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Research Article

Ursolic acid benzaldehyde chalcone leads to inhibition of cell proliferation and arrests cycle in G1/G0 phase in ovarian cancer

Ursolic acid benzaldehyde chalcone leads to inhibition of cell proliferation and arrests cycle in G1/G0 phase in ovarian cancer

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Abstract

In the present study, the effect of ursolic acid benzaldehyde chalcone (UABC) on ovarian carcinoma cells was studied. The results revealed that ovarian carcinoma cells on UABC treatment increased Sub-G1 cell population, increased rate of cell apoptosis and morphological changes in mitochondrial membrane. In OVCAR 432 cells treatment with UABC increased the Sub-G1 cell population to 72.3% and growth inhibition rate of >72%. Treatment with 20 μ M of UABC for 48 hours, led to an induction of apoptosis in 67.2% and induced morphological changes in OVCAR 432 cells. The Western blot results showed high concentration of cytochrome c in the cell cytosol after 48 hours of UABC treatment. Treatment of RMS-13 cells with UABC resulted in inhibition of *GLI1*, *GLI2*, *PTCH1*, and *IGF2* genes. In addition, we found a significant reduction in hedgehog activity of RMS-13 cells after UABC treatment by means of a hedgehog-responsive reporter assay. Therefore, UABC can be a promising agent for the treatment of ovarian carcinoma.

Introduction

Ovarian carcinoma because of late stage detection is the most lethal gynecological malignancy. It is usually diagnosed after tumor cells are widely metastasized within the peritoneal cavity. In spite of advancement in modern cancer treatments like surgery and combination therapy with paclitaxel and carboplatin the death rate of patients is still high (Cannistra, 2004; Lengyel, 2010). All the treatments for ovarian carcinoma at present are inefficient. Radiation therapy has led to an average survival rate of 5 years in 10% (Fuks, 1975; Dembo, 1984; Martinez et al., 1985; Fuks et al., 1987) while as chemotherapy has led to a response rate from 3 to 30% (Wiltshaw et al., 1986; Parker et al., 1980; Wharton et al., 1984; Williams et al., 1985; Louie et al., 1986). The patients with less than 25% and greater than 75% cytoreduction have an average difference in survival of 11 months (Bristow et al., 2001). Therefore, the need for new treatment regimens with roles in ovarian carcinoma treatment is highly desired. The 5-fluoro-

uracil (5-FU) and oral fluoropyrimidines have been shown to be effective in many cancers including gastrointestinal tract cancers (Takiuchi et al., 1998). New generations of oral fluoropyrimidine such as S-1, which inhibit the degradation of 5-FU and capecitabine have been developed and put into use in the treatment of several solid tumors (Schoffski, 2004). 5-FU and 4-HPR showed positive responses in combination with platinum based drugs or as a single agent in phase II trials respectively in ovary carcinoma (Rosa et al., 2008; Weiss, 1992; Garcia et al., 2004).

Ursolic acid, a pentacyclitriterpenoid present in abundance in apple peels (Liu, 1995) suppress tumorigenesis (Huang et al., 1994), inhibits tumor promotion (Tokuda et al., 1986; Ohigashi et al., 1986; Nishino et al., 1988) and angiogenesis (Sohn et al., 1995). Ursolic acid mainly exerts its anti-tumor effect by suppressing the expression of genes regulated by NF- κ B (Simon et al., 1995; Najid et al., 1992). Not only ursolic acid but its derivatives also induce apoptosis in a variety of cancer cells (Es-Saady et al., 1992;1996)



