were treated by β-lap and cell viability was determined by MTS assay. As shown in Fig. 1B, the efficiency of β-lap in altering cell viability of HN22 and HSC4 cells were assayed after 24 or 48 h of incubation in β-lap-containing medium at different concentration (1, 2, 3 or 4 µM). The cell viability of HN22 was 94.1±0.08, 73.3±0.05, 43.9±0.02 and 38.1±0.01% after treated with 1, 2, 3 and 4 µM of β-lap for 48 h, respectively, compared to untreated control cells. The cell viability of HSC4 was 82.5±0.05, 61.6±0.03, 52.0±0.01 and 48.1±0.02% after treated with 1, 2, 3 and 4 µM of β-lap for 48 h, respectively, compared to that of untreated control cells.

Next, the morphological changes were observed under an optical microscope after 48 h of treatment of β-lap. The apoptotic phenotype included rounded cells with cytoplasmic blebbing and irregular shape (Fig. 1C). These results showed that β-lap treatment effectively inhibited cell growth of human OSCC cells.
Figure 1. Inhibition of growth by β-lap in oral squamous cell carcinoma (OSCC) cells. (A) Chemical structure of β-lap; (B) HN22 and HSC4 cells were treated with 0, 1, 2, 3 and 4 µM β-lap for 24 and 48 h. The cell viability was monitored by MTT assay according to the manufacturer's instructions. The percentage of viability was calculated as follows: \(\text{viable cells} \% = \left(\frac{\text{OD of β-lap-treated sample}}{\text{OD of untreated sample}}\right) \times 100\). Mean ± SD. n=3. (C) Cellular morphological alterations in HN22 and HSC4 cells treated with or without β-lap for 48 h.

Figure 2. Apoptotic effect induced by β-lap in oral squamous cell carcinoma (OSCC) cells. Cells were treated with 0, 1, 2 and 3 µM β-lap for 48 h and then cells from each well were harvested for examination of DNA damage by (A) DAPI-staining, as described in Materials and methods. White arrows indicate DNA fragmentation and chromatin condensation. (B) Apoptotic DNA fragmentation and chromatin condensation were quantified. Data represent the mean percentage levels ± SD (n=3). (C) HN22 and HSC4 cell cultures were treated with 1, 2 and 3 µM β-lap or PBS (vehicle) for 48 h. Cell cycle analysis was performed as described in Materials and methods. Data shown are representative of at least three repeats.
**β-lap treatment induces apoptosis in OSCC cells.** In order to evaluate the mechanisms of growth suppression of HN22 and HSC4 cells by β-lap, we observed DNA condensation and apoptotic bodies in the nucleus. Confocal laser microscopic analysis of β-lap-treated HN22 or HSC4 cells was used to demonstrate apoptotic morphological changes using DAPI staining. The results presented in Fig. 2A indicate that β-lap treatment induced DNA condensation and decreased the cell number. Percentage of cells with DNA condensation in β-lap-treated group compared to DMSO-treated group are shown in Fig. 2B.

To gain insight into the mechanism of the anti-proliferative activity of β-lap, its effects on cell cycle distribution were analyzed by FACS analysis. As shown in Fig. 2C, significant increase of the number of sub-G1 cells in HN22 and HSC4 treated by β-lap compared to untreated control cells was found in a concentration-dependent manner. These data showed that β-lap treatment effectively inhibited cell proliferation, leading to apoptotic cell death in OSCC cells.

**β-lap suppresses expression of Sp1 in OSCC cells.** Because Sp1 plays an important role in oncogenesis, if the expression level of Sp1 protein is effectively modulated by a chemotherapeutic agent, then the agent may be a potent candidate as an anticancer drug by suppressing tumor progression. We therefore assessed the anti-proliferative response of β-lap in HN22 and HSC4 cells correlating its effect with Sp1. As shown in Fig. 3A and B, HN22 and HSC4 cells were treated with vehicle (DMSO) or different concentrations of β-lap for 48 h. Sp1 levels were dramatically decreased in treated cells, with a maximum decrease of 94.7±0.01% of HN22 compared to untreated group and 76.1±0.05% of decrease of HSC4 compared to untreated group. To further verify the apoptotic effect of the downregulation of Sp1 by β-lap, HN22 and HSC4 were treated with 3 µM β-lap for different time periods (0, 12, 24 and 48 h) (Fig. 3C and D). To understand the cellular effect of downregulation of Sp1 by β-lap in detail, we examined the expression levels of PARP and cleaved PARP, as indicators of apoptosis induction, in HN22 and HSC4 (Fig. 3C and D). Our results revealed that the downregulation of Sp1 by β-lap treatment leads to apoptotic cell death.

