Ursolic Acid from Oldenlandia diffusa Induces Apoptosis via Activation of Caspases and Phosphorylation of Glycogen Synthase Kinase 3 Beta in SK-OV-3 Ovarian Cancer Cells

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Although ursolic acid isolated from Oldenlandia diffusa (Rubiacae) was known to have anticancer activities in prostate, breast and liver cancers, the underlying mechanism of ursolic acid in ovarian cancer cells was not investigated so far. In the present study, the apoptotic mechanism of ursolic acid was elucidated in SK-OV-3 ovarian cancer cells by 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, cell cycle analysis and Western blotting. Ursolic acid exerted cytotoxicity against SK-OV-3 and A2780 ovarian cancer cells with IC50 of ca. 50 and 65 µm, respectively. Apoptotic bodies were observed in ursolic acid treated SK-OV-3 cells. Also, ursolic acid significantly increased ethidium homodimer stained cells and sub-G1 apoptotic portion in SK-OV-3 cells. Consistently, Western blotting revealed that ursolic acid effectively cleaved poly(ADP-ribose) polymerase (PARP), caspase-9 and -3, suppressed the expression of survival genes such as c-Myc, Bcl-xL and astrocyte elevated gene (AEG)-1, and upregulated phosphorylation of extracellular signal-regulated kinase (ERK) in SK-OV-3 cells. Interestingly, ursolic acid suppressed β-catenin degradation as well as enhanced phosphorylation of glycogen synthase kinase 3 beta (GSK 3β). Furthermore, GSK 3β inhibitor SB216763 blocked the cleavages of caspase-3 and PARP induced by ursolic acid and proteosomal inhibitor MG132 disturbed down-regulation of β-catenin, activation of caspase-3 and decreased mitochondrial membrane potential (MMP) induced by ursolic acid in SK-OV-3 cells. Overall, our findings suggest that ursolic acid induces apoptosis via activation of caspase and phosphorylation of GSK 3β in SK-OV-3 cancer cells as a potent anti-cancer agent for ovarian cancer therapy.

Key words ursolic acid; SK-OV-3; apoptosis; caspase; astrocyte elevated gene-1; glycogen synthase kinase 3β

Ovarian cancer is the seventh common cause of cancer deaths in women all over the world. In general the treatment consists of a combination of surgery and platinum-based chemotherapy, alone or in combination with paclitaxel. Although recently molecular based therapy developed novel agents, such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors for ovarian cancer treatment, the adverse effects of EGFR inhibitors including skin and gastrointestinal toxicity of erlotinib are still a hot issue to be overcome. Thus, many anticancer phytochemicals from herbal medicine are attractive with little side effects, since they have potentials to sensitize cancer cells to chemotherapy.

Ursolic acid is a pentacyclic triterpene acid, contained in apples, basil, bilberries, cranberry, elder flower, peppermint, rosemary, lavender, oregano, thyme, hawthorn, prunes and medicinal plants such as Oldenlandia diffusa, Erionotrya japonica, Rosmarinus officinalis and Glechoma hederacea. Ursolic acid was known to induce apoptosis in leukemia, prostate cancer, breast cancer, melanoma and colon cancer through various signaling pathways such as Akt, c-Jun N-terminal kinase (JNK), nuclear factor (NF)-κB and reactive oxygen species (ROS). Also, Wang and colleagues reported that ursolic acid increased Bax and mitogen-activated protein kinase phosphatase 1 (MKP-1) proteins but suppressed the proliferation and downregulated the phosphorylation of extracellular signal-regulated kinase (ERK) in CA-0A-3 ovarian cancer cells. Nonetheless, the underlying ovarian cancer mechanism of ursolic acid in SK-OV-3 cells still remains unclear. Thus, in the present study, the caspase-dependent apoptosis by ursolic acid in SK-OV-3 cells was examined in association with glycogen synthase kinase 3 beta (GSK 3β) signaling by 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, cell cycle analysis and Western blotting.

