Oroxylin A Exerts Its Antitumor Effects in Human Gallbladder Cancer via Inhibition of the PTEN/PI3K/AKT Signaling Pathway

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Gallbladder carcinoma (GBC) is one of the most common carcinomas of the biliary tract and is associated with aggressive malignancy and poor prognosis. Current therapeutic strategies, including surgery, radiotherapy, and chemotherapy, are not sufficient for the treatment of GBC, and new therapeutic strategies are urgently needed. The antitumor effects of oroxylin A (OrA), a natural flavonoid extracted from the dried roots of medicinal plants such as Scutellariae species (Radix Scutellariae), have been widely reported in various cancers. In this study, we first evaluated the antitumor activity and the underlying mechanism of action of OrA on GBC cells in vitro. Our results revealed that OrA significantly attenuated the proliferation, migration, and invasion of GBC cells, simultaneously promoting their apoptosis. Suppression of the phosphate and tension homology deleted chromosome ten (PTEN)/phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) signaling pathway was found to be the underlying mechanism involved in the antitumor activity of OrA. In addition, experiments using a tumor xenograft mouse model confirmed the antitumor effects of OrA in vivo. Taken together, our findings indicate that OrA could be a potential antitumor agent for the prospective treatment of GBC.

Key words oroxylin A; phosphatidylinositol-3 kinase (PI3K); protein kinase B (AKT); gallbladder carcinoma; phosphate and tension homology deleted on chromosome ten (PTEN)

INTRODUCTION

Gallbladder carcinoma (GBC) is considered the most common carcinoma of the biliary tract; however, compared to other cancers, it is still a rare disease with a poor prognosis and can lead to serious consequences. 1) Currently, surgical resection is considered the only effective and curative treatment strategy for GBC; however, the limitations and side effects of resection have always been the major drawbacks. 2) Additionally, therapeutic alternatives such as chemotherapy remain insufficiently developed for the treatment of GBC. Therefore, novel agents with therapeutic potential for the treatment of GBC are urgently needed.

The phosphate and tension homology deleted on chromosome ten/phosphatidylinositol-3 kinase/protein kinase B (PTEN/PI3K/AKT) signaling pathway plays an important role in modulating several vital biological processes related to cancer, such as proliferation, metabolism, and apoptosis. 3) Phosphatidylinositol-3,4,5-triphosphate (PIP3) is a lipid product of PI3K, 4) and through the involvement of PTEN in modulating the conversion of PIP3 into diphosphate product PIP2, PIP3 plays a vital role in modulating cell growth and survival via activating PDK1, subsequently promoting the activation of its downstream target AKT. 5) Further, the activation of AKT is responsible for tumor cell invasion by promoting the activation of matrix metalloproteinases (MMPs). 6) Moreover, accumulated evidences of the association between AKT and caspase-8 have revealed that AKT inhibition is highly associated with tumor cell apoptosis. 7) Previous studies also indicate that drugs targeting the PTEN/PI3K/AKT signaling pathway can attenuate tumor progression. 8, 9)

Oroxylin A (OrA) is a natural flavonoid extracted from several medicinal plants such as Radix Scutellariae, the dried root of the medicinal plant Scutellariae. 10) Previous studies have demonstrated the anti-inflammatory, 11) anti-oxidative 12) and anti-tumor effects of OrA in various cancers. For example, OrA was reported to suppress colon carcinoma via inhibiting the interleukin (IL)-6/signal transducer and activator of transcription (STAT) 3 signaling pathway. 13) Furthermore, OrA could inhibit human hepatocellular carcinoma via attenuating the PI3K/AKT signaling pathway. 14) Thus, we considered OrA a potential antitumor agent for human gallbladder cancer therapy.

In this study, we aimed to explore the antitumor effects and underlying mechanism of OrA on GBC cells in vitro and in vivo. These data could serve as evidences for OrA as a potential therapeutic agent for GBC treatment in the near future.

