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Licochalcone B induced apoptosis and autophagy in osteosarcoma tumor cells via the inactivation of PI3K/AKT/mTOR pathway

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Abstract

Licochalcone B (LicB) is a flavonoid derived from the Chinese medicinal herb Glycyrrhiza uralensis Fisch. Several previous studies have demonstrated the wide range of pharmacological activities shown by LicB. In this study, we investigated the anticancer effects of LicB in osteosarcoma (OS) tumor cells and its underlying mechanisms. According to the CCK8 analysis and EdU staining results, we found that LicB suppresses OS cells (MG-63 and U2OS) growth depending on its concentration. Furthermore, flow cytometry and western blot revealed that LicB promoted autophagy and apoptosis in OS cells in a dose-dependent manner. LicB treatment not only decreased the levels of Bcl-2, p62, Caspase-3, and Ki67 protein in MG-63 and U2OS cell lines but also increased the levels of Cleaved Caspase-3, Beclin1, Bax, Atg7, and LC3B. Mechanistically, LicB induced cell apoptosis by promoting the apoptosis-related cleavage of Caspase-3, while suppressing the PI3K/AKT/mTOR pathway to induce autophagy. The present work is the first to illustrate that LicB can serve as a potential drug candidate for tumor treatment owing to its ability to enhance autophagy and apoptosis, and suppress OS proliferation by inactivating the PI3K/AKT/mTOR pathway.

**Key words:** Osteosarcoma tumor, Licochalcone B, Apoptosis, Autophagy, Phosphatidylinositol 3-kinase.
Introduction

Osteosarcoma (OS) is a primary malignant bone tumor that usually affects children and adolescents.\textsuperscript{1, 2} Currently, OS cases have a poor 5-year survival rate of around 60%. In addition, a large proportion of survivors require amputation, seriously affecting their quality of life.\textsuperscript{3} To date, the most common treatments of OS include radiotherapy, surgery, and neoadjuvant chemotherapy.\textsuperscript{4} However, these approaches are unable to improve patient survival rate substantially, especially in cases of metastatic OS.\textsuperscript{5, 6} Therefore, it is essential to develop novel and efficient antitumor agents to treat OS and improve patient survival.

Certain natural products are shown to possess multiple effects like anti-inflammation, antitumor, and apoptosis induction on various cancer cells. \textit{Glycyrrhiza glabra}, an ancient Chinese medicine, has been demonstrated to play an effective role in the treatment of osteosarcoma.\textsuperscript{7} LicB is an extract of \textit{Glycyrrhiza glabra}, and belongs to the flavonoid family.\textsuperscript{8, 9} Previous research has highlighted the anti-oxidation, anticancer and anti-inflammation effects of LicB.\textsuperscript{10-12} LicB inhibits the proliferation of lung cancer cells and induces apoptosis by dual targeting of EGFR and MET.\textsuperscript{13} It also ameliorates liver cancer and breast cancer by targeting apoptotic genes, DNA repair systems, and cell cycle control.\textsuperscript{14, 15} We recently found that LicB inhibited the proliferation of osteosarcoma tumor cells in vitro. However, the precise mechanisms for its antitumor activity remain unclear.

Macroautophagy (also known as autophagy) is a process that involves the packaging of cells, degradation of cytosolic components, and recycling of the decomposed
Autophagy can suppress carcinogenesis in healthy cells, but tumor cells can hijack autophagy to facilitate cancer proliferation and provide resistance to antitumor treatments.\(^{19, 20}\) However, excess or abnormal autophagy induces the death of autophagic cells, a process referred to as type II programmed cell death.\(^{21-23}\) Autophagy-dependent cell death offers molecular implications and mechanisms for cancer treatment.

In this study, we determined the effects of LicB on the proliferation of osteosarcoma tumor cell lines in vitro. We also analysed apoptosis and autophagy level of osteosarcoma tumor cells during LicB treatment.

**Materials and Methods**

**Chemicals**

Licochalcone B was provided by Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China) with ≥98% purity. 3-MA was purchased from Sigma-Aldrich (MO, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Click-iT\textsuperscript{®} EdU Alexa Fluor\textsuperscript{®} 594 Imaging Kit was purchased from Thermo Fisher scientific (CA, USA). Antibodies for Ki67, Caspase-3, Cleaved Caspase-3, Bcl-2, Bax, Beclin1, Atg7, LC3A/B, p62 and β-actin were obtained from Cell Signaling Technology (MA, USA). p-PI3K (Tyr199), PI3K, p-AKT (Thr308), AKT, p-mTOR (Ser2448), mTOR, Goat anti-Rabbit IgG (H+L) secondary antibody, and Goat anti-Mouse IgG (H+L) secondary antibody were the products of Abcam (Cambridge, UK). All other chemicals used were of reagent grade.

