ANTICANCER RESEARCH *41*: 4365-4375 (2021)
doi:10.21873/anticanres.15241

Ouabain Suppresses Cell Migration and Invasion in Human Gastric Cancer AGS Cells Through the Inhibition of MMP Signaling Pathways

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Abstract. *Background/Aim: Ouabain has been shown to induce human cancer cell death via apoptosis. Still, its anti-metastatic effect on cell migration and invasion of human gastric cancer cells has not been addressed. Materials and Methods: Cell proliferation and viability were measured by the MTT assay and flow cytometry, respectively. Cell motitlity was analysed by wound healing assay. Cell migration and invasion were analysed by the transwell system. Protein expression was assayed by western blotting. Results: Ouabain decreased AGS cell proliferation, cell viability, and motility. In addition, ouabain inhibited AGS cell migration and invasion. Furthermore, ouabain decreased matrix metalloproteinase-2 (MMP-2) activity at 48 h. Ouabain reduced the levels of proteins associated with PI3K/AKT and p38/MAPK pathways. In addition, ouabain decreased the expressions of N-cadherin, tissue inhibitor of metalloproteinases-1 (TIMP-1), urokinase-type plasminogen activator (c-uPA), and MMP-2 at 48 h. Conclusion: Ouabain suppresses cell metastasis through multiple signaling pathways in AGS cells.*

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Key Words: Ouabain, AGS cells, matrix metalloproteinase-2 (MMP-2), migration, invasion.

Gastric cancer (GC) is a major health burden worldwide and the second cause of cancer deaths in the year 2010 (1); however, in 2018, it was the third leading cause of cancer-related deaths following lung and liver cancer worldwide (2). In addition, over 90% of GCs are adenocarcinomas. In

2018, an estimated 3,658 new GC cases and 2,379 GC deaths were recorded in Taiwan (3). Globally, over 70% of GCs occur in developing countries (1). However, overall, less than 30% of patients diagnosed with GC survive for more than 5 years because the early stages are clinically silent (4) and large numbers of GC patients are diagnosed with metastases. Therefore, the overall clinical outcome of GC patients remains unsatisfactory (5, 6). Currently, the treatment of GC patients include radiotherapy, chemotherapy, or concurrent chemo-radiotherapy, and the side effects such as late toxicities are still a concern. Therefore, we attempted to find novel agents from natural products for GC treatment.

Ouabain is a steroid hormone secreted by the adrenal gland, hypothalamus (7, 8), and adrenocortical gland (9). It can bind to Na^+/K^+-ATPase (NKA) (10, 11) and acts as a cardiotonic steroid hormone in higher mammals (12); thus, ouabain is commonly used to study NKA function. At high concentrations (300 nM or greater), ouabain inhibits the function of the NKA pump and affects endocytosis and degradation of the tight junction molecules, resulting in the opening of tight junctions in the cellular membrane and the induction of cell death (13). At subsaturating concentrations, it stimulates cell proliferation (14, 15), modulates cell-to-cell contacts (16), and protects the cell from apoptosis (17, 18). However, at the low concentration of 10 nM, ouabain increases the degree of tight junction sealing (19), cell-cell communication (20), and accelerates ciliogenesis (21). Ouabain has also been reported to be involved in the regulation of immunologic functions (22, 23). Recently, in our previous study, we found that ouabain impairs cell migration and invasion in human osteosarcoma U-2 OS cells (24).

The high death rate of GC patients is associated with cancer metastasis (25). At first, cancer cells must migrate from their original site into the nearby circulatory system and then to presumptive metastatic sites for successful migration and invasion (26). Currently, therapeutic approaches for advanced GC with invasion and metastasis are not satisfying (27); thus, the progression and metastasis mechanisms of GC should be investigated. Extracellular matrix (ECM) plays a critical role in the metastasis of tumors (28). Matrix metalloproteinases (MMPs) digest ECM for cells to migrate to new sites (29, 30). In the present study, we report that ouabain inhibits cell migration and invasion of human gastric cancer AGS cells *via* the inhibition of MMP-2 associated pathways *in vitro*.

