



Cis-3-O-p-hydroxycinnamoyl Ursolic Acid Induced ROS-Dependent p53-Mediated Mitochondrial Apoptosis in Oral Cancer Cells

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Abstract

Cis-3-O-p-hydroxycinnamoyl ursolic acid (HCUA), a triterpenoid compound, was purified from *Elaeagnus oldhamii* Maxim. This traditional medicinal plant has been used for treating rheumatoid arthritis and lung disorders as well as for its anti-inflammation and anticancer activities. This study aimed to investigate the anti-proliferative and apoptotic-inducing activities of HCUA in oral cancer cells. HCUA exhibited anti-proliferative activity in oral cancer cell lines (Ca9-22 and SAS cells), but not in normal oral fibroblasts. The inhibitory concentration of HCUA that resulted in 50% viability was 24.0 μ M and 17.8 μ M for Ca9-22 and SAS cells, respectively. Moreover, HCUA increased the number of cells in the sub-G1 arrest phase and apoptosis in a concentration-dependent manner in both oral cancer cell lines, but not in normal oral fibroblasts. Importantly, HCUA induced p53-mediated transcriptional regulation of pro-apoptotic proteins (Bax, Bak, Bim, Noxa, and PUMA), which are associated with mitochondrial apoptosis in oral cancer cells via the loss of mitochondrial membrane potential. HCUA triggered the production of intracellular reactive oxygen species (ROS) that was ascertained to be involved in HCUA-induced apoptosis by the ROS inhibitors YCG063 and N-acetyl-L-cysteine. As a result, HCUA had potential antitumor activity to oral cancer cells through eliciting ROS-dependent and p53-mediated mitochondrial apoptosis. Overall, HCUA could be applicable for the development of anticancer agents against human oral cancer.

Key Words: Cis-3-O-p-hydroxycinnamoyl ursolic acid, Oral cancer cells, ROS, Mitochondrial apoptosis

INTRODUCTION

Elaeagnus (*Elaeagnaceae*) has pharmacological effects as an anti-inflammatory, anti-edema, anti-nociceptive, and muscle relaxant. It has been widely used to treat indigestion and osteoarthritis, enhance blood alcohol clearance, and prevent coronary heart disease (Liang *et al.*, 2015; Du *et al.*, 2016; Panahi *et al.*, 2016). *Elaeagnus oldhamii* Maxim., a traditional herbal medicine in Taiwan, is utilized in the treatment of rheumatoid arthritis and lung disorder. Isolated triterpenoid compounds of *Elaeagnus oldhamii* Maxim. exhibit biological

activities including analgesic, anti-inflammation, and anti-lung cancer activities (Liao *et al.*, 2012, 2013, 2014). Triterpenoid compounds such as ursolic acid, oleanolic acid, lupeol, avicins, oleanane, pristimer, and ursane demonstrate a variety of antitumor activities against breast cancer, skin cancer, ovarian cancer, non-small cell lung cancer, and prostate cancer (Kim *et al.*, 2016; Mendes *et al.*, 2016; Nakanishi *et al.*, 2016; Ren *et al.*, 2016; Wu *et al.*, 2016; Yin *et al.*, 2016). Ursolic acid (UA) is an attractive compound with potent anticancer activity in several types of cancers such as melanoma, leukemia, colorectal, lung, and breast cancers (Cai *et al.*, 2017).

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Ursolic acid displays various anticancer actions by reducing oncogene expression, modulating cell cycle, triggering apoptosis, suppressing metastasis, and regulating drug resistance (Zhang *et al.*, 2007; Manu and Kuttan, 2008; Lin *et al.*, 2013; Ou *et al.*, 2014; Xiang *et al.*, 2015). Ursolic acid derivatives such as FZU-03,010, ursolic acid-benzylidene, UP12, and IUA have been demonstrated as potent anticancer agents against hepatocellular carcinoma, osteosarcoma, lung, colon, and breast cancer cells (Chen *et al.*, 2014; Dong *et al.*, 2015; Dar *et al.*, 2016; Li *et al.*, 2017). However, the antitumor activity of ursolic acid derivatives in oral cancers has been rarely reported. *Cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA), a novel ursolic acid derivative, has been identified in *Elaeagnus oldhamii* Maxim. (Liao *et al.*, 2013). Therefore, the antitumor activity of HCUA to oral cancers is still unclear and needs further investigations.

Oral cancer is one of the fastest moving malignancies with nearly 300,000 new cases per year in the world, and is ranked as the 12th most common cancer in the world (Misra *et al.*, 2008). Most oral cancers (near 90%) are oral squamous cell carcinoma (OSCC), which is a highly invasive and metastatic cancer with high mortality (Neville and Day, 2002; Parkin *et al.*, 2005). Its prevalence was markedly increased in Asia, correlating with betel quid chewing, alcohol consumption, and smoking (Ko *et al.*, 1995; Sturgis, 2004). The death rate of oral cancer ranks fourth out of the top 10 malignancies for men in Taiwan in 2015 (Chang *et al.*, 2016), which highlights the increased morbidity of oral cancer in males in recent years. The current treatment for oral cancer is surgical excision of the tumor tissues, combination radiation therapy with chemotherapy, and surgical excision with chemotherapy. However, less than 50% of cases of OSCC receiving surgery or radiotherapy plus chemotherapy survive more than five years (Vokes *et al.*, 1993; Su *et al.*, 2010; Lin *et al.*, 2011). Therefore, effective and affordable therapeutic agents are urgently needed for reducing mortality, inhibiting morbidity of OSCC, and improving the quality of life for patients with oral cancer.

