Original Article

Resibufogenin inhibits ovarian clear cell carcinoma (OCCC) growth in vivo, and migration of OCCC cells in vitro, by down-regulating the PI3K/AKT and actin cytoskeleton signaling pathways

Guannan Zhou1,2*, Zhongyi Zhu1,2*, Lihua Li3, Jingxin Ding1,2

1Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, China; 2Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China; 3Department of Cell Biology, Taizhou University, 1139 Shifu Road Jiaojiang District, Taizhou, China. *Equal contributors.

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Abstract: Patients diagnosed with ovarian clear cell carcinoma (OCCC), a rare histologic subtype of ovarian cancer, often experience poor prognosis owing to the chemoresistance of their disease. Thus, there is an urgent need to identify new therapeutic options for these patients. A drug screen of 172 traditional Chinese herbs identified resibufogenin as a compound that inhibited the growth of cultured OCCC cells. Resibufogenin, a bioactive compound originally isolated from toad venom, is used in traditional Chinese medicine to treat several malignancies. The current study examined the impact of resibufogenin treatment on proliferation, migration, and invasion of ES-2 and TOV-21G OCCC cells in vitro. Flow cytometric analyses were employed to determine if resibufogenin affects apoptosis in OCCC cells. Additionally, the ability of resibufogenin to inhibit tumor growth in vivo was evaluated in murine xenograft models. RNA sequencing, quantitative polymerase chain reactions (qPCR), immunohistochemical assays, and western blotting were used to identify and verify cellular pathways potentially targeted by resibufogenin. Resibufogenin inhibited proliferation, migration, and invasion of OCCC cells, and induced apoptosis in them. Resibufogenin also suppressed the growth of xenograft tumors, which consequently showed lower Ki-67 and higher terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) expression. We observed down-regulation of (a) PI3K and AKT in the PI3K/AKT signaling pathway, and (b) MDM2 and myosin in the actin cytoskeleton pathway upon resibufogenin treatment. Thus, resibufogenin inhibits growth and migration of OCCC cells in vitro and suppresses OCCC growth in vivo through the PI3K/AKT and actin cytoskeleton signaling pathways.

Keywords: Resibufogenin, ovarian clear cell carcinoma, apoptosis, migration, PI3K/AKT pathway, actin cytoskeleton

Introduction

Ovarian cancer is one of the leading causes of cancer-related deaths among women worldwide [1, 2], accounting for 5-6% of all cancer-related deaths. Ovarian clear cell carcinoma (OCCC) accounts for approximately 5-25% of epithelial ovarian cancers (EOC), and its incidence varies by population [3-5]. Although rare, it presents a unique treatment challenge; OCCC carries a very poor prognosis in large part due to the extremely high rate of resistance to standard cis-platinum and paclitaxel combination chemotherapy [5-8]. Epidemiologically, histologically, and clinically, OCCC is a distinct subtype of EOC, and is different from the commonest subtype of EOC, high-grade serous ovarian carcinoma (HGSOC).

While OCCC tends to present at earlier stages, women with advanced disease have a poorer prognosis compared to women with other EOC subtypes [9]. A median overall survival of 21.3 months has been reported for women with OCCC, compared to a median overall survival of 40.8 months for those with HGSOC. It is believed that the disparity in outcomes may be attributed, at least in part, to tumor resistance
to chemotherapy. While platinum-based chemotherapy is standard first-line treatment for EOC, OCCC does not respond well to this regimen. In a multi-center retrospective study of patients with stage III/IV OCCC with measurable disease after surgery, response to platinum-based chemotherapy was 11.1%, as compared with 72.5% in patients with HGSOC [9, 10]. Another analysis of data from multiple clinical trials for advanced ovarian cancer found that overall response rates were 45% for OCCC versus 81% for HGSOC [11, 12]. Thus, to improve outcomes for patients diagnosed with OCCC, it is critical to overcome chemo-resistance or identify new drugs for therapy.