Materials and Methods

Chemicals and reagents. Ouabain, dimethyl sulfoxide (DMSO), propidium iodide (PI), and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, and antibiotics (penicillin/streptomycin) were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, CA, USA). The primary antibodies anti-son of sevenless 1 (SOS1), -growth factor receptor-bound protein-2 (GRB2), -Ras, and -matrix metalloproteinase-2 (MMP-2), -N-cadherin, -E-cadherin, -nuclear factor κB [NF-κB (p65)], -tissue inhibitor of metalloproteinases-1 (TIMP1), -Vimentin, -urokinase-type plasminogen activator (c-uPA), and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-p-p38, -phosphorylated extracellular signal-regulated protein kinases 1 and 2 (p-ERK1/2), -p-JNK1/2, -MEK1, -phosphatidylinositol 3-kinase (PI3K), -phosphorylated Akt (Thr308) (p-AKT), and -β-actin antibodies were obtained from Santa-Cruz Biotechnology, Inc. (Dallas, TX, USA). Ouabain stock solutions were dissolved in DMSO and further diluted in the culture medium.

Cell culture. The human gastric cancer cell line AGS was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin (100 Units/ml penicillin and 100 μg/ml streptomycin), and 10% FBS, and were incubated at 37˚C and in a humidified atmosphere of 5% CO_2 and 95% air (31).

Cell proliferation and viability assays. AGS cells (1×10^5 cells/well) were cultured with RPMI-1640 medium in 12-well plates overnight and treated with ouabain at final concentrations of 0, 0.4, 0.8, 1.2, and 1.6 μM for 24 and 48 h. After treatment, cell proliferation was assayed by MTT assay as previously described (32). In addition, cells were incubated with 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 μM for 48 h, harvested, incubated with the PI solution (5 μg/ml), and analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) as previously described (32).

Wound healing assay. AGS cells (3×10^5 cells/well) were cultured with RPMI-1640 medium in 12-well plates overnight; cells were scraped using a 200-μl tip and washed with PBS. After washing, cells were treated with ouabain at the final concentrations of 0, 0.4, 0.8, and 1.6 μM for 12 and 24 h. After treatment, the healing area was monitored and photographed under microscopy (32).

Cell migration and invasion assay. The transwell (Boyden chamber) coated with collagen and Matrigel was used to investigate cell migration and invasion, respectively, as described previously (33, 34). Transwell culture chambers with 8-μm pore size membranes (Millipore, Temecula, CA, USA) were coated with collagen overnight. AGS cells at the density of 5×10^4 cells/well were added to the upper chamber, which was filled with low-serum RPMI-1640 medium (2% of FBS) and ouabain (0, 0.4, 0.8, and 1.6 μM), RPMI-1640 medium with 10% FBS was added to the lower chamber, and then cells were incubated for 48 h. Cells migrated to the lower surface (penetrated the filter) were fixed with methanol, washed with PBS, stained with 2% crystal violet, and were examined and photographed under light microscopy at 200×. Migrated cells were counted and compared to control to determine the percentage of inhibition as described previously (33, 34). Measurement of cell invasion, which was performed as described in cell migration assay except collagen was replaced with Matrigel.

Gelatin zymography. AGS cells (1×10^5 cells/well) were cultured with RPMI-1640 medium containing 2% FBS in 12-well plates overnight and treated with 0, 0.4, 0.8, and 1.6 μM ouabain for 24 and 48 h. After treatment, the conditioned media were collected for MMP activities and 15 μl of the medium was electrophoresed on 10% SDS-PAGE

containing 0.2% gelatin gel. After electrophoresis, the gel was treated with 2.5% Triton X-100 and then soaked in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100, pH 8.0) while gently shaking for 24 h at 37˚C. Subsequently, the gel was stained with 0.2% Coomassie blue (Bio-Rad, Hercules, CA, USA) in 10% acetic acid and 50% methanol and was photographed on a lightbox. Proteolysis was detected as a clear zone (MMP-2 gelatinolytic activities) in a dark blue field, as described previously (35).