MATERIALS AND METHODS

Isolation of Ursolic Acid Ursolic acid (molecular weight (MW=456) was isolated from Oldenlandia diffusa. In brief, the dried whole herbs (500 g) of O. diffusa were extracted with 90% MeOH at room temperature for 2 weeks. The extract was concentrated to dryness under reduced pressure to give a MeOH extract (42 g), which was partitioned with n-hexane (3.5 g) and 90% MeOH. The n-hexane soluble part was subjected to silica gel column chromatography using a mixture of hexane and EtOAc as an eluting solvent with a gradient of increasing polarity (20:1~2:1) to give five fractions (Fr. 1~Fr. 5). From two fractions (Fr. 4 and Fr. 5) some major compounds were observed using thin layer chromatography. Repeated silica gel column chromatography and crystallization in n-hexane and EtOAc (2:1) afforded 680 mg, which was identified as ursolic acid by NMR (1H and 13C) and Mass Spectrometry (Fig. 1A).

Cell Culture SK-OV-3 human ovarian cancer cells were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with fetal bovine serum and 10% FBS.

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serum (FBS, 10%) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO₂.

**Cytotoxicity Assay**  The cytotoxicity of ursolic acid was measured by XTT colorimetric assay. SK-OV-3 cells were seeded onto 96-well microplates at a density of 1×10⁴ cells per well and treated with ursolic acid for 24 h. XTT working solution was prepared just prior to culture application by mixing 1 mL of XTT stock solution (1 mg/mL in phosphate buffered saline (PBS)) with 10 µL of PMS (1.53 mg/mL in PBS). After incubation at 37°C in a humidified incubator for 24 and 48 h, a 50 µL of XTT working solution was added to each well. Cells were incubated at 37°C for 2 h and the optical density (OD) was measured using microplate reader (Sunrise, TECAN, Mannedorf, Switzerland) at 450 nm. Cell viability was calculated by employing the following equation:

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\text{cell viability} (%) = \frac{\text{OD (drug treatment)} - \text{OD (blank)}}{\text{OD (control)} - \text{OD (blank)}} \times 100
\]

**Ethidium Homodimer Assay**  To measure cell death, we stained SK-OV-3 cells with the 4',6-diamino-2-phenylindole (DAPI) and ethidium homodimer dye (Molecular Probes) using Live/Dead assay kit according to the manufacturer’s instructions. Ethidium homodimer dye can enter cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. In brief, cells were treated with 20 µM ursolic acid for 24 h. After incubation, cells were fixed in 4% methanol-free formaldehyde solution and stained with the 5 µM ethidium homodimer and then incubated at 37°C for 30 min in the dark. Then the cells were mounted with mounting medium containing DAPI and visualized under an Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc., Weimar, Germany).

**Cell Cycle Analysis**  Cell cycle analysis was performed as previously described. SK-OV-3 cells treated with ursolic acid were fixed in 75% ethanol at −20°C, resuspended in PBS containing RNase A (1 mg/mL), and incubated for 1 h at 37°C.
The cells were stained with propidium iodide (PI) (50 µg/mL) for 30 min at room temperature in dark. The DNA contents of the stained cells were analyzed using CellQuest Software with the FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

**Western Blotting** Whole cell lysates were prepared using lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na3VO4, 1 mM NaF, protease inhibitor cocktail). The protein contents in the supernatants were measured by using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, U.S.A.), separated on 4–12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, U.S.A.) and electro-transferred onto a Hybond ECL transfer membrane (GE Healthcare Bio-Science, Piscataway, NJ, U.S.A.). The membranes were blocked with 5% nonfat dry milk and immuno-blotted with anti-cleaved caspase-3, caspase-3, poly(ADP-ribose) polymerase (PARP), cleaved caspase-9, astrocyte elevated gene (AEG)-1, p-GSK 3β, GSK 3β, β-catenin, p-ERK and ERK (Cell Signaling, Danvers, MA, U.S.A.) antibodies. GSK 3β inhibitor SB216763 from Sigma-Aldrich (St. Louis, MO, U.S.A.) was used for confirming the role of GSK 3β in ursolic acid induced apoptosis in SK-OV-3 cancer cells.