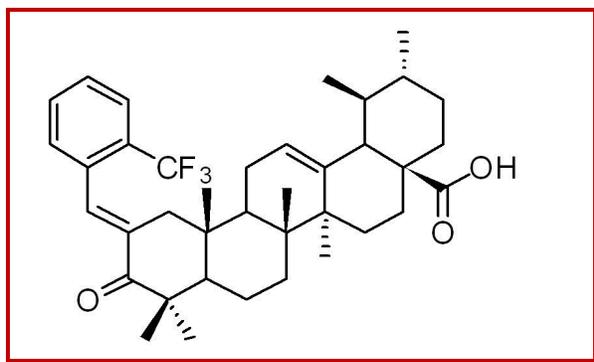


Figure 1: Structure of ursolic acid benzaldehyde chalcone

through DNA replication inhibition (Kim et al., 2000), caspase activation, (Choi et al., 2000) and tyrosine kinases inhibition (Hollosoy et al., 2000). In the present study we first time report the effect of ursolic acid chalcone (Figure 1) treatment of ovarian carcinoma.

Materials and Methods

Chemicals

Ursolic acid benzaldehyde chalcone was purchased from Sigma-Aldrich (St. Louis, MO, USA). L-glutamine from invitrogen (Carlsbad, CA, USA), 1% penicillin and streptomycin from Gibco, (Grand Island, NY, USA). Enhanced chemiluminescence (ECL), nitro-cellulose membrane and ECL prime Western blotting detection reagents were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), 1-methyl-4-phenylpyridinium (MPPp), dimethyl sulfoxide (DMSO) and β -actin were purchased from Sigma (St Louis, MO, USA).

Cell culture

The human ovarian cancer lines OVCAR 432, RMS-13 cell lines and NIH-3T3 fibroblasts were obtained from American Type of Collection centre (Manassas, VA, USA). The cells were maintained in minimum essential medium (MEM), containing 10% fetal bovine serum and antibiotics. The cell lines were maintained in a humidified incubator at 37°C and 5% CO₂.

Cytotoxicity assay

In 96-well culture plates 5 × 10³ cells were plated per well in MEM. To each well 200 μ L containing different concentrations of UABC in DMSO was added and the plates were incubated for 24 hours at 37°C. After 24 hours the medium was changed to drug free medium and incubation was continued for 5 days more. Alamar blue vital dye indicator assay (Ahmad et al., 1994) was used to measure the cell viability. Inhibition in cell growth is denoted as cytotoxicity and represented as

concentration-response curve (surviving percentage versus drug concentration). The IC₅₀ is determined from a least squares regression fit to the linear portion of the curve.

Flow cytometry

The cells distributed at a density of 8 × 10⁵ onto 100 mm dishes were incubated at 37°C for 24 hours. After 24 hours, the cells were treated with UABC at IC₅₀ concentration. Nuclear staining for DNA content was performed after harvesting the cells at 12, 24 and 48 hours from treatment. The untreated cells were also harvested at the same time points and used as control. The cells were treated with two solutions, a salt solution containing SDS and RNaseA, second solution containing sucrose and citric acid. Additionally both solutions contained ethidium bromide. Becton Dickinson FAC-Scan (cell Quest software) was used to acquire cell cycle data and Cell Cycle Muticycle (Phoenix Flow Systems, San Diego, CA) was used for analysis.

Assessment of apoptosis

The cells treated with UABC after harvesting were washed with PBS and fixed in formaldehyde at room temperature for 20 min. Then fixative was removed and cells were suspended in 50 μ L of 8 μ g/mL bisbenzimidazole trichloride dye (Hoechst-33258). On to the glass slides, 30 μ L cell aliquots were fixed. Five hundred cells were counted and scored for the incidence of apoptosis using fluorescence microscope. All the measurements were performed in triplicates.

