Antitumor and apoptosis-inducing effects of α-mangostin extracted from the pericarp of the mangosteen fruit (*Garcinia mangostana* L.) in YD-15 tongue mucoepidermoid carcinoma cells

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Abstract. α-mangostin is a dietary xanthone which has been shown to have antioxidant, anti-allergic, antiviral, antibacterial, anti-inflammatory and anticancer effects in various types of human cancer cells. In the present study, we aimed to elucidate the molecular mechanisms responsible for the apoptosis-inducing effects of α-mangostin on YD-15 tongue mucoepidermoid carcinoma cells. The results from MTT assays revealed that cell proliferation significantly decreased in a dose-dependent manner in the cells treated with α-mangostin. DAPI staining illustrated that chromatin condensation in the cells treated with 15 µM α-mangostin was far greater than that in the untreated cells. Flow cytometric analysis indicated that α-mangostin suppressed YD-15 cell viability by inducing apoptosis and promoting cell cycle arrest in the sub-G1 phase. Western blot analysis of various signaling molecules revealed that α-mangostin targeted the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) signaling pathways through the inhibition of ERK1/2 and p38 phosphorylation in a dose-dependent manner. α-mangostin also increased the levels of Bax (pro-apoptotic), cleaved caspase-3, cleaved caspase-9 and cleaved-poly(ADP-ribose) polymerase (PARP), whereas the levels of the anti-apoptotic factors, Bcl-2 and c-myc, decreased in a dose-dependent manner. The anticancer effects of α-mangostin were also investigated in a tumor xenograft mouse model. The α-mangostin-treated nude mice bearing YD-15 tumor xenografts exhibited a significantly reduced tumor volume and tumor weight due to the potent promoting effects of α-mangostin on cancer cell apoptosis, as determined by TUNEL assay. Immunohistochemical analysis revealed that the level of cleaved caspase-3 increased, whereas the Ki-67, p-ERK1/2 and p-p38 levels decreased in the α-mangostin-treated mice. Taken together, the findings of our study indicate that α-mangostin induces the apoptosis of YD-15 tongue carcinoma cells through the ERK1/2 and p38 MAPK signaling pathways.

Introduction

Cancer is one of the most life-threatening diseases worldwide, and the incidence and mortality of cancer in Korea have continuously increased due to various acquired risk factors, including a Western diet and environmental factors (1). Oral cancer accounts for approximately 3-5% of all cancer cases (2, 3); however, unlike cancers in other parts of the body, damage to the facial region can cause psychological disorders in patients and negatively affect their daily life, particularly in terms of eating or speaking. Thus, there is great interest in exploring treatments for oral cancer (4). Oral cancer poses a risk of invasion to adjacent organs and a higher possibility of metastatic relapse. Thus, surgical therapy, radiation and medication in combination are usually used to treat oral cancer; however, severe adverse effects and low treatment efficacy, as well as the ineffective inhibition of cancer cell growth and micrometastasis, results in patients with oral cancer tending to have a poor prognosis (5). It is therefore necessary to find naturally-derived substances to serve as anticancer drugs that are capable of specifically targeting cancer cells, with limited side-effects and potent anticancer effects.

Mangosteen (*Garcinia mangostana* L.) is well known as the ‘queen of tropical fruit’ due to its delicious taste, and has long been used for medicinal purposes, particularly for the treatment of dermatitis, ulcers and diarrhea, in parts of Southeastern South Asia such as Malaysia, Indonesia, Taiwan, Philippines, India and Sri Lanka (6). It has been reported that xanthone, a component contained within the pericarp (rind or peel) of the mangosteen fruit, has been shown to exert various biological effects, including antioxidant (7), anticancer (8), antibacterial (9, 10), anti-inflammatory (11), anti-allergic and antiviral effects (12). Xanthone has also been widely used as an inhibitor of enzymes involved in the oxidation of low-density...
lipoprotein (LDL) cholesterol (13), as well as those associated with infections, such as prostaglandin E$_2$ (PGE$_2$) and cyclooxygenase-2 (COX-2) (14). Thus far, various xanthones have been found in fruit, fruit skin, tree bark, moss and mold, and approximately 40 different xanthones have been found in the mangosteen fruit (15).