In recent years, researchers have noted the potential antitumor activity of natural products used in traditional Chinese medicines. We screened ethanol extracts of 172 traditional Chinese herbs against ES-2 and TOV-21G OCCC cells and found that the ethanol extracts of several traditional Chinese medicines inhibit growth of these cells. Then, the bioactive compounds were isolated from those Chinese medicines. One of them was resibufogenin, a traditional Chinese medicine that has been used to treat malignancies for several decades in China. Resibufogenin (3-hydroxy-14,15-epoxy-20,22-diennolide glycoside, C_{24}H_{32}O_{4}) is the bioactive component of toad venom and belongs to steroid family of compounds [13]. Its chemical structure is shown in Figure 1A. Resibufogenin is mainly obtained from the skin and parotid venom glands of toads, including Bufo Bufo gargarizans Cantor and Bufo melanostictus Schneider. Numerous physiological and pharmacological effects including cardio tonic effects, platelet inhibition, vascular contraction, antiepileptic, and local anesthetic actions have been reported for resibufogenin [14]. Resibufogenin inhibits the growth of tumor cells such as human hepatocellular cancer cells, and human colon cancer cells [13-16]. However, the precise molecular mechanism of cancer cell growth inhibition by resibufogenin is still unknown.

Therefore, this study was designed (1) to examine the effects of resibufogenin on proliferation and migration of OCCC cells in vitro, (2) to examine the ability of resibufogenin to inhibit tumor growth in vivo in xenograft models, and (3) to explore the molecular mechanism underlying the anti-cancer activity of resibufogenin in OCCC.

Materials and methods

Cell lines and reagents

We obtained the ES-2 and TOV-21G OCCC cell lines from the American Type Culture Collection. These cells were grown in Roswell Park Memorial Institute (RPMI) 1640 Medium (Hy clone, Pittsburgh, PA) with 10% fetal bovine serum (FBS; Hy clone), 1% penicillin, and 1% streptomycin, at 37°C in 5% CO_2. Resibufogenin was obtained from MedChem Express Chemical Co. (Shanghai, China) and dissolved in DMSO, to a final concentration (v/v) of 0.1%.

Cell viability assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, approximately 5 × 10^3 cells were seeded in 96-well plates. After cells had adhered overnight, they were either treated with DMSO (as control) or with 20 μM resibufogenin for the indicated durations, followed by addition of 10 μl CCK-8 reagent per 100 μl culture medium. Cells were cultured for an additional hour at 37°C before absorbance was measured at 450 nm using a spectrophotometer. All experiments were repeated three times.

Migration assay

ES-2 and TOV-21G OCCC cells were starved for 12 hours in serum-free medium. A total of 1 × 10^5 cells were resuspended in 200 μl of serum-free medium before being seeded into the upper Transwell chamber (Corning, New York, NY) with DMSO (as control) or 20 μM resibufogenin. Then, 600 μl medium with 10% FBS was added to the lower chamber to act as the chemotactrant, and cells were cultured at 37°C. After 12 hours, cells on the upper surface of the chamber were carefully cleansed with a cotton swab to remove culture medium and cells that had not migrated through the insert. Cells that had migrated through the filter pores to the underside of the insert were fixed with 4% paraformaldehyde for 30 minutes, and then stained with 0.1% crystal violet for 10 minutes. Finally, an upright metallurgical microscope was used to photograph five random fields in the membrane underside, and cells that had migrated were counted. All experiments were repeated in triplicate.
Inhibition of ovarian clear cell carcinoma by resibufogenin

ES-2 and TOV-21G OCCC cells were starved in serum-free medium for 12 hours. The Transwell chamber was pre-applied with 50 μl Matrigel (BD Biosciences, San Jose, CA). A total of 1 × 10^5 cells were resuspended in 200 μl of serum-free medium and seeded into the upper Transwell chamber (Corning) with DMSO (as control) or 20 μM resibufogenin. To act as a chemoattractant, 600 μl medium containing 10% FBS was added to the lower chamber, and cells were cultured at 37°C. After 24 hours, invaded cells on the lower surface were fixed with 4% paraformaldehyde for 30 minutes, and then stained with 0.1% crystal violet for 10 minutes. Finally, an upright metallurgical microscope was used to photograph five random fields in the underside of the insert, and cells that had successfully invaded were counted. All experiments were repeated in triplicate.

