Fomitoside-K from *Fomitopsis nigra* Induces Apoptosis of Human Oral Squamous Cell Carcinomas (YD-10B) via Mitochondrial Signaling Pathway

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In this study, a new lanostane triterpene glycoside (fomitoside-K) having biologically active molecules was isolated from a mushroom *Fomitopsis nigra* to test its anticancer activity on human oral squamous cell carcinomas (YD-10B). We focused on the effect of fomitoside-K on apoptosis, the mitochondria-mediated death pathway and the accumulation of reactive oxygen species (ROS) in YD-10B cells. Fomitoside-K could induce a dose and time-dependent apoptosis in YD-10B cells as characterized by cell morphology, cell cycle arrest, inhibition of survival, activation of poly(ADP-ribose) polymerase (PARP), caspase-3, -9 and an increased expression ratio of Bax/Bcl-2. The mitochondria membrane potential loss and cytochrome c (Cyt C) release from mitochondria to cytosol were observed during the induction. Moreover, fomitoside-K caused dose-dependent elevation of intracellular ROS level and increase phosphorylation of c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) in YD-10B cells. To further investigate the mechanisms, we examined the effects of ROS scavenger N-acetyl-l-cysteine (NAC) and selective inhibitors for mitogen activated protein kinase (MAPK) pathways on the cell death. The fomitoside-K induced cell death by ROS was significantly inhibited by NAC, ERK (PD98059) and JNK inhibitor (SP600125). In addition, fomitoside-K has a synergistic effect with Adriamycin in suppressing the growth of YD-10B cells. These data suggest that fomitoside-K induces apoptosis in YD-10B cells through the ROS-dependent mitochondrial dysfunction pathway and provides a mechanistic framework for further exploring the use of fomitoside-K against the proliferation of human oral cancer.

**Key words** fomitoside-K; anti-cancer drug; apoptosis; cytochrome c; oral squamous cell carcinoma; reactive oxygen species

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, and accounts for approximately 4% of all carcinomas in men and 2% in women worldwide, with geographical variation in frequency. In recent years, the development of new anticancer drugs is a key issue for cancer chemotherapy, because of the reality that cancer cells, which are resistant to current chemotherapy, will eventually dominate the cell population and cause mortality. Furthermore, traditional or folk herbal medicine as alternative cancer therapy has recently attracted a great deal of attention due to its low toxicity and cost. Mushroom has been used as a traditional medicinal treatment in China, Korea, Japan and other Asian countries for the treatment of various diseases, including oral ulcer, gastroenteric disorder, lymphatic disease and various cancers. It was reported that mushroom has the effect of being antioxidant, anti-inflammatory and inhibiting tumor growth and metastasis. Mushrooms produce a large variety of secondary metabolites with unique chemical structures and interesting biological activities. Recently, a new compound fomitoside-K from the fruiting body of *Fomitopsis nigra* has been isolated. *Fomitopsis* belonging to Polyporaceae, is known to produce various triterpenoids and triterpene glycosides. A new lanostane triterpene glycoside, designated as fomitoside-K, has been isolated from the methanolic extract of the fruiting body of *Fomitopsis nigra*. However, the mechanism underlying this new compound extracted from mushroom in oral cancer effect is not clear.

Apoptosis, also known as programmed cell death, is characterized by typical cellular morphology and biochemical features such as cell shrinkage, cytoplasm vacuolization, chromatin condensation, DNA fragmentation, and finally the cellular breakdown into apoptotic body. It has been shown that mitochondria play essential roles in triggering apoptosis through the mitochondrial permeability transition. The mitochondrial permeability transition leads to a loss of mitochondrial membrane potential and produces translocation of pro-apoptotic Bax to mitochondria and cytochrome c (Cyt C) from mitochondria to cytosol resulting in the activation of caspase cascades. Furthermore, recent studies have proposed that cellular reactive oxygen species (ROS) may mediate the mitochondria-initiated apoptosis. Many anti-cancer drugs and chemo preventive agents can elevate cellular ROS production, which target mitochondria and eventually lead to the activation of apoptosis.

