Anti-tumor effect of licochalcone-E is mediated by caspase-dependent apoptosis through extrinsic and intrinsic apoptotic signaling pathways in KB cancer cells

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ABSTRACT

This study investigates the anti-cancer effects of licochalcone-E (Lico-E), a phenolic chalconoid derived from the genus Glycyrrhiza, in the human KB squamous cancer cell. Concentration-dependent cytotoxicity in KB cells increased following 24 hours of treatment with 12.5, 25, or 50 µg/ml Lico-E, with an estimated IC₅₀ value of approximately 25 µg/ml. Chromatin condensation, a typical apoptotic phenomenon, was observed in KB cells treated with Lico-E. Consistent with this finding, Lico-E increased caspase-3 activity in KB cells. FasL, a death ligand associated with extrinsic apoptotic signaling pathways, was significantly up-regulated by Lico-E treatment. Subsequently, the pro-apoptotic factor caspase-8, a part of the extrinsic apoptotic signaling pathway, was activated in a concentration-dependent manner by Lico-E treatments. Expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL, components of the mitochondria-dependent apoptotic signaling pathway, significantly decreased following Lico-E treatment. Conversely, expression of pro-apoptotic factors such as Bax, Bad, Apat-1, and caspase-9 increased with Lico-E concentrations. Finally, Lico-E activated caspase-3 and Poly(ADP-ribose) polymerase (PARP) to induce cell death. Z-VAD-fmk significantly inhibited cell death through suppression of caspase-3 expression in KB cells treated with Lico-E. Taken together, Lico-E induces KB cell death through death receptor and mitochondria-dependent apoptotic signaling pathways.

KEY WORDS: Apoptosis, Cancer, Caspases, Chemotherapeutic agent, Licochalcone-E

Introduction

Cancer is a disease with high mortality due to the invasion or metastasis of abnormal cells to other parts of the body [1]. Worldwide, the most commonly reported types of cancer are breast cancer, colorectal cancer, lung cancer, cervical cancer, and oral cancer [2]. The number of newly diagnosed cancer patients is increasing annually all over the world.

Recent studies associated with the development of chemotherapeutic agents have focused on producing cancer-specific cell death to overcome numerous side-effects such as fatigue, pain, mouth and throat sores, diarrhea, nausea and vomiting, blood disorders, nervous system effects, loss of appetite and hair loss, among others [3, 4]. Thus, to overcome the side effects associated with current chemotherapies, drug developers have sought natural compounds that cause cancer-specific cell death from plants traditionally used in either folk or oriental medicine [5]. Furthermore, some medicinal plant-derived compounds that induce cancer-specific cell death have recently been approved by the United States Food and Drug Administration for chemotherapeutic use in cancer patients [6].

Licorice, the root from Glycyrrhiza species, is one of the most commonly used traditional oriental medicines [7]. Recently, licochalcone-A (Lico-A), a phenolic chalconoid purified from Glycyrrhiza species, has been reported to show anticancer effects in human lung cancer, human
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breast cancer, human cervical cancer, human gastric cancer, and head and neck squamous carcinoma [7]. Licochalcone-E (Lico-E, (E)-3-[5-(1,1-Dimethyl-2-propenyl)-4-hydroxy-2-methoxyphenyl]-1-(4-hydroxyphenyl)-2-propen-1-one, C_{21}H_{22}O_{4}) has also been isolated from liquorice roots. As shown in Fig. 1, Lico-E is a flavonoid belonging to the retrochalcone family. In this study, we examined the anti-cancer effects of Lico-E and the potential cellular signaling pathways associated with its mechanism of apoptosis in the KB human squamous cancer cell.

Materials and Methods

Cell culture

The KB human squamous cancer cell was obtained from the American Type Culture Collection (ATCC), and cultured according to the instructions provided. Briefly, KB cells were maintained in minimum essential medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37°C in 5% CO₂.