$\alpha$-mangostin is a key, physiologically active substance contained within the fruit skin of mangosteens that has been demonstrated to inhibit the cell cycle and induce the apoptosis of various cancer cell lines, including colorectal, mammary, liver and prostate cancer cells (8,16-19). In particular, the anticancer effects and the inhibitory effects on lymph node metastasis of $\alpha$-mangostin have been reported using tumor xenograft mouse models of mammary cancer (19).

The mitogen-activated protein kinase (MAPK) cascade, a pathway used to send external signals to internal cells, is involved in various processes, including cell proliferation and fragmentation, apoptosis and survival. There are also subgroups of MAPKs, which include extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). Each group is controlled by its own pathway and performs distinct functions. ERK is mainly involved in cell survival, whereas SAPK and p38 kinase mainly regulate apoptosis (20).

However, the anticancer effects of $\alpha$-mangostin on oral cancer remain unknown. Thus, in this study, we aimed to investigate the anticancer effects of $\alpha$-mangostin on oral (tongue) cancer, which is a type of cancer with severe adverse effects and lower treatment efficacy compared with other types of cancer. The naturally-derived substance, $\alpha$-mangostin, was evaluated in YD-15 cells, a tongue mucoepidermoid carcinoma cell line, in order to examine its inhibitory effects on cancer progression in terms of apoptosis. Accordingly, we focused on the ERK1/2 and p38 MAPK signaling pathways in an aim to elucidate the underlying molecular mechanisms.

Materials and methods

Chemicals, drugs and antibodies. $\alpha$-mangostin (chemical structure shown in Fig. 1) was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. RPMI-1640 medium, penicillin-strep-tomycin, trypsin-EDTA and fetal bovine serum (FBS) were purchased from HyClone Laboratories, Inc. (Logan, UT, USA). 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were obtained from Sigma-Aldrich. Cell lysis buffer and 4,6-diamidino-2-phenylindole (DAPI) were purchased from InVitrogen Life Technologies (Carlsbad, CA, USA). The fluorescein isothiocyanate (FITC)-conjugated Annexin V Apoptosis Detection kit was purchased from BD Biosciences (San Diego, CA, USA). Anti-β-actin (#4967), anti-Bax (#2772), anti-Bcl-2 (#2876), anti-caspase-3 (#9662), anti-cleaved caspase-3 (#9661), anti-caspase-9 (#9502), anti-poly(ADP-ribose) polymerase (PARP; #9542), anti-ERK1/2 (#9102), anti-phosphorylated (p)-ERK1/2 (#4376), anti-p38 (#9212), anti-p-p38 (#4631), anti-c-myc (#9027), Anti-Ki-67 (#9027) and goat anti-rabbit horseradish peroxidase (HRP)-conjugated (#7074) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The DeadEnd™ fluorometric terminal deoxynucleotidyl trans-

ferase-mediated dUTP nick-end labeling (TUNEL) assay kit was purchased from Promega Corp. (Madison, WI, USA).

Cell lines and culture. The human tongue mucoepidermoid carcinoma cell line, YD-15, was purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO$_2$ atmosphere. The culture medium was replaced every 2-3 days. For $\alpha$-mangostin treatment, the YD-15 cells were seeded at a density of approximately 3x10$^4$ cells/cm$^2$ in a 175-cm$^2$ flask and were allowed to adhere overnight.

Cell viability assay. The effects of $\alpha$-mangostin on YD-15 cell survival were determined by MTT assay. The YD-15 cells were seeded in 96-well plates at a density of 2x10$^4$ cells/ml in a volume of 200 µl/well. Following 24 h of incubation, the cells were treated with 10, 15, 20, 25 or 30 µM $\alpha$-mangostin for 24 h in triplicate. Following treatment, the medium was discarded and 40 µl 5 mg/ml MTT solution were added followed by incubation for an additional 2 h. The medium was then aspirated, and the formazan product generated by the viable cells was solubilized by the addition of 100 µl DMSO. The absorbance of the solutions at 595 nm was determined using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of viable cells relative to the untreated (control) cells was estimated.