Although there are many therapeutic strategies to treat cancer, including chemotherapy, high systemic cytotoxic toxicity and drug resistance have limited the successful outcomes in most cases. In addition, the combination treatment of anti-cancer drugs and natural products dramatically augments the therapeutic effects against cancer. It has been reported that some mushroom extraction activated apoptosis through ROS generation. Therefore, mushroom with strong apoptosis...
inducing activity would be expected to have potential utility as anti-cancer drugs. We found that the mushroom extract of fomitoside-K has significant growth inhibition against human oral cancer cell YD-10B cells. For exploring the mechanism of the anti-oral cancer effect for this mushroom, experiments demonstrated that fomitoside-K could induce human YD-10B cell apoptosis accompanied by the collapse of mitochondrial membrane potential, the release of Cyt C, and the activation of Caspase-9 and -3. Further, fomitoside-K has the ability to synergize the inhibitory action of adriamycin in YD-10B cell lines. We herewith hypothesized that fomitoside-K is able to induce apoptosis via mitochondrial membrane potential loss, thereby releasing Cyt C into the cytosol and increasing the level of ROS in YD-10B cells. This is the first report showing that fomitoside-K induces cytotoxicity in YD-10B which involves apoptosis induction and production of ROS.

MATERIALS AND METHODS

Cell Lines and Cell Culture  YD-10B and KB (OSCC) cells were grown in RPMI 1640, HELA (human cervical cancer cells) were grown in minimal essential medium (MEM), CT26 (murine colon adenocarcinoma cell), MCF-7 (human breast cancer cells), U2OS (human osteosarcoma), HDF (human diploid fibroblast) and HGF (human gingival fibroblast) were grown in Dulbecco's modified Eagle's medium (DMEM) and MC3T3-E1 (mouse osteoblast) cells were grown in α-MEM (Gibco BRL Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with heat inactivated 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, U.S.A.), 300 μg/mL glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate (Gibco BRL Life Technologies). The cells were maintained in a humidified atmosphere of 37°C and 5% CO₂.

Isolation and Purification of Fomitoside-K  Isolation and purification of fomitoside-K was evaluated from our previous publication. Briefly, fomitoside-K was obtained from the fruiting bodies of Fomitopsis nigra through consecutive solvent partitioning, silica gel and Sephadex LH-20 column chromatographies and preparative reversed-phase HPLC. Its chemical structure was determined as a new lanostane triterpenoid. The formazan crystals in each well were dissolved in MTT stock solution (5 mg/mL in serum-free medium) were added to each well to reach a total reaction volume of 200 μL.

Western Blot Analysis  Western blot analysis was performed as previously described. The samples were mixed with the sample buffer, and separated by 10–15% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis under denaturing conditions, followed by electro blotting onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in a Tris-buffered saline-Tween 20 (TBS-T) buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, and incubated with each of the following antibodies: Bax (sc-493), Bcl-2 (sc-7382), Cyt C (sc-7159), Caspase-3 (sc-7148) from Santa Cruz (Santa Cruz, CA, U.S.A.) and Survivin (#2808), caspase-9 (#9508), a phospho-type ERK (#4377), JNK (#9251), and p38 (#9215) from Cell Signaling (Danvers, MA, U.S.A.). The membranes were washed with phosphate-buffered saline (PBS) and incubated with horseradish peroxidase conjugated secondary antibody. The signals were visualized by chemiluminescent detection according to the manufacturer’s protocol (Amersham Pharmacia Biotech, London, U.K.). The membrane was reprobed with the anti-actin antibody to verify an equal protein loading.

In Vitro Cytotoxic Assay  The cytotoxicity of fomitoside-K and adriamycin on the viability of YD-10B cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which relies on the ability of viable cells to metabolically reduce the tetrazolium salt MTT to a purple formazan product, which can be quantified colorimetrically. Briefly, equal amounts of cell suspensions were seeded in 12-well plates and incubated at 37°C to allow for cell attachment. At the end of each exposure time, 150 μL of the MTT stock solution (5 mg/mL) in serum-free medium were added to each well to reach a total reaction volume of 200 μL. After incubation for 2h at 37°C, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL dimethylsulfoxide, and the absorbance was read on a scanning multi well spectrophotometer. The relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

Nuclear Morphology Assessment  YD-10B cells were subcultured for 24 h on coverslip in six-well culture plates. After treatment with 15 μM fomitoside-K for 24 h, the nuclear morphology changes were observed. Cells were washed with ice-cold PBS, and then fixed with 2% formaldehyde and 0.2% glutaraldehyde for 30 min. The nuclei were stained with Hoechst33258 (0.1 mg/mL in PBS). After washing with PBS, cells were mounted under glass cover slips with Vectashield (Brsuschwig, Amsterdam, The Netherlands). Photographs were visualized by fluorescence microscopy (Olympus [I/IX71-21PH] Tokyo, Japan).

