Ouabain Induces DNA Damage in Human Osteosarcoma U-2 OS Cells and Alters the Expression of DNA Damage and DNA Repair–associated Proteins

JIUN-LONG YANG[1*], MEI-DUE YANG[2*], JAW-CHYUN CHEN[3], KUNG-WEN LU[4],
YI-PING HUANG[5], SHU-FEN PENG[6,7], FU-SHIN CHUEH[8], KUO-CHING LIU[9],
TZU-SHUN LIN[1,10], PO-YUAN CHEN[6#] and WEI-JEN CHEN[11,12#]

[1]Department of Nursing, Saint Mary's Junior College of Medicine, Nursing and Management, Yilan, Taiwan, R.O.C.;
[2]Department of Surgery, China Medical University Hospital, Taichung, Taiwan, R.O.C.;
[3]Department of Medicinal Botany and Health Applications, Da-Yeh University, Changhua, Taiwan, R.O.C.;
[4]College of Chinese Medicine, School of Post-Baccalaureate Chinese Medicine,
China Medical University, Taichung, Taiwan, R.O.C.;
[5]Department of Physiology, School of Medicine, China Medical University, Taichung, Taiwan, R.O.C.;
[6]Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, R.O.C.;
[7]Department of Medical Research, China Medical University Hospital, Taichung, Taiwan, R.O.C.;
[8]Department of Food Nutrition and Health Biotechnology, Asia University, Taichung, Taiwan, R.O.C.;
[9]Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung, Taiwan, R.O.C.;
[10]Department of Pharmacy, Saint Mary's Hospital Luodong, Yilan, Taiwan, R.O.C.;
[11]Department of Orthopedics, Chang Bing Show-Chwan Memorial Hospital, Changhua, Taiwan, R.O.C.;
[12]Department of Orthopedics, Show-Chwan Memorial Hospital, Changhua, Taiwan, R.O.C.

Abstract. *Background/Aim: Ouabain, isolated from natural plants, exhibits anticancer activities; however, no report has presented its mechanism of DNA damage induction in human osteosarcoma cancer cells in vitro. The aim of this study was to investigate whether ouabain induces DNA damage and repair, accompanied with molecular pathways in human osteosarcoma cancer U-2 OS cells in vitro. Materials and Methods: The percentage of viable cell number was measured by flow cytometric assay; DNA damage was assayed by DAPI staining, comet assay, and agarose gel electrophoresis. DNA damage and repair associated protein expressions were assayed by western blotting assays. Results: Ouabain reduced total cell viability, induced chromatin condensation, DNA fragmentation, and DNA damage in U-2 OS cells. Ouabain increased p-ATM^{Ser1981}, p-ATR^{Ser428}, and p53 at 2.5-10 μM, increased p-p53^{Ser15} at 10 μM; however, it decreased p-MDM2^{Ser166} at 2.5-10 μM. Ouabain increased p-H2A.X^{Ser139}, MDC-1, and PARP at 2.5-10 μM and BRCA1 at 5-10 μM; however, it decreased DNA-PK and MGMT at 2.5-10 μM in U-2 OS cells at 48 h treatment. Ouabain promoted expression and nuclear translocation of p-H2A.X^{Ser139} in U-2 OS cells and this was confirmed by confocal laser microscopy. Conclusion: Ouabain reduced total viable cell number through triggering DNA damage and altering the protein expression of DNA damage and repair system in U-2 OS cells in vitro.*

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*,#These Authors contributed equally to this study.

Correspondence to: Po-Yuan Chen, Ph.D., Department of Biological Science and Technology, China Medical University, No. 100, Section 1, Jingmao Road, Beitun District, Taichung, Taiwan, R.O.C. Tel: +886 422053366 ext. 2525, Fax: +886 422053764, e-mail: pychen@mail.cmu.edu.tw; and Wei-Jen Chen, Dr., Department of Orthopedics, Show Chwan Memorial Hospital, No. 542, Section 1, Chung-Shan Road, Changhua, Taiwan, R.O.C. Tel: +886 975617171, e-mail: weijen128@yahoo.com.tw

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Osteosarcoma (OS) originates from long bones, including the humerus, femur, and tibia (1, 2) and it is the most frequent type of malignant bone cancer. OS contributes to cancer-

related mortality in adolescents (3, 4) and accounts for ~5% of cases of childhood cancer (2, 5, 6). Patients with osteosarcoma often have also a metastatic or recurrent disease, leading to a lower percentage of survival (7). Currently, the treatment of OS includes surgery, radiotherapy, and multiagent chemotherapy or a combination of chemotherapy, radiation therapy, and surgery, which have been shown to improve patient survival, which is still unsatisfactory (8-10). Therefore, developing effective and safe compounds for improving the survival rate of OS patients is urgent, and many studies are focusing on finding new compounds from natural products.