Western blot assay

In 6-well plates 1 × 10⁵ cells were plated per plate for 12 hours and then treated with UABC in the presence or absence of 50 μ Mz VAD.fmk. Vehicle treated cells were used as negative and cycloheximide (Sigma-Aldrich, Taufkirchen, Germany) as positive control. After 48 hours, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 1% NP-40, and 5 mM cocktail) and the lysate was centrifuged to remove cell debris. Protein Assay System (Bio-Rad, Hercules, CA, USA) was used to determine concentration of proteins. The protein were loaded and resolved by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. The semidry method was used to transfer proteins onto a PVDF membrane which was then blocked with 5% non-fat dry milk overnight. Incubation of membranes with mouse anti-human caspase-3, rabbit antihuman cleaved caspase-3, rabbit antihuman β -actin (Cell Signalling Technology, Danvers, MA, USA), mouse anti-human COX4, rabbit antihuman cytochrome c (Clontech, Mountain View, CA, USA) or goat antihuman GLI1 (Santa Cruz Biotechnology, Santa

Cruz, CA, USA) antibodies was performed for 2 hours. Then the incubation was continued for 1.5 hours with horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse, or rabbit anti-goat IgG secondary antibodies (DakoCytomation, Hamburg, Germany). ECL chemiluminescence detection system (GE Healthcare) was used for visualising the signal.

Hedgehog reporter and activation assays

5×10^5 RMS-13 cells or NIH-3T3 fibroblasts were transfected with 900 ng of the reporter plasmid p11 x Gli or the empty control pGL3-TK (Beer et al., 2003) and 100 ng of the reference plasmid pRL-TK using FuGene 6 transfection reagent (Roche Diagnostics). 1 μ g of an expression plasmid containing murine sonic hedgehog gene (Karolinska Institute, Stockholm, Sweden) was transfected into fibroblasts. After 24 hours transfection, cells were treated with UABC, 10 μ M cyclopamine (Toronto Research Chemicals, Toronto, Canada), or vehicle and cultured for 24 hours. A Dual-Glo Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) was used for determination of

reporter gene activity. Firefly luciferase activity was normalized to Renilla luciferase activity. After 24 hours of transfection total RNA was isolated using Trizol (Invitrogen) from fibroblasts.

Statistical analyses

The vehicle treated control cells were arbitrarily assigned 100% and other data were expressed in comparison to control. Data were analyzed by Dunnett's multiple comparison test (Sigma Stat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $p < 0.05$.

Result

The cytotoxicity effect of UABC in OVCAR 432 cells was determined by cytometric analysis of Sub-G1 cell population. OVCAR 432 cells on treatment with various concentrations of UABC for different time periods exhibited cytotoxicity in a dose-and time-dependent manner. Among the range of concentrations (5-30 μ M)