Cell viability assay

KB squamous cancer cells were cultured at a density of 5x10^5 cells/well in 96-well plates, and allowed to attach to the well overnight. After incubation, the cultured cells were treated with 12.5, 25, or 50 μg/ml Lico-E in triplicate, and incubated at 37°C in a 5% humidified CO₂ incubator for 24 h. MTT assay was then added to each well and incubation was continued for an additional 4 h at 37°C. To dissolve the resulting formazan, the cells were resuspended in 200 μL dimethyl sulfoxide, and the optical density (OD) of the solution was determined using a spectrometer at an incident wavelength of 570 nm. The experiments were repeated three times, independently. The mean OD ± standard deviation (SD) for each group of replicates was calculated. The percentage of cell growth inhibition was calculated using the equation: % growth inhibition = [(1 – OD extract-treated cells) / (OD negative control cells)] × 100.

Cell survival assay

Cell survival was verified using calcein-AM to stain living cells, and ethidium bromide homodimer-1 to stain dead cells. These reagents were obtained from Molecular Probes (Eugene, OR, USA). For the cell survival assay, KB cells were cultured at a density of 5 × 10^5 cells/well in an 8-well chamber slide, and incubated with 12.5, 25, or 50 μg/ml Lico-E for 24 h, then stained with green calcein-AM and ethidium homodimer-1 according to the manufacturer’s protocol. The cells were then observed and photographed using an inverted phase-contrast microscope (Eclipse TE2000; Nikon Instruments, Melville, NY, USA).

Nucleus staining using DAPI

Cells were treated with Lico-E and incubated for 24 h, then fixed with 4% paraformaldehyde before washing with PBS. The washed cells were stained with 1 mg/ml [4',6-diamidino-2-phenylindole dihydrochloride (DAPI); Roche Diagnostics] for 20 min. Nuclear condensation was observed by fluorescence microscopy (Eclipse TE2000; Nikon Instruments, NY, USA).

Caspase-3/-7 activity assay

The apoptotic activity of executioner caspase-3/-7 was determined using the cell-permeable fluorogenic substrate, PhiPhiLux-G1,D2 (OncoImmunin Inc., Gaithersburg, MD, USA), according to the manufacturer’s instructions.
Western blot analysis

KB cells plated on culture dishes at a density of 5 × 10⁶ cells/ml were incubated for 24 h in a humidified incubator at 37°C in 5% CO₂, then treated with Lico-E for 24 h. Thereafter, the cells were lysed using a cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease and phosphatase inhibitor cocktails, and incubated for 1 h at 4°C. Lysates were centrifuged at 14,000 × g for 10 min at 4°C. The supernatant contained the cytosolic fraction. Total protein concentrations of the cell lysates were determined using bicinchoninic acid protein assays (Thermo Scientific, Rockford, IL, USA). Loading buffer (5×) was added to equal amounts of protein, and the mixture was boiled at 90°C for 10 min. Total proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking for 2 h with 5% bovine serum albumin in Tris-buffered saline containing Tween-20 at room temperature, membranes were incubated with primary antibody at 4°C overnight, and then incubated with horseradish peroxidase-conjugated secondary antibody. The antibodies used to study the apoptotic signaling pathways included antibodies against Fas ligand (FasL, 48 kDa), cleaved caspase-3 (17 and 19 kDa), cleaved caspase-8 (18 kDa), cleaved caspase-9 (37 kDa), poly (ADP ribose) polymerase (PARP, preform 116 kDa and cleaved form 85 kDa), B-cell lymphoma 2 (Bcl-2, 26 kDa), B-cell lymphoma extra-large (Bcl-xL, 26 kDa), Bcl-2-associated X protein (Bax, 21 kDa), Bcl-2-associated death promoter (Bad, 23 kDa), apoptotic protease activating factor 1 (Apaf-1, 13 kDa), and β-actin (45 kDa), purchased from Cell signaling Technology (Danvers, MA, USA). The immunoreactive bands were visualized using the ECL System (Amersham Biosciences, Piscataway, NJ, USA) and were exposed on radiographic film.