Agents and anticancer drugs inducing DNA single-strand break (SSB) or double-strand break (DSB) lesions may lead to genomic instability and cause cancer development. Etoposide induces double-strand damage of cancer cells by acting as an inhibitor of DNA topoisomerase II (11). Temozolomide, the first-line monotherapeutic agent for glioblastoma patients (12), exerts its cytotoxic effect by the induction of O^6-methylguanine and leads to DAN damage, including the formation of DNA double-strand breaks (DSB) and cell death (13). Tobacco smoke contains over 60 documented cancer-causing agents and some of these agents have been documented to induce DNA damage and mutations in human cells (14). Eukaryotic cells exhibit a network of biochemical pathways to sense, signal, and repair the DNA damage for regulating DNA damage response (15, 16). After being exposed to DNA damage agents, cells can repair DNA damage and remain alive. Otherwise, this may lead to cell death (17). Numerous anticancer drugs have been obtained from natural products. Therefore, these products induce DNA damage or affect DNA repair systems of cancer cells and this may play important roles in their anticancer activities.

Ouabain, a cardiotonic steroid, is derived from the *Strophanthus gratus* and *Acokanthera schimperi* and acts like a hormone (18, 19) that regulates cell adhesion (20). Ouabain is also secreted endogenously by the adrenal glands (21) and exerts Na^+/K^+-ATPase binding activity (22). Besides, ouabain regulates blood pressure and Na^+ homeostasis (23, 24) and it has been also used for the treatment of heart failure and atrial fibrillation (25). Recently, it was shown that ouabain represents an attractive agent due to improved survival rate of gastric cancer patients (26, 27) and it exerts various biological functions in human cancer cells. It suppressed proliferation of breast cancer, including MCF-7 and MDA-MB-231 cells (28), induced TRAIL-mediated cell death in human lung cancer cells and prostate DU 145 cancer cells (29, 30), and triggered cell apoptosis in Jurkat cells (human acute T-cell lymphoblastic leukemia cells) (31). Besides, ouabain suppressed human renal cancer OS-RC-2 cell proliferation by targeting to the NKA α3 isoform (32). Furthermore, ouabain reduced IL-2 secretion and resulted in a decrease in regulatory T cell numbers in mice (33).

Currently, there are several reports regarding ouabain decreasing total viable cell number *via* inducing cell apoptosis in human osteosarcoma U-2 OS cells. However, the effects and possible signaling pathways associated with the expression of DNA damage and repair-associated proteins which were induced in human osteosarcoma cancer cells are still unclear. Therefore, herein, its effects on U-2 OS human osteosarcoma cancer cells were investigated. It was found that ouabain induced DNA damage and suppressed DNA repair associated protein expressions in U-2 OS cells *in vitro*.

Materials and Methods

Chemicals and reagents. Ouabain, dimethyl sulfoxide (DMSO), propidium iodide (PI), phosphate buffered saline (PBS), and trypsin-EDTA used in this study were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). McCoy's 5A medium, fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Anti-p-ATM^Ser1981, -PARP, and -p-H2A.X^Ser139 were obtained from GeneTex Inc. (Irvine, CA, USA). Anti-p-ATR^Ser428, -p-MDM2^Ser166, -p-p53^Ser15, and -BRCA1 were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-MGMT, -p53, and -β-actin were obtained from Sigma-Aldrich Corp.. Anti-DNA-PK was purchased from Calbiochem (San Diego, CA, USA). Anti-MDC1 was purchased from Millipore (Billerica, MA, USA). The anti-mouse IgG was obtained from Amersham Pharmacia Biotech, Inc (Piscataway, NJ, USA).

Cell line and cell culture. The human osteosarcoma cancer U-2 OS cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 90% McCoy's 5A medium supplemented 2 mM L-glutamine, 10% FBS, and antibiotics (100 Units/ml penicillin and 100 μg/ml streptomycin) in 75 cm^2 tissue culture flasks. Cells were cultured in a humidified incubator at 37°C and 5% CO_2 atmosphere (34).

Cell viability. U-2 OS cells were seeded at 1.6×10^5 cells/well in 12-well plates for 24 h and then treated with 0, 2.5, 5, and 10 μM of ouabain for 48 h. After incubation, all cells from each well were harvested and re-suspended in PBS containing 5 μg/ml of PI for measuring the percentage of cell viability by using flow cytometer, as cited previously (34).

4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. U-2 OS cells were grown at 1.6×10^5 cells/well in 12-well plate for 24 h and treated with 0, 2.5, 5, and 10 μM of ouabain for 48 h. At the end of incubation, 3.7% paraformaldehyde (v/v) in PBS was used to fix cells for 15 min, followed by being permeabilized with 0.1% Triton X-100 in PBS for 5 min, and stained with DAPI solution (2 μg/ml) for 10 min. All cells in each well were examined and photographed using a fluorescence microscope at 100×, as described previously (35).

Comet assay. The comet assay was used to determine the phenomenon of DNA damage in U-2 OS cells after exposed to ouabain, as described previously (36). U-2 OS cells (1.6×10^5 cells/well) were incubated with 0, 2.5, 5, and 10 μM of ouabain or H_2O_2 (as a positive control) for 48 h. All cells from each sample

were examined for DNA damage by using comet assay (single-cell electrophoresis). The comets were randomly captured at a constant depth of the gel, and comet tail length was measured and quantified by using the Tri Tek Comet Score™ software image analysis system (TriTek Corp, Sumerduck, VA, USA) as described previously (36).

DNA gel electrophoresis. U-2 OS cells (2×10^6 cells) in 10-cm dishes were exposed to 0, 2.5, 5, and 10 μM of ouabain for 48 h. After treatment, cells were harvested from each treatment and lysed in ice-cold lysis buffer and then DNA was extracted. The resulting DNA was quantitated and electrophoresed on a 1.5% agarose gel, examined and photographed as previously described (37).