Preparation of Cytosolic and Mitochondrial Fractions  The YD-10B cells (5×10⁴) were seeded on a 100 mm dish and cultured for 24 h. The cells were treated with fomitoside-K for the indicated times, and the cytosolic and mitochondrial fractions were then obtained according to our previous report. Briefly, the cells were harvested and washed with ice-cold PBS and then resuspended in a hypertonic buffer (20 mM N- (2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), pH=7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of pepstatin A, 10 μg/mL of aprotenin and 10 μg/mL leupeptin) on ice for 30 min. The cells were disrupted by 20 passages through a 26-G needle and then centrifuged at 10000×g for 20 min at 4°C. The cytosolic supernatant was removed, and the pellet containing the mitochondria was resuspended in a whole-cell lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tri-HCl (pH=8.0), 1%–NP 40, 1 mM apotinin, 0.1 mM leupeptin and 1 mM pepstatin) on ice for 30 min. After centrifugation at 12000×g for 20 min at 4°C, the supernatant was collected as the mitochondrial fraction.

FACScan Analysis for Cell Cycle  The YD-10B cells in 10% FBS-RPMI medium were seeded at a density of 5×10⁴/plate. Twenty-four hours after plating, the cells were treated with fomitoside-K, as described above, for the indicated times. The cells were trypsinized and fixed in cold 70% ethanol for at least 30 min on ice, washed in PBS and stained with propidium iodide (6 μg/mL) and RNase A (15 μg/mL) for at least 30 min. The samples were analyzed using an EPIC profile analyzer.

Measurement of Intracellular ROS by Flow Cytometry  Production of intracellular ROS was detected by flow cytometry using the oxidation sensitive fluorescent probe,
2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma, St. Louis, MO, U.S.A.). YD-10B cells were cultured in 60-mm cell-culture dishes for 24 h. This culture medium was replaced with a new medium and then exposed to indicate concentration of fomitoside-K. After treatment, the cells were treated with 10 μM DCFH-DA for 30 min in the dark, washed once with PBS, detached by trypsinization, collected by centrifugation, and suspended in PBS. The intracellular ROS, as indicated by the fluorescence of dichlorodihydrofluorescein (DCF), were measured with a Becton-Dickinson FACScan flow cytometry (Becton-Dickinson, U.S.A.). Results were analyzed by the WinMDI 2.9 program.

**Inhibitor Treatment** To investigate the effects of reactive oxygen species scavenger and mitogen activated protein kinase (MAPK) inhibitors on fomitoside-K induced apoptosis and anti-proliferative viability in YD-10B cells, confluent cells were preincubated for 1 h with one of the following inhibitors before the addition of fomitoside-K: 1 mM NAC, 20 μM SP600125: a JNK-specific inhibitor and 20 μM, PD98059: a ERK-specific inhibitor.

**Statistical Analysis** For every events, triplicate experiments were performed, each of which yielded comparable results. Significance was assessed by analysis of variance (ANOVA). All pair-wise comparisons were performed by the post hoc Scheffé test using ORIGIN 7.0. A p-value <0.05 was considered to indicate statistical significance.