This study investigated the antitumor activity of HCUA in oral cancer cell lines Ca9-22 and SAS using assays for cell proliferation, cell cycle, and apoptosis. The anti-proliferative mechanism of HCUA against oral cancer cells was further examined by assessing the mitochondrial membrane potential, caspase activity, p53-mediated transcription, and reactive oxygen species (ROS) production. Eventually, the ROS inhibitors were used to confirm the ROS-dependent apoptosis of oral cancer cells in response to HCUA treatment.

MATERIALS AND METHODS

Cells and HCUA

Two human oral cancer cell lines, OSCC SAS and gingival Ca9-22 cells, were analyzed in this study. Human normal oral fibroblasts (OF), which were isolated from human oral tissue, were kindly provided from Dr. Ming-Ching Kao at China Medical University. Oral cancer cells were cultured in DMEM medium (HyClone Laboratories, Logan, UT, USA), as described in our prior report (Jou *et al.*, 2015). HCUA, previously identified in the leaves of *E. oldhamii* Maxim, was purified from re-separation by silica gel column chromatography and semi-preparative HPLC, as described in our prior report (Liao *et al.*, 2013). ¹³C-NMR and ¹H-NMR spectra of purified HCUA were

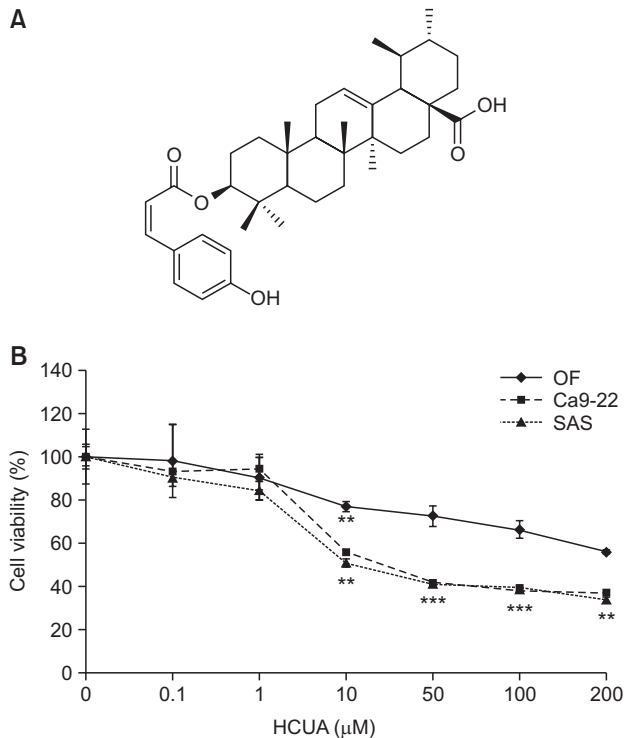


Fig. 1. Survival rates of oral cancer cell lines (Ca9-22 and SAS) and normal oral fibroblasts (OF) in response to *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA). The structure of HCUA is shown in (A). Ca9-22, SAS, and OF cells were treated with HCUA for 48 h, and then the survival rate of treated cells was calculated based on MTT assays (B). ** $p < 0.01$, *** $p < 0.001$ compared with mock cells.

further examined to confirm the structure of HCUA (Fig. 1A).

Anti-proliferative assay

The anti-proliferative activities of HCUA in Ca9-22, SAS, and OF cells were assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Ca9-22, SAS, or OF cells (5×10^4 cells/mL) were placed in 96-well plates and then treated with HCUA at concentrations of 0.1, 1, 5, 10, 20, 50, 100, and 200 μ M at 37°C in a 5% CO₂ incubator. After a 48-h incubation, cells were treated with 10 μ L of MTT solution (5 mg/mL) for 4 h, and then mixed with 100 μ L stop solution for dissolving formazan crystals. The optical density (OD) at 570–630 nm for each well was measured with the BioTek FLx800 micro-ELISA reader. The 50% inhibitory concentrations (IC₅₀) of HCUA for Ca9-22, SAS, and OF cells were calculated based on survival rate of the treated cells.

Sub-G1 arrest and apoptosis assays by flow cytometry

Cells were treated with HCUA (0, 1, 10, and 20 μ M) for 48 h, and then collected for sub-G1 arrest and apoptosis analyses. In the sub-G1 arrest assay, cells were fixed in 70% ethanol overnight, washed in cold PBS, and stained by propidium iodide (PI, 1 μ g/mL). For the apoptosis assay, viable cells were directly stained using Annexin V-FITC (200 μ g/ml) and PI. After staining, cells were analyzed by flow cytometry as described in our prior report (Jou *et al.*, 2015).