Western blotting. U-2 OS cells (2×10^6 cells) in 10-cm dishes were exposed to 0, 2.5, 5, and 10 μM of ouabain for 48 h, and cells from each treatment were collected. The total cellular proteins from each sample were extracted by RIPA buffer [50 mM Tris-HCI (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA containing both 1% protease inhibitor and 1% phosphatase inhibitor mixture II] (Sigma-Aldrich Corp.) as described previously (36, 37) and extracted proteins were quantitated using BioRad assay kit (BSA as a protein standard) as described previously (36, 37). Each protein samples (30 μg) were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA, USA). The membranes were blocked with 2.5% BSA and probed with indicated primary antibodies: anti-p-ATM^{Ser1981}, -p-ATR^{Ser428}, -PARP, -MGMT, -p-MDM2^{Ser166}, -p53, -p-p53^{Ser15}, -p-H2A.X^{Ser139}, -BRCA1, -DNA-PK, -MDC1, and -β-actin (1:1,000). The appropriate secondary antibodies (goat anti-mouse IgG coupled to HRP used at a 1:5,000 dilution) and the bound antibodies were detected by Chemiluminescent HRP substrate (Millipore Corp.) and quantified by ImageJ software as described previously (36, 37).

Confocal laser scanning microscopy. U-2 OS cells were grown on coverslips at 5×10^4 cells/slide and exposed to 0, 2.5, 5, and 10 μM of ouabain for 48 h. After exposure, cells were fixed with 4% formaldehyde in PBS and were permeabilized by using 0.2% Triton-X 100 in PBS for 15 min. Cells were washed and probed with anti-p-H2A.X^{Ser139} primary antibody, followed by FITC-conjugated goat anti-mouse IgG (green fluorescence), and PI (red fluorescence) was used to stain the nucleus. After washed with PBS, all samples were examined and photographed under a Leica TCS SP2 Confocal Spectral Microscope as described previously (36, 37).

Statistical analysis. The data from each experiment are presented as the mean±standard deviation (S.D.) as indicated in the figures and were analyzed using one-way ANOVA. $p < 0.05$ is considered as significant differences between the ouabain-treated and -untreated (control) groups.

Results

Ouabain decreased cell viability. U-2 OS cells were exposed to ouabain at a final concentration of 0, 2.5, 5, and 10 μM for 48 h. All samples were collected and measured for total viable cell number (cell viability) by the PI exclusion method, and results are presented in Figure 1. Results
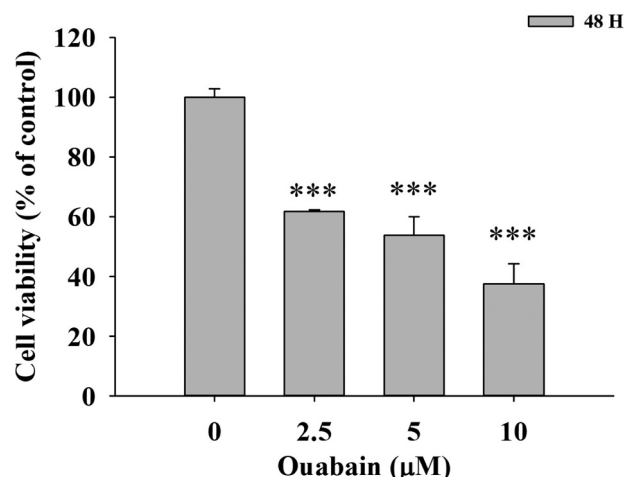

Figure 1. *Ouabain decreased the percentage of viable cells in U-2 OS cells. Cells were incubated with 0, 2.5, 5, and 10 μM of ouabain for 48 h and were collected for measuring the percentage of viable cells by flow cytometry. Experiments were performed in triplicate as described in Materials and Methods. Data represent mean±S.D. *p<0.05 was significantly different between ouabain-treated and control groups.*

indicated that increased concentrations (2.5-10 μM) of ouabain led to decreasing total cell viability from 61.74, 53.79 and 37.48%, respectively, and these effects were observed in a dose-dependent manner (Figure 1).

Ouabain induced chromatin condensation. To understand whether ouabain reduced total cell viability *via* induction of chromatin condensation which is one of the character of cell apoptosis, U-2 OS cells were incubated with various concentrations (0, 2.5, 5, and 10 μM) of ouabain for 48 h. Subsequently, cells were stained with DAPI solution, and the results are shown in Figure 2. Cells treated with ouabain showed a lighter staining (DAPI dyed; Figure 2A) and higher intensity of DAPI fluorescence (Figure 2B) than that of the control group in U-2 OS cells. Thus, results indicated that ouabain at 10 μM for 48 h significantly induced chromatin condensation.