**Measurement of Mitochondrial Membrane Potential** SK-OV-3 cells were treated with ursolic acid (20 µM) in the absence or presence of MG132 (20 µM). Cells were stained with tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37°C. Mitochondrial membrane potential was detected by flow cytometry (FACS Vantage SE, Becton Dickinson) at 582 nm.

**Statistical Analysis** Statistical analysis of the data was conducted using Sigmaplot version 12 software (Systat Software Inc., San Jose, CA, U.S.A.). All data were expressed as means±standard deviation (S.D.). The statistically significant differences between control and drug-treated cells were calculated by the Student’s t-test.

**RESULTS**

**Ursolic Acid Exerted the Cytotoxicity in SK-OV-3 Cells**

Ovarian Cancer Cells The cytotoxicity of ursolic acid was evaluated against SK-OV-3 cancer cells using XTT assay. As shown in Figs. 1B and C, ursolic acid exerted cytotoxicity against SK-OV-3 and A2780 ovarian cells with IC50 of ca. 50 and 65 µM, respectively. We observed apoptotic bodies in ursolic acid treated SK-OV-3 cells by ethidium homodimer assay, indicating apoptotic feature, while intact morphology was shown in untreated control (Fig. 2).

**Ursolic Acid Increased Sub-G1 Apoptotic Portion in SK-OV-3 Cancer Cells** To confirm the cytotoxicity of ursolic acid against SK-OV-3 cells was due to apoptosis induction, cell cycle analysis was carried out. As shown in Fig. 3A, ursolic acid accumulated sub-G1 apoptotic portion to 2.25±0.01%, 8.14±1.02%, 24.96±5.12%, and 41.94±2.87% at the concentrations of 5, 10, 20 and 40 µM, respectively, compared to untreated control (1.20±0.38%).

**Ursolic Acid Activated Caspase-9 and -3 and Cleaved PARP in SK-OV-3 Cells** Generally, apoptosis is induced via two distinctive pathways such as cell death extrinsic pathway and mitochondrial dependent intrinsic pathway. Western blotting revealed that ursolic acid cleaved PARP and also activated caspase-9 and -3 in SK-OV-3 cells as shown in untreated control (Fig. 2).

**Ursolic Acid Down-Regulated the Expression of Survival Genes in SK-OV-3 Cells** AEG-1 first cloned as an human immunodeficiency virus (HIV)- and tumor necrosis factor (TNF)-α-inducible gene in primary human fetal astrocytes, is involved in proliferation, angiogenesis and metastasis in cancers. AEG-1 protects from sTNS-α-induced apoptosis by activating phosphatidylinositol 3 kinase (PI3K)/Akt signaling. Conversely, by activating Akt, AEG-1 down-regulates proapoptotic Bad and p21 and upregulates MDM2, nullifying p53 function, thereby exerting anti-apoptotic effect as one of survival genes.10 The ERK1/2, one of mitogen activated protein kinases (MAPKs), is a central signaling pathway that regulates various cellular processes such as proliferation, differentiation, and survival.11 c-Myc is the most commonly overexpressed gene in human cancers. In mammalian cells,

![Fig. 2. Ursolic Acid Mediates Cell Death in SK-OV-3 Cells](Image)

Cells were treated with or without 20 µM ursolic acid for 24 h. Ethidium homodimer dye staining was performed to detect cell death. DAPI was used for counter staining. Stained cells were visualized under fluorescence microscopy at ×200 magnification.

![Control](Image)

![Ursolic acid (20 µM)](Image)
c-Myc expression is highly regulated and closely tied to cell growth, apoptosis and differentiation. Anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL are frequently overexpressed in cancers. The principal site of apoptosis regulation by Bcl-2 family proteins is probably the mitochondrial membrane. Anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) are mainly in mitochondria, protecting against mitochondrial membrane permeabilization. In current study, Western blotting showed that ursolic acid suppressed the expression of AEG-1, c-Myc, Bcl-xL as anti-apoptotic genes, while it upregulated the phosphorylation of ERK in SK-OV-3 cells (Figs. 4A, B).