**Cells and cell treatment**

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Human osteosarcoma cell lines (143B, MG-63 and U2OS) and normal osteoblast cell line (hFOB 1.19) were obtained from China Center for Type Culture Collection (Shanghai, China). MG-63 cells were incubated with α-MEM along with 10% fetal bovine serum (FBS) (Gibco, CA, USA) and 1% penicillin–streptomycin (Invitrogen, CA, USA). U2OS cells were incubated with McCoy's 5A containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Invitrogen). 143B and hFOB 1.19 were cultured with the RPMI-1640 medium (Invitrogen, CA, USA) containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Invitrogen). All cells were maintained at 37°C with 5% CO₂.

**Assay for cell viability**

CCK-8 assay was used to measure cell viability. We positioned cells onto 96-well microtiter plates at 5 × 10³ cells per well, followed by overnight incubation. Subsequently, cells were treated with varying LicB concentrations for 24 h. Then, we added 10 µL CCK-8 into every well for extra 4 h incubation at 37 °C in the dark. The absorbance (OD) values at 450 nm were measured using a microplate reader (Thermo Fisher Scientific). The rate of cell viability was measured using the equation:

Cell viability rate (%) = (test sample OD - blank OD)/(control OD - blank OD) ×100%

**Estimation of DNA replication in osteosarcoma tumor by EdU labeling**

We used the Click-iT® EdU imaging kit to measure osteosarcoma tumor proliferation ability. MG-63 and U2OS cells were inoculated on 6-well plates at 1 × 10⁵/well density and incubated overnight at 37 °C. After LicB treatment, 10 µM EdU was
added to the medium. After fasting, 4% formaldehyde was added, and cells were incubated in the dark with the Click-iT reaction cocktail. Corresponding nuclei were counterstained using DAPI (Beyotime, Shanghai, China) and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Detection of apoptosis by flow cytometry

MG-63 and U2OS cells were seeded in 6-well plates at $1 \times 10^5$ cells per well for 24 h. Then, the cells were treated with various concentrations of LicB (0, 2.5, 5, 10 and 20 μM). After the treatment, the treated cells were harvested and washed with cold PBS buffer. The cells were resuspended in binding buffer and stained with 5 μl Annexin V-FITC plus 5 μl propidium iodide (Beyotime, Shanghai, China) in 4 °C for 30 min in dark. The stained cells were washed by binding buffer for 3 times to remove excess dyes and then resuspended in 500 μl binding buffer. The percentage of apoptosis were analyzed by using flow cytometry (BD FACS Calibur, NJ, USA) within 1 h. The experiment was repeated three times.

Assessment of mitochondrial depolarization.

According to the manufacturer's instructions, pre-treated MG-63 and U2OS cells were suspended in 1 ml of complete medium containing 10 μg/ml of JC-1 (Beyotime) at 37°C for 30 min. The cells were analyzed on FACS Calibur flow cytometer (BD Biosciences).

Intracellular ROS measurements.

To detect intracellular hydrogen peroxide levels, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich) was used to measure intracellular ROS levels.
Cell fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences).

**Immunofluorescence**

Cells were inoculated into 24-well culture plates with aseptic cover slides and treated with corresponding drugs. After washing twice with PBS, the cells were fixed in 4% paraformaldehyde for 30 min, rinsed 3 times for 5 min with PBS, and 0.1% Triton-X-100 (PBST) statically placed at room temperature for 15 min. The cells were blocked with 5% BSA for 1 h, after which the blocking solution was discarded, primary antibody was added, and the cells were incubated overnight at 4 °C. The cells were then rinsed 3 times for 5 min in PBST and incubated with fluorescent secondary antibody. After incubation at room temperature for 1 h in the dark, the cells were stained with DAPI for 3 min in the dark, washed 3 times for 5 min with PBST, and then sealed and observed under a fluorescence microscope (Olympus, Tokyo, Japan). Five representative fields were captured at × 200 magnification, and the numbers of cells expressing target proteins in the cytoplasm and the nucleus (overlapping with DAPI) were counted.