**RESULTS**

**Structure of Fomitoside-K and Effects on Cell Proliferation and Morphological Changes** Figure 1A shows the structure of fomitoside-K containing the compound new la nostane triterpene glycoside. The structure of this compound was determined as mentioned in Materials and Methods sections. At first we analyzed the anti-proliferative effects of fomitoside-K in various cancer cells and also in normal cells by MTT assay. Fomitoside-K at a dose of 15 μM for 24 h was found to be cytotoxic towards KB, CT26, HELA, MCF-7 and U2OS cells. After treating fomitoside-K for 24 h, YD-10B possessed the most pronounced anti-proliferation activity. However, it has less anti-proliferative effects in normal cells like HGF, HDF and MC3T3-E1 compare to cancer cells (Fig. 1B). Further, as shown in Fig. 1C, fomitoside-K inhibited the proliferation of YD-10B cell in a dose- and time-dependent manner. Inhibition of proliferation was observed as early as 24 h when cells were treated with fomitoside-K at a concentration of 10 μM and the strongest inhibition was detected when cells were treated with fomitoside-K at a concentration higher than 20 μM for 48 h. About 15 μM of fomitoside-K inhibited 50% of cell growth after 24 h, and for all subsequent experiments 15 μM was used.

**Effects of Fomitoside-K on Cell Morphological Changes and Inducing Apoptosis in YD-10B by Altering the Cell Cycle** To further examine the morphological changes in responding to the fomitoside-K treatment, both control and
fomitoside-K treated cells were stained with the fluorescent dye Hoechst 33342 and visualized by a fluorescent microscope. The control cells showed normal, intact cellular morphology, and the nuclei were round and homogeneous (Fig. 2A), while the cells treated with fomitoside-K that were undergoing apoptosis, after 24 h exhibited the typical characteristics of apoptosis, such as membrane blebbing and/or bubble formation, apoptotic bodies, nuclear condensation and fragmentation (Fig. 2B). These are the characteristics that were seen in cells dying via apoptosis. We therefore selected a concentration of 15 $\mu$M for the next experiment because obvious DNA damages could be observed at this concentration.

To examine whether fomitoside-K induced apoptosis by altering the cell cycle, the number of cells accumulated in the sub-G1 phase of the cell cycle was counted by flow cytometric analysis. As shown in Figs. 2C and D, the sub-G1 population of fomitoside-K treated cells accumulated compared to the vehicle control. YD-10B cells were treated with or without various concentrations of fomitoside-K for 24 h before the cell cycle and apoptosis were examined and analyzed by flow cytometric methods. The results demonstrated that 24 h treatment of fomitoside-K induced a G0/G1 phase arrest.

Release of Cyt C Induced by Fomitoside-K Regulate the Intrinsic Apoptotic Pathway Cyt C is localized in the intermembrane space and loosely attached to the surface of the inner mitochondrial membrane. Release of Cyt C from the intermembrane spaces of the mitochondria into the cytosol is a key event in apoptosis. To examine this step in the apoptotic cell death pathway initiated by fomitoside-K, we measured the Cyt C content in cytosol and the mitochondrial of YD-10B cells treated with fomitoside-K through 0–36 h. As shown in Fig. 3A, progressive accumulation of Cyt C in cytosolic compartment was observed but decreased in mitochondrial Cyt C. The release of Cyt C was initiated as early as 6 h of fomitoside-K treatment. The release of Cyt C suggests that it might be important in the execution of fomitoside-K induced apoptosis.

In addition, total cell lysates were analyzed to detect the expression of caspase-9, caspase-3 and poly(ADP-ribose) polymerase (PARP) by Western blotting. As shown in Fig. 3B, cleaved/activated caspase-9, caspase-3 and PARP were detected in the fomitoside-K treated cells. These results indicate that treatment with fomitoside-K resulted in activation of the intrinsic mitochondrial apoptotic pathway in YD-10B cells.

The release of Cyt C from mitochondria is tightly regulated by a variety of factors. Among them, Bcl-2 family proteins, including anti-apoptotic members (such as Bcl-2) and pro-apoptotic members (such as Bax) play a pivotal role. To further elucidate the possible mechanism underlying the fomitoside-K-induced apoptosis, we examined the expression of Bcl-2 and Bax in YD-10B cells after fomitoside-K treatment. As shown in Fig. 3C, exposure of YD-10B cells to...
µM fomitoside-K led to an obvious decrease of Bcl-2 and survivin protein expression, a known survival molecule, but a drastic increase of Bax protein expression in a time-dependent manner.

