ANTICANCER RESEARCH *38*: 169-178 (2018)
doi:10.21873/anticanres.12205

Ouabain Induces Apoptotic Cell Death Through Caspase- and Mitochondria-dependent Pathways in Human Osteosarcoma U-2 OS Cells

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Abstract. *Background/Aim: Ouabain, a plant-derived product/substance with Na^+/K^+-ATPase inhibiting properties, has been shown to exert anti-cancer activity on human cancer cells. This is the first study to investigate the effect of ouabain on apoptotic cell death of human osteosarcoma-derived U-2 OS cells. Materials and Methods: Flow cytometry was used to examine cell viability, cell cycle, and reactive oxygen species (ROS), Ca^{2+}, mitochondrial membrane potential (MMP) and caspase activity. Morphological changes were examined by contrast-phase microscopy, while apoptosis-associated protein levels were analyzed by western blot. Results: Ouabain, at concentrations of 5-60 μM, significantly decreased the total viable cells and induced cell morphological changes in a time-dependent manner. It also time-dependently decreased G_0/G_1 phase and increased S and G_2/M phase in U-2 OS cells. The production of ROS and the levels of MMPs ($\Delta\Psi_m$) were inhibited, while Ca^{2+} production in U-2 OS cells was increased. Regarding cell apoptosis, flow cytometry assay revealed increased caspase-3, -8, and -9 activities in U-2 OS cells. Moreover, western blot results showed that ouabain increased the expression of pro-apoptotic protein Bax and decreased the expression of anti-apoptotic protein Bcl-2 in U-2 OS cells. Furthermore, results also showed that ouabain increased cytochrome c release, apoptosis-inducing factor (AIF) and endonuclease (Endo) G that is associated with apoptosis through caspase-dependent and -independent pathway in U-2 OS cells. Conclusion: Our findings provide important insight into the cytotoxic effects of ouabain on U-2 OS cells, in vitro, which are mediated at least partly via cell apoptosis induction.*

Osteosarcoma is a type of bone cancer, the most common primary bone malignancy in children and adolescent population (1). According to the Ministry of Health and Welfare, bone cancer mortality rate was 0.4 per 100,000 people in Taiwan, in 2016 (2). Currently, therapeutic approaches for patients with osteosarcoma include a combination of surgery and intensive multi-agent chemotherapy. Chemotherapy for osteosarcoma patients increased the five-year survival rate to 60-75% (3).

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Key Words: Ouabain, caspase-dependent pathways, mitochondria-dependent pathways, apoptosis, human osteosarcoma U-2 OS cells.

However, high-dose chemotherapy on patients with osteosarcoma has been shown to exhibit severe side effects, limiting further use of these drugs (4, 5). Therefore, it is urgent to reduce the side-effects of chemotherapy on patients with osteosarcoma. Many studies have focused on natural products in order to improve the efficiency of clinical anticancer drugs.

Ouabain, a natural compound derived from *Acocanthera ouabaio* and *Strophanthus gratus*, has been used as a therapeutic agent for congestive heart failure, due to its positive ionotropic effect on cardiac muscle (6, 7). Ouabain has been reported to promote TRAIL-mediated cancer cell death, *via* Mcl-1 down-regulation (8). Moreover, inhibition of Na^+/K^+-ATPase activity by ouabain led to selective death of drug-resistant T98G glioblastoma cells and disruption of K^+ homeostasis in a time- and concentration-dependent manner (9). Ouabain has also been shown to induce antiproliferative effects on SW13 and H295R adrenocortical tumor cell lines and primary adrenocortical tumor cells (10). Furthermore, ouabain induced apoptotic cell death in HeLa cells *via* the activation of Rho-associated kinases (Rho/ROCK) pathway, which is differentially regulated in cancer cell lines and normal cells during cell death induction (11).

Numerous studies have shown that ouabain exerts anticancer activities on many human cancer cell lines and also inhibits migration and invasion of cancer cells. However, this is the first study to investigate the effects of ouabain on U-2 OS human osteosarcoma cells and the underlying molecular mechanisms, *in vitro*. Our results showed that ouabain induced apoptotic cell death *via* caspases- and mitochondria-dependent pathways.

Materials and Methods

Chemicals and reagents. Dimethyl sulfoxide (DMSO), propidium iodide (PI), ouabain, L-glutamine and penicillin-streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). McCoy's 5A medium and fetal bovine serum (FBS) were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, California, USA).

Cell culture. U-2 OS cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in McCoy's 5A medium containing 10% FBS, 2 mM L-glutamine, and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37˚C, as previously described (12, 13).

Cell's morphological changes and viability assays. U-2 OS cells (1.6×10^5 cells/well) were maintained in 12-well plate with McCoy's 5A medium for 24 h then were treated with 1% DMSO (control) or ouabain at a final concentration (5, 10, 20, 40, and 60 μM). Cells were treated trypsin for 5 min, and collected by centrifugation at 1500 rpm for 5 min, and stained with PI (5 μg/ml) for total viable cell examination by flow cytometry (BD Biosciences, FACS Calibur, San Jose, CA, USA), as previously described (12). Otherwise, cells were treated with 1% DMSO (control) or 5 μM of ouabain for 0, 12, 24 and 48 h and were examined with contrast-phase microscopy (magnification ×400) (Olympus, Tokyo, Japan) (12, 14).