**Ursolic Acid Induced Apoptosis in SK-OV-3 Cells via GSK 3β Regulation**

GSK 3β as a regulator of glycogen metabolism is involved in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility and apoptosis. GSK 3β plays a critical role in Wnt αβ catenin signaling pathway. Here, ursolic acid suppressed β-catenin degradation and also enhanced phosphorylation of GSK 3β at Ser 9 to inhibit its enzyme activity in SK-OV-3 cells (Fig. 5A). Furthermore, GSK 3β selective inhibitor SB216763 blocked the cleavages of caspase-3 and PARP induced by ursolic acid.

Fig. 3. Ursolic Acid Induces Apoptosis in SK-OV-3 Cells

(A) Cells were treated with various concentrations of ursolic acid (0, 5, 10, 20, or 40 µM) for 24 h and stained with propidium iodide (PI). Cell cycle distribution was analyzed by flow cytometry. Bar graphs represent the percentages of sub-G1 DNA contents undergoing apoptosis (right). Data represent means±S.D. *p<0.05 vs. untreated control. (B) Cells were treated with ursolic acid (0, 5, 10, or 20 µM) for 24 h. Cell lysates were prepared and subjected for Western blotting for PARP, cleaved caspase-3 and cleaved caspase-9.

Fig. 4. Ursolic Acid Down-Regulates the Expression of Survival Proteins in SK-OV-3 Cells

Cells were treated with ursolic acid (0, 5, 10, or 20 µM) for 24 h. Cell lysates were prepared and subjected for Western blotting with antibodies for indicated proteins. (A) AEG-1, p-ERK and ERK. (B) c-Myc and Bcl-xL.
in SK-OV-3 cells (Fig. 5B).

The level of β-catenin protein is regulated by ubiquitin-dependent proteolysis pathway.\(^{19}\) We utilized MG132, a cell-permeable proteasome inhibitor, to examine whether ursolic acid-induced suppression of β-catenin is mediated by the proteasome. As shown in Fig. 5C, MG132 treatment attenuated ursolic acid-induced down-regulation of β-catenin and activation of caspase-3.

To confirm ursolic acid-induced apoptosis is linked to mitochondria-dependent apoptosis, we analyzed mitochondrial membrane potential (MMP) by flow cytometry. Cells were treated with ursolic acid in the absence or presence of MG132. As shown in Fig. 5D, fluorescence intensity reflecting MMP was significantly reduced by ursolic acid from 84.9±1.1% to 71.4±3.6%. In contrast, MG132 treatment prevented the loss of MMP in ursolic acid-treated cells, suggesting ursolic acid induces mitochondria-dependent apoptosis in SK-OV-3 cells.

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Fig. 5. Ursolic Acid Induces Apoptosis in SK-OV-3 Cells via GSK 3β Regulation

(A) Cells were treated with ursolic acid (0, 5, 10, or 20 µM) for 24 h. Cell lysates were prepared and subjected for Western blotting for p-GSK 3β (Ser 9), GSK 3β and β-catenin. (B) Cells were treated with ursolic acid (20 µM) and/or GSK 3β inhibitor (10 µM) for 24 h. Cell lysates were prepared and subjected for Western blotting for p-GSK 3β (Ser 9), GSK 3β, PARP and cleaved caspase-3. (C and D) Cells were treated with ursolic acid (20 µM) and/or MG132 (20 µM) for 8 h. C: Western blotting was performed for β-catenin and cleaved caspase-3. D: Cells were stained with tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37°C and mitochondrial membrane potential (MMP) was detected by flow cytometry. ** p<0.01 vs. untreated control. * p<0.05 vs. ursolic acid-treated group.
DISCUSSION