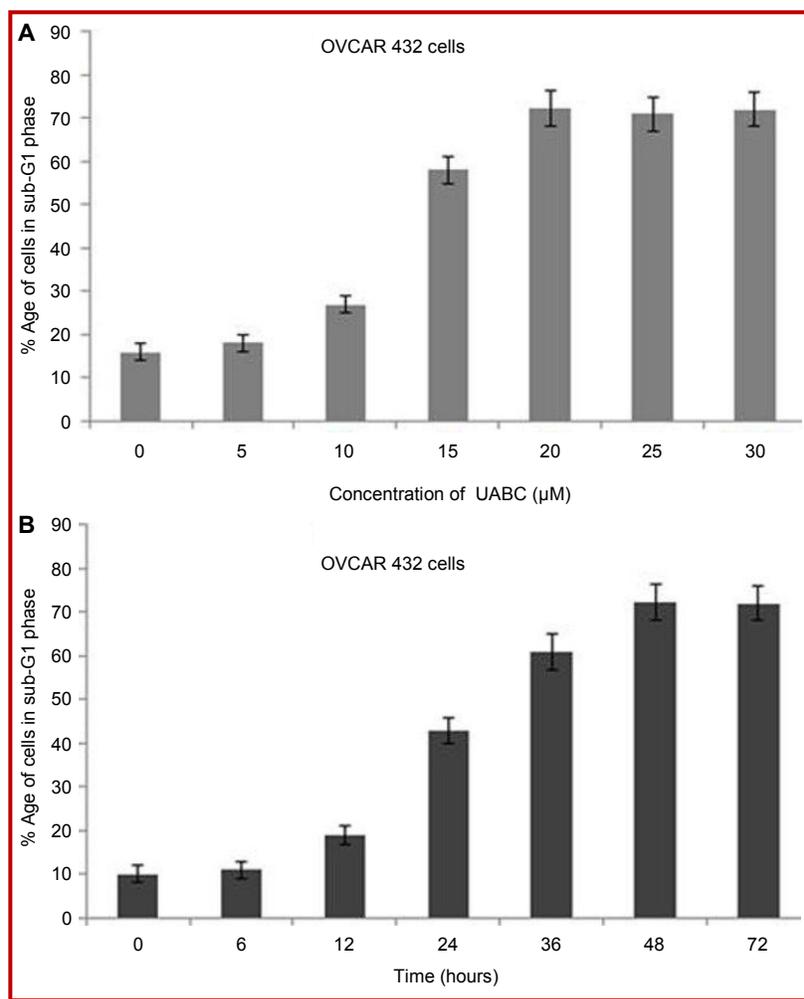


Figure 2: Cytotoxic activity of UABC against OVCAR 432 cells

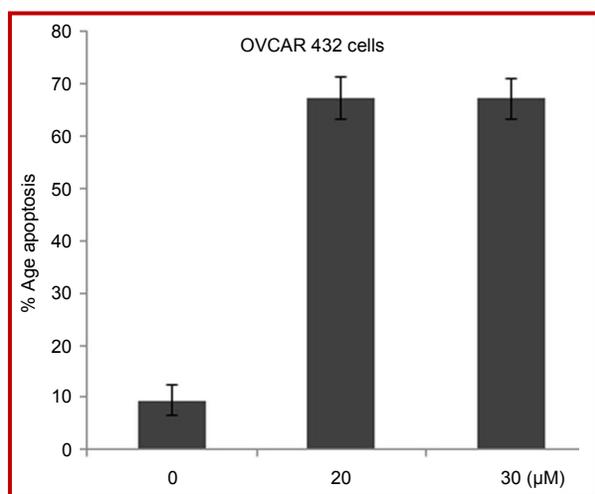


Figure 3: Rate of apoptosis in OVCAR 432 cells using quantitative fluorescence microscopy. All the results are mean of triplicate measurements

used, the cytotoxicity was significant at 20 μM of UABC after 48 hours. In OVCAR 432 cells population of cells in Sub-G1 phase increased to 72.3% compared to only 16.2% in control (Figure 2).

We used quantitative fluorescence microscopy to observe apoptotic chromatin condensation in OVCAR 432 cells. The results of the analysis showed that UABC induces a higher rate of apoptosis compared to untreated control cells (Figure 3). Treatment of OVCAR 432 cells with 20 μM UABC for 48 hours, caused an induction of apoptosis in 67.2% cells compared to 9.5% in control cells.

We also analysed the effect of UABC on morphological

changes in OVCAR 432 cells. The results from flow cytometry showed that UABC induced significant rate of apoptosis after 48 hours. When OVCAR 432 cells were treated with UABC along with caspase inhibitor, zVAD.fmk there was inhibition in rate of apoptosis (Figure 4A). This indicated involvement of caspases in UABC-induced apoptosis. Therefore, UABC induces apoptosis through caspase dependent pathway.

The results from Western blot analysis showed high concentration of cytochrome c in the cell cytosol after 48 hours of UABC treatment. On the other hand cytochrome c concentration in the mitochondrial fraction inversely decreased (Figure 4B). Together, these results clearly indicate that UABC-induced apoptosis in OVCAR 432 cells is mediated by the intrinsic apoptotic pathway.