Western blotting analysis. The AGS cancer cell extracts were produced using a PRO-PERP™ protein extraction solution (Intron Biotechnology, Inc., Gyeonggi, Republic of Korea) as described previously (36-38). AGS cells (1×10^6 cells/dish) were cultured onto 10-cm dishes for 24 h and incubated with 1.6 μM ouabain for 0, 6, 12, 24 and 48 h. At the end of incubation, cells were collected, total proteins extracted and protein concentration was quantified by using the Bio-Rad protein assay kit (Hercules, CA, USA) as described previously (36-38). Protein samples were resolved by 8-12% (w/v) SDS-PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Belford, MA, USA) in transfer buffer (20% methanol, 15.6 mM Tris base, and 120 mM glycine) according to standard methods. The membrane was blocked with Tris-buffered saline and Tween-20 (TBST) containing 2.5% BSA for 2 h and probed with primary antibodies [anti-β-catenin, -β-actin, -c-uPA, -E-cadherin, -GRB2, -GSKα/β, -MEK1, -MMP-2, -N-cadherin, -NF-κB (p65), -p-AKT(Thr308), -p-ERK1/2, -PI3K, -p-JNK1/2, -p-p38, -Ras, -SOS1, -TIMP-1, and -Vimentin] at 4˚C overnight. Followed TBST wash for 10 min, the membrane was incubated with peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology), and protein bands were visualized using an ECL detection kit (Amersham ECL™, GE Healthcare, Chicago, IL, USA) and quantified as described previously (33, 34).

Statistical analysis. Data are presented as mean±SD. Statistical significance between ouabain-treated and control group values was analyzed by ANOVA. *p*-Values <0.05 were considered significant and designated by an asterisk (*).

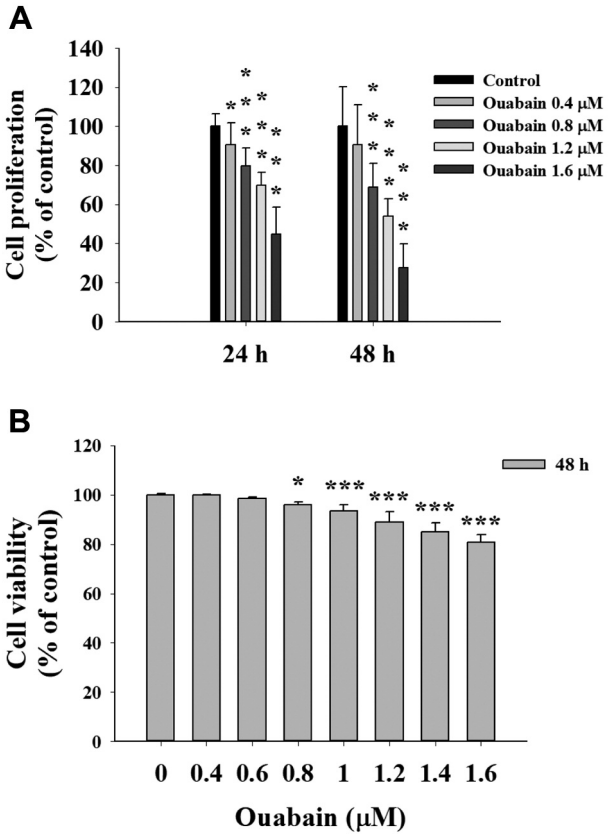

Figure 1. *Ouabain affected cell proliferation and viability of AGS cells. Cells (1×10^5 cells/well) were incubated with 0, 0.4, 0.8, 1.2 and 1.6 μM ouabain for 24 and 48 h and cell proliferation was examined by MTT assay (A). Cells were treated with 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 μM ouabain for 48 h, harvested, and cell viability was measured by flow cytometry (B) as described in Materials and Methods. *p<0.05, *p<0.001 indicates a significant difference between ouabain-treated groups and the control, as analyzed by one-way ANOVA.*

Results

Ouabain affects proliferation and viability in AGS cells. AGS cells were incubated with 0, 0.4, 0.8, 1.2, and 1.6 μM of ouabain for 24 and 48 h, and cell proliferation was assayed by MTT assay (Figure 1A). At both time treatments, ouabain inhibited cell proliferation dose-dependently. Furthermore, cells were treated with 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 μM ouabain for 48 h and cell viability was measured by flow cytometrty (Figure 1B). Ouabain was found to significantly decrease total cell viability at 0.8-1.6 μM compared to the control group. These effects of ouabain in AGS cells were dose-dependent.