Caspase dependent cell survival assay

Cells were plated at a density of 1 × 10⁵ cells/ml in 96-well plates and allowed to attach overnight. After incubation, cultured cancer cells were treated with Lico-E in the presence or absence of 50 μM of the caspase-3 inhibitor (Z-VAD-FMK, N-Benzylxoycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone) (Sigma-Aldrich, St. Louis, MO, USA) and were incubated for 24 h at 37°C. After incubation, cytotoxicity was measured using the MTT assay.

Statistical analysis

Data are reported as the mean ± standard deviation of three individual experiments performed in triplicate. Statistical analysis was carried out using a Student's t-test, and a p-value < 0.05 was considered statistically significant.

Results

Lico-E induces KB cell death in a concentration-dependent manner

To verify whether Lico-E causes cytotoxicity, the MTT assay was performed on KB cells treated with various concentrations of Lico-E for 24 h. As shown in Fig. 2A, the percentage of cell growth inhibition was measured as
19.7±2.4 \( (p<0.01) \), 53.4±2.8 \( (p<0.01) \), and 89.6±0.5% \( (p <0.01) \) for KB cancer cells treated with 12.5, 25, and 50 μg/ml Lico-E, respectively, compared with untreated controls.

Based on these results, the IC\textsubscript{50} value for Lico-E-induced cytotoxicity in KB cancer cells was estimated to be approximately 25 μg/ml. Furthermore, to confirm Lico-E-induced KB cell death, a living and dead cell assay was performed using permeable green calcein AM, which stains living cells with green fluorescence, and ethidium homodimer-1, which stains dead cell with red fluorescence. As shown in Fig. 2B, after 24 h treatment the number of dead KB cells stained with red fluorescence by ethidium homodimer increased with Lico-E concentration (12.5, 25, and 50 μg/ml). Taken together, these results indicate that Lico-E induces KB cell death in a concentration-dependent manner.

Lico-E-induced KB cell death is mediated by apoptosis

To investigate whether Lico-E-induced KB cell death is mediated by apoptotic mechanisms, DAPI staining was performed to observe chromatin condensation, a representative phenomenon of programmed cell death, or apoptosis, in KB cells treated with 12.5, 25, and 50 μg/ml Lico-E for 24 h. As shown in Fig. 3A, the number of KB cells with condensed nuclei increased with Lico-E treatment concentrations. Furthermore, to verify whether Lico-E-induced KB cell death is mediated by apoptosis, caspase-3/-7 activity was measured using the cell-permeable fluorogenic substrate PhiPhiLux-G1D2. As shown in Fig. 3B, the cleavage of PhiPhiLux-G1D2 by activated caspase-3 was significantly upregulated in KB cells treated with 25 μg/ml Lico-E, compared with non-treated control cells. These results indicate that Lico-E-induced KB cell death is mediated by the activation of the pro-apoptotic factor caspase-3.

Lico-E-induced apoptosis is mediated by extrinsic death receptor-dependent and intrinsic mitochondria-dependent apoptotic signaling pathways

To investigate the cellular apoptotic pathways associated with Lico-E-induced KB cell death, western blotting was performed, as shown in Fig. 4. FasL (48 kDa), a death receptor ligand associated with the extrinsic death receptor-mediated apoptosis pathway, was concentration-dependently up-regulated in KB cells treated with 12.5 and 25 μg/ml Lico-E, compared with non-treated control cells. Sequentially, up-regulated FasL induced a concentration-dependent increase in cleaved caspase-8 (18 kDa), a downstream target of the pro-apoptotic factor FasL, in KB cells treated with Lico-E. These results indicate that Lico-E-induced apoptosis involves the extrinsic death receptor-mediated apoptosis pathway.