Nuclear staining. To assess apoptosis, the nuclei of the YD-15 cells were stained with DAPI. The cells were seeded onto 60-mm dishes at a density of 1x10$^4$ cells/ml and incubated with 10 or 15 µM $\alpha$-mangostin for 24 h. Following treatment, the cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 15 min in an incubator. Following fixation, the cells were washed twice with PBS and the cell nuclei were stained with DAPI in PBS. Fluorescence signals were visualized using a fluorescence microscope (BX41; Olympus Co., Tokyo, Japan) at x200 magnification.

Annexin V staining for the analysis of apoptosis. The Annexin V/propidium iodide (PI) assay was performed according to the manufacturer’s instructions (BD Biosciences). Briefly, the YD-15 cells were treated with or without 10 or 15 µM $\alpha$-mangostin for 24 h, washed twice with cold PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and phycoerythrin (PE)-conjugated PI in binding buffer at room temperature for 15 min in the dark. The samples were analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).
Flow cytometric analysis of the cell cycle. Cell cycle progression was assayed by measuring DNA fragmentation with PI staining. The YD-15 cells were treated with or without 10 or 15 µM α-mangostin for 24 h, washed twice with PBS and fixed with 70% ethanol for 30 min. Following fixation, the DNA fragments were stained in PBS containing PI and RNase (Sigma-Aldrich) for 30 min at room temperature. After sorting out the viable cells, the fluorescence intensity was measured using a FACSCalibur™ flow cytometer (BD Biosciences).

Western blot analysis. The cells were grown in culture flasks under the same conditions as described above and treated with 10 or 15 µM α-mangostin for 24 h. The cells were washed with PBS and treated with trypsin-EDTA for 1 min. Cell pellets were obtained by centrifugation, lysed in lysis buffer (Invitrogen Life Technologies) and centrifuged at 13,000 rpm for 5 min at 4°C to obtain whole-cell lysates. Protein concentrations were determined using a Bradford Protein assay kit (Bio-Rad Laboratories Inc.). The samples were stored at -80°C. The proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories Inc.). The membranes were blocked with Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween-20 at 4°C for 2 h. After blocking, the membranes were incubated with anti-β-actin, anti-Bax, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-PARP, anti-ERK1/2, anti-p-ERK1/2, anti-p38, anti-p-p38 and anti-c-myc antibodies overnight and incubated for 1 h at 4°C, with gentle shaking. Following incubation with the primary antibodies, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies for 2 h at room temperature with gentle shaking. After washing the membranes 3 times for 10 min in TBS containing 0.1% Tween-20, bands were detected using enhanced chemiluminescence (ECL) western blotting detection reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. β-actin was used as a loading control. Band density was measured using the ImageJ software (NIH, Bethesda, MD, USA) program.

Animal experiments. Five-week-old male BALB/c nude (nu/nu) mice were purchased from the animal production company of Orient Bio, Inc. (Gyeonggi-do, Korea) and maintained in a controlled environment at 23±5°C with 40±10% relative humidity with artificial lighting from 8:00 a.m. to 8:00 p.m. in facilities approved by the Companion and Laboratory Animal Science Department of Konju National University (Chungnam, Korea). The animals were housed in cages and allowed access to sterilized water and commercial rodent chow (Bipia, Seoul, Korea) ad libitum. All animal experiments were performed following the approval of the Institutional Animal Care and Use Committee according to the guidelines of Konju National University.