Flow cytometric analysis of apoptosis

Phycoerythrin (PE) Annexin V Apoptosis Detection Kit I (BD Biosciences) was used to detect apoptotic cells following the manufacturer’s protocol. After ES-2 and TOV-21G cells were treated with 20 μM resibufogenin for 24 hours, they were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 1 × Binding Buffer at a concentration of 1 × 10^6 cells/ml. Then, 5 μl PE Annexin V and 5 μl 7-Amino-Actinomycin (7-AAD) were added to the Binding Buffer and cells were incubated at room temperature for 15 minutes in the dark. Thereafter, apoptotic cells were detected via flow cytometry (FV500, Beckman Coulter, Brea, USA). The PE Annexin V-positive, 7-AAD-positive cell population were considered as the apoptotic cell population. FlowJo 7.6 (FlowJo, LLC, Ashland, OR) was used to analyze the flow cytometry data. Each experiment was conducted in triplicate.

Mouse xenograft assay

Four weeks-old female athymic nude mice (BALB/c, nu/nu) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). They were housed and maintained in a standard environment and the protocol was approved by the Animal Care and Research Committee of Fudan University. The mice were injected in their flanks subcutaneously with 1 × 10^7 TOV-21G cells suspended in 200 μl of RPMI 1640 medium. Mice were randomly divided into 2 groups (n = 3 per group)-the control group and the resibufogenin treatment group. Mice in the two groups were injected intraperitoneally with PBS or resibufogenin (20 mg/kg/day), respectively, for 21 consecutive days. We
Inhibition of ovarian clear cell carcinoma by resibufogenin

measured tumors sizes using calipers every day. Tumor volumes were calculated as follows: volume = 0.5 × length × width². After treatment for 21 days, the mice were euthanized and tumors were removed carefully, photographed, and fixed with 4% paraformaldehyde for the following immunohistochemistry assay.

**Immunohistochemistry assay**

Formalin-fixed paraffin-embedded murine tumor tissues were sectioned into 5-mm-thick sections. After deparaffinization and dehydration, the sections were subjected to antigen retrieval at 95°C for 30 minutes and were blocked with goat serum for 1 hour. Then, the sections were incubated with primary antibodies at 4°C overnight and with horseradish peroxidase (HRP)-conjugated secondary antibodies at 37°C for 45 minutes. Antibodies against Ki-67, PI3K, AKT, and MDM2 were purchased from Abcam (Cambridge, MA), and cell apoptosis was assessed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an In-Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Five images at 400 × magnification were selected at random to evaluate the average number of Ki-67-, TUNEL-, PI3K-, AKT-, and MDM2-positive cells.

**RNA sequencing and analysis of differential mRNA expression**

TOV-21 cells were seeded in 6-well plates at a density of 1 × 10⁶ cells/well and incubated with 20 μM resibufogenin or PBS for 6 hours and 12 hours, respectively. After incubation, total RNA of cells belonging to the four experimental groups was isolated using Trizol. After the mRNA library was established and checked to ensure that the mRNA quality was acceptable, differential mRNA expression was analyzed. Tophat software (version 2.0.9) was used to align the RNA-seq reads to the *Homo sapiens* reference genome. Cufflinks software (version 2.1.1) was used to assemble these mapped reads into possible transcripts and generate a final transcriptome assembly. The transcripts of different samples were consolidated by Cuffmerge. Comprehensive and non-redundant transcript information of each sample was thus obtained. Differential gene expression was analyzed by Cuffdiff software and genes that were differentially expressed in the treated samples were identified.

**KEGG pathway analysis of differentially expressed genes**

Different genes in organisms coordinate to perform various biological functions. Pathway analysis helps to further understand these biological functions. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a major public database used for pathway mapping and analysis to identify key biochemical and signal transduction pathways regulated by genes of interest. For resibufogenin-treated TOV-21G cells, KEGG enrichment analysis was carried out with differentially expressed genes as the foreground, and all the remaining transcripts as the background. Hypergeometric test/Fisher's exact test was performed to calculate the $P$-value of foreground genes with a certain branch in pathway classification, which was corrected for False Discovery Rate (FDR).

**Real-time fluorescence quantification PCR test**

The impact of resibufogenin on the expression levels of genes involved in the PI3K-AKT and actin cytoskeleton pathways was determined by real time fluorescence quantitative PCR (qRT-PCR) that was performed using SYBR green (Invitrogen, Waltham, MA). TOV-21 cells were incubated with 20 μM resibufogenin for 24 hours, after which total RNA was isolated using Trizol. Equal quantities of RNA (2 μg) from each sample were used to synthesize cDNA with PrimeXcript RT Master Mix (Takara, Mountain View, CA). qRT-PCR was performed with the 7900HT Fast Real-Time PCR System (Thermo Fisher, Waltham, MA). The polymerase chain reaction conditions were as follows: 50°C for 2 minutes and 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The primers for PI3K, MDM2, GRLF1, Myosin-II, RTK, and SOS were synthesized by BioTNT (GuanTai, China).