**Fomitoside-K Induced ROS in YD-10B Cells by Activating Extracellular Signal Regulated Protein Kinase (ERK) and c-Jun N-Terminal Kinase (JNK)**

Many drugs induce apoptosis in cancer cells partially by increasing intracellular ROS levels and it is known to be an important element in the induction of apoptosis; it would therefore be interesting to determine whether fomitoside-K stimulates ROS generation in YD-10B cells. We evaluated the stimulation of the production of intracellular ROS by fomitoside-K in the YD-10B cells. As shown in Fig. 4A, fomitoside-K caused a dose dependent increase in intracellular ROS production after 24 h treatment as detected by DCF fluorescence.

As it has been shown, the ROS mediated apoptosis triggers activation of MAPK pathway and subsequent cell death. We examined the status of ERK, JNK and p-38 phosphorylation after fomitoside-K treatment. As shown in Fig. 4B, phosphorylation of ERK and JNK were significantly induced in a time dependent manner but p-38 was not. Furthermore, studies have shown that the MAPK signaling pathway plays an important role in the action of chemotherapeutic drugs. Therefore, we determined whether the MAPKs were activated in the YD-10B cells by Western blot analysis using specific antibodies against the phosphorylated (activated) forms of the kinases. It was found that fomitoside-K treatment induced differential phosphorylation of ERK and JNK.

To determine whether ROS and MAPK is involved in apoptosis induced by fomitoside-K, PD98059, SP600125 and NAC, a general free radical scavenger, were used to block ROS generation. As shown in Fig. 4C, ROS generation induced by 15 µM fomitoside-K was almost completely inhibited by MAPK and ROS inhibitor. In addition, PD98059, SP600125 and NAC, attenuated fomitoside-K induced apoptosis as observed by MTT assay (Fig. 4D). These results indicate that ROS generation may mediate the observed effects of fomitoside-K on MAPKs activation as well as apoptosis in YD-10B cells.

**Fomitoside-K with Adriamycin at a Low Dose, Synergized to Induce Apoptosis in YD-10B Cells by Inhibiting Cell Proliferation and Increase of ROS**

To investigate the synergies effect of fomitoside-K on YD-10B cells, we tested the effect of fomitoside-K or adriamycin treatment on human YD-10B. YD-10B cells were either left untreated, or treated with fomitoside-K or low doses of adriamycin for 24 and 48 h. After treatment alone, a moderate growth inhibition effect was observed. Low doses of adriamycin (500 ng/mL) had a minor apoptotic effect on the cells. Combined treatment of adriamycin with fomitoside-K caused more than 80% of the cells to undergo apoptosis (Fig. 5A). Since low doses of adriamycin had a low rate in order to induce apoptosis, we tested the effect of the combination treatment of these two compounds, in the low dose range, on YD-10B cells that have demonstrated resistance to adriamycin treatment. Interestingly, the combination of low doses of 500 ng/mL of adriamycin had a significantly greater inhibitory effect than that of fomitoside-K or adriamycin alone. Moreover, the growth-inhibitory effect of the combination treatment on YD-10B cells was found to be time-dependent, because the inhibitory effect became gradually stronger with the passage of time after treatment, the most significant effect was observed at 48 h. Further, the ROS increment level of adriamycin in single treatment is low. However, in combined treatment with fomitoside-K, the level of ROS is dramatically increased (Fig. 5B). This shows that fomitoside-K has a capacity to inhibit cancer cell proliferation and its high action trigger when combined with anti-cancer drug.
Fig. 4. (A) Flow Cytometric Analysis of Reactive Oxygen Species (ROS) in YD-10B Cells with Fomitoside-K for 24 h Time Periods; (B) Fomitoside-K Affects the Levels of Phosphorylated Mitogen-Activated Protein Kinase; (C, D) Effects of PD98059, SP600125 and NAC on Fomitoside-K Induced ROS Generation and Growth Inhibition

(A) The cells were treated with 10, 15 and 20 µM of fomitoside-K for 24 h and the changes of ROS were detected. The zero concentration was defined as the control without the fomitoside-K treatment. The X-axis shows the number of cells and Y-axis shows the fluorescence intensity. The cells that were stained by DCFH-DA dye were determined by flowcytometry as described in Materials and Methods. (B) YD-10B cells were incubated with 15 µM of fomitoside-K for the indicated time and the levels of phospho-ERK, -JNK and -p38 were determined by Western blotting with phospho-specific antibodies. Anti actin was used as a loading control. The total ERK1/2, JNK1/2 and p38 levels were used as the internal controls in the subsequent experiment. (C, D) Cells were pretreated with an inhibitor at 1 h prior to the treatment with 15 µM fomitoside-K for 24 h (total inhibitor exposure time was 25 h). Generation of ROS and Cell viability was determined by flow cytometry and MTT assay, respectively as mentioned above. Bars with different characters are statistically different at the p<0.05 level.