**Western blotting assay**

Western blotting assay was performed following standard protocol. Briefly, MG-63 and U2OS cells were treated with DMSO (<0.1%) or different concentrations of LicB, then collected and lysed with RIPA lysis buffer (Amresco, PA, USA). The BCA protein detection kit (Beyotime) was used to measure corresponding protein content. Equivalent protein content in total cell lysates was separated by sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (BioRad Laboratories, CA, USA) and transferred onto polyvinylidene difluoride membrane (BioRad Laboratories) through wet transfer. Then, 5% skimmed milk in tris-buffered saline (containing 0.1% Tween 20, pH 7.6) was utilized to block the membrane under room temperature (RT) for 1 h, followed by overnight incubation with primary antibodies and 1 h incubation using appropriate secondary antibodies at RT. The ECL system (CLINX, Shanghai, China) was used to expose protein bands. Band intensity was examined with Image lab (version 3.0, BioRad Laboratories).

**Statistical analysis**

Overall trials were conducted at least three times. For statistical analysis, Prism software (GraphPad v6) was utilized. One-way ANOVA was used to analyze the statistical differences among three or more groups while unpaired Student’s t test was applied to analyze the statistical differences between two groups. Overall quantitative data were indicated by “mean ±S.D.” Statistical significance was expressed as *P < 0.05.

**Results**

*LicB inhibited proliferation of osteosarcoma cells*

To investigate the effect of LicB on OS cell growth, we conducted EdU staining and CCK-8 assays following a 24-hour treatment with LicB at different doses to detect OS cell viability (143B, U2OS, MG-63). Figure 1B suggests that LicB suppresses proliferation dose-dependently in OS cells but has no effect on the normal osteoblast cell line (hFOB 1.19). In comparison to 143B cells, LicB induced growth inhibition
by about 50% in U2OS and MG-63 cells at varying concentrations of 5, 10, and 20 µM. Therefore, we used them for subsequent assays. Western blotting (WB) analysis revealed that LicB decreased Ki67 protein levels in MG-63 and U2OS cells in a dose-dependent manner (Figure 1C and D). Furthermore, EdU staining showed that LicB exposure remarkably inhibits EdU-positive cells dose-dependently (Figure 1E and F). Based on the above findings, we can conclude that LicB has the ability to inhibit MG-63 and U2OS cell growth.

**LicB promotes mitochondrial dysfunction and induces apoptosis in OS cells.**

To estimate the effect of LicB in apoptosis, flow cytometry analysis was performed in MG-63 and U2OS cells with annexin V-FITC/PI. Following a 24-hour treatment with LicB (0, 2.5, 5, 10, and 20 µM), treated cells showed a markedly elevated apoptosis rate in a dose-dependent manner relative to their untreated counterparts (Figure 2A and B). Exposure to 20 µM LicB caused the most number of MG-63 and U2OS cells to enter the early apoptotic stage. To illustrate the mechanism underlying the effect of LicB on cell apoptosis induction, we utilized a WB assay to detect the levels of caspase-3 and cleaved caspase-3. Results revealed that LicB enhanced the cleavage of caspase-3 in MG-63 and U2OS cells in a dose-dependent manner as compared to controls (Figure 2C and D). Furthermore, a reduction was observed in the Bcl-2 levels while Bax levels showed a clear elevation in MG-63 and U2OS cells dose-dependently relative to controls (Figure 2C and E). In addition, LicB decreased the mitochondrial membrane potential (Δψm), which was confirmed by JC-1 probe application (Figure 2F). With the increase in LicB concentration, the intensity of
green fluorescence increased gradually. Besides, we evaluated the impact of LicB on ROS production by staining cells with the ROS indicator DCFH-DA. The results showed that the ROS production in the two cell lines was increased in a dose-dependent manner after treatment with LicB (Figure 2G). The above findings indicate that LicB increased the ROS production and cleavage of Caspase-3, thereby leading to increased apoptosis.

**LicB activated the autophagy pathway in osteosarcoma tumor cell**

To predict the role of LicB in autophagy, we measured the autophagy of MG-63 and U2OS cells using a WB assay. A remarkable up-regulation of autophagy-related proteins (ARPs, ATG7, Beclin1) was observed in MG-63 and U2OS cells in a dose-dependent manner. In addition, LicB significantly reduced the decomposition of p62 and LC3B (Figure 3A, B and C). The above results indicate that LicB promotes autophagy. Furthermore, immunofluorescence staining revealed that LicB up-regulated LC3B expression in MG-63 and U2OS cells in a dose-dependent manner and that it had a positive correlation with autophagosome formation (Figure 3D and E). To verify the effect of LicB on autophagy activation in MG-63 and U2OS cells, we utilized 3-MA, an autophagy inhibitor. Results showed a significant decrease in the ARPs (Atg7, Beclin1 levels along with a remarkable suppression of the p62 and LC3B decomposition turnover in MG-63 and U2OS cells treated with 3-MA (P < 0.05) (Figure 4A, B and C). On simultaneous administration of LicB and 3-MA to cells, Atg7 and Beclin1 levels increased significantly, and the p62 and LC3B decomposition turnover recovered in comparison to cells exposed to 3-MA alone (P < 0.05) (Figure
This phenotype was verified further using immunofluorescence staining (Figure 4D and E). The above findings indicate that LicB leads to autophagy in MG-63 and U2OS cells.