The qRT-PCR primers were as follows: PI3K forward: 5'-GAGATTGCAAGCAGTATGTTG-3' and PI3K reverse: 5'-TAATTTTGGCAGTGGTGGGG-3'; MDM2 forward: 5'-CTCTCTAGAGAGATTTGTTGCG-3' and MDM2 reverse: 5'-CTCTCTAGAGAGATTTGTTGCG-3'; GRLF1 forward: 5'-GAGATTGCAAGCAGTATGTTG-3' and GRLF1 reverse: 5'-TAATTTTGGCAGTGGTGGGG-3'; Myosin-II forward: 5'-GAGATTGCAAGCAGTATGTTGCG-3' and Myosin-II reverse: 5'-CTCTCTAGAGAGATTTGTTGCG-3'; RTK forward: 5'-GAGATTGCAAGCAGTATGTTGCG-3' and RTK reverse: 5'-GAGATTGCAAGCAGTATGTTGCG-3'.
Inhibition of ovarian clear cell carcinoma by resibufogenin

TCAAGACTAACCAGA-3'; SOS forward: 5'-CAGCTTTTGAGATGTTGTGGA-3' and SOS reverse: 5'-GGGAGCTTGGTAATGTAAGA-3'; GAPDH was chosen as the internal control gene, and the results were analyzed by comparing 2^ΔΔCT values.

Western blotting analysis

OCCC cell lines ES-2 and TOV-21G cells were cultured with or without 20 μM resibufogenin for 24 hours. Cells were harvested and lysed with RIPA (Beyotime, Shanghai, China), supplemented with phenylmethyl sulfonyl fluoride (PMSF) and phosphotransferase inhibitor (Beyotime, Shanghai, China). Protein concentration was determined with a Bicinchoninic Acid (BCA) kit (Beyotime, Shanghai, China), and 30 μg of protein was loaded onto SDS-PAGE gels for electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% fat-free milk for 1 hour at room temperature and incubated in primary antibodies at 4°C overnight. Membranes were incubated with HRP-conjugated secondary antibodies (1:4000) (Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature and detected with Immobilon Western substrate (Millipore, Burlington, MA). Anti-PI3K, anti-AKT, anti-MDM2, anti-Myosin-II, and anti-actin antibodies were purchased from Abcam. All primary antibodies were monoclonal and applied at a dilution of 1:1000. All experiments were repeated three times.

Statistical analysis

SPSS 23.0 software was used for statistical analysis. The experimental data were presented as mean ± standard deviation. T tests were used for comparisons of means between two groups, and one-way ANOVA tests were used for comparisons of more than two groups. Differences were considered to be significant at a P-value of less than 0.05.

Results

Resibufogenin inhibits proliferation of OCCC cells

The in vitro cytotoxicity of resibufogenin (chemical structure shown in Figure 1A) against ES-2 cells and TOV-21G cells was measured using the CCK-8 method. As shown in the phase contrast images in Figure 1A and 1C, resibufogenin in treatment resulted in rounded and shrunken cells, and a decreased number of cells as compared to the control group. Furthermore, this decrease in cell viability was concentration-dependent (Figure 1B and 1D). Based upon this assay, the IC50 value for resibufogenin was around 20 μM.

Resibufogenin inhibits migration and invasion of OCCC cells

To evaluate the effect of resibufogenin on the migratory abilities of OCCC cells, ES-2, and TOV-21G cells were treated with resibufogenin (20 μM) and subjected to transwell migration assay without Matrigel. Resibufogenin significantly inhibited migration of treated cells as compared to the control group in both the ES-2 and TOV-21G cell lines (Figure 2A and 2B). In addition, to determine if resibufogenin treatment affects invasion in OCCC cells, ES-2, and TOV-21G cells were treated with 20 μM resibufogenin and subjected to transwell invasion assay with Matrigel. Resibufogenin significantly inhibited cell invasion as compared to control group in both ES-2 and TOV-21G cells (Figure 2C and 2D). Our results show that resibufogenin (20 μM) effectively inhibits cell migration and invasion in OCCC cells.