Treatment of RMS-13 cells with UABC resulted in inhibition of *GLI1*, *GLI2*, *PTCH1*, and *IGF2* genes (Figure 5A). We did not observe any significant effect in two *GLI1*-negative RMS cell lines RH-30 and RD (Figure 5A). Moreover, we found a significant reduction in hedgehog activity of RMS-13 cells after treatment by means of a hedgehog-responsive reporter assay. To examine whether this inhibition is dependent on hedgehog signalling components upstream of *GLI1*, RMS-13 cells were treated with UABC in the presence or absence of 10 μM cyclopamine, a specific hedgehog signalling inhibitor (Taipale et al., 2000). However, the inhibitory effect of UABC on *GLI1* activity was found to be independent of cyclopamine treatment (Figure 5B), indicating that UABC is able to selectively target hedgehog signalling on the level of *GLI1*.

In order to activate hedgehog pathway in

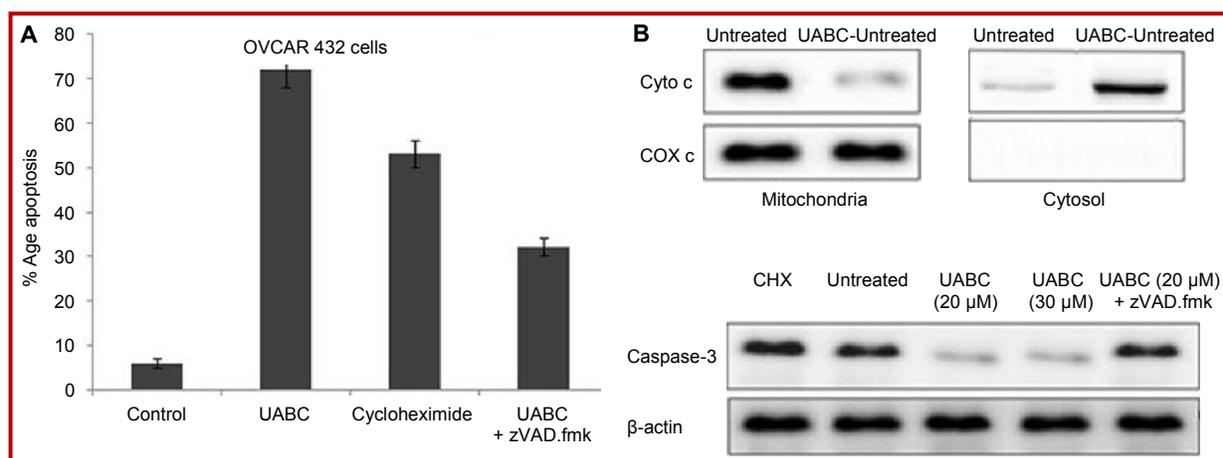


Figure 4: Effect of UABC on apoptosis in RMS cell lines. (A) The RMS cells were treated for 48 h with UABC in the presence or absence of 50 μM of the zVAD.fmk. Apoptosis-specific DNA fragmentation was determined by FACS analysis of propidium iodide-stained nuclei; 100 μg/mL cycloheximide (CHX) was used as a positive control for apoptosis induction. (B) The RMS-13 cells were treated with UABC. Mitochondria were isolated, lysed, and the expression of cytochrome c and COX4 in the mitochondrial and cytosolic fraction was detected by western blot analysis. The RMS-13 cells were treated for 48 hours with UABC in the absence or presence of zVAD.fmk. Expression of caspase-3 and β-actin was detected by Western blot analysis. 100 μg/mL cycloheximide (CHX) was used as a positive control for apoptosis induction