**LicB inhibited PI3K/AKT/mTOR pathway**

To investigate the mechanism underlying the effect of LicB on inducing autophagy, we applied a WB assay to detect the PI3K/AKT/mTOR pathway. The results revealed a remarkable decrease in the p-mTOR/mTOR, p-AKT/AKT, and p-PI3K/PI3K ratios in cells treated with LicB in a dose-dependent manner, relative to their untreated counterparts ($P < 0.05$) (Figure 5A). In addition, when cells were treated with 3-MA and LicB simultaneously, the above ratios were found to be significantly lower than cells treated with either drug alone ($P < 0.05$) (Figure 5B). Furthermore, EdU staining revealed that LicB combined with 3-MA treatment suppressed cell growth significantly while promoting cell apoptosis in comparison to either drug treatment ($P < 0.05$) (Figure 5C). Based on the above results, LicB was found to suppress cell growth via the PI3K/AKT/mTOR pathway.

**Discussion**

Several edible plant-originated natural extracts are being examined as potential antitumor agents due to their effectiveness and safety.$^{24}$ Chalcones are widely distributed natural products that possess various biological activities, including antiviral, antibacterial, anti-inflammatory and antitumor effects.$^{25}$ LicB, a chalcone found in the roots of Glycyrrhiza species, has been reported to inhibit tumor cell
proliferation by inducing S phase arrest and apoptosis. LicB has also been shown to suppress non-small-cell lung cancer (NSCLC) cell growth through dual targeting of EGFR and MET and inducing cell apoptosis. Although LicB is known to inhibit the growth of different cancer cells, no data on its effect on OS cell growth has been published yet. This study suggests that LicB suppresses cell growth and tumor proliferation in vitro by accelerating cell autophagy and apoptosis. The findings of this work offer novel evidence supporting the effect of LicB on suppressing OS cell proliferation.

Cell apoptosis is a key cell death form linked to the occurrence of tumors. Bcl-2 family proteins play a critical role in the mitochondria-regulated apoptosis pathway. Bcl-2 and Bax are the essential components regulating apoptosis. According to our findings, we found that LicB exposure up-regulated the expression of Bax and cleaved-caspase-3 while down-regulating the expression of Bcl-2 in vitro, thereby increasing the Bax/Bcl-2 ratio significantly. This results in LicB-mediated cell death, regulated by the Bax/Bcl-2 ratio and caspase-mediated apoptosis. Further, the additional mechanisms responsible for LicB-induced suppressed OS cell growth were investigated. Autophagy can suppress carcinogenesis in healthy cells, but tumor cells can hijack autophagy to facilitate cancer proliferation and provide resistance to antitumor treatments. However, deregulated or excessive autophagy could cause autophagic cell death, also known as the type II programmed cell death. According to previous research, PI3K signaling is involved in a variety of human disorders, such as proliferative disorders and cancers. Furthermore, the
PI3K/AKT/mTOR pathway has been shown to modulate tumor cell autophagy and apoptosis. As a result, inhibiting PI3K signaling has been identified as a potential treatment alternative for these disorders, and PI3K serves as a major target for drug development. Our study shows a remarkable suppression of the PI3K/Akt/mTOR pathway by LicB. Moreover, LicB elevated the expression of ARPs (ATG7, Beclin1) in a dose-dependent manner and promoted the p62 and LC3B decomposition turnover in MG-63 and U2OS cells. The above findings suggest that LicB induces autophagy. Some recent studies indicate that the role of autophagy in promoting death or survival is still controversial. Excessive autophagy results in autophagic cell death. The PI3K/Akt/mTOR pathway is involved in the initial stage of autophagy, and its suppression can lead to the autophagy activation, thereby inducing tumor cell death. This phenomenon also demonstrates that LicB combined with 3-MA (the class I/III PI3K kinase inhibitor) treatment suppresses cancer cell growth remarkably relative to treatment with LicB alone. The similar phenomenon could be observed when chloroquine (CQ) was used in combination with LicB as an autophagy inhibitor (Supplemental Figure).