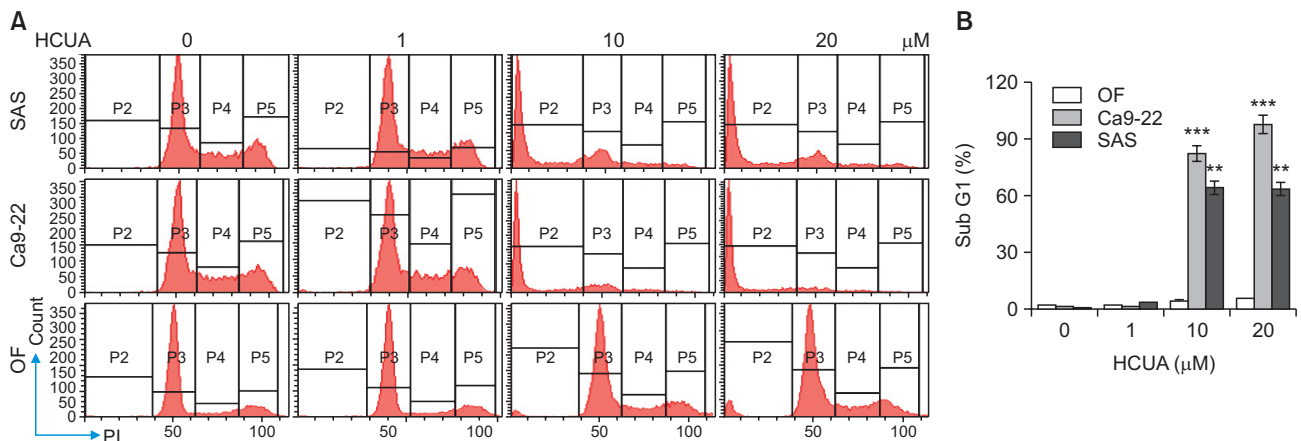


Fig. 2. Effect of *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA) on cell cycle profile of oral cancer cell lines (Ca9-22 and SAS) and normal oral fibroblasts (OF). Cells were harvested 48 h post-treatment, stained with propidium iodide, and then analyzed by flow cytometry (A). The percent of Ca9-22, SAS, and OF cells in the sub G1 (apoptotic) phase after treatment with HCUA was determined (B). ** $p < 0.01$, *** $p < 0.001$ compared with mock cells.

Mitochondrial membrane potential (MMP) assay

Ca9-22 and SAS cells were treated with HCUA at 1, 10, and 20 μM for 48 h, harvested, and then stained with the 3,3'-di-hexyloxacarbocyanine iodide (DiOC6[3]) solution (1 $\mu\text{g}/\text{mL}$ in PBS) at 37°C for 1 h. The cells were subsequently examined using flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Western blotting analysis

Ca9-22 and SAS cells were collected 24 and 48 h post-treatment, and then suspended in RIPA lysis buffer (Roche Diagnostics, San Francisco, CA, USA). The lysate was mixed with the sample buffer, boiled for 8 min, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then analyzed by western blotting assays, as described in our prior report (Yang *et al.*, 2013). The blots were probed with specific primary antibodies against phospho-p53 (Ser15), p53, Bax, Bak, and β -actin (Cell signaling, Danvers, MA, USA). Next, they were reacted with HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA, USA). Immunoreactive bands were developed using chemiluminescent western blotting detection reagents, and then imaged.

Real-time RT-PCR

Total RNA of oral cancer cells was extracted using the RNA purification kit 24 h post-treatment (Invitrogen), and then reverse-transcribed into cDNA with oligo dT primers and SuperScript III reverse transcriptase (Thermo Fisher, Waltham, MA, USA). Two-step RT-PCR was performed using SYBR Green I as described in our prior report (Wang *et al.*, 2012). Oligonucleotide primer pairs were as follows: (1) 5'-AGTGGG-TATTTCTCTTTTGACACAG-3' and 5'-GTCTCCAATACGC-CGCAACT-3' for human Bim, (2) 5'-GACGACCTCAACG-CACAGTA-3' and 5'-CACCTAAT TGGGCTCCATCT-3' for human PUMA, (3) 5'-GAGATGCCT GGAAGAAGG-3' and 5'-TTCTGCCGGAAGTT CAGTTT-3' for human Noxa, and (4) 5'-CCACCCATGGCAAATTCC-3' and 5'-TGGGATTTCATT-GATGACAAG-3' for human GAPDH. Relative mRNA levels of the indicated genes were normalized by housekeeping gene

GAPDH.

Detecting intracellular ROS by flow cytometry

HCUA-treated Ca9-22 cells were harvested and stained with 10 μM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 30 min in a dark room. Later, the DCF fluorescence in stained cells was analyzed using flow cytometry with an excitation wavelength of 485 nm and emission wavelength of 530 nm. The DCF fluorescence intensity reflected the ROS activity with the oxidation of DCFH-DA. To confirm whether the ROS production played a role in HCUA-induced apoptosis, Ca9-22 cells were also co-treated with HCUA and ROS inhibitors (YCG063 and NAC [N-acetyl-L-cysteine]) that were purchased from Sigma-Aldrich, and then assessed by DCFH-DA staining plus flow cytometry analysis, as mentioned above.

Statistical analysis

All data from three independent experiments were represented as the mean \pm standard error (mean \pm SE), and evaluated by comparisons using the Student's *t*-test. $p < 0.05$ indicated statistically significance.

RESULTS

Anti-proliferative effect of HCUA on OSCC cell lines Ca9-22 and SAS

To evaluate the proliferative inhibition effect of HCUA on oral cancer cells, the survival rate of Ca9-22 and SAS cells treated with HCUA were assessed using the MTT assay. HCUA inhibited the growth of both oral cancer cell lines in a concentration-dependent manner (Fig. 1B). The IC_{50} of HCUA on cell proliferation was 24.0 and 17.8 μM for Ca9-22 and SAS cells, respectively. However, HCUA had a small inhibitory effect ($\text{IC}_{50} > 200 \mu\text{M}$) on the growth of human OF cells (Fig. 1B). The result indicated that HCUA significantly inhibited the growth of oral cancer cell lines Ca9-22 and SAS, exerting an anti-proliferative activity against oral cancer cells at low concentrations.