Furthermore, the expression of Bcl-2 (26 kDa) and Bcl-xL (26 kDa), anti-apoptotic factors associated with the intrinsic mitochondria dependent apoptosis pathway, were concentration-dependently down-regulated in KB cells treated with 12.5 and 25 μg/ml Lico-E, compared with non-treated control cells. Whereas, the expression of mitochondria dependent pro-apoptotic factors such as Bax (21 kDa), Bad (23 kDa) and Apaf-1 (130 kDa) were concentration-dependently increased in KB cells treated with Lico-E. Sequentially, Lico-E increased the expression of cleaved caspase-9 in KB cells. As shown in Fig. 4B, these results therefore indicate that Lico-E-induced KB cell death involves the intrinsic mitochondria-dependent apoptosis pathway. Subsequently, both cleaved caspase-8 and caspase-9, located both in the extrinsic death receptor mediated and intrinsic mitochondria-dependent apoptosis pathways, induced con-
Licochalcone-E-induced apoptosis is mediated by the extrinsic death receptor-mediated and the intrinsic mitochondria-dependent apoptotic signaling pathways in KB cells. KB cells were treated with 12.5 or 25 μg/ml Lico-E for 24 h. Thereafter, total proteins were extracted and western blots verified altered expressions of pro-apoptotic and anti-apoptotic factors associated with apoptotic signaling pathways. In Fig. 4A, Lico-E upregulated the death receptor ligand FasL and subsequently induced the extrinsic death receptor-mediated apoptotic signaling pathway through the cleavage of caspase-8 in KB cells. In Fig. 4B, Lico-E induced the intrinsic mitochondria-dependent apoptotic signaling pathway in KB cells. In Fig. 4C, cleaved caspase-8 and caspase-9 induced the activation of caspase-3 and PARP in KB cells treated with Lico-E.

Fig. 4. Licochalcone-E-induced apoptosis is mediated by the extrinsic death receptor-mediated and the intrinsic mitochondria-dependent apoptotic signaling pathways in KB cells. KB cells were treated with 12.5 or 25 μg/ml Lico-E for 24 h. Thereafter, total proteins were extracted and western blots verified altered expressions of pro-apoptotic and anti-apoptotic factors associated with apoptotic signaling pathways. In Fig. 4A, Lico-E upregulated the death receptor ligand FasL and subsequently induced the extrinsic death receptor-mediated apoptotic signaling pathway through the cleavage of caspase-8 in KB cells. In Fig. 4B, Lico-E induced the intrinsic mitochondria-dependent apoptotic signaling pathway in KB cells. In Fig. 4C, cleaved caspase-8 and caspase-9 induced the activation of caspase-3 and PARP in KB cells treated with Lico-E.

Lico-E-induced apoptosis depends on the activation of caspases in KB cells

To verify whether Lico-E-induced apoptosis in KB cells requires caspase activation, Lico-E treated KB cells, with or without 50 μM pan caspase inhibitor Z-VAD-fmk, were incubated for 24 h. Thereafter, MTT staining and western blot analysis were performed, as shown in Fig. 5. Compared to untreated control cells, 25 μg/ml Lico-E treatment decreased the percentage of cell growth inhibition by 43±6%. On the other hand, the relative survival rates were measured as 65±8% in the KB cells co-treated with Lico-E and 50 μM pan caspase inhibitor Z-VAD-fmk for 24 h. Furthermore, the cleaved form of pro-apoptotic factors such as caspase-3 and PARP were significantly down-regulated in the KB cells co-treated with Lico-E and 50 μM pan caspase inhibitor Z-VAD-fmk, compared with Lico-E treatment alone. These data indicate that Lico-E-induced apoptosis depends upon the activation of caspases in KB cells.