Tumor xenografts. The YD-15 cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin at 3°C in a humidified 5% CO₂ atmosphere. The YD-15 cells were harvested by exposure to trypsin-EDTA. The cells were then washed twice and resuspended in RPMI-1640 medium. The YD-15 cells were then injected subcutaneously (1x10⁷ cells/0.2 ml medium/animal) into the left and right flanks of the mice using a 27-gauge needle. When the tumors were palpable, the mice were assigned randomly into 3 groups with 3 mice in each (the vehicle-treated controls, and the groups treated with 10 or 20 mg/kg body weight α-mangostin). The doses of α-mangostin (10 and 20 mg/kg) were selected for the in vivo experiments using mice based on the results of a previous study by Akao et al (8), in which mice administered >20 mg/kg α-mangostin exhibited a significant increase in natural killer (NK) cell activity. Therefore, we selected 20 mg/kg as the dose for use in the present study, as this is the dose that others have reported has no harmful effect. For administration, α-mangostin was dissolved in 0.1% DMSO and further diluted in PBS before injection. α-mangostin was administered intraperitoneally 5 times per week at a dose of 10 or 20 mg/kg body weight, while the control group mice were administered the vehicle only (DMSO in PBS). Tumor weight and size were monitored twice each week. Tumor size was measured using Vernier calipers (Mitutoyo, Kawasaki, Japan). The mice were sacrificed by ether inhalation at 22 days following treatment and the tumors were excised for the measurement of tumor weight. A portion of the tumor was embedded in paraffin and used for TUNEL assays and immunohistochemical analysis.

TUNEL assay. Apoptotic cell death was quantified using a Promega DeadEnd Colorimetric TUNEL system kit according to the manufacturer's instructions (Promega Corp.). Briefly, the tumor tissues were fixed in 10% formalin overnight and embedded in paraffin. The blocks were then cut into 5-µm-thick slices. The tissue sections attached to microscopic slides were then deparaffinized by immersion in xylene and the slides were then washed with 100% ethanol. The samples were rehydrated by sequential immersion in a graded ethanol series (95, 85, 70 and 50%). The tumor sections were visualized using 3'-diaminobenzidine tetrahydrochloride (DAB) solution, treated with mounting reagent, and observed under a microscope (BX41; Olympus Co.) at x200 magnification.

Immunohistochemical analysis. The tumor sections were deparaaffinized with two changes of xylene for 10 min, rehydrated with two changes each of 100 and 95% ethanol for 1 min, and rinsed with tap water for 10 min. The sections were then incubated at 4°C with anti-cleaved caspase-3, anti-p-ERK1/2, anti-p-p38 and anti-Ki-67 antibodies overnight and incubated for 1 h at room temperature with a HRP-conjugated goat anti-rabbit antibody followed by incubation for 1 h. The tumor sections were visualized using DAB solution, treated with mounting reagent, and observed under a microscope (x200 magnification).

Statistical analysis. The results are all expressed as the means ± standard deviations (SD). Differences between mean values for the individual groups were assessed by one-way analysis of variance (ANOVA) with Dunnett's t-tests. A P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory effects of α-mangostin on the cell survival rate and morphological changes in YD-15 cells. An MTT assay was conducted to examine the effects of α-mangostin on YD-15 cell viability. The YD-15 cells were treated with 0, 10, 15,
20, 25 and 30 µM α-mangostin for 24 h. Cell viability was inhibited at a concentration of 10 µM (Fig. 2A). Compared with the untreated control cells, the cells treated with 10 µM α-mangostin displayed approximately 15% inhibition, whereas those treated with 15 µM α-mangostin exhibited approximately 58% inhibition. Moreover, the number of viable cells decreased in a concentration-dependent manner. DAPI staining was performed to identify the morphological changes associated with nuclear and chromosomal condensation. The YD-15 cells were treated with 0, 10 and 15 µM α-mangostin for 24 h. DAPI staining was utilized to observe the cells by fluorescence microscopy. As a result, increased apoptosis (indicated by increased chromatin condensation) was observed in the cells treated with 10 and 15 µM α-mangostin compared to the control group (Fig. 2B). These results indicate that α-mangostin decreased the viability of the YD-15 cells, creating apoptotic bodies, thus leading to cellular apoptosis.