Ouabain induced DNA damage. For further exploring the reduction of cell viability of U-2 OS cells mediated by induction of DNA damage, cells were exposed to ouabain (0, 2.5, 5, and 10 μM) for 48 h, collected, and measured using their comet tail by single-cell electrophoresis (comet assay). Ouabain at 5 and 10 μM significantly increased comet tail length to that of control groups. The positive-control group (H_2O_2) has the highest comet tail (Figure 3A). These observations indicated that ouabain induced DNA damage at 48 h treatment, and these effects are dose-dependent in U-2 OS cells (Figure 3B).
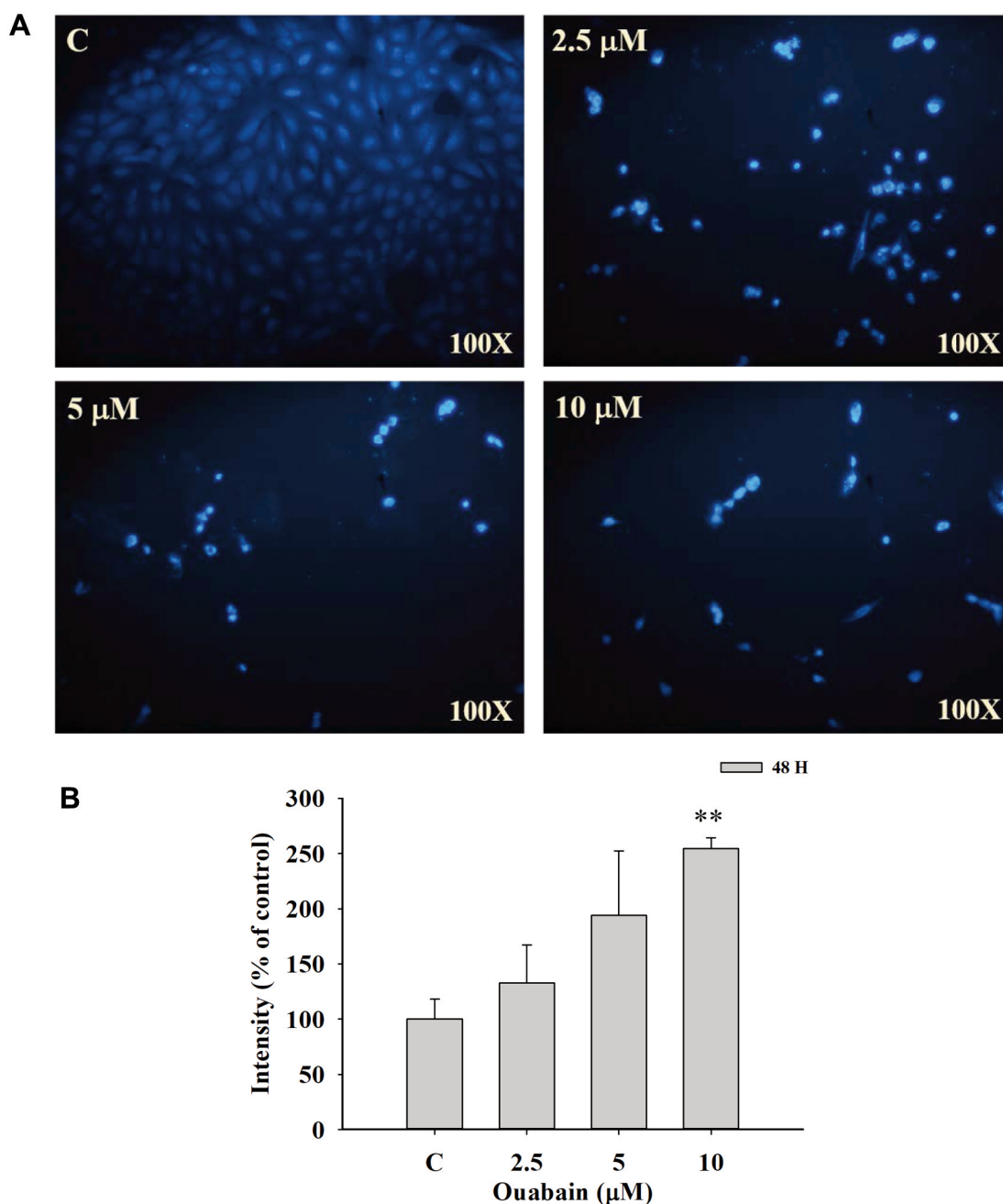
Figure 2. *Ouabain affected DNA condensation in U-2 OS cells. Cells (1.6×10^{5} cells/well) were placed in 12-well plates and incubated with 0, 2.5, 5, and 10 μM of ouabain for 48 h. Cells were fixed, permeablized and nuclei were stained with 2 μg/ml of DAPI for 10 min. All samples were examined and photographed using a fluorescence microscope at 100× (A) and were measured for the intensity of fluorescence (B) as described in Materials and Methods. Data represent mean±S.D. *p<0.05 was significantly different between ouabain-treated and control groups.*