Ouabain inhibits mobility of AGS cells. AGS cells were grown in 12-well plates until monolayer formation, wounded by scraping, and then incubated with 0, 0.4, 0.8, and 1.6 μM of ouabain for 12 and 24 h. As shown in Figure 2A, ouabain inhibited scrape wound closure in AGS cells. The percentage of migration inhibition was significantly increased following treatment with 0.4-1.6 μM ouabain for 12 and 24 h compared to the control (Figure 2B).

Ouabain inhibits migration and invasion in AGS cells. The Boyden chambers were coated with collagen or Matrigel and used to measure cell migration and invasion, respectively, *in vitro*. AGS cells were incubated with 0, 0.4, 0.8, and 1.6 μM ouabain for 48 h, fixed, permeabilized, and stained. The cells present in the lower surface of the filter membrane were photographed under phase-contrast microscopy. The percent inhibition of cell migration and invasion was calculated and these results are shown in Figure 3 and Figure 4, respectively. Ouabain (0.8 and 1.6 μM) significantly suppressed the cell migration (Figure 3) and invasion (Figure 4) of AGS cells *in vitro*.

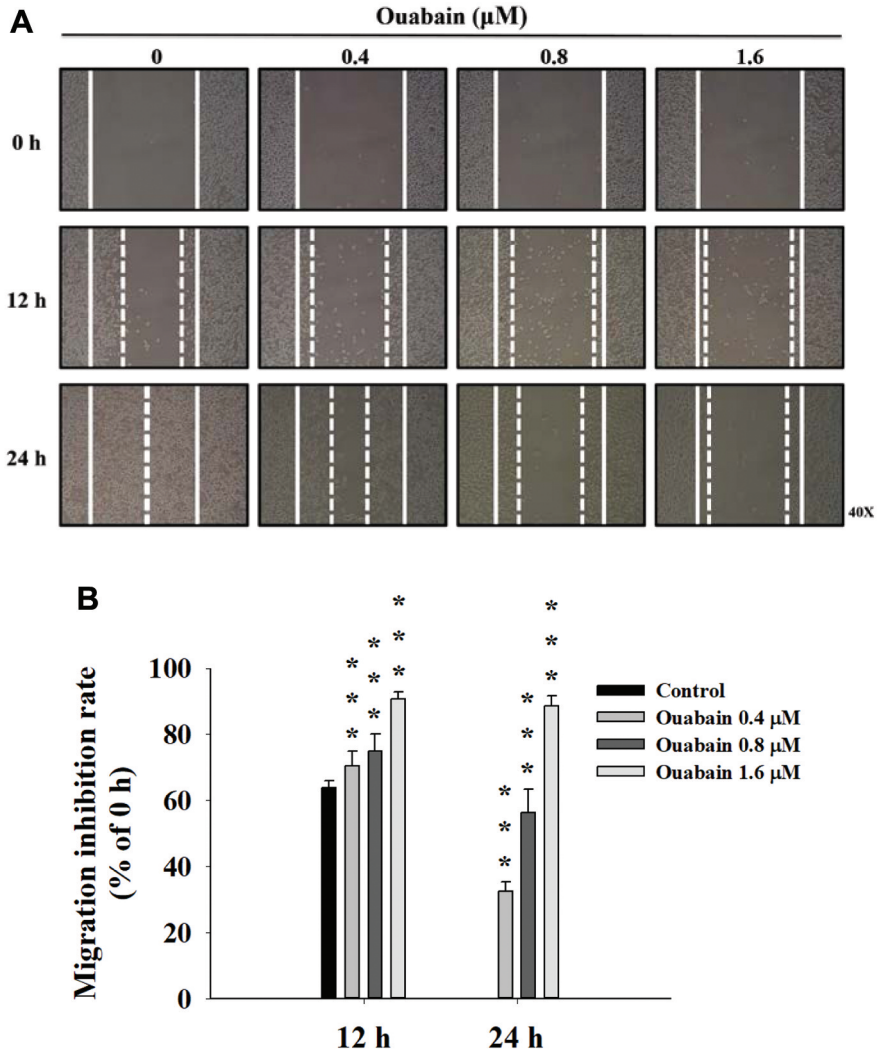

Figure 2. *Ouabain suppressed the mobility of AGS cells. Cells (3×10^5 cells/well) were cultured in 12-well plates overnight, scraped using 200-μl tips and washed with PBS. After washing, cells were treated with 0, 0.4, 0.8, and 1.6 μM ouabain for 12 and 24 h. The wound healing area was monitored and photographed at 12 and 24 h under a light microscope at 40× (A), and the healing area was quantified (B) as described in Materials and Methods. **p<0.001 indicates a significant difference between ouabain-treated groups and the control, as analyzed by one-way ANOVA.*