**β-lap regulates cell cycle arrest- and apoptosis regulating proteins.** To determine the regulatory role of β-lap, we focused on the expression levels of Sp1 downstream targets and apoptosis-related proteins. We found that cell cycle arrest proteins, including p27 and p21, were significantly enhanced in a concentration-dependent manner by β-lap, whereas cell proliferation and survival associated proteins, including cyclin D1 and survivin, were decreased by β-lap treatment (Fig. 4). Additionally, we tested the expression levels of several pro-apoptotic and anti-apoptotic proteins in HN22 and HSC4 cell lines. As shown in Fig. 5, downregulation of Bcl-2 and Bcl-xL and upregulation of Bax appeared to be involved in the apoptotic cell death induced by β-lap. In addition, PARP and caspase were decreased by β-lap treatment. Cleaved PARP and
cleaved caspase-3 were induced by β-lap in a concentration-dependent manner. Our results demonstrated that the treatment of OSCC cells with β-lap induced the downregulation of Sp1, resulting in cell cycle arrest and induction of apoptotic cell death.

Discussion

Natural derived or originated compounds are a rich source of agents of value to medicine and have been the mainstay for cancer chemotherapy for the past 30 years (22). This situation is accompanied by increasing interest from drug companies and institutions devoted to the search for new drugs (23). Thus, the evaluation of natural products has been considered to play an important role in development of chemotherapeutic agents. Here, we focused on the anticancer effects of β-lap, a naturally occurring quinone found in the bark of lapacho tree (Tabebuia avellanedae) native to South America (5). β-lap has been also identified to be a topoisomerase I and II inhibitor (14,15). Previous studies have reported that inhibition of the enzymes of topoisomerase I and II can strongly inhibit lymphocyte-, neutrophil- and macrophage-associated joint inflammatory processes and reduce the severity of collagen-induced arthritis (24,25). Therefore, topoisomerase inhibitors can not only be used as anticancer drugs to inhibit the proliferation of tumor cells, as they have the potential to inhibit similar proliferative processes, including rheumatoid arthritis (RA), synoviocytes and angiogenesis.

Previously, several mechanisms involved in the anticancer effect of β-lap were demonstrated. β-lap was reported to induce sub-G1 cell population of cell cycle and induce apoptosis in various cancer cells, including human leukemic, breast...
carcinoma, prostate carcinoma, and lung carcinoma (5,26-29), suggesting that β-lap could interfere with proliferation and induce apoptosis in close association with sub-G1 arrest. Nevertheless, the anticancer activities of β-lap on human OSCC cells have not yet been fully characterized.

Here, we clearly demonstrated that β-lap could induce apoptosis in human OSCC (HN22 and HSC4) cells. The induction of apoptotic cell death by β-lap was also associated with characteristic morphological changes, such as DNA condensation and rounded cells, with cytoplasmic blebbing and irregularities in shape. We observed an increase of DNA condensation and perinuclear apoptotic bodies and sub-G1 population by β-lap treatment in a concentration-dependent manner, supporting the progression of apoptosis in β-lap-treated cells.

Sp1 is known as a transcription factor that is regulated by the molecular target genes in various biological processes, including survival, invasion, metastasis, and angiogenesis (30). Previous reports have shown that Sp1 is accumulated in cancer cells (31). Transcriptional activity primarily contributes to the increase of Sp1 during cancer formation (32). Therefore, other studies have examined whether downregulation of Sp1 by anti-proliferative agents could modulate cell cycle- and apoptosis-associated proteins and lead to growth inhibition and apoptosis (33-36). For these reasons, Sp1 has been suggested as therapeutic molecular target as well as potential predictor of poor prognosis in cancer. Other studies have shown that Sp1 level was highly increased and that the inhibition of Sp1 plays a role in growth inhibition and induction of apoptosis in malignant pleural mesothelioma and oral cancer cells (20,21). Our results showed that Sp1 was significantly reduced in β-lap-treated cells and that apoptosis-related proteins were regulated by Sp1.

Both p21 and p27 are negative regulator of cyclin-dependent kinases and in this function they are negative check-point regulators of the cell cycle. Their functional roles in sub-G1 phase arrest result from the interaction of cyclins and cyclin-dependent kinase (CDKs) complexes (37,38). Cyclin D1 was also regulated by β-lap treatment. Cyclin D1 is the first cyclin to be upregulated by growth factors during G1 phase and it is considered to be a key intracellular mediator of extracellular signal, that regulates proliferation (39). Thus, its expression level is related to tumorigenesis and cell maintenance (40). A previous study showed that increased level of cyclin D1 is frequently observed in human cancers (41). In addition, Sp1 was reported to be related to a variety of tumor-related genes. For example, survivin contains Sp1 sites in the promoter region (42). We confirmed that β-lap could modulate expression of anti- and pro-apoptotic proteins toward apoptosis. In addition, β-lap positively regulates p21 and p27 and negatively regulates cyclin D1 and survivin in OSCCs, resulting in the activation of caspase-dependent apoptosis pathway through activated caspase-3 and PARP.

Based on the present data, we examined the cancer chemoprevention effect and mechanisms of β-lap on OSCCs. Our results indicated that β-lap was able to inhibit cell proliferation and induce apoptosis by Sp1 through regulation of cell cycle-related downstream target of Sp1 protein and apoptosis associated proteins. Taken together, β-lap induced both anti-proliferative and apoptotic effects by inhibiting Sp1-regulated gene products.

Acknowledgements

This study was carried out with the support of ‘Cooperative Research Program for Agriculture Science and Technology Development’ (Project No. PJ009274) Rural Development Administration, Republic of Korea.

References