Resibufogenin induces apoptosis in OCCC cells

To determine if resibufogenin treatment affects cell survival/apoptosis in OCCC cells, flow cytometric analysis of PE-Annexin V- and 7-AAD-stained resibufogenin-treated and untreated cells was performed. As shown in Figure 3A and 3C, after a 24-hour treatment with resibufogenin (20 μM), both ES-2 and TOV-21G cells showed an increase in the apoptotic cell population as compared to the control group (Figure 3B and 3D). These results indicate that resibufogenin increases apoptosis of OCCC cells.

Resibufogenin suppresses in vivo tumor growth in xenograft models of OCCC

Nude mice with TOV-21G cell tumor xenografts were continuously injected intraperitoneally with PBS (control) or resibufogenin (20 mg/kg/day) until study termination. After 21 days, a significant decrease in tumor volumes was observed in the resibufogenin-treated mice when compared to tumor volumes in the vehicle-treated group (Figure 4A-C). Immunohistochemical staining of mouse tumor sections show-
Inhibition of ovarian clear cell carcinoma by resibufogenin

Figure 2. Resibufogenin inhibits cell migration and invasion in ES-2 and TOV-21G cell lines. ES-2 and TOV-21G cells were divided into two groups: the control group and resibufogenin-treated group (20 μM). Transwell assays were used to measure cell migration and invasion after the treatment. Photomicrographs (left) and scatter plots (right) show that cell migration (A and B) and invasion (C and D) were inhibited by resibufogenin. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Inhibition of ovarian clear cell carcinoma by resibufogenin

Figure 3. Effects of resibufogenin on apoptosis in ES-2 and TOV-21G cell lines. Apoptosis was evaluated using PE Annexin V and 7-AAD staining in ES-2 and TOV-21G OCCC cells after treatment with resibufogenin (20 μM) for 24 hours. Two-parameter (dual color fluorescence) dot plots (A and C) were obtained via flow cytometric analysis of cells from the indicated experimental groups. Bar graphs (B and D) depict mean ± SD of three replicates. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. Resibufogenin inhibits tumor growth in a TOV-21G mouse xenograft model. A. Photomicrographs showing mice from the two experimental groups in the xenograft experiments. B. Photomicrographs showing the xenograft tumors resected from the mice after they were euthanized. C. Graphs showing tumor volumes in control mice and resibufogenin-treated mice. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5. Resibufogenin inhibits proliferation of OCCC cells and induces apoptosis in vivo. Representative photomicrographs (100 × and 400 × magnification) showing immunohistochemically stained sections of xenograft tumor tissues stained for Ki-67 (A, left) and TUNEL (B, left) expression. Scatter plots show the percentage of Ki-67-positive cells (A, right) and TUNEL-positive cells (B, right) in the xenograft tumors resected from mice belonging to the two experimental groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Inhibition of ovarian clear cell carcinoma by resibufogenin

KEGG pathway analysis of genes differentially expressed upon resibufogenin treatment

Figure 6. Microarray analysis of mRNA extracted from TOV-21G cells treated with resibufogenin for 6 hours and 12 hours. TOV-21G cells were treated with resibufogenin (20 μM), and cells were hierarchically clustered into four groups based on the treatment durations. The heat map shows gene expression levels in each group (horizontal axis) plotted by genes (vertical axis). The tree displays the log-transformed value of average fold changes. Red color indicates up-regulated levels, whereas blue color indicates down-regulated levels of genes. Each mapped gene was divided into four segments that represent the different durations of treatment with resibufogenin; from left to right: control-6 hours, resibufogenin-treated-6 hours, control-12 hours, and resibufogenin-treated-12 hours.

RNA sequencing and analysis of differential gene expression in resibufogenin-treated OCCC cells

To gain insights into the signaling pathways targeted by resibufogenin, we performed RNA sequencing analysis of TOV-21G cells treated with resibufogenin for 6 hours and 12 hours. A total of 814 genes were differentially expressed between the resibufogenin-treated group and the control group (Figure 6). The expression levels of 335 genes were downregulated, whereas 479 genes were upregulated.