Ouabain induced DNA fragmentation. To confirm the effects of ouabain on DNA damage, DNA fragmentation was analyzed by agarose gel electrophoresis, which displayed the presence of laddered DNA in U-2 OS cells. After exposure to 0, 2.5, 5, and 10 μM of ouabain for 48 h, DNA was extracted from each treatment and electrophoresed in agarose gel. As shown in Figure 4, ouabain induced DNA fragmentation at 48 h treatment, indicating the development of apoptotic cell death in U-2 OS cells.
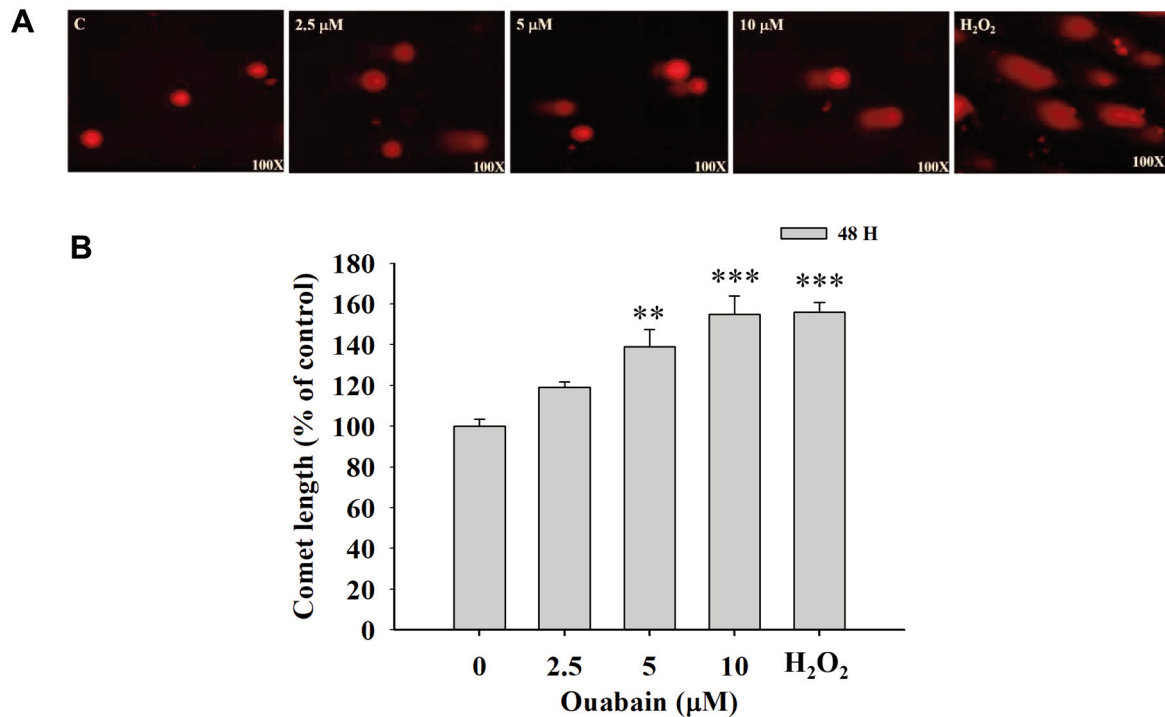
A

B

Figure 3. *Ouabain induced DNA damage in U-2 OS cells. Cells were incubated with 0, 2.5, 5, and 10 μM of ouabain and H_2O_2 for 48 h and were measured by the Comet assay (A) and evaluated for intensity of fluorescence (B) as described in Materials and Methods. Data represent mean±S.D. *p<0.05 was significantly different between ouabain-treated and control groups.*

Ouabain affected the levels of DNA damage and repair-associated proteins. To further confirm ouabain altered DNA damage and repair-associated proteins, cells were exposed to 0, 2.5, 5, and 10 μM of ouabain for 48 h and collected for western blotting analysis. As shown in Figure 5, ouabain increased p-ATM^{Ser1981}, p-ATR^{Ser428}, and p53 at 2.5-10 μM and increased p-p53^{Ser15} at 10 μM. However, ouabain decreased p-MDM2^{Ser166} at 2.5-10 μM (Figure 5A). Furthermore, ouabain increased p-H2A.X^{Ser139}, MDC-1, and PARP at 2.5-10 μM, and increased BRCA1 at 5-10 μM, but decreased DNA-PK and MGMT at 2.5-10 μM in U-2 OS cells at 48 h treatment (Figure 5B).

Ouabain affected nuclear translocation of p-H2A.X^{Ser139}. Results from western blotting already showed that ouabain at 2.5-10 μM treatment increased p-H2A.X^{Ser139} protein levels in U-2 OS cells. We further investigated whether the induction of DNA damage by ouabain is also involved in the nuclear translocation of p-H2A.X^{Ser139} in U-2 OS cells. Cells were exposed to 0, 2.5, 5, and 10 μM of ouabain for 48 h, probed with p-H2A.X^{Ser139} antibody, and observed under confocal laser microscopy. As shown in Figure 6, ouabain promoted the expression and enhanced the nuclear translocation of p-
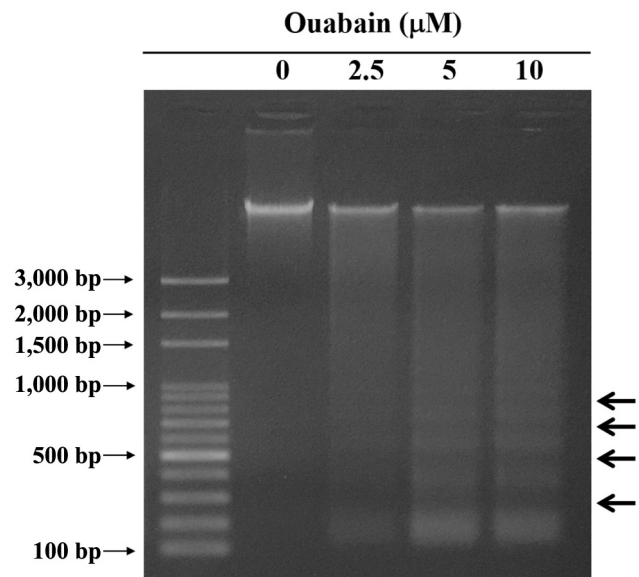