Measurement of cell cycle distribution by flow cytometry. U-2 OS cells (1.6×10^5 cells/well) were treated with 1% DMSO (control) or 5 μM of ouabain for 0, 12, 24 and 48 h and were collected for cell cycle distribution assessment using flow cytometry. Analysis was performed with CellQuest and Mod Fit computer programs (BD Biosciences Clontech, Palo Alto, CA) as described previously (15, 16). The proportions of cells in the G_0/G_1, S, and G_2/M phases were represented in DNA histograms.

ROS, intracellular Ca^{2+}, and MMP ($\Delta\Psi_m$) assays by flow cytometry. U-2 OS cells (1.6×10^5 cells/well) were treated with 1% DMSO (control) or 5 μM of ouabain for 0, 12, 24 and 48 h. Cells were harvested, centrifuged, and resuspended in 500 μl of dihydrodichlorofluorescein diacetate (H_2DCF-DA, 10 μM), 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$, 4 μM), or fluo-3 acetoxymethyl ester (Fluo-3/AM, 2.5 μg/ml) for 30 min, for the measurement of ROS (H_2O_2), $\Delta\Psi_m$, levels or intracellular Ca^{2+}, respectively, using flow cytometry as previously described (12, 17).

Measurements of caspase-3, caspase-8, and caspase-9 activities. Activity of caspases was measured by flow cytometry using commercially available fluorescent caspase substrates (OncoImmunin, Inc., Gaithersburg, MD, USA). Initially, U-2 OS cells (1.6×10^5 cells/well) were treated with 1% DMSO (control) or 5 μM of ouabain for 0, 12, 24 and 48 h. Cells were harvested, centrifuged and then were resuspended in 25 μl of 10 μM substrate solution CaspaLux8-L1D2 (for caspase-8), PhiPhiLux-G1D1 (for caspase-3), or CaspaLux9-M1D2 (for caspase-9) and were incubated at 37˚C for 60 min. Cells were assayed as described previously (12, 18).

Western blot analysis. U-2 OS cells (2.4×10^6 cells) were treated with 1% DMSO (control) or 5 μM of ouabain for 0, 12, 24 and 48 h and were collected and resuspended in lysis buffer [40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40] for measuring total protein as described previously (12). An equal amount of total protein (30 μg) was separated by polyacrylamide gel electrophoresis and was transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). After washed, blots were incubated with primary antibodies. The primary antibodies used were: anti-endonuclease G (Endo G), anti-apoptosis-inducing factor (AIF), anti-cytochrome c, anti-growth arrest- and DNA damage-inducible gene 153 (GADD153), anti-activating transcription factor 6α (ATF6α), anti-glucose-regulated protein (GRP78) and anti-calpain 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-B-cell lymphoma 2 (Bcl-2), anti-Bax, anti-poly(ADP-ribose) polymerase (PARP), anti-apoptotic protease activating factor-1 (Apaf-1) (Cell Signaling, St Louis, MO, USA), anti-superoxide dismutase 1 (SOD1), anti-superoxide dismutase 2 (SOD2), anti-catalase, anti-Fas-associated protein with death domain (FADD), anti-Fas-Ligand (FasL) (Millipore, Temecula, CA, USA), anti-Fas and anti-caspase-4 (BD Biosciences, San Jose, CA, USA) and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). Membranes were stained with the appropriate secondary antibodies (GeneTex, Irvine, CA, USA) and were visualized with enhanced chemiluminescence detection (Millipore, Temecula, CA, USA) as described previously (12, 19). ImageJ 1.490 software (National Institutes of Health, Bethesda, MD, USA) was used to quantify changes in protein expression by densitometry

analysis and using β-actin as the loading control.

Statistical analysis. All data are expressed as the mean±standard deviation (S.D.) from at least 3 experiments. Differences between groups were analyzed by one-way analysis of variance and Dunnett test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA). Comparisons were made between groups of ouabain-treated cells and untreated cells (control). Differences with $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***) were considered statistically significant.

Results

Ouabain induced cell morphological changes and decreased the cell viability of U-2 OS cells. U-2 OS cells treated with 5-60 μM ouabain for 24 h showed a significant reduction of viable cell number [24 h: 5 μM (72.47±5.67%, $p<0.001$); 10 μM (65.40±5.46%, $p<0.001$); 20 μM (60.44±6.23%, $p<0.001$); 40 μM (57.88±1.41%, $p<0.001$); 60 μM (54.95±5.69%, $p<0.001$)] compared to untreated control cells (100.00±0.5%). The 48-h group had a lower viable cell number compared to 24-h. treated U-2 OS cells [48 h: 5 μM (52.53±2.15%, $p<0.001$); 10 μM (43.02±4.50%, $p<0.001$); 20 μM (38.45±6.19%, $p<0.001$); 40 μM (34.90±0.56%, $p<0.001$); 60 μM (34.18±4.37%, $p<0.001$)] compared to untreated control cells (100.00±0.85%) (Figure 1A). Thus, the 5 μM concentration (IC_{50}) of ouabain was selected for further cell morphological changes examination and results are presented in Figure 1B. Treatment of U-2 OS cells with ouabain at 5 μM significantly induced cell morphological changes and decreased total viable cell number.