Ouabain inhibits the MMP activity in AGS cells. Gelatin zymography was used to measure the gelatinolytic activity of MMP-2 in the conditioned media of AGS cells. AGS cells were incubated with 0, 0.4, 0.8, and 1.6 μM ouabain for 24 and 48 h, and MMP activity in the conditioned medium of cells was assayed by gelatin zymography. Ouabain significantly inhibited the gelatinolytic activity of MMP-2 at 48 h treatment compared to the control (0 h) (Figure 5).

Ouabain affects key metastasis-related proteins in AGS cells. AGS cells were treated with 1.6 μM ouabain for 0, 6, 12, 24, and 48 h, harvested, and total proteins were quantitated for western blot analysis (Figure 6A-D). Ouabain (1.6 μM) significantly decreased the levels of SOS-1, GRB2, PI3K, p-AKT(Thr308) (Figure 6A), Ras, MEK-1, p-p38, NF-κB (p65) (Figure 6B), N-cadherin (Figure 6C), TIMP-1, c-uPA, and MMP-2 (Figure 6D). However, ouabain increased the levels of p-ERK1/2, p-JNK (Figure 6B), E-cadherin, Vimentin, GSK3α/β, and β-catenin (Figure 6C) in AGS cells. Based on these findings, ouabain suppresses cell metastasis of AGS cells through multiple signaling pathways.

Discussion

Numerous reports have demonstrated that the major reason for failure of treatment of cancer patients is cancer metastasis and the side effects of anti-cancer drugs. GC is a biologically