Figure 4. *Ouabain induced DNA fragmentation in U-2 OS cells. Cells were incubated with 0, 2.5, 5, and 10 μM of ouabain for 48 h. Cells were collected, lysed and DNA was extracted for DNA gel electrophoresis as described in Materials and Methods.*
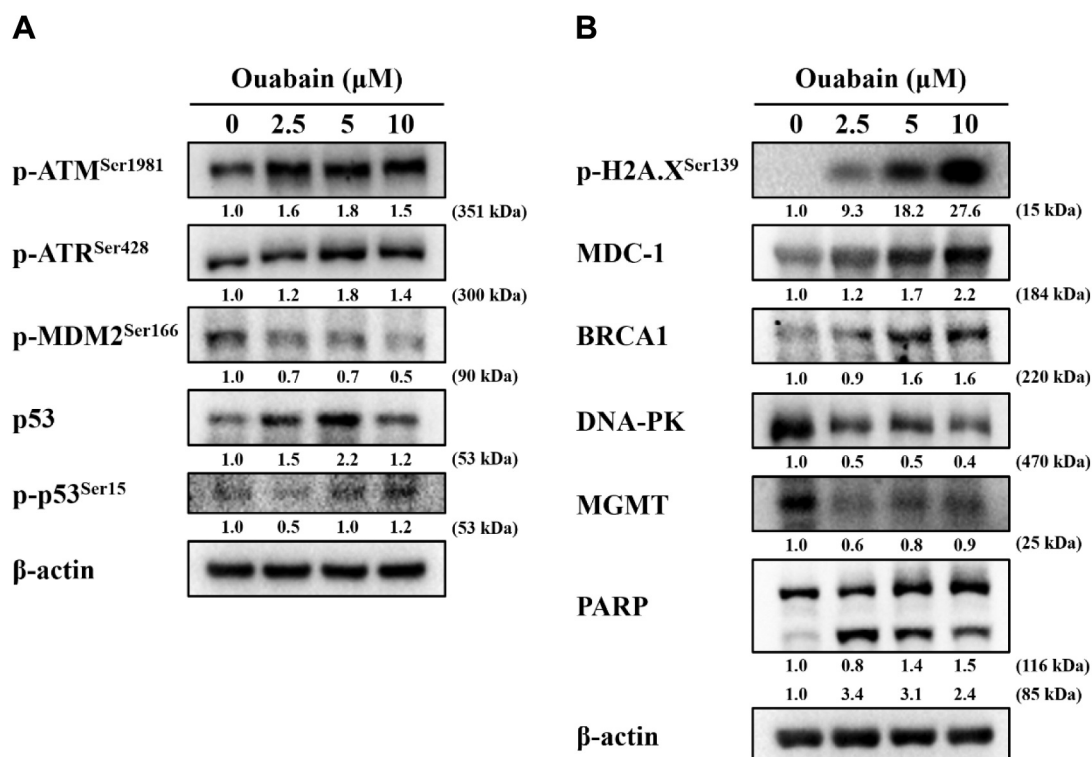
A

Ouabain (μM)

	0	2.5	5	10	
p-ATM^{Ser1981}					
	1.0	1.6	1.8	1.5	(351 kDa)
p-ATR^{Ser428}					
	1.0	1.2	1.8	1.4	(300 kDa)
p-MDM2^{Ser166}					
	1.0	0.7	0.7	0.5	(90 kDa)
p53					
	1.0	1.5	2.2	1.2	(53 kDa)
p-p53^{Ser15}					
	1.0	0.5	1.0	1.2	(53 kDa)
β-actin					

B

Ouabain (μM)

	0	2.5	5	10	
p-H2A.X^{Ser139}					
	1.0	9.3	18.2	27.6	(15 kDa)
MDC-1					
	1.0	1.2	1.7	2.2	(184 kDa)
BRCA1					
	1.0	0.9	1.6	1.6	(220 kDa)
DNA-PK					
	1.0	0.5	0.5	0.4	(470 kDa)
MGMT					
	1.0	0.6	0.8	0.9	(25 kDa)
PARP					
	1.0	0.8	1.4	1.5	(116 kDa)
	1.0	3.4	3.1	2.4	(85 kDa)
β-actin					

Figure 5. *Ouabain altered the DNA damage and repair associated protein expressions in U-2 OS cells. Cells were placed in 10-cm dishes and were incubated with 0, 2.5, 5, and 10 μM of ouabain for 48 h. Cells were collected for western blotting and the resulting membranes were used to probe anti-p-ATM^{Ser1981}, -p-ATR^{Ser428}, -p-MDM2^{Ser166}, -p53 and -p-p53^{Ser15} (A), -p-H2A.X^{Ser139}, -MDC1, -BRCA1, -DNA-PK, -MGMT and -PARP (B) as described in Materials and Methods. β-actin was used as an internal control.*

H2A.X^{Ser139} in U-2 OS cells and these effects were dose-dependent (Figure 6).