MATERIALS AND METHODS

Reagents OrA was obtained from MedChem Express (NJ, U.S.A.) and dissolved in dimethylsulfoxide (DMSO) as a 100mM stock solution and stored at −20°C for subsequent experiments. SC79 (an AKT activator) and Cell Counting Kit (CCK)-8 were purchased from MedChem Express. High-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (NY, U.S.A.). Streptomycin and penicillin were obtained from HyClone (UT, U.S.A.). The primary antibodies against MMP-2 (#40994), MMP-9 (#13667), B-cell lymphoma-2 (Bcl-2) and other related antibodies were purchased from Cell Signaling Technology (MA, U.S.A.).

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Cells with chromatin condensation appearing in PBS prior to examination using a fluorescence microscope stained cells. After incubation in dark for 15 min, the Hoechst 33258 (10 µg/mL) staining was performed to visualize the apoptotic cells. Following incubation in dark for 15 min, the Hoechst 33258 reagent was removed, and cells were washed three times with PBS prior to examination using a fluorescence microscope (Nikon, Japan). Cells with chromatin condensation appearing bright blue were considered apoptotic cells, and the apoptosis rate was calculated as follows:

\[
\text{Apoptosis} (\%) = \left( \frac{\text{Apoptotic cells}}{\text{Total cells}} \right) \times 100
\]

Flow Cytometric Analysis NOC cells (5 × 10^6 cells/well) were cultured on 6-well plates and adhered. Cells were washed three times with PBS, followed by fixation with 4% paraformaldehyde for 20 min at 25°C. Then, Hoechst 33258 (10 µg/mL) staining was performed to visualize the apoptotic cells. After incubation in dark for 15 min, the Hoechst 33258 reagent was removed, and cells were washed three times with PBS prior to examination using a fluorescence microscope (Nikon, Japan). Cells with chromatin condensation appearing bright blue were considered apoptotic cells, and the apoptosis rate was calculated as follows:

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Matrigel Invasion Assay A transwell assay (8-µm pore size) was conducted to evaluate the inhibitory effect of ORA on the invasive potential of NOC cells. All the upper chambers were precoated with Matrigel, and after solidification, the NOC cells (5 × 10^4) were plated onto the Matrigel in a serum-free medium containing ORA at varying concentrations (0, 10, 20, and 40 µM). Meanwhile, 200 µL of high-glucose DMEM containing 20% FBS was added into the lower chambers. After 48 h of incubation, the invasive cells were fixed with 4% paraformaldehyde for 20 min at 25°C. Subsequently, the invasive cells were stained with crystal violet for 10 min at 25°C.

Western Blotting Proteins in the treated NOC cells were extracted using a RIPA buffer containing protease and phosphatase inhibitors. All the protein samples were boiled on ice for 30 min prior to centrifugation at 12000 × g for 10 min at 4°C. Finally, the samples were normalized using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

Colony Formation Assay NOC cells (1 × 10^3 cells/well) were cultured on 6-well plates at 37°C with 5% CO_2. After cell adherence, the medium was replaced with fresh medium containing different concentrations of ORA (0, 4, 8, 16, 32, 64, and 128 µM), and the cells were incubated for 48 h. Thereafter, 10 µL of CCK-8 was added into each well. Subsequently, the plates were incubated for 4 h. Finally, absorbance was measured at 450 nm (OD450), using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

Hoechst 33258 Staining Assay NOC cells (1 × 10^4 cells/well) were cultured on 96-well plates at 37°C with 5% CO_2. After cell adherence, the medium was replaced with fresh medium containing 10% FBS, supplemented with 100 mg/mL streptomycin and 100 units/mL penicillin, in an incubator with 5% CO_2 at 37°C.

Cell Toxicity Assay NOC and GBC-SD cells (5 × 10^3 cells/well) were cultured on 12-well plates followed by treatment with ORA (0, 10, 20, and 40 µM) for additional 10 d, and the medium was replaced every 2 d. At the end of the experiment, medium was removed, and the cells were washed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at 25°C. Finally, crystal violet staining was performed to stain the colonies.