Resibufogenin inhibited OCCC proliferation and migration via the PI3K-AKT and actin cytoskeleton pathways

To investigate the molecular mechanism whereby resibufogenin inhibits proliferation and migration, we focused on the PI3K-AKT and actin cytoskeleton pathways. The effects of resibufogenin on these two pathways were confirmed at the mRNA and protein levels using qRT-PCR and Western blotting, respectively, to check the relative expression levels of key genes and proteins in these pathways. The qRT-PCR assay showed that the gene expression levels of PI3K, MDM2, PI4P5K, GRLF1, and Myosin-II were significantly decreased upon exposure to resibufogenin (Figure 8). Meanwhile, the Western blotting results also revealed that resibufogenin significantly inhibited PI3K, AKT, MDM2, and Myosin-II expression (Figure 9). These findings were further corroborated by immunohistochemical staining of tumor tissues excised from mice; the xenograft tumors from the resibufogenin-treated group showed a significant decrease in the expression of PI3K, AKT, and MDM2 compared to their expression levels in the control group (Figures 10, S1, and S2). These results are consistent with the KEGG enrichment results, which revealed that PI3K-AKT and actin cytoskeleton signaling pathways were transcriptionally down-regulated by resibufogenin in OCCC cells. We also observed that the gene expression levels of RTK and SOS was decreased upon resibufogenin treatment.

Figure 5A and 5B). These data suggest that resibufogenin inhibits OCCC proliferation and induces apoptosis in vivo.
Inhibition of ovarian clear cell carcinoma by resibufogenin

Taken together, these results suggest that resibufogenin inhibits the expression of a number of genes responsible for cell survival, proliferation, and migration through down-regulation of the PI3K-AKT and actin cytoskeleton pathways.

Discussion

The study presented herein strongly suggests that the bioactive compound resibufogenin inhibits proliferation, migration, and invasion of OCCC cells, and induces apoptosis effectively both in vitro and in vivo through down-regulation of the PI3K/AKT and actin cytoskeleton pathways.

Although previous studies have reported the apoptosis-enhancing effects of resibufogenin in different types of human cancers including liver cancer, colon cancer, and osteosarcoma, no studies so far have performed an in-depth evaluation of the effect of resibufogenin on the proliferation, migration, and invasion of OCCC cells and the molecular underpinnings of these anti-neoplastic effects. Other studies have
Inhibition of ovarian clear cell carcinoma by resibufogenin

Figure 8. Resibufogenin down-regulates expression of genes in the PI3K/AKT and actin cytoskeleton pathways. ES-2 and TOV-21G cells were divided into 2 groups: the control group, and the resibufogenin-treated group (20 μM). qRT-PCR was performed to detect the mRNAs levels of PI3K (A and E), MDM2 (B and F), GRLF1 (C and G), Myosin-II (D and H), RTK (I and J), and SOS (K and L) after 12 hours of treatment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 9. Resibufogenin down-regulates expression of proteins in the PI3K/AKT and actin cytoskeleton pathways in vitro. ES-2 and TOV-21G cells were divided into 2 groups: the control group, and the resibufogenin-treated group.
Inhibition of ovarian clear cell carcinoma by resibufogenin

reported that high dosages of resibufogenin may result in significant toxicities. In our studies, we used relatively less cytotoxic dosages of 20 μM for in vitro assays, and 20 mg/kg/day for in vivo experiments to evaluate the effect of resibufogenin on OCCC. In addition, mRNA sequencing technology and KEGG pathway enrichment analyses were used to identify the pathways targeted by resibufogenin and advance our understanding of the molecular mechanisms through which resibufogenin affects OCCC cells. Resibufogenin down-regulated the PI3K/AKT and actin cytoskeleton signaling pathways, resulting in the inhibition of proliferation, migration, and invasion in OCCC cells, and the robust induction of apoptosis both in vitro and in vivo.

An imbalance in apoptotic and survival signaling pathways leads to cancer progression [19]. Recent studies have identified potential driver genes and aberrant signaling pathways involved in development of OCCC [20-22], including genes such as ARID1A, PTEN, RTK, and numerous components in the PI3K/AKT pathway. PI3K/AKT signaling pathway [20, 23] is a major signaling pathway leading to recruitment and activation of the AKT serine/threonine kinase, which plays an important role in tumorigenesis by regulating critical cellular functions including survival, proliferation, and angiogenesis. Previous studies have showed that the overall survival of patients with activated PI3K/AKT and RTK/Ras signaling pathways was significantly higher than that of patients in whom these pathways were down-regulated, and that activation of the PI3K/AKT and RTK/Ras signaling pathways was significantly and independently associated with better prognosis in patients with OCCC [9, 24]. Moreover, the PI3K/AKT signaling pathway is believed to be a potential therapeutic target in OCCC [7, 11, 25].