Fig. 5. Licochalcone-E-induced apoptosis is dependent on the activation of caspases in KB cells. In Fig. 5A, pretreatment with a pan-caspase inhibitor, Z-VAD-fmk, counteracts Lico-E-induced KB cell death. KB cells were cultured and treated with 25 μg/ml Lico-E in the presence or absence of 50 μM Z-VAD-fmk for 24 h. Thereafter, the MTT assay was performed to measure cytotoxicity. In Fig. 5B, A pan-caspase inhibitor Z-VAD-fmk inhibited the activation of caspase-3 and PARP in KB cells treated with Lico-E. Thereafter, total proteins were extracted and western blots verified the alterations in expression of caspase-3 and PARP.
Discussion

Cancer, characterized by abnormal cell hyperplasia, is a chronic worldwide health problem [8, 9]. Recent studies report that more than 100 types of cancer have been classified according to cell type and their pathological characteristics [10]. Although clinical interventions associated with cancer treatment, such as surgery, radiotherapy, and chemotherapy, have rapidly progressed due to huge investments in the past half-century, resulting increases in the long-term survival rates of cancer patients have been modest [7, 11]. Recently, chemotherapy has demonstrated the greatest clinical efficacy among treatments for cancer patients [10]. However, despite the clinical efficacy of chemotherapy in treating cancer, clinical applications are often limited by various side effects, such as high toxicity to healthy cells and multidrug resistance, among other side effects [12]. Therefore, to discover new clinical applications of potential chemotherapeutic agents, recent drug development studies have focused on medicinal plants with known biological safety to overcome limitations imposed by negative side effects [11].

Licorice is the root from the genus Glycyrrhiza, including the species Glycyrrhiza glabra, Glycyrrhiza inflata, and Glycyrrhiza uralensis, which are commonly used in traditional oriental medicine [11]. Recent studies have reported that licorice roots contain multiple bioactive components such as saponins, flavonoids, and coumarins [13-15]. Furthermore, licorice has been shown to have anticancer effects in various types of cancer such as colon [16], lung [17, 18], breast [19, 20], prostate [21], and oral cancers [11] through metastatic inhibition [19], anti-proliferative properties [20], increased apoptosis [11, 22], and cell cycle arrest [23].

Recently, six retrochalcone compounds, which are structurally distinguished from typical chalcones by the lack of oxygen functionalities at C-2’ and C-6’, have been isolated and characterized from the roots of Glycyrrhiza species [24, 25]. Among these six retrochalcones, the chemical structure of Lico-E ((E)-3-[4-hydroxy-2-methoxy-5-[(2R)-3-methylbut-3-en-2-yl]phenyl]-1-(4-hydroxyphenyl)propl-2-en-1-one) is extremely similar to Lico-A ((E)-3-[4-hydroxy-2-methoxy-5-[2-methylbut-3-en-2-yl]phenyl]-1-(4-hydroxyphenyl)prop-2-en-1-one). Furthermore, although studies associated with the biological effects of Lico-E have reported lower efficacy than that of Lico-A, the biological effects of both licochalcones exhibit similar properties. According to recently reported studies associated with Lico-A, various biological effects such as neuroprotective [26], chondroprotective [27, 28], antithrombotic [29], antimicrobial [30], and antiviral activities [30] have been reported. In addition, recent studies have also reported reduction in chronic allergic contact dermatitis as well as anti-diabetic, anti-inflammatory, and antimicrobial effects. Anticancer effects of Lico-A have been prominently reported for various types of cancers, such as A549 and H460 human lung cancer cells [17], T24 bladder cancer cells [31], human cervical cancer cells [32], human BGC-823 gastric cancer cells [33], malignant pleural mesothelioma [34], human FaDu head and neck squamous carcinoma cells [22], and human esophageal carcinoma [35] among others. On the other hand, although recent studies associated with the biological effects of Lico-E have been reported anti-diabetic effect [36], the reduction of chronic allergic contact dermatitis [37], neuronal protection [38], anti-inflammation [39] and antimicrobial effects [40], but the anticancer effects of Lico-E reported in the 4T1 mammary orthotopic cancer [41] and human head and neck squamous carcinoma FaDu cells [11]. As shown in various studies associated with biological effects of both licochalcone, the physico-chemical properties of Lico-E are quite similar to those of Lico-A. Furthermore, we have recently reported that the anti-cancer effects of Lico-A and Lico-E in the human pharyngeal squamous carcinoma FaDu are mediated through extrinsic and intrinsic apoptotic mechanisms [11, 22]. Therefore, we hypothesized that Lico-E may have anti-cancer effects similar to Lico-A in KB cells. In support of our hypothesis, the present study demonstrated that Lico-E induces apoptosis in KB cancer cells through activation of caspases in both the extrinsic death receptor-mediated and the intrinsic mitochondria-dependent apoptotic pathways.