**Effects of α-mangostin on YD-15 cell apoptosis.** Apoptotic cells were analyzed quantitatively by Annexin-V FITC/PI staining to examine the effects of α-mangostin on YD-15 cells. The percentage of apoptotic cells in the untreated (control) group was 16.06% (early apoptotic cells, 4.30%; late apoptotic cells, 11.76%). However, the number of apoptotic cells increased significantly with the increasing concentration of α-mangostin. The percentages of apoptotic cells following treatment with 10 and 15 µM α-mangostin were 28.1 (early apoptotic cells, 10.79%; late apoptotic cells, 17.31%) and 33.65% (early apoptotic cells, 9.84%; late apoptotic cells, 23.81%), respectively (Fig. 3A). Flow cytometry was used to examine the effects of α-mangostin on the YD-15 cell cycle. The percentage of sub-G1 cells in the control group was 0.28% (Fig. 3B). The number of cells in the sub-G1 phase tended to increase in a concentration-dependent manner (10 µM, 1.38% and 15 µM, 6.08%; Fig. 3B). These results suggest that the inhibitory effects of α-mangostin on cell viability are caused by sub-G1 arrest related to apoptosis.

**Effects of α-mangostin on the expression of Bcl-2 family proteins.** The expression of Bcl-2 family proteins, responsible for controlling apoptosis, was determined by western blot analysis in order to elucidate the mechanisms responsible for the apoptosis induced by α-mangostin in YD-15 cells. The α-mangostin-treated group exhibited an increased expression of the pro-apoptotic factor, Bax, and a decreased expression of the anti-apoptotic factor, Bcl-2, in a concentration-dependent manner (Fig. 4).
Figure 3. Effects of α-mangostin on apoptosis of YD-15 cells. YD-15 cells were treated with α-mangostin (0, 10 and 15 µM) in complete medium for 24 h. (A) Annexin V/PI staining was performed to detect apoptosis. The lower right quadrant of the fluorescence activated cell sorting (FACS) histogram indicates the percentage of early apoptotic cells (Annexin V-stained cells), while the upper right quadrant indicates the percentage of late apoptotic cells (Annexin V+/PI-stained cells). (B) The sub-G1 fraction was assessed by PI staining and flow cytometry. Representative staining profiles for 10,000 cells per experiment are shown.

Figure 4. α-mangostin alters the levels of Bax and Bcl-2 in YD-15 cells. YD-15 cells were treated with α-mangostin (0, 10 and 15 µM) for 24 h. Cell lysates were prepared as described in the Materials and methods and analyzed by 12% SDS-PAGE followed by western blot analysis. (A) Membranes incubated with the anti-Bax antibody. (B) Membranes incubated with the anti-Bcl-2 antibody. Blots were also probed with the anti-β-actin antibody to confirm equal sample loading. Data analysis was performed using ImageJ software by measuring the integrated band densities following background subtraction. Each bar represents the mean ± SD calculated from 3 independent experiments. Significance was determined by Dunnett's t-test. *P<0.05, significant difference compared to the untreated controls.
These results imply that the apoptotic mechanism induced by α-mangostin is associated with Bcl-2 family proteins.

Effects of α-mangostin on caspase activation. The expression levels of caspase-3, caspase-9 and PARP were measured by western blot analysis in order to determine the association between the induction of apoptosis by α-mangostin and caspase activation in YD-15 cells. The cells treated with α-mangostin exhibited increased caspase-3 and -9 activity in a concentration-dependent manner (Fig. 5A). The level of cleaved-PARP increased as well. Cleaved caspase-3 was visualized by immunohistochemical analysis in order to examine the effects of α-mangostin on caspase expression in tumor tissues collected from mice with tumor xenografts (Fig. 5B). The tumor tissue of the mice treated with α-mangostin exhibited decreased levels of p-ERK1/2 and p-p38 in a concentration-dependent manner.