Ouabain induced G_2/M arrest in U-2 OS cells. Cell cycle distribution results are shown in Figure 2. Results indicated that ouabain induced G_2/M phase arrest in a time-dependent manner (Figure 2B; 12 h: 1.41-fold, $p=0.002$; 24 h: 2.47-fold, $p<0.001$; 48 h: 3.25-fold, $p<0.001$) compared to untreated controls. Also, ouabain was shown to induce sub-G1 phase (apoptotic cell death) in U-2 OS cells after 12-24 h treatment (Figure 2C; 12 h: 1.48-fold, $p=0.618$; 24 h: 3.02-fold, $p=0.011$; 48 h: 10.43-fold, $p<0.001$) compared to untreated controls.

Ouabain affect ROS, intracellular Ca^{2+}, and MMP ($\Delta\Psi_m$) levels in U-2 OS cells. Treatment of U-2 OS cells with 5 μM of ouabain for 0, 12, 24 and 48 h revealed that ouabain decreased ROS production (Figure 3A; 12 h: 0.34-fold, $p<0.001$; 24 h: 0.28-fold, $p<0.001$; 48 h: 0.25-fold, $p<0.001$) and $\Delta\Psi_m$ levels (Figure 3B; 12 h: 0.67-fold, $p<0.001$; 24 h: 0.59-fold, $p<0.001$; 48 h: 0.59-fold, $p<0.001$), while increased Ca^{+2} productions (Figure 3C; 12 h: 1.63-fold, $p<0.001$; 24 h: 1.79-fold, $p<0.001$; 48 h: 1.61-fold, $p<0.001$) in U-2 OS cells compared to untreated control cells.

Ouabain increased caspase activity in U-2 OS cells. U-2 OS cells were treated with 5 μM for 0, 12, 24 and 48 h. Assays of the intracellular activity of caspase -3, -8, and -9 showed that ouabain increased the activity of examined caspases. More specifically, ouabain treatment for 24-48 h significantly increased caspase-8 (24 h: 1.29-fold, $p<0.001$; 48 h: 1.52-fold, $p<0.001$) and caspase-3 (24 h: 1.42-fold, $p<0.001$; 48 h: 1.50-fold, $p<0.001$) activity, though caspase-9 activity was significantly increased after 12h of ouabain treatment (12 h: 1.43-fold, $p<0.001$; 24 h: 1.47-fold, $p<0.001$; 48 h: 1.59-fold, $p<0.001$) in U-2 OS cells compared to untreated control cells (Figure 4B).

Ouabain alters levels of proteins associated with apoptotic cell death of U-2 OS cells. Western blot analysis revealed that ouabain treatment altered the expression levels of apoptosis-associated proteins in U-2 OS cells in a time-dependent manner (Figure 5). Ouabain significantly increased the levels of proteins associated with estrogen receptor (ER) stress, such as SOD1 (24 h: 1.6-fold, $p<0.05$; 48 h: 2.0-fold, $p=0.001$), SOD2 (24 h: 1.8-fold, $p<0.01$; 48 h: 1.6-fold, $p<0.01$) and catalase (24 h: 1.9-fold, $p<0.001$; 48 h: 2.0-fold, $p<0.001$) and Calpain 1 (48 h: 1.3-fold, $p<0.001$), caspase-4 (48 h: 1.3-fold, $p<0.001$), GRP 78 (48 h: 1.4-fold, $p=0.001$) and GADD153 (48 h: 1.4-fold, $p<0.001$). Ouabain effects on apoptosis-related proteins were observed after 12 h of treatment. Specifically, ouabain increased the expression levels of pro-apoptotic proteins, such as Bax (12 h: 1.9-fold, $p<0.05$; 24 h: 2.9-fold, $p=0.001$; 48 h: 3.6-fold, $p<0.001$), AIF (24 h: 5.9-fold, $p<0.001$; 48 h: 5.5-fold, $p<0.001$), Endo G (12 h: 2.5-fold, $p<0.001$; 24 h: 3.8-fold, $p<0.001$; 48 h: 3.6-fold, $p<0.001$), cytochrome *c* (24 h: 3.5-fold, $p<0.001$; 48 h: 2.0-fold, $p<0.01$), Apaf-1 (12 h: 1.3-fold, $p=0.471$; 24 h: 1.8-fold, $p<0.05$; 48 h: 2.4-fold, $p<0.01$) and PARP (12 h: 2.2-fold, $p<0.001$; 24 h: 4.6-fold, $p<0.001$; 48 h: 3.7-fold, $p<0.001$); however, it decreased anti-apoptotic protein Bcl-2 (24 h: 0.3-fold, $p<0.001$; 48 h: 0.3-fold, $p<0.001$) in U-2 OS cells. Furthermore, ouabain treatment for 24-48 h increased FADD (24 h: 1.3-fold, $p<0.01$; 48 h: 1.3-fold, $p<0.01$), Fas (24 h: 1.6-fold, $p<0.01$; 48 h: 2.0-fold, $p<0.001$), and FasL (24 h: 1.3-fold, $p<0.05$; 48 h: 1.5-fold, $p<0.01$) that are associated with Fas receptor for the induction of cell apoptosis. The protein levels in U-2 OS cells were significantly affected in ouabain-treated cells compared with untreated-cells.