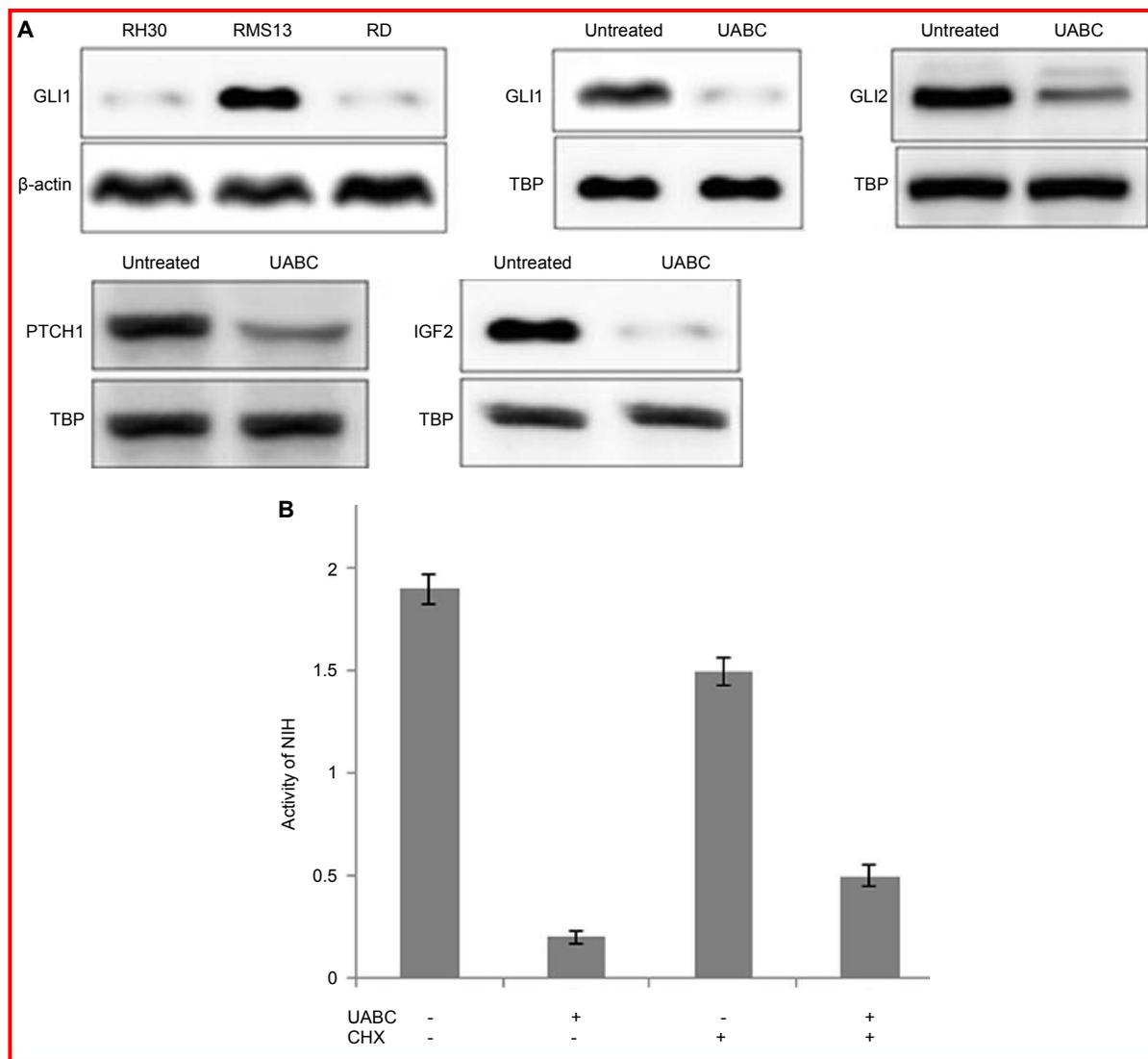


Figure 5: (A) UABC blocks hedgehog signalling, GLI1 expression of rhabdomyosarcoma cell lines. The RMS cell lines were lysed and the expression of GLI1 and β -actin was detected by western blot analysis. For hedgehog signalling dependency, the RMS cells were treated for 48 hours with UABC. GLI1, GLI2, PTCH1, and IGF2 mRNA expression from untreated and treated RMS cells was measured by quantitative real-time PCR in relation to the house-keeping gene TBP as a calibrator. (B) Hedgehog pathway activity. The RMS-13 cells were transiently transfected with 900 ng hedgehog-responsive reporter plasmid (p11 \times Gli) or control plasmid (pGL3-TK) and treated for 24 hours with UABC and 10 μ M cyclopamine or vehicle. Reporter assay experiments were repeated three times and transfections performed in duplicate