Hoechst 33258 Staining Assay NOC cells (1 × 10^3 cells/well) were cultured on 96-well plates at 37°C with 5% CO_2. After cell adherence, the medium was replaced with fresh medium containing 10% FBS, supplemented with 100 mg/mL streptomycin and 100 units/mL penicillin, in an incubator with 5% CO_2 at 37°C.

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Flow Cytometric Analysis NOC cells (5 × 10^5 cells/well) were cultured on 6-well plates and adhered. Cells were washed three times with PBS, followed by fixation with 4% paraformaldehyde for 20 min at 25°C. Next, Hoechst 33258 (10 µg/mL) staining was performed to visualize the apoptotic cells. After incubation in dark for 15 min, the Hoechst 33258 reagent was removed, and cells were washed three times with PBS prior to examination using a fluorescence microscope (Nikon, Japan). Cells with chromatin condensation appearing bright blue were considered apoptotic cells, and the apoptosis rate was calculated as follows:

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RESULTS

Cytotoxic Effects of OrA on GBC Cells  The structure of OrA is shown in Fig. 1A. CCK-8 assay was first performed to examine the cytotoxic effects of OrA on GBC cells. GBC-SD and NOZ cells were treated with different concentrations of OrA (0, 4, 8, 16, 32, 64, 128, and 256 µM) for 48 h, and CCK-8 assay was conducted to evaluate the cell cytotoxicity. (C) Plate clone formation assay of NOZ cells exposed to varying concentrations of OrA (0, 10, 20, and 40 µM). (D) Colony formation rate (%). All experiments were repeated at least three times, and the data were reported as mean ± standard deviation. *p < 0.05, **p < 0.01 or ***p < 0.001 vs. untreated group.

Fig. 1. OrA Suppressed the Proliferation of GBC Cells

(A) The chemical structure of OrA. (B) Cytotoxic effects of OrA on GBC-SD and NOZ cells. GBC-SD and NOZ cells were treated with different concentrations of OrA (0, 4, 8, 16, 32, 64, 128, and 256 µM) for 48 h, and CCK-8 assay was conducted to evaluate the cell cytotoxicity. (C) Plate clone formation assay of NOZ cells exposed to varying concentrations of OrA (0, 10, 20, and 40 µM). (D) Colony formation rate (%). All experiments were repeated at least three times, and the data were reported as mean ± standard deviation. *p < 0.05, **p < 0.01 or ***p < 0.001 vs. untreated group.

OrA Inhibits NOZ Cells Proliferation  Based on the confirmed concentration of OrA, we further explored the effects of OrA on the proliferation of GBC cells. A colony formation assay was used to evaluate the effects of OrA on the proliferation of NOZ cells. OrA treatment significantly inhibited the colony forming ability of NOZ cells as compared to that of the untreated cells (Fig. 1C) with the colony formation rate indicated in Fig. 1D.

OrA Promotes Apoptosis of NOZ Cells  Cell apoptosis was first investigated by using Hoechst 33258 staining method. Normal-blue fluorescence represented normal cells, whereas bright-blue fluorescence in the condensed nuclei represented apoptotic cells. Results indicate that OrA promoted the percentage of apoptotic NOZ cells in a dose dependent manner (Figs. 2A, B).

An Annexin V-FITC/PI detection kit was used to evaluate the OrA induced NOZ cell apoptosis. As indicated (Figs. 2C, D), treatment with OrA dramatically promoted NOZ cell apoptosis.

Previous studies indicate that Bcl-2 as an important factor in modulating cell apoptosis. The high expression levels of pro-apoptotic protein BAX have also been reported to promote apoptosis in GBC cells. We thus explored the effect of
OrA treatment on BAX and Bcl-2 in NOZ cells. Our findings indicate that OrA treatment inhibited the expression of Bcl-2 but promoted the expression of BAX in a dose-dependent manner (Fig. 2E).

Cleaved-caspase-3 and cleaved-caspase-8 play a master role in modulating NOZ cell apoptosis. We thus further explored the effect of OrA on cleaved-caspase-3 and cleaved-caspase-8. OrA treatment increased the expression levels of cleaved-caspase-3 and cleaved-caspase-8 in a dose-dependent manner (Fig. 2F).