Discussion

Ouabain has been shown to induce cytotoxic effects in many human cancer cell lines and has also been reported to inhibit migration and invasion of human liver cancer A549 cells (15). However, there is not any report for human bone cancer cells. Herein, we investigated the *in vitro* cytotoxicity of ouabain

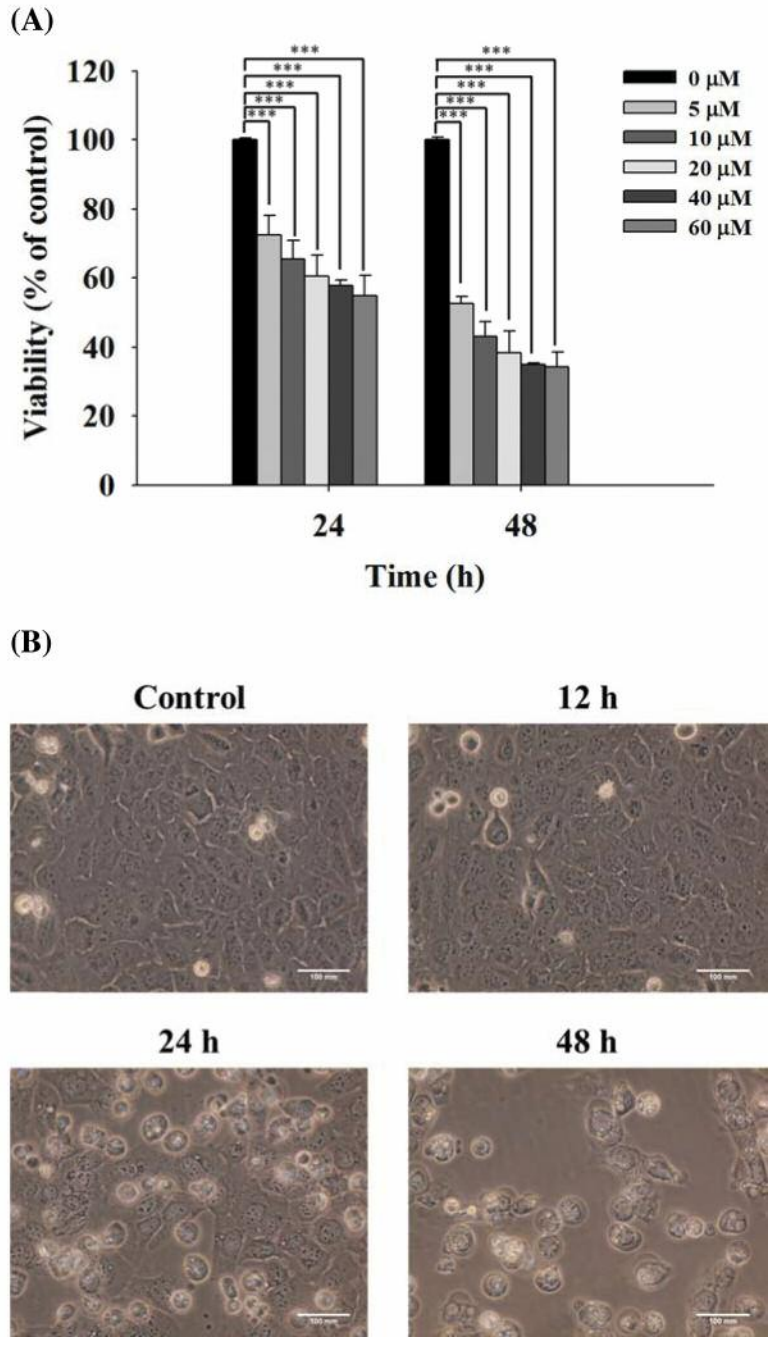

Figure 1. *Ouabain affects the morphology and percentage of viable U-2 OS cells. Cells treated with ouabain (0, 5, 10, 20, 40, and 60 μM) for 24 and 48 h were examined by flow cytometry. Bars (A) indicate the percentage of viable cells. Morphological changes of U-2 OS treated with ouabain (5 μM) were examined via phase-contrast microscopy at magnification ×400 (B). Asterisks denote the statistical significance of the experimental group compared to the corresponding control group (*p<0.05, **p<0.01 ***p<0.001).*

on human osteosarcoma-derived U-2 OS cells, focusing on the induction of apoptosis. Results indicated that i) ouabain at concentrations 5-60 μM significantly reduced the total viable cell number and induced cell morphological changes (at a concentration of 5 μM); ii) ouabain decreased G_0/G_1 phase and increased G_2/M phase and sub-G_1 phase in cell cycle distribution; iii) ouabain decreased ROS production and the levels of $\Delta\Psi_m$, though increased the production of Ca^{2+};