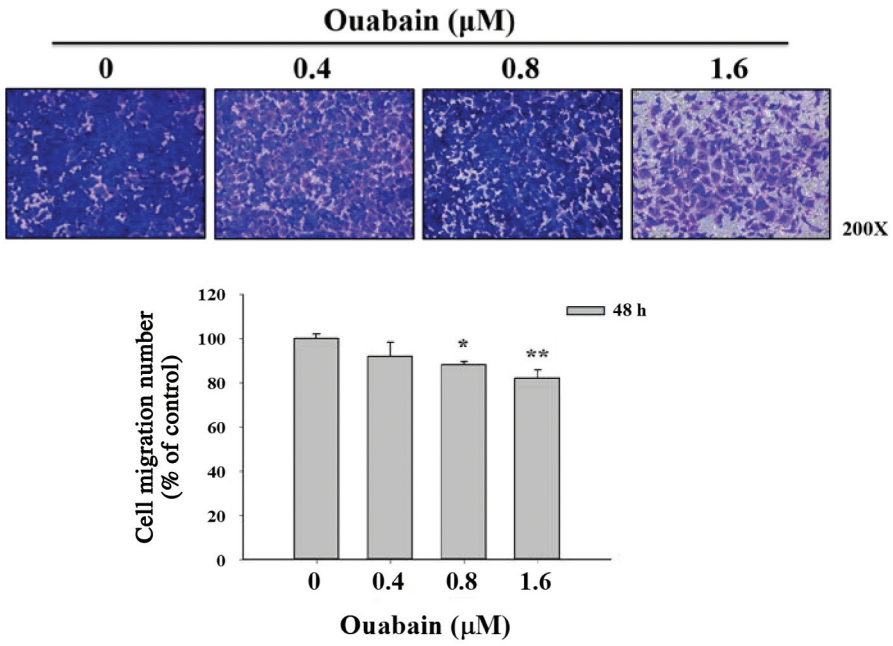

Figure 3. *Ouabain suppressed the migration of AGS cells. Cells (5×10^4 cells/insert) were plated in transwell inserts coated with collagen and treated with 0, 0.4, 0.8, and 1.6 μM ouabain for 48 h. The cells that penetrated to the lower surface of the transwell membrane were stained with crystal violet, photographed under a light microscope at 200× (A), and counted (B) as described in Materials and Methods. *p<0.05, **p<0.01 indicates a significant difference between ouabain-treated groups and the control, as analyzed by one-way ANOVA.*

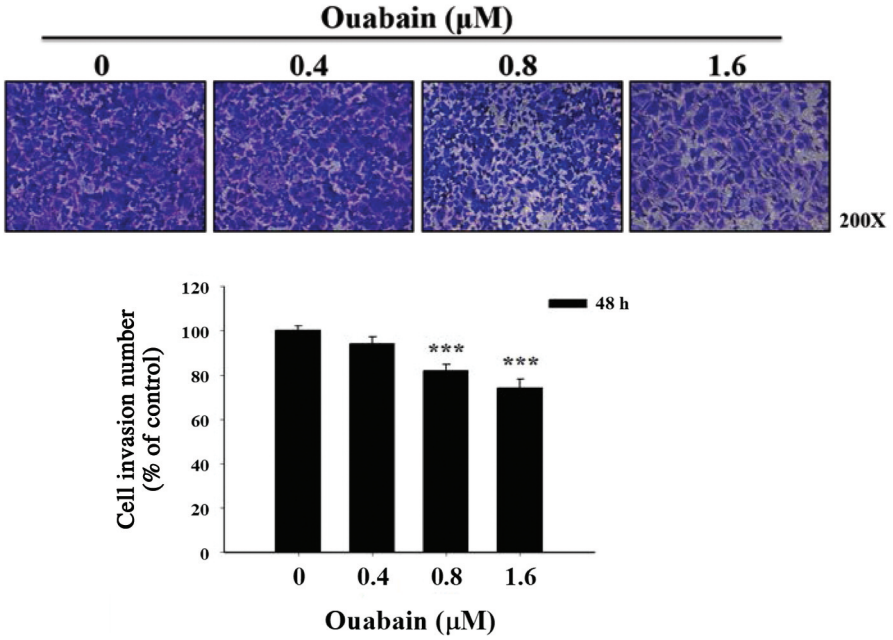

Figure 4. *Ouabain suppressed the invasion of AGS cells. Cells (5×10^4 cells/insert) were plated in transwell inserts coated with Matrigel and treated with 0, 0.4, 0.8, and 1.6 μM ouabain for 48 h. The cells that penetrated to the lower surface of the transwell membrane were stained with crystal violet, photographed under a light microscope at 200× (A), and counted (B) as described in Materials and Methods. ***p<0.001 indicates a significant difference between ouabain-treated groups and the control, as analyzed by one-way ANOVA.*