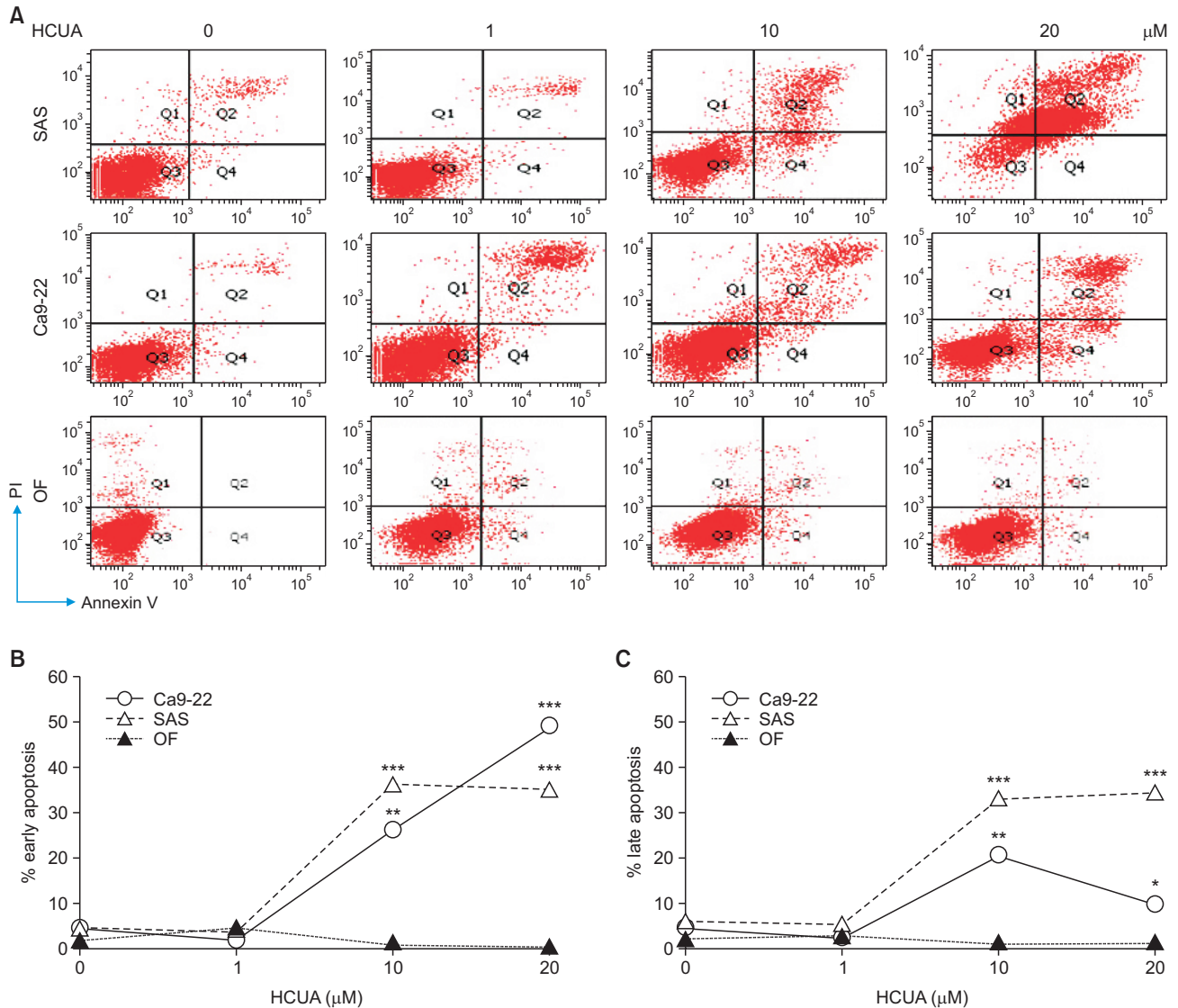


Fig. 3. Induction of apoptosis in oral cancer cells by *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA). Ca9-22 and SAS cells as well as oral fibroblasts were harvested 48 h post-treatment, double-stained with Annexin V-FITC/PI, and then analyzed by flow cytometry (A). The percentage of cells that were Annexin V-positive/PI-negative (early phase of apoptosis) is shown (B). The percentage of cells that were Annexin V-positive/PI-positive (late apoptosis) is shown (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with mock cells.

Selective induction of sub-G1 arrest and apoptosis induced by HCUA

To examine the sub-G1 arrest and apoptosis induction effect of HCUA on oral cancer cells and normal OFs, the cell cycle profiles of Ca9-22, SAS and OF cells were analyzed using PI staining with flow cytometry 48 h post-treatment (Fig. 2). HCUA at 10 and 20 μM significantly increased the population of cells in the sub-G1 arrest phase in Ca9-22 and SAS cells, but not OF cells. The percentage of the sub-G1 population was 82.1% and 63.9% in Ca9-22 and SAS cells, respectively, in the presence of 10 μM HCUA. In order to further confirm the apoptotic event induced by HCUA, treated cells were double-stained using annexin V-FITC and PI, and then analyzed by flow cytometry (Fig. 3). The result showed that HCUA triggered apoptosis of oral cancer cells in a concentration-dependent manner. After treatment with 10 μM HCUA, the

percentages of cells in early apoptosis (annexin V positive/PI negative) were 26.4% and 36.7% in Ca9-22 and SAS cells, respectively (Fig. 3B). Moreover, the percentages of cells in late apoptosis (annexin V positive/PI positive) were 20.3% and 33.4% in Ca9-22 and SAS cells treated with 10 μM HCUA, respectively (Fig. 3C). However, HCUA had no significant effect on cells in sub-G1 arrest and apoptosis in OF cells (Fig. 2, 3). These results indicated that HCUA initiated sub-G1 arrest and induced apoptosis of oral cancer cells in a concentration-dependent manner.