Fig. 5. Synergistic Cell Growth Inhibition and Apoptosis in YD-10B Cells Treated with Fomitoside-K in Combination with Adriamycin

(A) The cells viability incubated with adriamycin and combination with fomitoside-K for 24 and 48 h was measured by the MTT reduction assay, as described in Materials and Methods. (B) Combined treatment of fomitoside-K and adriamycin increase the ROS in YD-10B. An equal number of cells were seeded, and after 24 h, adriamycin combined with fomitoside-K was treated for a further 24h. The cellular ROS production by YD-10B was measured and analyzed as mentioned in Materials and Methods.
DISCUSSION

Oral cancer is one of the most risky cancers. Prevention and early treatment of oral cancer could significantly improve the quality of life for individuals at risk. It has been demonstrated that many mushroom extraction products have been found to possess anticancer activity. Therefore, searching for new anticancer and other medicinal substances from mushrooms and studying the medicinal value of these mushrooms has become a matter of great significance. In this work, new lanostane triterpene glycoside (Fomitoside-K), an active component of a medicinal mushroom, was extracted from the fruiting bodies of Fomitopsis nigra. It has a significant growth inhibition and could induce apoptosis in YD-10B cells.

In this study, we found that fomitoside-K inhibited cell growth of various cancer cell lines including KB, CT26, HELO, MCF-7, and U2OS. High cytotoxicity was shown in YD-10B cells and less toxicity in normal cells (Figs. 1B, C). Further, the induction of apoptosis by fomitoside-K in YD-10B cells was confirmed by several biochemical markers such as a loss in the level of mitochondrial function, induction of caspase-3, -9 activity and morphological examination of the cells. Apoptosis, characterized by morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation and nuclear fragmentation with the formation of apoptotic bodies, is a form of programmed cell death that occurs naturally in cells and can be beneficial to cancer therapy as previously studied. To explain whether cytotoxicity induced by the treatment with fomitoside-K was a result of the apoptotic or necrotic effect, we performed a morphological analysis. It was found that fomitoside-K induced cell shrinkage and chromatin condensation (Fig. 2B). Therefore, the morphological observations of fomitoside-K induced apoptosis in YD-10B cells have been confirmed. Cellular morphology of YD-10B cells progressively changed with the increasing duration of fomitoside-K exposure. Prolonged drug incubation resulted in the appearance of disintegrated cells as evidenced by apoptotic bodies and cells with condensed nuclear chromatin. These morphological observations together with the appearance of sub-G1 populations (Fig. 2D) confirmed the presence of a considerable amount of apoptosis in YD-10B cells. Inhibition of deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth. Our results demonstrate that treatment of YD-10B cells with fomitoside-K induces the G1 phase arrest of cell cycle progression indicating that one of the mechanisms by which fomitoside K inhibits the proliferation of YD-10B cells is the alteration of cell cycle progression.