Apoptosis is a mechanism of cell death far distinct from necrosis. Every second, almost one million cells are physiologically removed in our body by apoptosis and replaced with new cells. However, apoptosis is a therapeutic target for cancer cells, while cell death or apoptosis induced by harmful stimuli should be prevented in normal cells. GSK 3β, an isoform of GSK 3 family, was subsequently shown to function in a wide range of cellular processes including differentiation, growth, motility and apoptosis. Aberrant regulation of GSK 3β has been implicated in a range of human pathologies including Alzheimer’s disease, non-insulin-dependent diabetes mellitus and cancer. In general, detection of balanced levels of inhibitory phospho-serine9 (pSer9; inactive form) and stimulatory phospho-tyrosine216 (pY216; active form) of GSK 3β is considered a hallmark of its transient kinase regulation in normal CD34+ cells and in a broad range of human cell types. Previous studies demonstrate that GSK 3β inhibition was mainly a target for anticancer therapy in these cancer cells, since GSK 3β activation is associated with the survival of colorectal cancer, prostate cancer, and leukemia and melanoma cells. In contrast, as for GSK 3β activation target studies, Soto-Cerrato et al. reported GSK 3β activation induces apoptosis and cytotoxicity via up-regulation of death receptors 4 and 5 expression in MCF7 breast cancer cells. Urbanska et al. demonstrated that BsB8 medulloblastoma cells were partially rescued from NVP-AEW541 by GSK 3β inhibitor, lithium chloride and were sensitized by GSK 3β activator, sodium nitroprusside. Likewise, Li et al. also revealed that activation of GSK 3β induced nuclear translocation of survivin leading to GI cell-cycle arrest and apoptosis in A549 lung cancer cells, suggesting seemingly contradictory roles of GSK 3β as a mediator of both cell survival and apoptosis in different cancer cell lines.

Nonetheless, there were not much evidences on the role of GSK 3β in ovarian cancer apoptosis except Arafa et al.’s report that tangeretin-induced apoptosis in A2780/CP70 cells was reduced by upregulation of P38/Mkt pathway and downregulation of GSK 3β signaling. Thus, in the current study, the role of GSK 3β in ursolic acid-induced apoptosis was investigated in SK-OV-3 ovarian cancer cells.

Ursolic acid showed better cytotoxic effect in SK-OV-3 more than in A2780 ovarian cells. Thus, we performed supplementary experiments using SK-OV-3 cells. We found that the cytotoxic effect of ursolic acid was induced by apoptosis, not necrosis, by observing apoptotic bodies in ursolic acid treated SK-OV-3 cells. Consistently, ursolic acid significantly increased cell death biomarker ethidium homodimer stained cells and sub-G1 apoptotic portion in SK-OV-3 cells, implying the apoptotic activity of ursolic acid. Western blotting revealed that ursolic acid effectively induced apoptotic PARP cleavages and also activated caspase-9 and -3. Furthermore, ursolic acid suppressed the expression of survival genes such as c-Myc, Bcl-xL, and AEG-1, and enhanced ERK phosphorylation in SK-OV-3 cells, suggesting anti-survival gene regulation by ursolic acid in SK-OV-3 cells. We also observed that ursolic acid enhanced the loss of MMP, indicating that the alteration of the MMP is involved in ursolic-induced apoptosis in SK-OV-3 cells. Interestingly, ursolic acid also suppressed β-catenin degradation and enhanced phosphorylation of GSK 3β (Ser 9) of Wnt signaling pathway, indicating the apoptosis induction by ursolic acid can be mediated by β-catenin degradation and GSK 3β phosphorylation in SK-OV-3 cells. Conversely, GSK 3β inhibitor SB216763 blocked the cleavages of caspase-3 and PARP induced by ursolic acid and proteosomal inhibitor MG132 disturbed down-regulation of β-catenin, activation of caspase-3 and decreased MMP induced by ursolic acid in SK-OV-3 cells, demonstrating that GSK 3β phosphorylation and β-catenin degradation mediate ursolic acid induced apoptosis in SK-OV-3 ovarian cancer cells. Likewise, Overall, our findings suggest that ursolic acid induces apoptosis via activation of caspase and phosphorylation of GSK 3β in SK-OV-3 cancer cells as a potent anti-cancer agent for ovarian cancer treatment.

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