untransformed NIH-3T3 cells, we expressed the ligand sonic hedgehog by transient transfection. This resulted in strong expression of the hedgehog target genes Gli1, Gli2, Ptc1, and Igf2 (Figure 6). But exposure to hedgehog inhibitor, cyclopamine resulted in inhibition of expression of these hedgehog target genes. The use of UABC led to inhibition of Gli1 and Gli2 expression without affecting Ptc1 and Igf2. These results led us to conclude that UABC inhibits hedgehog signalling through GLI family transcription factors selectively.

Discussion

This study was carried out in order to investigate the potential of UABC in the treatment of ovary carcinoma. Angiogenesis has been reported as a prerequisite for tumor growth beyond certain size as well as for metastatic spread and, therefore, is an attractive target for clinical tumor biologists (Folkman, 2002). There are reports that anti-angiogenic treatments synergise with traditional chemotherapeutic and radiotherapeutic

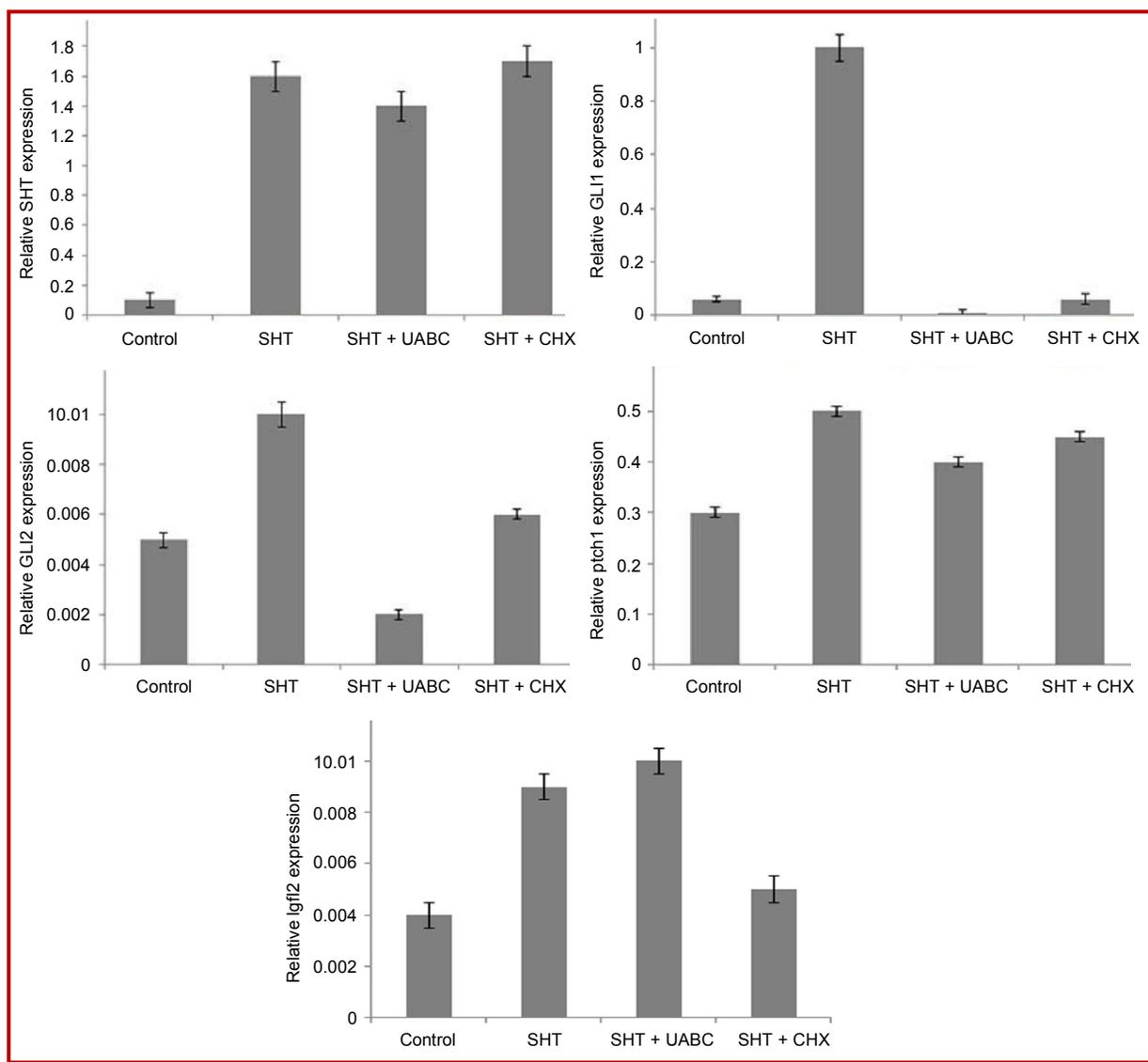


Figure 6: Hedgehog activation assay. The NIH-3T3 cells were transiently transfected with 1 μ g sonic hedgehog (Shh) expression plasmid. For the inhibition of hedgehog signalling, cells were additionally exposed to UABC and 10 μ M cyclopamine (CHX) after 48 hours transfection. Expression of the murine genes SHT, Gli1, Gli2, Ptc1, and Igf2 was determined after an incubation period of 48 hours using quantitative real-time PCR in relation to the house-keeping gene TBP as a calibrator

regimens. Ursolic and betulinic acids not only exhibit cytotoxic effect against endothelial cells, but also inhibits tube-like structure formation of aortic endothelial cells (Kwon et al., 2002; Mukherjee et al., 2004). In the present study treatment of OVCAR 432 cells with UABC increased the Sub-G1 cell population to 72.3%. The concentration of UABC that is effective in enhancing cytotoxicity is 20 μ M after 48 hours. Treatment of OVCAR 432 cells with 20 μ M UABC for 48 hours, induced apoptosis in 79.7% cells.