Discussion

Numerous evidence has shown that certain clinical anticancer drugs not only induce cancer cell apoptosis but also cause DNA damage and alter the expression of DNA repair associated proteins. After further animal studies, anticancer drugs also reduced the tumor size of a tumor-xenografted animal model *in vivo* (38-40). Furthermore, DNA damage is a critical parameter for examining the carcinogenicity of chemicals (41, 42). Numerous studies have shown that ouabain induced cytotoxic effects such as reducing total viable cell number *in vitro* and inducing cell apoptosis *in vitro*. Therefore, in the present study, the aim was to further investigate whether or not ouabain induces DNA damage in human osteosarcoma U-2 OS cells *in vitro*.

In the first experiments, we evaluated the cytotoxic effects of ouabain on U-2 OS cells and results indicated that ouabain reduced total cell viability dose-dependently (Figure 1). These results are also consistent with the report that ouabain decreased

the total viable numbers of breast cancer cells (28). Our earlier studies have shown that ouabain reduced viable cell numbers *via* apoptotic cell death in U-2 OS cells (43). However, no report is available on whether ouabain reduces cell viability concerning DNA damage; therefore, we used DAPI staining, comet assay, and DNA electrophoresis to confirm these. As shown in Figure 2, ouabain induced DNA condensation time-dependently. Many reports have shown that anticancer drugs induce DNA condensation (one of the aspects of cell apoptosis) in cancer cells, which were also able to be approved by DAPI staining (44, 45). Figure 3A and B indicate that ouabain induced the development of DNA comet tailing, indicating DNA damage in U-2 OS cells. Cells exposed to genotoxic agents may have induced error replications of genomic DNA resulting in genomic instability and cancer development (15). The plasticity of chromatin structure provides cellular responses to DNA damage (46, 47) and chromosome is also the physiological template for DNA repair machinery to maintain and restore genome integrity for cell survival (36). Furthermore, DNA damage induced by ouabain in U-2 OS cells was observed in agarose gels, showing the presence of DNA fragmentation (laddered DNA), which is a marker of cell apoptosis (15, 48).
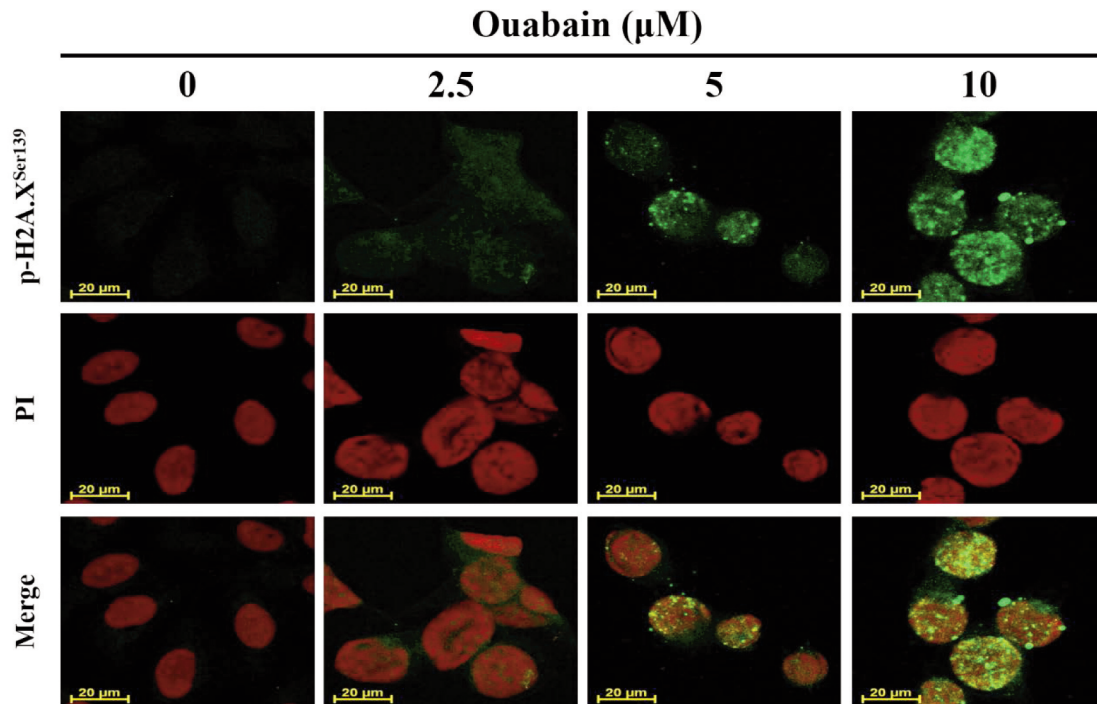
Ouabain (μM)

Figure 6. *Ouabain altered the translocation of p-H2A.X^{Ser139} in U-2 OS cells. Cells (5×10^4 cells/well) were placed on 4-well chamber slides and incubated with 0, 2.5, 5, and 10 μM of ouabain for 48 h. Cells were stained by anti- p-H2A.X^{Ser139} (green fluorescence) and were stained with FITC-conjugated goat anti-mouse IgG. All cells were stained by PI (red fluorescence) for nucleus examination and were photographed under a Leica TCS SP2 Confocal Spectral Microscope as described in Materials and Methods.*