The phosphorylation of p53 at Ser15 modulates its function to serve as an upstream regulator of cell cycle arrest and apoptosis (Dumaz and Meek, 1999; Shanaz *et al.*, 2005; Long *et al.*, 2007; Takeba *et al.*, 2007). The protein levels of phospho-p53 (Ser15), p53, Bak, and Bax in treated cells were further measured by western blotting (Fig. 4). Quantitative analysis of

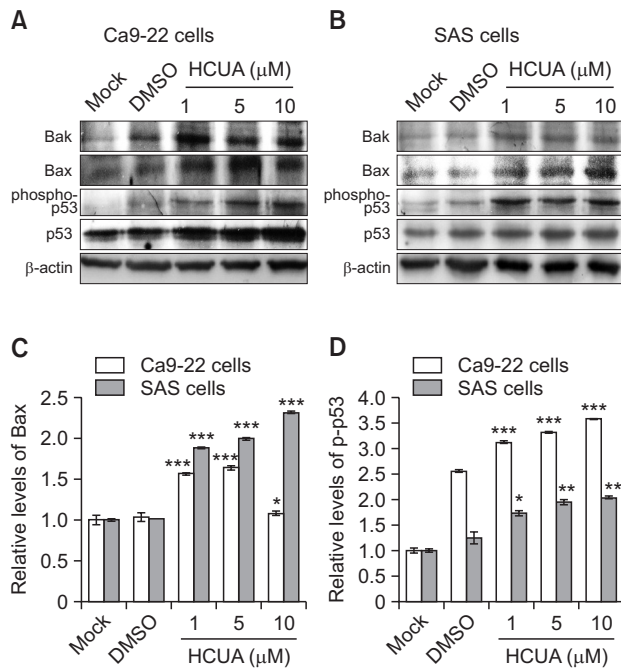


Fig. 4. Relative protein of pro-apoptotic genes in *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA)-treated cells. The protein levels of Bak, Bax, phospho-p53 (Ser15), and p53 in Ca9-22 (A) and SAS (B) cells treated with HCUA were characterized 48 h post-treatment by western blot analysis. The intensity of each immuno-reactive band for Bax or phospho-p53 (Ser15) was quantified using Image J; their relative intensities were normalized by β -actin (C, D), respectively. * p <0.05, ** p <0.01, *** p <0.001 compared with mock cells.

western blot data revealed that HCUA stimulated significant increases of phospho-p53 (Ser15), p53, Bak, and Bax in both oral cancer cell lines (Fig. 4). Therefore, HCUA significantly induced the p53 phosphorylation at Ser15 and increased the protein amount of Bak and Bax in both oral cancer cell lines. The results revealed that HCUA might activate p53-mediated apoptosis in oral cancer cells.

The p53-mediated transcriptional regulation of pro-apoptotic proteins in oral cancer cells by HCUA

To examine whether HCUA affected p53-mediated transactivation of pro-apoptotic proteins, the mRNA and protein levels of Bim, Noxa, and PUMA in treated cells were determined by real-time RT-PCR and western blot analysis at 24 h post-treatment (Fig. 5). HCUA-induced phosphorylation of p53 at Ser15 correlated with upregulation of p53-mediated transcriptional activities on the mRNA expressions of Bim, Noxa, and PUMA in both oral cancer cell lines treated with 10 μ M HCUA (Fig. 5A, 5B). In addition, an increased amount of Noxa and PUMA proteins were detected in cells treated with 10 μ M HCUA (Fig. 5C, 5D). Significant increases in the mRNA and protein levels of Bim, Noxa, and PUMA were mainly detected in cells treated with high concentrations of HCUA. This may have been due to the short incubation period (24 h), which allowed for an obvious change in the early regulators of apoptosis in cells treated with low concentrations of HCUA. Since the pro-apoptotic proteins (Bax, Bim, Noxa, and PUMA) were the effectors of mitochondrial apoptosis via triggering the permeabilization of the mitochondrial outer membrane (Chipuk *et al.*, 2010), the