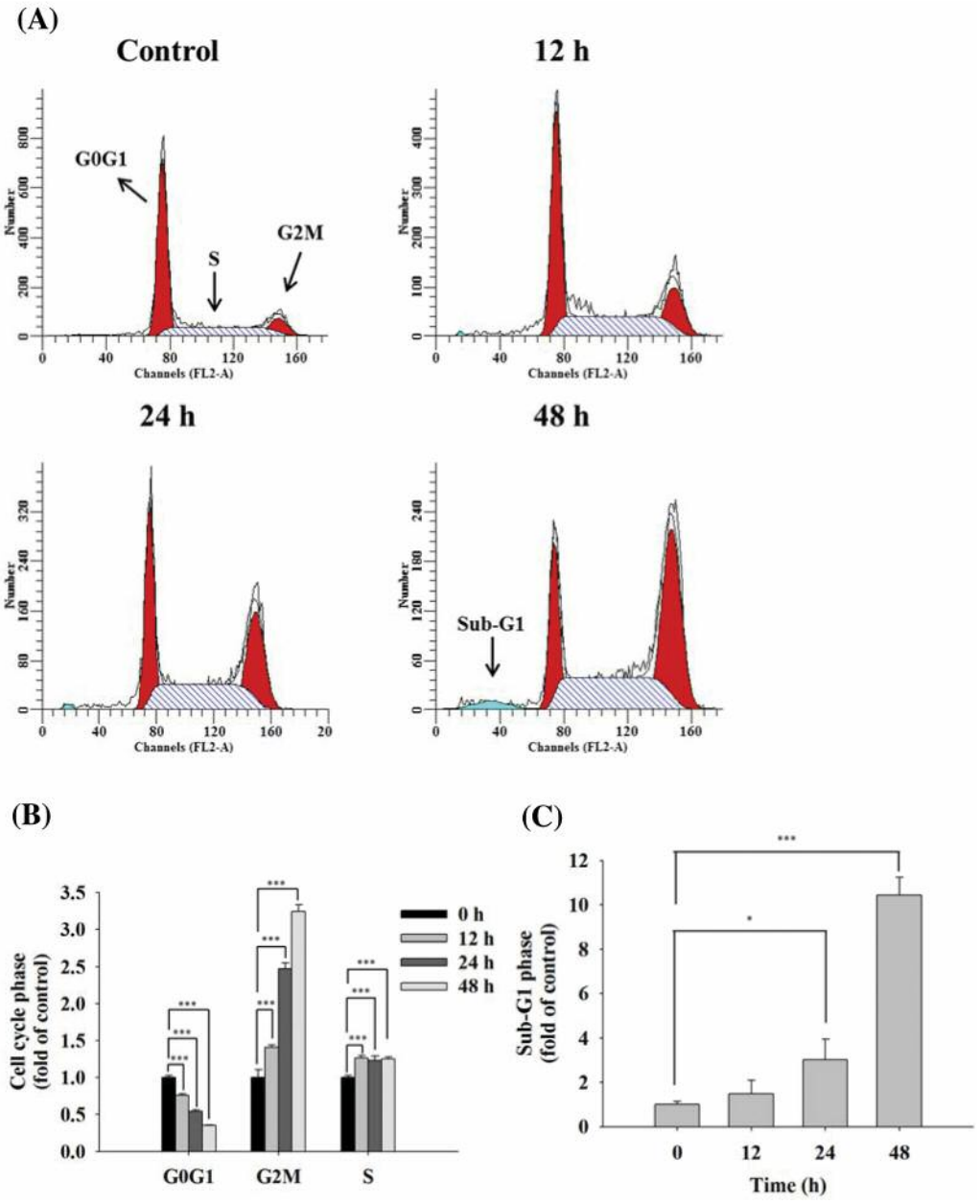

Figure 2. *Ouabain affects cell-cycle distribution in U-2 OS cells. Cells were treated with 5 μM for 0, 12, 24 and 48 h and cell-cycle distribution using flow cytometry. The proportions of cells in the G_0/G_1, S, and G_2/M phases were represented in DNA histograms (A). The percentages of cell-cycle distribution were measured (B) and sub-G_1 phase were quantified (C). Asterisks denote the statistical significance of the experimental group compared to the corresponding control group (*p<0.05, **p<0.01 ***p<0.001).*

iv) ouabain increased caspase-3, -8 and -9 activity; v) ouabain induced apoptosis *via* the Fas/FasL pathway, as indicated by the protein expression of FADD, Fas, FasL, the increased pro-apoptotic Bax and decreased anti-apoptotic Bcl-2 protein levels on U-2 OS cells. This is in agreement with previous reports showing that ouabain induced cytotoxic effects on human cancer cell lines (10, 11). Thus, concentration of 5 μM of ouabain was selected to examine the expression of apoptotic pathway-related proteins in U-2 OS cells.