Effects of α-mangostin on ERK1/2 MAPK and p38 MAPK expression. The levels of ERK1/2, p38 and c-myc were examined by western blot analysis in order to determine whether the induction of apoptosis by α-mangostin involves the MAPK pathway. The inhibition of ERK1/2 and p38 activation, and decreased c-myc expression were observed in the cells treated with α-mangostin in a concentration-dependent manner (Fig. 6A). The levels of p-ERK1/2 and p-p38 were determined by immunohistochemical analysis to determine whether the effects of α-mangostin involve the MAPK pathway in tumor tissue from mice with tumor xenografts (Fig. 6B). The tumor tissue of the mice treated with α-mangostin exhibited decreased levels of p-ERK1/2 and p-p38 in a concentration-dependent manner.

Effects of α-mangostin on mouse tumor xenografts. The effects of α-mangostin on tumor volume in mice with YD-15 tumor xenografts were investigated. α-mangostin was administered by intraperitoneal injection at the dose of 10 mg/kg (low-dose) and 20 mg/kg (high-dose). The mice in the control group received the vehicle (0.5% DMSO in PBS) with the same timing and dosing schedule used for the treatment group. A significant difference in tumor volume emerged in the control group from day 8 onwards following treatment. The mice treated with 20 mg/kg α-mangostin exhibited a tumor volume inhibition rate of 89.9% compared with the control group (Fig. 7A). The sizes of the final tumors in the control and α-mangostin-treated (10 and 20 mg/kg body weight) mice were 639, 105 and 43 mm^3, respectively. Tumor weights in the experimental nude mice were also deter-
mined to be 0.25, 0.08 and 0.05 g in the control, and 10 and 20 mg/kg α-mangostin-treated groups, respectively, indicating a decreasing trend in tumor weight upon the administration of α-mangostin (Fig. 7B). A TUNEL assay was performed on the extracted tumor tissue to examine cell apoptosis. As a result, many apoptotic cells were confirmed in the mice treated with α-mangostin compared with the control group (Fig. 7C).

Immunohistochemical analysis was also performed to examine the expression of Ki-67, the protein responsible for the rate of tumor growth (Fig. 8). The tumor samples from the mice treated with α-mangostin exhibited a decreased Ki-67 expression in a concentration-dependent manner, suggesting that α-mangostin induced the apoptosis of YD-15 cells (in the tumor xenografts), thus inhibiting tumor growth.

**Discussion**

Surgical therapies, radiation therapies, medications and other current treatments for oral cancer are associated with a low treatment efficacy and severe adverse effects; thus, in general, oral cancer has a poor prognosis. Thus, it is important to develop a treatment strategy that selectively destroys cancer cells without inducing any toxic effects. In some studies, it was reported that several naturally derived substances induce apoptosis and inhibit cancer cell growth (21-24). α-mangostin, a naturally-derived substance, is extracted from the pericarp of the mangosteen fruit. α-mangostin was initially reported to be a substance that induces the apoptosis of cancer cells, particularly that of mammary, colorectal and liver cancer cells (16,18,19); however, the mechanisms responsible for the apoptosis induced by α-mangostin in oral cancer cells remain unknown.

In this study, an MTT assay was performed to confirm the inhibitory effects of α-mangostin on cell viability (Fig. 2A). The YD-15 cells treated with α-mangostin exhibited a concentration-dependent decrease in viability; at a concentration of 15 µM α-mangostin, there was an approximate inhibition rate of 58%. According to a previous study by Shibata et al (19), proliferation was significantly decreased in BJMC3879luc2 cells treated with 12 µM α-mangostin for 24 and 48 h. In another study by Hsieh et al (16), an inhibition rate of 50% was observed in
SK-Hep-1 cells treated with 24.8 μM α-mangostin for 24 h. The cells treated with 19.6 μM α-mangostin for 48 h also exhibited an inhibition rate of 50% (16). The findings of these other studies were similar to those from our study, in which α-mangostin decreased the viability of YD-15 tongue cancer cells in a concentration-dependent manner; therefore, α-mangostin is believed to effectively inhibit cancer cell proliferation.