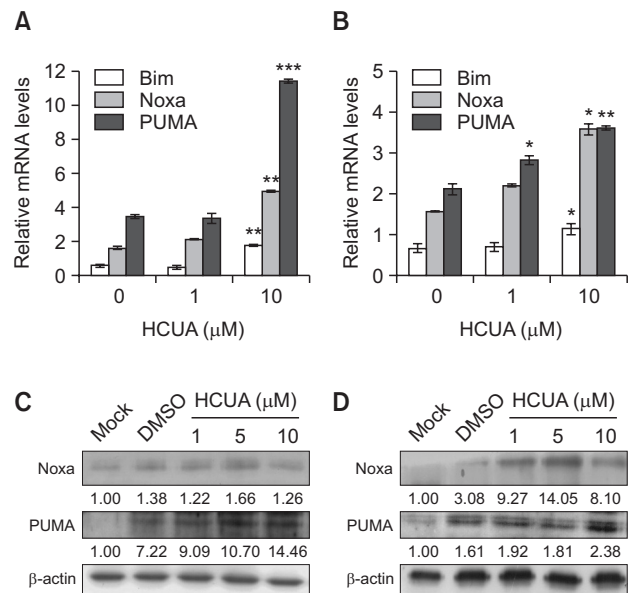


Fig. 5. Relative mRNA and protein levels of p53-mediated transcriptional genes in *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA)-treated cells. Relative mRNA and protein levels of Bim, Noxa, and PUMA in HCUA-treated Ca9-22 (A, C) and of SAS (B, D) cells were measured using real-time RT-PCR and western blotting, respectively. Their relative levels were normalized by GAPDH mRNA or β -actin protein. * p <0.05, ** p <0.01, *** p <0.001 compared with mock cells.

mitochondrial membrane potential (MMP) in treated cells was further examined using DiOC6 staining and flow cytometry (Fig. 6). HCUA at 10 μ M but not 1 μ M significantly decreased the MMP in both oral cancer cell lines at 48 h post-treatment, correlating with the concentration effect on the expression of Noxa and PUMA. The results illustrated that HCUA triggered p53-mediated mitochondrial apoptosis via the upregulation of pro-apoptotic proteins in oral cancer cells.

ROS-dependent apoptosis induced by HCUA

Since the generation of ROS influences on p53-mediated transcription of proapoptotic proteins in mitochondrial apoptosis (Liu *et al.*, 2005; Macip *et al.*, 2013), accumulation of intracellular ROS in treated cells was measured using DCFH-DA staining and flow cytometry (Fig. 7). DCF fluorescence intensity (the oxidation of DCFH-DA by ROS) indicated that HCUA significantly produced intracellular ROS in oral cancer cells in concentration dependent manners, but not in normal OF (Fig. 7). To ascertain the association of HCUA-induced apoptosis with the ROS production, oral cancer cells were treated with ROS inhibitor (YCG063 or NAC) alone or in combination with HCUA, and then cell cycle changes were examined using PI staining plus flow cytometry (Fig. 8, 9). The ROS inhibitor alone did not change the cell cycle profile in oral cancer cells. Importantly, YCG063 resulted in a reduction in HCUA-induced apoptosis of oral cancer cells in a concentration-dependent manner (Fig. 8). NAC at low concentrations also significantly diminished the percentage of HCUA-induced apoptotic cells (Fig. 9). The result demonstrated that the ROS production played a critical role in HCUA-induced apoptosis in oral cancer cells.

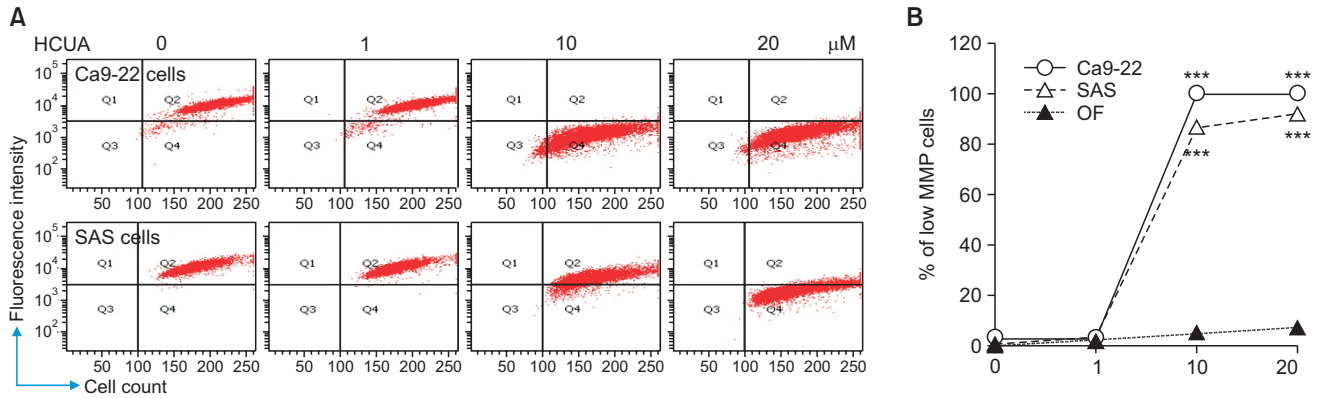


Fig. 6. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) in Ca9-22 and SAS cells treated with *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA). Oral cancer cells and fibroblasts were treated with HCUA and then harvested at 48 h post-treatment, stained with DiOC6(3), and then analyzed by flow cytometry (A). The relative changes in the percentage of low MMP cells in response to HCUA are shown (B). *** $p < 0.001$ compared with mock cells.