The present results indicated that ouabain significantly decreased total viable cell number and induced morphological changes on treated U-2 OS cells, in a dose-

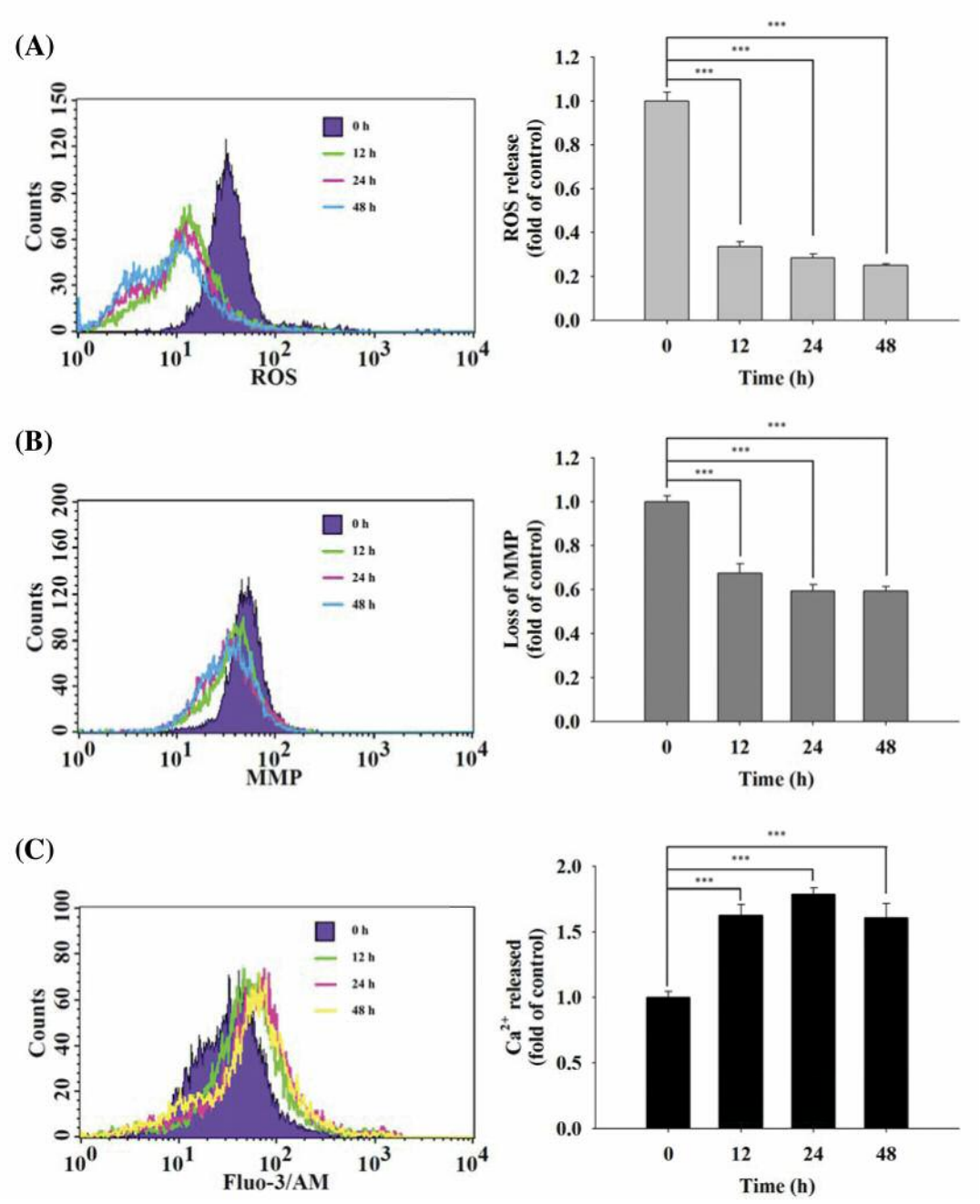

Figure 3. *Ouabain affects reactive oxygen species (ROS), intracellular Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$) in U-2 OS. Cells (1.6×10^5 cells/well) were treated with 5 μM for 0, 12, 24 and 48 h. Ouabain treatment resulted in reduced release of ROS (H_2O_2) (A), and of $\Delta\Psi_m$ levels (B), while increased intracellular Ca^{2+} release (C). Asterisks denote the statistical significance of the experimental group compared to the corresponding control group (*p<0.05, **p<0.01, ***p<0.001).*

and time-dependent manner, respectively. This finding is in agreement with previous studies (10, 11). Results also showed that ouabain significantly decreased G_0/G_1 phase and increased G_2/M phase and sub-G_1 arrest in cell-cycle distribution on U-2 OS cells (Figure 2) and both effects of ouabain are time-dependent. This is in line with other reports that ouabain induced G_2/M phase arrest and increase S phase in hepatoma cell lines (HepG2 and SMMC-7721) cancer cells (20). Ouabain inhibits neuroblastoma SH-SY5Y cells *in vitro* and reducing by >50% tumor growth when SH-