To summarize, this work suggests that the anti-proliferation activity of LicB observed in OS cells was possibly achieved by promoting the cleavage of caspase-3 to induce apoptosis and suppressing the PI3K/Akt/mTOR pathway to induce autophagy. This was evidenced by the decreased p-Akt, p-PI3K, and p-mTOR protein levels measured via the WB assay. Further, this study supports LicB as a candidate antitumor drug for OS treatment.
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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

This article contains supplementary materials.
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Fig.1. Suppression of osteosarcoma tumor cell viability by LicB

(A) The chemical structure of Licochalcone B. (B) The cell viability was measured by CCK-8 assay after 24-hour LicB treatment. (C) Ki67 protein levels in U2OS and MG-63 cells were measured via WB assay after 24-hour LicB treatment. (D) Ki67 protein levels in (C). (E) The ratio of EdU-positive cells in MG-63 and U2OS cells visualized by EdU staining following 24-hour LicB treatment, Scale bar: 50 µm. (F)
Quantification of EdU-positive cells in (E). Results are indicated in the form of mean ±SD from 3 trials. Group 0 is the control group, and all groups are compared with the control group, *P<0.05.
Fig. 2. LicB promotes mitochondrial dysfunction and induces apoptosis in OS cells. (A) MG-63 and U2OS cell apoptosis measured by flow cytometry following 24-hour LicB exposure. (B) Quantification of cell apoptosis in (A). (C) Levels of caspase-3, cleaved caspase-3, Bcl-2, Bax, and GAPDH in MG-63 and U2OS cells treated with different concentrations of LicB. (D) Bcl-2/Bax ratio in MG-63 and U2OS cells treated with different concentrations of LicB. (E) JC-1 red fluorescence to green fluorescence ratio in MG-63 and U2OS cells treated with different concentrations of LicB. (F) DCFH-DA fluorescence intensity in MG-63 and U2OS cells treated with different concentrations of LicB.
apoptosis-related proteins (Bcl-2, Bax, Caspase-3) in MG-63 and U2OS cells were measured via WB assay following 24-hour LicB exposure. (D) Measurement of Caspase-3 activity in (C). (E) Bcl-2/Bax ratio in (D). (F) JC-1 was used to evaluate Dym in MG-63 and U2OS cells after LicB treatment. (G) DCFH-DA was used to evaluate ROS in MG-63 and U2OS cells after LicB treatment. Results are indicated in the form of mean ±SD from 3 trials. Group 0 is the control group, and all groups are compared with the control group, *P<0.05.
Fig. 3. LicB promoted osteosarcoma cell autophagy.

(A) Protein expression of ARPs (Atg7, Beclin1) and autophagy substrates (p62, LC3) was examined in MG-63 and U2OS cells via WB assay following 24-hour LicB exposure. (B and C) Quantification of protein levels in (A). (D) LC3 puncta measured in MG-63 and U2OS cells through immunofluorescence staining (×200) following 24-hour LicB exposure. (E) Quantification of LC3 puncta in (A). Results are indicated in the form of mean ±SD from 3 trials. Group 0 is the control group, and all groups are compared with the control group, *P<0.05.
Fig. 4. LicB reversed the inhibitory effects of 3-MA on autophagy

(A) Protein expression of ARPs (Atg7, Beclin1) and autophagy substrates (p62, LC3) in MG-63 and U2OS cells measured via WB assay following 24-hour treatment with LicB, 3-MA or LicB combined with 3-MA. (B) and (C) Quantification of the protein expression level in (A). (D) LC3 puncta measured in MG-63 and U2OS cells through immunofluorescence staining (×200) following 24-hour treatment with LicB, 3-MA or LicB combined with 3-MA. (E) Quantification of LC3 puncta in (D). Results are displayed in the form of mean ±SD from 3 independent assays. In comparison to LicB (10 µM), *P < 0.05; In comparison to 3-MA + LicB (10 µM), #P < 0.05.
Fig.5. LicB inhibited PI3K/AKT/mTOR pathway

(A) AKT, mTOR, and PI3K phosphorylation degrees measured via WB assay in MG-63 cells following 24-hour LicB exposure. (B) AKT, mTOR, and PI3K phosphorylation degrees measured via WB assay in A2708 cells following 24-hour
treatment with LicB, 3-MA or LicB combined with 3-MA. (C) EdU-positive cell proportion in MG-63 cells measured through EdU staining following 24-hour treatment with LicB, 3-MA or LicB combined with 3-MA, Scale bar: 50 µm. Results are expressed in the form of mean ±SD from 3 assays. In comparison to LicB (10 µM), *P < 0.05; In comparison to 3-MA + LicB (10 µM), ‡P < 0.05.