Apoptosis, otherwise known as programmed cell death, is characterized by a number of well-defined features, such as condensation and fragmentation of chromatin, internucleosomal DNA cleavage, caspase activation and the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane (25). In the present study, these morphological changes were further examined by DAPI staining, in order to examine the inhibitory effects of α-mangostin on YD-15 cell viability. Based on DAPI staining, the number of apoptotic bodies in the cells treated with α-mangostin increased in a concentration-dependent manner compared to the control group (Fig. 2B). Thus, the distinct characteristics of apoptosis (i.e., shrinking of the cytoplasm, chromosomal condensation and apoptotic body formation) were observed.

The apoptotic cells were analyzed quantitatively by flow cytometry to determine whether the morphological changes observed in the DAPI-stained chromosomes were caused by apoptosis. In addition, the cell cycle was analyzed to examine the inhibitory effects of α-mangostin on cell cycle progression.
The apoptotic cells were first analyzed by Annexin V FITC/PI staining. The percentage of apoptotic cells was 16.06% in the control group and increased to 28.1 and 33.65% in the groups treated with 10 and 15 µM α-mangostin, respectively (Fig. 3A). Li et al (26) previously examined apoptotic MCF-7 and MDA-MB-231 cells treated with 0, 1, 2 and 4 µM α-mangostin for 24 h; they detected 4.19, 5.42, 8.89 and 27.96% apoptotic cells among the MCF-7 cells and 6.69, 7.97, 11.42 and 42.34% among the MDA-MB-231 cells, respectively. Moreover, the observed increases were concentration-dependent. Based on the findings of previous studies and those of our study, we concluded that α-mangostin induced cancer cell apoptosis in a concentration-dependent manner.

From the perspective of cell proliferation, cancer cells can be defined as being resistant to cell cycle control (27). Thus, as regards the development of particular anticancer drugs and preventive medications, the extent that a substance affects cancer cell cycle progression should be identified. In general, if apoptosis is induced in vitro and in vivo, an increase in the sub-G1 population is observed along with DNA fragmentation (28,29). In other words, an increase in the proportion of sub-G1 cells reflects an increase in apoptosis. In the present study, flow cytometry was used to analyze the cell cycle to examine the inhibitory effects of α-mangostin on different cell cycle phases. No significant differences were observed in the distribution of G1, S or G2/M cells, whereas the sub-G1 cell populations in the control group and groups treated with 10 and 15 µM α-mangostin were 0.28, 1.38 and 6.08%, respectively (Fig. 3B). Based on these results, we concluded that the inhibitory effects of α-mangostin on cell proliferation, particularly on the sub-G1 cell cycle arrest, were induced by apoptosis.

Apoptosis occurs via an organic reaction of various proteins controlled by internal/external cellular pathways. Bcl-2 family proteins control membrane permeability and are located in the mitochondrial membrane or move to the mitochondrial membrane to induce apoptotic cell death (30). Bax and Bad are pro-apoptotic factors that promote apoptosis, whereas Bcl-2 is an anti-apoptotic factor (31,32). In this study, the expression levels of Bax and Bcl-2 were measured by western blot analysis, which revealed that the expression of the pro-apoptotic factor, Bax, was increased and the expression of Bcl-2 was decreased in the α-mangostin-treated cells in a concentration-dependent manner (Fig. 4). This result implied that α-mangostin increased Bax expression in the YD-15 cells and decreased Bcl-2, ultimately leading to apoptosis.

Caspases are key factors that control apoptosis and are involved in a common pathway of various apoptotic signals. Caspases are further classified into initiator and effector caspases. Initiator caspases are activated by death signals to further activate the effector caspases (33). Caspase-3, which is activated by caspase-9, can cleave proteins involved in damaged DNA recovery or PARP. Caspase activation and PARP cleavage are typical characteristics of apoptosis (34). In this study, the levels of caspase-9 (initiator caspase), caspase-3 (effector caspase) and PARP were measured by western blot analysis in order to confirm the effects of α-mangostin on caspase activity in YD-15 cells. Both caspase-3 and -9 were activated, as evidenced by the increased levels of the respective cleaved forms, as well as PARP segmentation (Fig. 5A). The results of immunohistochemical analysis of cleaved caspase-3 were similar to those of western blot analysis (Fig. 5B). Considering these results, it appears that caspase plays a significant role in the apoptosis induced by α-mangostin in YD-15 tongue carcinoma cells, and the activation of caspase-9 and caspase-3 leads to PARP segmentation.