As shown in Fig. 2, Lico-E effectively induced cytotoxicity in KB cells in a concentration-dependent manner. In our previous study, Lico-E did not affect cell viability in healthy human oral keratinocytes used as controls [11]. Furthermore, the IC50 value of Lico-E cell growth inhibition was estimated to be approximately 25 µg/ml in KB cells. This value is a significantly lower concentration compared to the reported 50 µg/ml IC50 value for Lico-E growth inhibition of FaDu human pharyngeal squamous carcinoma cells [11]. These data indicate that Lico-E may more effectively induce cytotoxicity in KB cells than in...
FaDu human pharyngeal squamous carcinoma.

Next, to verify whether Lico-E-induced KB cell death involved apoptosis, DAPI staining was performed to observe nuclear condensation, a hallmark of apoptosis [42]. As shown in Fig. 3A, the number of KB cells with condensed nuclei was increased by Lico-E in a concentration-dependent manner. Although the exact role of nuclear condensation in apoptotic cells remains unknown, it may be closely related to chromosomal DNA degradation by endonucleases [42]. To further verify Lico-E-induced apoptosis in KB cells, we measured the activation of caspase-3, a central component of the apoptotic cascade. As shown in Fig. 3B, the activity of caspase-3 was significantly increased in KB cells treated with Lico-E. Caspase-3 pro-apoptotic factors lead to apoptosis after their activation through the cleavage of inactive precursors expressed in cells [42]. Therefore, these results are consistent with the hypothesis that Lico-E-induced cell death is mediated by apoptosis in KB cells.

However, apoptosis occurs through both extrinsic and intrinsic apoptotic pathways. The extrinsic apoptosis pathway is triggered by the binding of ligands such as the Fas ligand (FasL or CD95L), and/or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) onto death domain-containing tumor necrosis factor-superfamily receptors, including CD95/Fas, TNF-R1, TRAIL-R1 (DR4), and TRAIL-R2 (DR5) [42]. Thereafter, intracellular adaptor proteins, such as the Fas-associated death domain (FADD) and/or the TNF-R-associated death domain (TRADD), bind onto the cytosolic death domain of the death receptor to induce the cleavage of procaspase-8. Subsequently, the cleaved (active) form of caspase-8 induces the cleavage of procaspase-3 to form cleaved (active) caspase-3 [42, 43]. As shown in Fig. 4A, the expression of FasL was significantly and concentration-dependently elevated in KB cells treated with Lico-E. Sequentially, the expression of cleaved caspase-8 was concentration-dependently increased in KB cells treated with Lico-E. Although we did not investigate the interaction with intracellular adaptor proteins and up-regulated FasL in KB cells treated with Lico-E, these results indicate that Lico-E-induced apoptosis is mediated by the extrinsic death receptor-mediated apoptosis pathway through the up-regulation of FasL in KB cells.