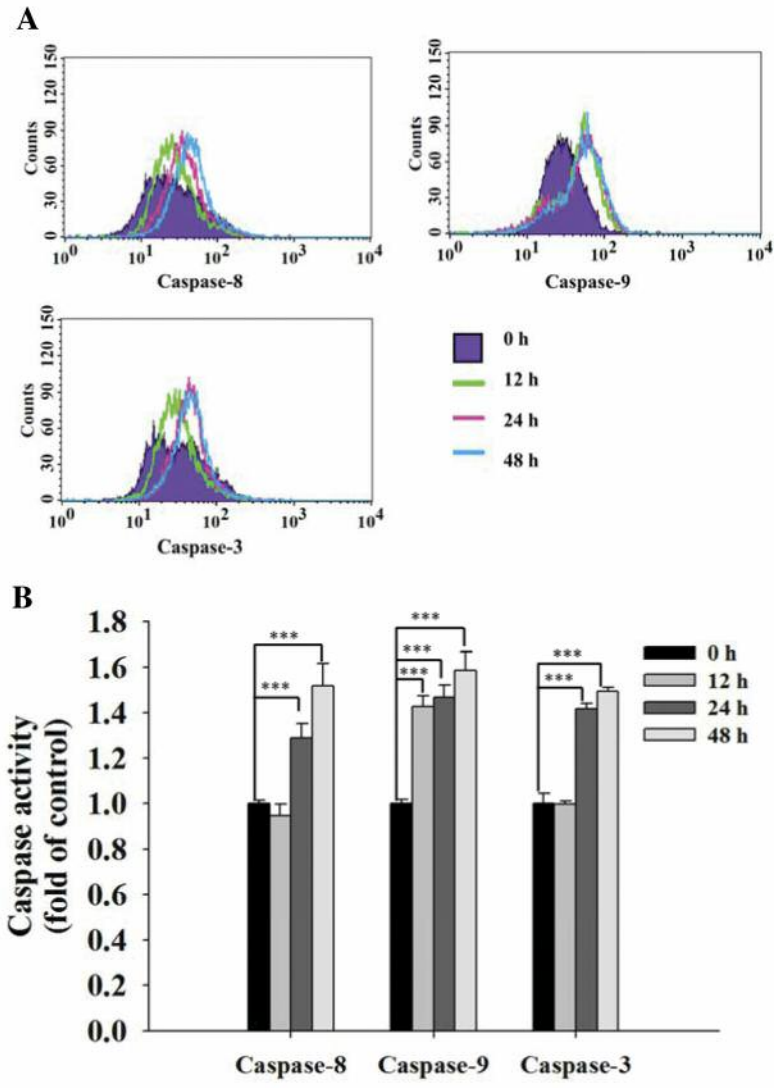

Figure 4. *Ouabain affects caspase activity in U-2 OS cells. Cells were treated with 5 μM for 0, 12, 24 and 48 h and intracellular activity caspase-8, caspase-9, and caspase-3 was measured (A). Results (quantified) are presented in the graph (B). Asterisks denote the statistical significance of the experimental group compared to the corresponding control group (*p<0.05, **p<0.01, ***p<0.001).*

SY5Y neuroblastoma cells were xenograft into immune-deficient mice (21). Cell-cycle arrests also have been recognized to be one of the mechanisms of anticancer drugs action (22, 23).

It is well-known that anticancer agent-induced apoptosis through ER stress involves production of ROS and Ca^{2+} release accompanied by mitochondrial dysfunction such as decreased $\Delta\Psi_m$ levels (24, 25). Herein, we found that ouabain increased Ca^{2+} production, but decreased ROS production and the level of $\Delta\Psi_m$ in U-2 OS cells (Figure 3). Other reports have shown ouabain to induce Ca^{2+} production (26) and ROS generation (27) that are involved in glioblastoma cell apoptosis. The contrary results might be due to the different cancer cell types, and further investigations are necessary. Though, the negative effect of ouabain on the level of $\Delta\Psi_m$ in U-2 OS cells is in agreement with other reports on prostate cancer PC-3 cells (6).

Apoptosis pathway also can be divided into caspase-dependent (28) and -independent (necroptosis) pathway (29). The caspase-dependent pathway is triggered by Fas and FasL interaction or death receptor for the activation of caspase-8 and -3 to induce apoptosis (28). Otherwise, it can be triggered by dysfunction of mitochondria and subsequent release of AIF and Endo G to induce cell apoptosis without