The MAPK signaling pathway is a core factor controlling various pathways, including cell growth, proliferation, segmentation and apoptosis. One of the key players in the MAPK pathway, ERK1/2, is activated by growth factors, such as those that promote apoptosis and further control cell growth, survival and division. Another key component of the MAPK pathway, p38 MAPK, is activated by chemical and environmental stresses and inflammatory factors that affect cellular levels (35). ERK1/2 and p38 activation through various pathways results in their translocation to the nucleus, where they function as transcription factors for early response proteins, such as c-myc and c-jun (36). Among the genes activated by the ERK1/2 signaling pathway, c-myc plays a major role in tumorigenesis (37). The c-myc protein level is strictly regulated by ERK1/2 through post-translational mechanisms (38). In this study, the levels of ERK1/2, p38 and c-myc were investigated by western blot analysis in order to verify the involvement of the ERK1/2 and p38 MAPK pathways in the apoptosis induced by α-mangostin. We determined that both ERK1/2 and p38 were deactivated via reduced phosphorylation. The expression of c-myc also decreased. Based on immunohistochemical analysis, the levels of p-ERK1/2 and p-p38 were found to decrease in a concentration-dependent manner (Fig. 6). According to a previous study, no significant differences were observed with respect to p-ERK1/2 and p-JNK1/2 activation in SK-Hep-1 cells treated with 10, 20 and 30 µM α-mangostin for 24 h, compared with the control group, whereas p-p38 decreased in a concentration-dependent manner (16). These results confirmed that the inhibition of p38 MAPK played a crucial role in apoptosis induced by α-mangostin in cancer cells (16). Moreover, in another study, no significant changes were observed in SW1353 cells treated with 20 µg/ml α-mangostin for 0, 3 and 6 h with respect to p-p38 activation, whereas p-ERK1/2 tended to increase after 3 h and decrease after 6 h, along with p-JNK. Thus, the inhibition of p-ERK1/2 was associated with the apoptosis induced by α-mangostin (39). Taking into consideration the findings of previous studies, as well as those from our study, we hypothesized that α-mangostin inhibited the activation of ERK1/2 and p38 MAPK signaling pathways, which further inhibited the expression of the c-myc oncogene.

In this study, YD-15 cells were also administered to nude mice to confirm the in vivo anticancer efficacy of α-mangostin. Mice were divided into 3 groups: the control (vehicle-treated) and the 10 and 20 mg/kg α-mangostin treatment groups. α-mangostin was administered intraperitoneally 5 times/week. A marked difference between the treated and control groups was observed beginning on day 8 following treatment (Fig. 7A). On day 22, tumors in the 20 mg/kg α-mangostin treatment group exhibited an inhibition rate of 89.9%. A TUNEL assay was performed on tumors extracted from the experimental nude mice, which revealed a significant increase in the expression of TUNEL-positive cells in the α-mangostin-treated group (Fig. 7C).

A Ki-67 antibody was used to differentiate nuclei in proliferating cells (G1, S, G2 and M phases) from those in resting cells, as previously described (40). Immunohistochemical analysis
confirmed the expression of Ki-67, a protein used to determine the rate of proliferation of cancer cells, and demonstrated that its expression was decreased in the α-mangostin-treated mice (Fig. 8). According to these results, α-mangostin induced the apoptosis of YD-15 cells and inhibited cell proliferation.

In conclusion, in the present study, we demonstrated that treatment with α-mangostin leads to cellular apoptosis through the inhibition of ERK1/2 and p38 MAPK signaling in YD-15 tongue carcinoma cells, indicating the potential use of α-mangostin as an anticancer treatment, particularly for the treatment of oral cancer.

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