In the other parts of the experiment, we evaluated the expression of proteins associated with DNA damage and repair in U-2 OS cells after exposure to various concentrations of ouabain by western blotting. Results from Figure 5A indicated that ouabain increased phospho-ataxia telangiectasia mutation (p-ATM^{Ser1981}), phosphor-ataxia telangiectasia and rad3-related (p-ATR^{Ser428}), p53, and p-p53^{Ser15} but decreased p-MDM2^{Ser166}. It was reported that anticancer drugs such as etoposide could induce DNA double-strand breaks (DSBs), which can further activate ATM and ATR and then led to activating their target proteins, CHKs and p53, by phosphorylation to repair DSBs. If its DSB repair fails, cells will develop apoptosis (11, 49, 50). Furthermore, if cells are exposed to genotoxic agents (or anticancer drugs) and result in DNA damage, ATM could phosphorylate p53 to stabilize and transactivate the p53 target genes for cell surviving; otherwise, cell would die (51). It was reported that the lack of ATM is involved in lymphoid malignancies in human and mice (52, 53).

Herein, ouabain significantly increased the expression of p-p53^{Ser15} at 2.5-5 μM (Figure 5A). p53 maintains genomic stability when stress occurs, including hypoxia, DNA damage, and oncogene activation (54). After the signals of stress activate p53 *via* post-transcriptional mechanisms, p53 is phosphorylated to form p-p53 (55). Our results also indicate that ouabain suppress the expression of p-MDM2^{Ser166} in U-2 OS cells (Figure 5A). Recently, the mouse double minute 2 (MDM2) oncogene has been attractive for cancer therapy and the anti-MDM2 antisense oligo would be indicated as cancer therapeutic agents in the future (56).

Ouabain increased p-H2A.X^{Ser139}, MDC-1, and PARP at 2.5-10 μM and BRCA1 at 5-10 μM; however, it decreased DNA-PK and MGMT at 2.5-10 μM in U-2 OS cells at 48 h treatment (Figure 5B). It is reported that the phosphorylation of H2A.X (p-H2A.X^{Ser139}) is a highly specific and sensitive molecular marker for DNA damage (57). Thus, in the present study, ouabain promoted the levels of p-ATM^{Ser1981} and p-p53^{Ser15} (Figure 5A) and increased p-H2A.X^{Ser139} (Figure 5B). That is in agreement with other reports that the activated ATM possesses kinase activity, which could phosphorylate its substrates, including p53 and histone H2A.X (58). Breast Cancer 1 (BRCA1) is associated with an increased risk of breast and ovarian cancer, and its expression regulates the response to cancer therapies (59). BRCA1 protein has been reported to directly participate in double-strand DNA (dsDNA) break repair (60). DNA breaks phosphorylate substrates to activate ATM, ATR, and DNA-PK, which jointly orchestrate DNA repair and cell recovery (61). Poly-ADP
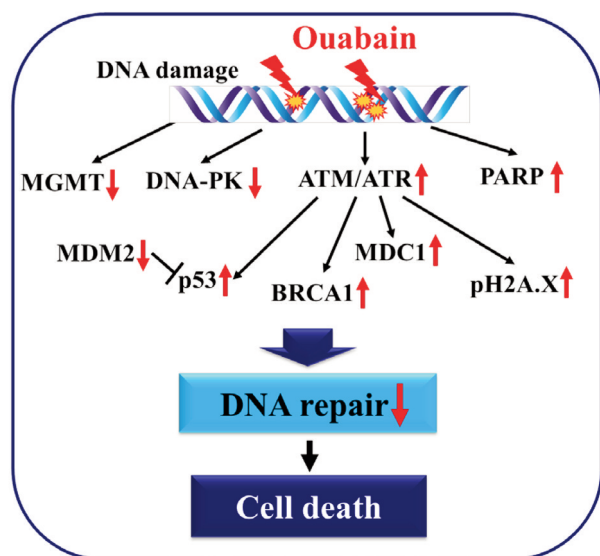
Figure 7. *The possible signaling pathways for ouabain-induced DNA damage and repair in U-2 OS cells in vitro.*

ribose polymerase (PARP) is involved in the DNA damage and repair system and in maintaining genome stability. Three related protein kinases, ataxia-telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK), are involved in the cellular response to DSBs (62). When DNA damage occurs, H2A.X, one the substrates of kinases, is phosphorylated on Ser139 at DSB sites and then referred to as γH2A.X (63) which is recognized by the mediator of DNA damage checkpoint 1 (MDC-1) (64) which acts as a platform for recruitment of various DNA damage response factors to mediate DNA repair.

In conclusion, based on these findings and observations, we suggested that ouabain decreased the total viable cell number of U-2 OS cells *in vitro via* induction of DNA damage and alteration of expression of DNA repair associated proteins. These results were also consistent with the results from the comet assay and DAPI staining, and were furthermore also confirmed ouabain-affected expression of DNA damage repair associated proteins such as p-ATM, p-ATR, BRCA1, MDC-1, p-H2A.X, p-p53, and MGMT in U-2 OS cells *in vitro* (Figure 7).

Conflicts of Interest

The Authors confirm that there are no conflicts of interest.

Authors' Contributions

Study conception and design: JLY, and MDY; Acquisition of data: JCC, KWL, YPH, and SFP; Analysis and interpretation of data:

Acknowledgements

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