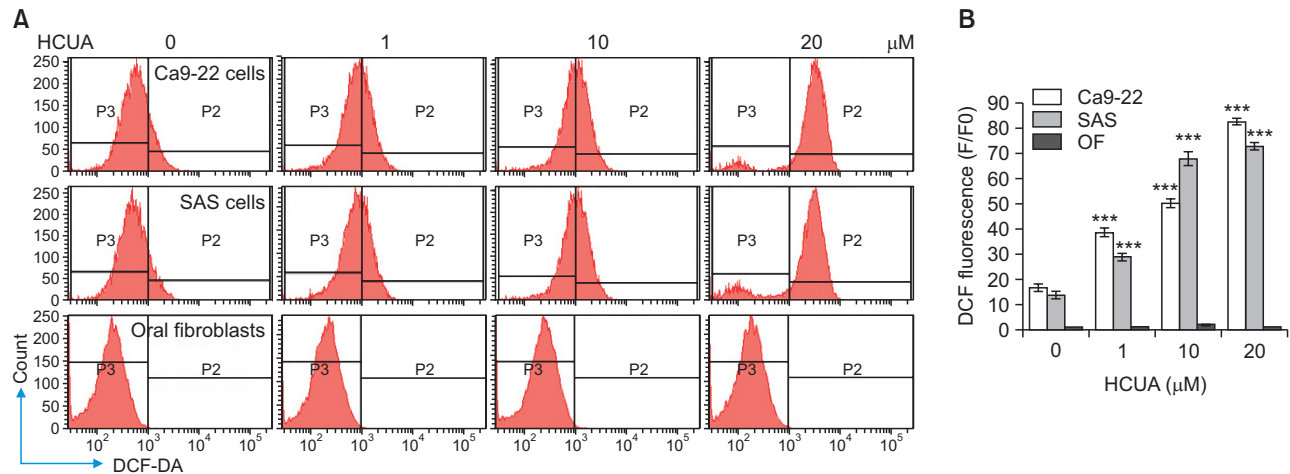


Fig. 7. Increase of intracellular ROS production in *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA)-treated cells. Ca9-22, SAS, and OF cells were harvested 48 h post-treatment with HCUA, stained with 2,7-dichlorodihydrofluorescein diacetate (DCF-DA), and then analyzed by flow cytometry (A). Relative fluorescent intensity of DCF in treated-cells was subsequently measured (B). *** $p < 0.001$ compared with mock cells.

DISCUSSION

HCUA exhibited an anti-proliferative activity in oral cancer cells ($IC_{50} < 25 \mu M$) (Fig. 1), and induced ROS-dependent and mitochondria-mediated apoptosis in oral cancer cells (Fig. 2-8). HCUA resulted in sub-G1 arrest and apoptosis of oral cancer cells via activating the p53-mediated transcriptional activity in a concentration-dependent manner (Fig. 2-5). Up-regulation of p53-mediated pro-apoptotic proteins (Bax, Bim, Noxa, and PUMA) was associated with a decrease of mitochondrial membrane potential in HCUA-treated oral cancer cells (Fig. 5, 6). HCUA also triggered the increase of ROS production in oral cancer cells (Fig. 7). The relationship between HCUA-elicited ROS production and apoptosis in oral cancer cells was ascertained by the ROS inhibitors YCG63 and NAC (Fig. 8, 9). Interestingly, HCUA was less toxic and apoptotic to normal OFs (Fig. 1-3). The finding might be correlated with a low ROS induction in normal OFs by HCUA (Fig. 7). The anti-proliferative and apoptosis-inducing activities of HCUA from

Elaeagnus oldhamii Maxim. in oral cancer cells.

HCUA is a novel ursolic acid derivative with anti-oral cancer activity via the induction of ROS-dependent and mitochondria-mediated apoptosis. ROS was predominantly generated in the mitochondria and reportedly related to the early stages of apoptosis (Samhan-Arias *et al.*, 2004; Yen *et al.*, 2012). The anti-oral cancer mechanism of HCUA was similar to the report in that triterpenoid pristimerin induced apoptosis in human hepatocellular carcinoma HepG2 cells and U87 glioma cells through ROS-mediated mitochondrial dysfunction (Guo *et al.*, 2013; Yan *et al.*, 2013). In addition, HCUA increased the phosphorylated levels of p53 at Ser15 in both oral cancer cell lines in a concentration-dependent manner (Fig. 4). The p53 Ser15 phosphorylation allowed p53 to interact with the p300 coactivator and MDM2 that was linked with cell cycle arrest and mitochondrial apoptosis (Dumaz and Meek, 1999; Shanaz *et al.*, 2005; Long *et al.*, 2007; Takeba *et al.*, 2007). The ROS generation has been shown to promote the phosphorylation of p53 at Ser15 that elicited p53-mediated mitochondrial apop-