Moreover, the mitochondrial outer membrane is protected by anti-apoptotic factors including Bcl-2, Bcl-xL, and Mcl-1, which neutralize the pro-apoptotic BH3-only members of the Bcl-2 family, such as Bax and Bak. Therefore, the down-regulation of anti-apoptosis factors promote mitochondrial outer membrane permeability (MOMP) by the up-regulation of Bax and Bak [44, 45]. Apart from this, cleaved caspase-8, which is a proapoptotic factor associated with the extrinsic death receptor-mediated apoptosis pathway, also increased MOMP through the cleavage of Bid to a truncated form tBid [44]. Thereafter, cytochrome c, released from mitochondrial outer membranes to the cytosol through the promoted MOMP form apoptosomes composed of a procaspase-9 and Apaf-1 [44]. Sequentially, procaspase-3 is activated through the cleavage of procaspase-9 induced by the interaction with Apaf-1. As shown in Fig. 4B, the expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL were down-regulated in KB cells treated with Lico-E. However, the expression of proapoptotic factors such as Bax and Bad in KB cells were significantly increased by Lico-E. These results consistently indicate that the MOMP of KB cells treated with Lico-E is promoted by the imbalance between anti-apoptotic factors (Bcl-2 and Bcl-xL) and proapoptotic factors (Bax and Bad). Furthermore, the expression of Apaf-1 was concentration-dependently increased in KB cells treated with Lico-E. These results indicated Lico-E treated KB cells formed apoptosomes from a complex of cytochrome c released from the mitochondrial outer membrane, Apaf-1, and procaspase-9, in the presence of dATP. Subsequently, the expression of cleaved caspase-9 was gradually increased in KB cells treated with Lico-E in a concentration-dependent manner. Furthermore, these results indicate that Lico-E-induced apoptosis in KB cells involves an intrinsic mitochondria-dependent apoptosis pathway. Finally, as shown in Fig. 4C, following Lico-E treatment in KB cells both cleaved caspase-8 and caspase-9 increased cleaved caspase-3 through the cleavage of procaspase-3. Then, cleaved caspase-3 triggered the cleavage of PARP to induce apoptosis through the access of endonucleases to chromatin, as shown in Fig. 4C [42]. Taken together, these results consistently indicate that Lico-E-induced KB cell death is mediated by apoptosis through both the extrinsic death receptor mediated and intrinsic mitochondria-dependent apoptosis pathways.

However, apoptotic cell death through both the extrinsic death receptor mediated and intrinsic mitochondria-dependent apoptosis pathways is closely associated with the cleavage of caspases such as caspase-8, caspase-9 and caspase-3. Therefore, to verify whether Lico-E-induced KB cell death is governed by the cleavage of procaspases,
we pretreated KB cells with Z-VAD-fmk, a cell-permeable fluoromethyl ketone (FMK)-derivatized peptide that acts as an irreversible caspase inhibitor with no cytotoxic effects, prior to Lico-E treatment. Thereafter, MTT assays were performed to measure cytotoxicity. As shown in Fig. 5A, Lico-E-induced KB cell death was significantly inhibited in the presence of Z-VAD-fmk compared to Lico-E only. Furthermore, the cleavage of caspase-3 and PARP were completely suppressed in KB cells co-treated with Lico-E and Z-VAD-fmk. Therefore, these results consistently indicate that Lico-E-induced KB cell death depends on the cleavage of procaspases in both the extrinsic death receptor mediated and the intrinsic mitochondria-dependent apoptosis pathways.

Our previous study showed Lico-E exhibits apoptotic effects in FaDu human pharyngeal squamous carcinoma cells through both extrinsic death receptor-mediated and intrinsic mitochondria-dependent intrinsic apoptosis pathways triggered by upregulated expression of FasL. Similarly, in KB cells, Lico-E induced apoptosis through both extrinsic death receptor-mediated and intrinsic mitochondria-dependent intrinsic apoptosis pathways triggered by upregulated expression of FasL in KB.

In conclusion, these results suggest that the Lico-E derived from the roots of traditional medicinal plants of the genus *Glycyrrhiza* may be a potential chemotherapeutic drug lead. Our present study demonstrated Lico-E-induced apoptotic cell death in KB cells; however, further studies, including in vivo studies using xenograft animal models, are required to fully characterize the potential side effects and effective dose range of Lico-E.

Conflict of Interest

The authors declare that they have no competing interests.

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