Mitochondria have been reported to play a critical role in the regulation of apoptosis. The loss of apoptogenic factors, such as Cyt C from mitochondria into the cytosol is associated with apoptosis induced by chemotherapeutic drugs. In the present study, the rapid loss of mitochondrial and release of Cyt C were both observed in a time-dependent manner in fomitoside-K treated YD-10B cells (Fig. 3A). At the execution phase of apoptosis, Caspase-3 and -9 are believed to be the most commonly involved in the various cell types. Caspase-3 is a member of the cysteine–aspartic acid protease (caspase) family, and plays a central role in inducing apoptotic phenomena such as plasmatic alteration, chromatin condensation, DNA fragmentation and apoptotic body formation. Caspase-9 induces caspase-3 activation through the formation of an apoptosome complex with Cyt C released from the mitochondria. Our data showed that the activity of caspase-3 and -9 was increased in a time-dependent pattern after fomitoside-K treatment. Further, the results showed that fomitoside-K is effective to activate PARP leading to apoptosis. These findings suggested that Caspases activation through the release of Cyt C from mitochondria, participates in the induction of apoptosis in fomitoside-K treated YD-10B cells. Further, the majority of apoptotic signaling pathways is involved in the mitochondria and induction of the disruption in mitochondrial via the changes in the ratio of Bax/Bcl-2. Pro-apoptotic Bax induces permeation of the outer mitochondrial membrane and elicits a pro-apoptotic response by stimulating the release of Cyt C, which is blocked by Bcl-2. The results suggest that fomitoside-K may induce apoptosis in YD-10B cell lines by causing an increase in BAX and decrease in Bcl-2 levels that leads to the mitochondrial Cyt C release and activation of caspases. Furthermore, survivin, which is highly upregulated in malignancy, is functionally involved in apoptosis as well as proliferation. Hence, it is currently attracting considerable interest both as a potential cancer biomarker and as a new target for cancer treatment. Therefore, in this study we also examined the expression of survivin in YD-10B cells. Our study depicts that the treatment of fomitoside-K significantly inhibits the expression of survivin in a time dependent manner.

Many anticancer compounds exert intracellular ROS overproduction to induce apoptosis in cancer cells. Once intracellular ROS production is increased, many of the intracellular macromolecules, including DNA, proteins, and lipids, would be damaged. Our results demonstrated that fomitoside-K could induce significant intracellular ROS production in YD-10B cells (Fig. 4A). These results seem to explain that the ROS production is one of the main causes for the mushroom-extract-induced apoptosis. In addition, the enhancement of ROS production has been associated with the apoptotic response induced by various chemotherapeutic agents. ROS can cause apoptotic cell death via a variety of mechanisms, among which the activation of ERK and JNK plays a key role. Here, we show that ROS production in fomitoside-K treated YD-10B cells is markedly increased and functionally linked to ERK and JNK activation during apoptosis via the mitochondrial-dependent pathway. The generation of ROS in response to fomitoside-K was further supported by the finding that pretreatment with PD98059, SP600125 and NAC blocked the ROS mediated MAPK activation and prevented fomitoside-K induced apoptosis in YD-10B cells. Furthermore, a member of the MAPK family, JNK is rapidly phosphorylated and subsequently activated by a diverse spectrum of different cell stimuli. The JNK pathway has been shown to be closely linked to apoptosis by activation of the mitochondria-mediated apoptotic pathway. Accordingly, a number of studies have shown the importance of the ERK signaling pathway in regulating apoptosis. Although ERK pathway delivers a survival signal, several recent studies have linked the activation of ERK with the induction of apoptosis by various chemopreventive and chemotherapeutic agents. We found that exposure of YD-10B cells with an apoptosis inducing concentration of fomitoside-K results in a rapid and sustained activation of ERK and JNK in a time dependent manner.
Since cancer development involves many aspects of the cell, treatment with a single agent is rarely effective.\(^\text{6(0)}\) Two or more cellular processes are usually targeted in therapy. Therefore, combination therapy is now a prominent approach in cancer chemotherapy. There are many advantages of this approach including targeting more than one critical molecular process, delivering lower dose agents with lower toxicity and increasing patient tolerance. Currently, there is growing interest in the use of combination chemotherapy, allowing the delivery of lower drug dosages each with different modes of activity.\(^\text{3(1)}\) In this study, our data showed that fomitoside-K has synergistic effects with adriamycin in suppressing the growth of YD-10B cells by increasing the ROS pathway. This suggests that fomitoside-K in combination with the commercially used chemotherapeutic agents has the potential to decrease the side effects caused by high doses of currently used chemotherapeutic agents.

In conclusion, our study clearly showed that fomitoside-K could inhibit the proliferation of YD-10B cells by the mechanism involved in the induction of apoptosis. We further proved that apoptosis of YD-10B cells was induced by the activation of caspase-9 and caspase-3, release of Cyt C from mitochondria, decrease of the Bcl-2 level and increase of the Bax and ROS levels by increments of the phosphorylation of the ERK and JNK pathways. The present study suggested that fomitoside-K could be a promising compound for the effective growth suppressor of human oral cancer cell.

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REFERENCES