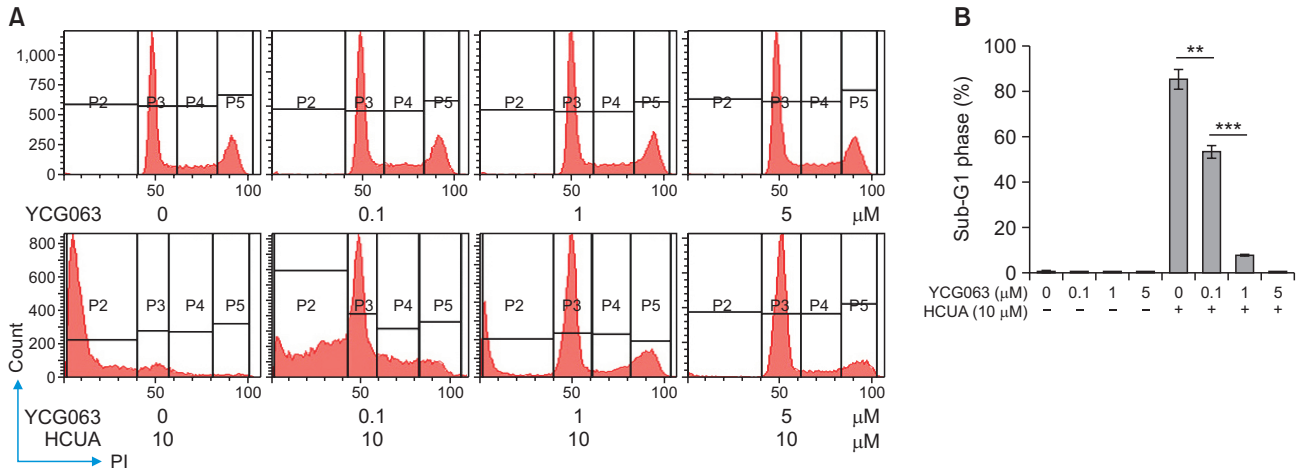


Fig. 8. Reduction of *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA)-induced apoptosis in Ca9-22 cells by YCG063. Cells were treated with YCG063, HCUA, or a combination of both, harvested 48 h post-treatment, fixed by 70% ethanol, stained with a propidium iodide solution, and then examined by flow cytometry (A). The percentage of cells in the sub-G1 (apoptotic) phase is shown in (B). ** $p < 0.01$, *** $p < 0.001$.

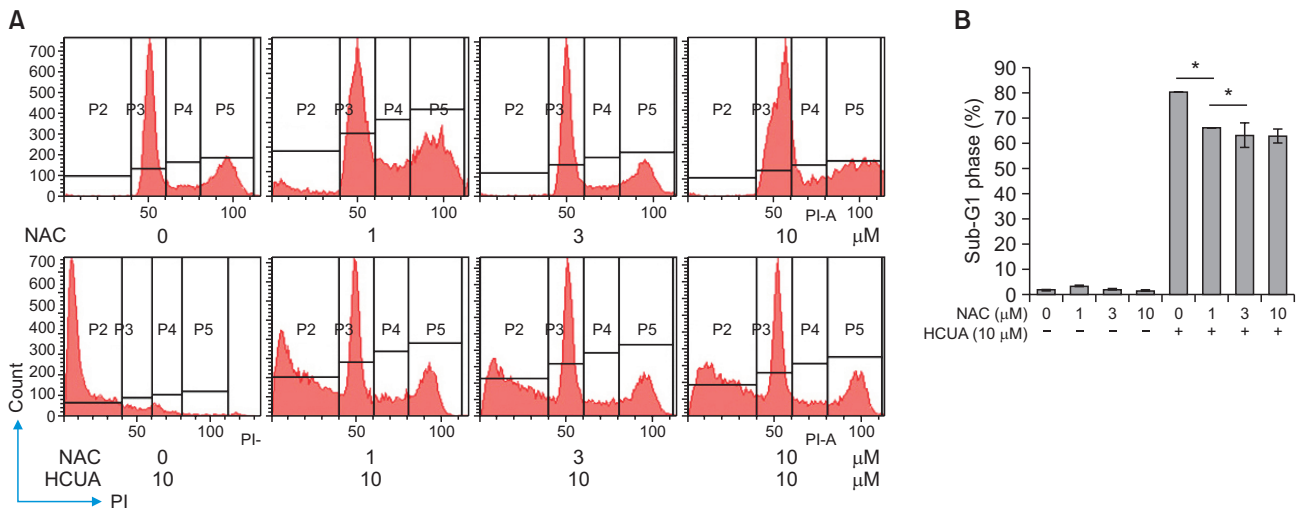


Fig. 9. Inhibitory effect of NAC on *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (HCUA)-induced apoptosis. Ca9-22 cells were treated with NAC, HCUA, or a combination of both, harvested 48 h post-treatment, stained with propidium iodide, and then examined by flow cytometry (A). The percentage of cells in the sub-G1 (apoptotic) phase is shown in (B). * $p < 0.05$.

tosis (Ito *et al.*, 2004; Li *et al.*, 2007; Shi *et al.*, 2014). Thus, the ROS production might be associated with the activation of p53-mediated transcriptional targets (Bax, Bim, Noxa, and PUMA) in the mitochondrial apoptosis of oral cancer cells treated with HCUA (Fig. 4-9). Furthermore, p53 regulated the induction of apoptosis by modulating the expression of pro-apoptotic genes such as PUMA, Noxa, and Bim (Mancini and Moretti, 2011; Jou *et al.*, 2016). These pro-apoptotic genes are three important members in the BH3-only family and are critical mediators of apoptosis in response to a variety of stimuli such as DNA damage and hypoxia (Oda *et al.*, 2000; Nakano and Vousden, 2001). In addition, ursolic acid has been reported to activate p53-mediated apoptosis in melanoma cells and p53-mediated, mitochondria- and caspase-dependent human endothelial cell apoptosis (Manu and Kuttan, 2008). Previous studies have supported an anticancer mechanism of HCUA via ROS-dependent and mitochondria-mediated apoptosis in

oral cancer cells.

This study demonstrated that HCUA isolated from *Elaeagnus oldhamii* Maxim. serves the anti-proliferative and apoptosis-inducing activities against oral cancer cells. HCUA has an effective inhibitory activity on the growth of oral cancer cells via the induction of apoptosis. Moreover, HCUA induces ROS-dependent and mitochondria-mediated apoptosis in oral cancer cells through p53-mediated transactivation of pro-apoptotic proteins (Bax, Bim, Noxa, and PUMA). The apoptotic pathways induced by HCUA provide insights into anti-oral cancer mechanisms. The study would be useful for developing potential anticancer agents for the treatment of oral cancers.

CONFLICT OF INTEREST

All authors had no conflict of interest.

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