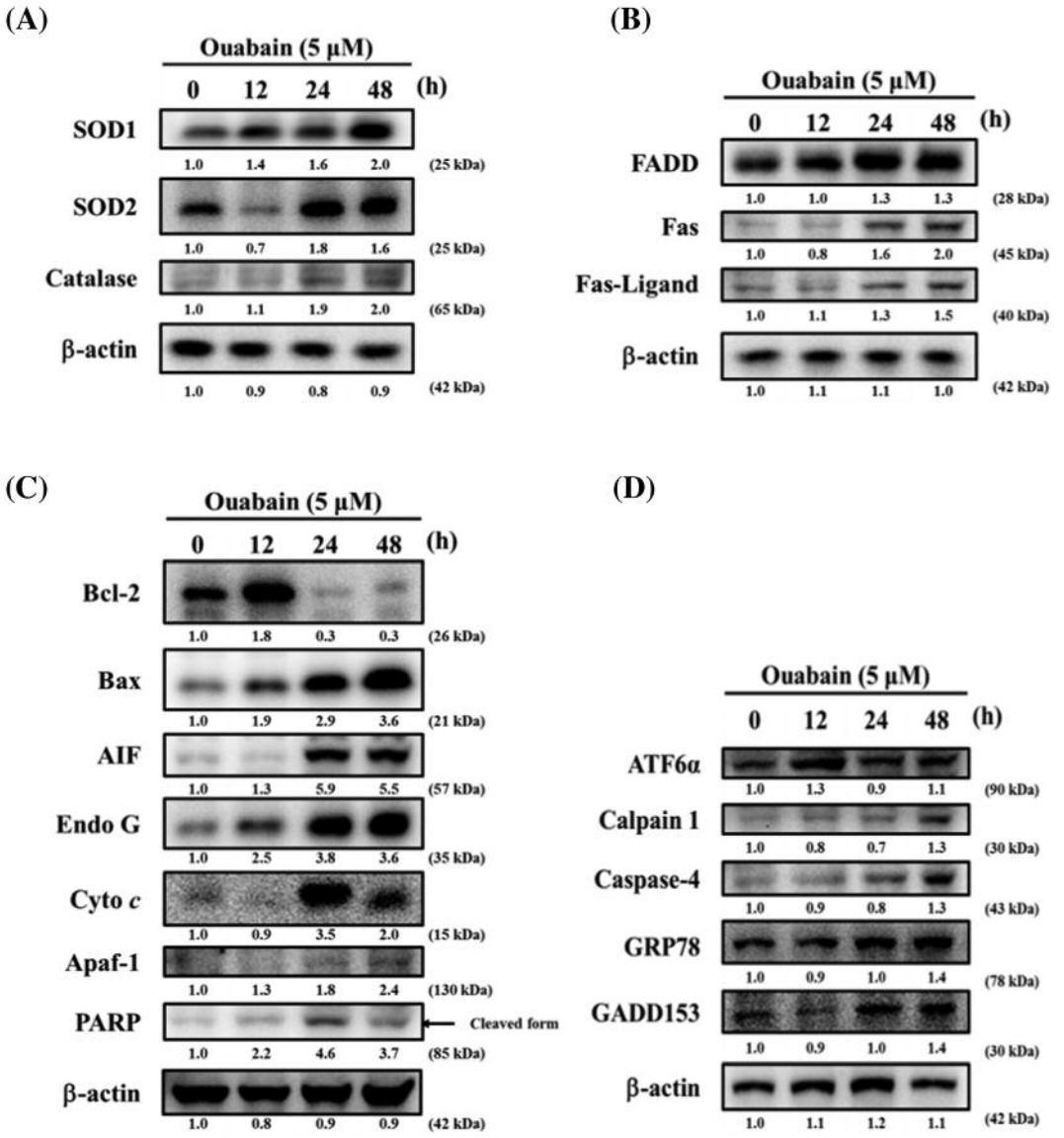

Figure 5. *Ouabain affects the levels of apoptosis associated proteins in U-2 OS cells. The levels of SOD1, SOD2 and catalase (A); FADD, Fas, and Fas-ligand (B), Bcl-2, Bax, AIF, Endo G, cytochrome c, Apaf-1 and PARP (C), ATF6α, Calpain 1, Caspase-4, GRP78 and GADD153 (D) expressions were estimated by western blotting. Fold-changes values are displayed below each band.*

caspase involvement (29). In the present study, we found that ouabain induced the activities of caspase-8, -9, and -3 in U-2 OS cells (Figure 4).

In order to further confirm ouabain-induced apoptotic cell death and whether it affects apoptosis-associated protein expression in U-2 OS cells, we examined protein expression in U-2 OS cells after exposed to 5 μM of ouabain for various time periods. Results demonstrated that ouabain significantly inhibited the expression of anti-apoptotic protein Bcl-2 (Figure 5C) and increased pro-apoptotic protein Bax (Figure 5C) in U-2 OS cells. Previous evidence has shown that the ratio of the apoptosis-related proteins, Bax/Bcl-2, is associated with the level of $\Delta\Psi_m$ (30, 31). Herein, we clearly showed that ouabain increased the expression of cytochrome c, Afpa-1, PARP, AIF and Endo G (Figure 5C), indicating that ouabain-induced apoptosis was associated with mitochondrial dysfunction in U-2 OS cells. Furthermore, we also found that ouabain increased the expression of FADD, Fas, and FasL (Figure 5B) indicating that ouabain-induced cell apoptosis in U-2 OS cells might involve the activation of Fas and FasL.

Thus, we may suggest that ouabain induced apoptosis of U-2 OS cells through caspase-dependent and also mitochondria-dependent pathway.

In conclusion, ouabain induced S-G_2/M phase cell-cycle arrest in U-2 OS cells, *in vitro*. Furthermore, ouabain induced apoptotic cell death with the involvement of caspase-dependent and -independent pathways, accompanied also by mitochondrial dysfunction. Thus, ouabain should be further examined *in vivo*, in order to justify its effectiveness in the prevention and treatment of osteosarcoma cancer.

Conflicts of Interest

The Authors do not have any conflicts of interest to disclose.

Acknowledgements

This study was supported by the grant 105-15 from Cheng Hsin General Hospital and SKH-8302-106-NDR-09 from Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan; R.O.C.. Experiments and data analysis were performed in part through the use of the Medical Research Core Facilities Center, Office of Research & Development at China medical University, Taichung, Taiwan, R.O.C.

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Received September 22, 2017
Revised October 25, 2017
Accepted October 30, 2017