The migration and invasion processes in cancer cells are regulated by several signaling pathways including actin cytoskeleton pathway [26, 27]. The cytoskeleton maintains the shape and internal organization of cells, and also

(20 μM). Western blotting assay was performed to detect the protein levels of PI3K (A and B), AKT (A), MDM2 (D), and Myosin-II (D) after 24 hours of treatment. The relative expression levels of the indicated proteins (relative to the expression level of actin) were quantitated using densitometry and are depicted bar graphically in (B, C, E, and F). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 10. Resibufogenin inhibits proliferation and invasion of OCCC cells in vivo through the PI3K/AKT and actin cytoskeleton pathways. Representative photomicrographs showing immunohistochemically stained xenograft tumor sections (100 × and 400 × magnification) showing PI3K, AKT and MDM2 expression.
functions in cell division and migration. Cell migration is initiated by an actin-dependent protrusion of the cell’s leading edge, which is composed of structures called lamellipodia and filopodia. Therefore, the actin cytoskeleton plays a fundamental role in cell migration, a critical step in cancer metastasis and invasion [18, 27].

Our results showed that the expression levels of key components of the PI3K/AKT and actin cytoskeleton pathways were downregulated in OCCC cells after treatment with resibufogenin. Combined with the earlier viewpoints, the results from our current study suggest that resibufogenin (a) induces apoptosis in OCCC cells via inhibition of the PI3K/AKT signaling pathway and (b) inhibits migration and invasion abilities of OCCC cells by targeting the actin cytoskeleton signaling pathway.

To our knowledge, this is the first pre-clinical study investigating the effect of resibufogenin on OCCC, and we identified putative pathways targeted by this compound (Figure 11). However, a limitation of this study is that the effects of resibufogenin were studied separately, and not compared with those of cis-platinum alone or with those of combination therapy alongside cis-platinum. Furthermore, the down-regulation of PI3K/AKT and actin cytoskeleton pathways was detected via mRNA-sequencing, qRT-PCR, immunohistochemistry, and western blotting assays. We need to further confirm that resibufogenin mediates its effects in OCCC via the PI3K/AKT and actin cytoskeleton pathways through well-designed experiments involving overexpression and knockdown of key components of these signaling pathways, followed by in vitro and in vivo functional assays involving the overexpression/knockdown OCCC cell lines. In conclusion, resibufogenin shows significant potential therapeutic value for OCCC based on its ability to inhibit OCCC cell growth and migration through the PI3K/AKT and actin cytoskeleton signaling pathways.

Disclosure of conflict of interest

None.
Inhibition of ovarian clear cell carcinoma by resibufogenin

Address correspondence to: Jingxin Ding, Department of Gynecology, The Obstetrics and Gynecology Hospital of Fudan University, 419 Fang-Xie Road, Shanghai 200011, China. E-mail: djxdd@sina.com; Lihua Li, Department of Cell Biology, Taizhou University, 1139 Shifu Road Jiaojiang District, Taizhou, China. E-mail: lilihua1018@sina.com

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Inhibition of ovarian clear cell carcinoma by resibufogenin


Inhibition of ovarian clear cell carcinoma by resibufogenin

**Figure S1.** Photographs of the original western blot membranes showing expression levels of the key proteins in this study. The samples from left to right: ES-2 (Control), TOV-21G (Control), ES-2 (RBG), TOV-21G (RBG), the protein bands from top to bottom: PI3K (110 kDa), AKT (56 kDa), Actin (42 kDa). Resibufogenin decreased the protein levels of PI3K and AKT. **Figure S1** shows representative membranes for the data shown in **Figure 9A**.

**Figure S2.** Photographs of the original western blot membranes showing expression levels of the key proteins in this study. The samples from left to right: ES-2 (Control), ES-2 (Control), TOV-21G (Control), ES-2 (RBG), ES-2 (RBG), TOV-21G (RBG), the protein bands from top to bottom: Myosin-II (200 kDa), MDM2 (55 kDa), Actin (42 kDa). Resibufogenin decreased the protein levels of MDM2 and Myosin-II. **Figure S2** shows representative membranes for the data shown in **Figure 9B**.