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OrA Inhibits the Migration and Invasion of NOZ Cells

Wound healing assays were performed to examine the protective effect of OrA on cell migration. OrA treatment dramatically attenuated the migration of NOZ cells in a dose-dependent manner compared to that in the control (untreated) group (Figs. 3A, B).

Transwell assay is a classical assay to evaluate the invasive ability of cancer cells. As shown (Figs. 3C, D), the control (untreated) group had a higher rate of cell invasion, whereas the OrA-treated NOZ cells showed a decrease in the invasion
MMPs are highly associated with cancer cell invasion and migration because of their ability to degrade the extracellular matrix.\textsuperscript{19} We thus further explored the effects of OrA treatment on MMP-2 and MMP-9 expression. Results revealed that OrA treatment attenuated the expression of MMP-2 and MMP-9 in a dose-dependent manner compared to that in the control (untreated) group (Fig. 3E).

\textbf{OrA Exerts Its Antitumor Effects via Suppressing the PTEN/PI3K/AKT Signaling Pathway} To investigate the underlying mechanism of action of OrA exerting its antitumor effects on human GBC cells, Western blotting was performed to examine the expression levels of PTEN, PI3K, AKT, and Thr\textsuperscript{308}P-AKT. As shown in Fig. 4A, OrA significantly up-regulated the expression of PTEN and suppressed the expression of Thr\textsuperscript{308}P-AKT in a dose-dependent manner. Hence, to confirm the association between OrA and AKT, we examined...
whether OrA-mediated suppression of the activation of AKT could be rescued via treatment with SC79, a specific AKT agonist. As the results of Western blotting indicated, SC79 significantly rescued the OrA-induced downregulation of AKT (Fig. 4B). Our findings indicate that OrA exerts its anti-tumor effects on GBC cells via suppressing the PTEN/PI3K/AKT signaling pathway.

**OrA Suppresses the Growth of NOZ Xenografts in Nude Mice**  With the confirmed antitumor effects of OrA on GBC cells in vitro, we further examined the anti-tumor effects of OrA on GBC in vivo. NOZ cells were subcutaneously injected into nude mice to establish a GBC tumor model. Mice in the treatment group were administered OrA (50 mg/kg) intraperitoneally every other day, while mice in the control group were administered an equal volume of saline intraperitoneally. Tumors removed from the nude mice are shown in Fig. 5A, and the mean weight and volume are shown in Figs. 5B and C. These results indicate a significant inhibition of gallbladder tumor growth using OrA treatment in mice.
DISCUSSION

Although GBC is a rare type of carcinoma, it is considered the most common biliary tract malignancy, leading to approximately 80–95% of biliary tract cancers. 10) GBC is characterized as a “silent” cancer because it is asymptomatic until it progresses into a non-curable stage. Only a few patients (less than 10%) are able to get the tumor tissues resected, and the prognosis is often abysmal. 20) In addition, alternative therapeutic strategies including chemotherapy and radiotherapy remain inadequately developed for GBC treatment, and they can only delay GBC progression. Therefore, there is an urgent need to develop potential therapeutic agents targeting GBC to improve the prognosis of patients with GBC.

OrA is a natural flavonoid, which is extracted from several medicinal plants such as Scutellariae species (Radix Scutellariae). 10) There is a plethora of accumulated evidence demonstrating the antitumor effects of OrA in various diseases. OrA suppresses human breast cancer invasion and proliferation via inhibiting the activation of extracellular signal-regulated kinase (ERK). 21) Besides, OrA attenuates lung cancer progression via suppressing the activation of AKT. 21) Thus, we considered OrA a potential candidate for GBC therapy and explored its antitumor effects in vitro and in vivo.