The purpose of the present study was to provide experimental evidence in order to help develop chemotherapeutic regimens for ovary carcinoma. The results from flow cytometry showed that UABC-

induced apoptosis was significant after 48 hours. When the OVCAR 432 cells were treated with UABC along with caspase inhibitor zVAD.fmk, there was inhibition in rate of apoptosis. This led to the conclusion that caspases were involved in UABC-induced apoptosis. The Western blot results showed high concentration of cytochrome c in the cell cytosol after 48 hours of UABC treatment. On the other hand cytochrome c concentration in the mitochondrial fraction inversely decreased.

Treatment of RMS-13 cells with UABC resulted in inhibition of GLI1, GLI2, PTCH1, and IGF2 genes. Moreover, we found a significant reduction in hedgehog activity of RMS-13 cells after UABC

treatment by means of a hedgehog-responsive reporter assay. To examine whether this inhibition is dependent on hedgehog signalling components upstream of GLI1, RMS-13 cells were treated with UABC in the presence or absence of 7.5 μ M cyclopamine, a specific hedgehog signalling inhibitor (Taipale et al., 2000). However, the inhibitory effect of UABC on GLI1 activity was found to be independent of cyclopamine treatment, indicating that UABC is able to selectively target hedgehog signalling on the level of GLI1.

In conclusion, UABC has an important role in ovarian carcinoma treatment by inducing apoptosis through mitochondrial pathway and specifically targeting the hedgehog signalling pathway.

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