In the present study, we first determined the effect of OrA on the proliferation of GBC cell lines. Our data show that OrA significantly attenuated the plate clone formation in a dose-dependent manner, indicating that OrA could suppress the proliferation of GBC cell lines. Next, Hoechst 33258 staining, flow cytometry, and Western blotting were performed to assess if OrA treatment could affect GBC cell apoptosis. OrA dramatically promoted the apoptotic induction rate in GBC cells in a dose-dependent manner. As Bcl-2 and BAX have been shown to be responsible for modulating cancer cell apoptosis, 23) we evaluated the effect of OrA on BAX and Bcl-2. Western blotting revealed that OrA upregulated the expression of BAX but downregulated the expression of Bcl-2, and the BAX/Bcl-2 ratio increased after stimulation with OrA. Caspases have been reported as vital mediators modulating cell death (apoptosis). 24) The expression levels of activated caspases, including cleaved-caspase 3 and cleaved-caspase 8, are highly associated with GBC cell apoptosis. 25, 26) Hence, we then investigated the effect of OrA on cleaved-caspase 3 and cleaved-caspase 8 and found that OrA suppressed cleaved-caspase 3 and cleaved-caspase 8 in a dose-dependent manner. Next, a series of experiments were performed to detect the effects of OrA on the migration and invasion of GBC cells. Our results revealed the inhibitory effects of OrA on GBC cell migration. Furthermore, transwell assay, which was carried out to evaluate the anti-invasion function of OrA, also demonstrated that OrA could significantly attenuate the invasive potential of GBC cells. Previous studies have indicated that MMPs promote cancer cell migration and invasion 27, 28), we thus evaluated the effect of OrA on two main MMPs: MMP-2 and MMP-9. Our results suggest that OrA mediated the inhibition of MMPs expression. Taken together, our study reveals that OrA can significantly attenuate the proliferation, migration, and invasion of GBC cells while also promoting apoptosis; however, the underlying mechanism is still unclear.

Several studies have highlighted the role of the PTEN/PI3K/AKT signaling pathway in regulating a variety of biological effects, including cell proliferation, metabolism, and apoptosis. 29) PTEN has been reported as a negative regulator of the PI3K/AKT signaling pathway, and the activation of PTEN negatively correlates with the activation of AKT, thereby suppressing tumor progression. 29) Furthermore, previous studies have indicated that suppression of the PI3K/AKT signaling pathway can also attenuate GBC cell proliferation, 30) migration, and invasion 31); thus, we further explored the effects of OrA on the PTEN/PI3K/AKT pathway. Treatment with OrA promoted the expression of PTEN and also blocked the activation of AKT in a dose-dependent manner. Next, to confirm the direct association between OrA and active AKT, a specific AKT agonist-SC79 was utilized. Treatment with SC79 rescued OrA-induced downregulation of AKT. Thus, these data show that OrA can suppress human GBC via modulating the PTEN/PI3K/AKT signaling pathway.

After the in vitro studies, nude mice were subcutaneously injected with NOZ cells to develop an NOZ tumor xenograft model, and OrA was administered to treat the tumor growth. An obvious suppression of tumor growth occurred after OrA treatment, and both the tumor weight and volume decreased significantly compared to those in the control (untreated) group. These data indicate OrA as a potential agent with antitumor effects both in vitro and in vivo.

Our study has several limitations as well. First, we only evaluated the anti-tumor effects of OrA on one type of GBC cell line due to the limitation of our experimental design. Second, we only evaluated the antitumor effects of OrA on the PTEN/PI3K/AKT signaling pathway, which is a classical signaling pathway, and further exploration of other cancer-related signaling pathways is crucial.

In conclusion, our study demonstrated that OrA is an effective antitumor agent that can attenuate the proliferation, migration, and invasion of human GBC cells while also promoting cell apoptosis. The underlying mechanism of action involves suppression of the PTEN/PI3K/AKT signaling pathway, especially the direct inhibition of AKT activation. Besides, the in vivo results revealed that OrA can decrease gallbladder tumor growth in vivo. These results suggest that OrA could be a potential antitumor agent for human GBC therapy in the near future.

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Conflict of Interest The authors declare no conflict of interest.

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