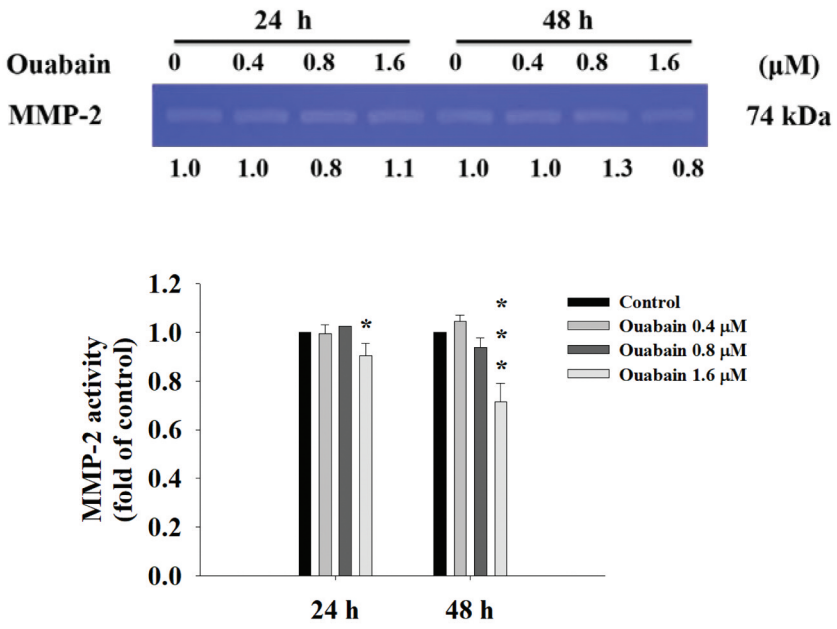

Figure 5. *Ouabain suppressed MMP gelatinase activity in AGS cells. Cells (1×10^5 cells/well) were plated in 12-well plates and treated with 0, 0.4, 0.8, and 1.6 μM ouabain for 24 and 48 h. The conditioned medium was loaded into SDS-PAGE containing gelatin. The proteins were then stained with coomassie blue, photographed under a lightbox (A) and then quantified (B) as described in Materials and Methods. *p<0.05, ***p<0.001 indicates a significant difference between ouabain-treated groups and the control, as analyzed by one-way ANOVA.*

heterogeneous disease; thus, comprehensive understanding of the molecular variables that affect GC disease pathways will help develop appropriate treatments (6). Ouabain is not only a natural bioactive component of *Strophanthus gratus*, but also a human hormone. It can also be found in low amounts in human plasma and tissue, which vary in the range of 0.002-0.77 nM (12, 36, 37). It has been reported that ouabain induces human cancer cell apoptosis (38-40) and also exhibits anti-metastatic effects in various human cancer cells, such as lung cancer (41, 42) and osteosarcoma U2OS cells (24). Although the anti-metastatic effect of ouabain has been shown *in vitro*, the mechanism of ouabain has not been fully revealed in human gastric cancer cells. In the present study, we showed that ouabain inhibited migration and invasion of human gastric cancer cells and uncovered its associated mechanism for the first time. Herein, we showed that 1) ouabain significantly inhibited cell proliferation and reduced the number of viable cells, and (Figure 1A and B); 2) inhibited cell mobility (Figure 2); 3) inhibited cell migration and invasion (Figure 3 and Figure 4); 4) decreased the gelatin enzymatic activity (Figure 5); 5) decreased the expression of p-p38, NF-kB (p65), N-cadherin, TIMP-1, c-uPA and MMP-2 but increased that of p-ERK1/2 and p-JNK (Figure 6).

At first, we investigated the cytotoxic effects of ouabain on AGS cells *in vitro*. Our results indicated that the ouabain concentration ranging from 0.4-1.6 μM and from 0.6-1.6 μM significantly inhibited cell proliferation and reduced the total number of viable cells, respectively. Therefore, we selected the ouabain concentrations 0.4, 0.8, and 1.6 μM for cell migration and invasion experiments.

Boyden chambers coated with collagen and Matrigel were used for measuring the effect of ouabain on the migration and invasion AGS cells *in vitro*, respectively. The results indicated that 0.4-1.6 μM ouabain significantly suppressed cell migration and invasion of AGS cells in a dose-dependent manner (Figures 3 and 4). It has been reported that tumor metastasis accounts for 90% of cancer-associated deaths, in which invasion of tumor cells plays a vital role in metastasis (43). Recently, GC metastasis has has been the focus of intense research due to the high death rate of patients with cancer metastasis (44, 45). It has been shown that ouabain possesses anti-metastatic activity through the inhibition of Na^+/K^+ ATPase in human breast cancer cells (46). Therefore, we further investigated cancer cell metastasis-associated protein expression in AGS cells after exposure to ouabain. We found that ouabain (1.6 μM) inhibited the expression of SOS1, GRB2, PI3K, p-AKT (Figure 6A), Ras, MEK1, p-p38, NF-kB (p65) (Figure 6B), N-cadherin (Figure 6C), TIMP-1, c-uPA, and MMP-2 (Figure 6D). However, ouabain increased p-ERK1/2, p-JNK (Figure 3B), E-cadherin, Vimentin,

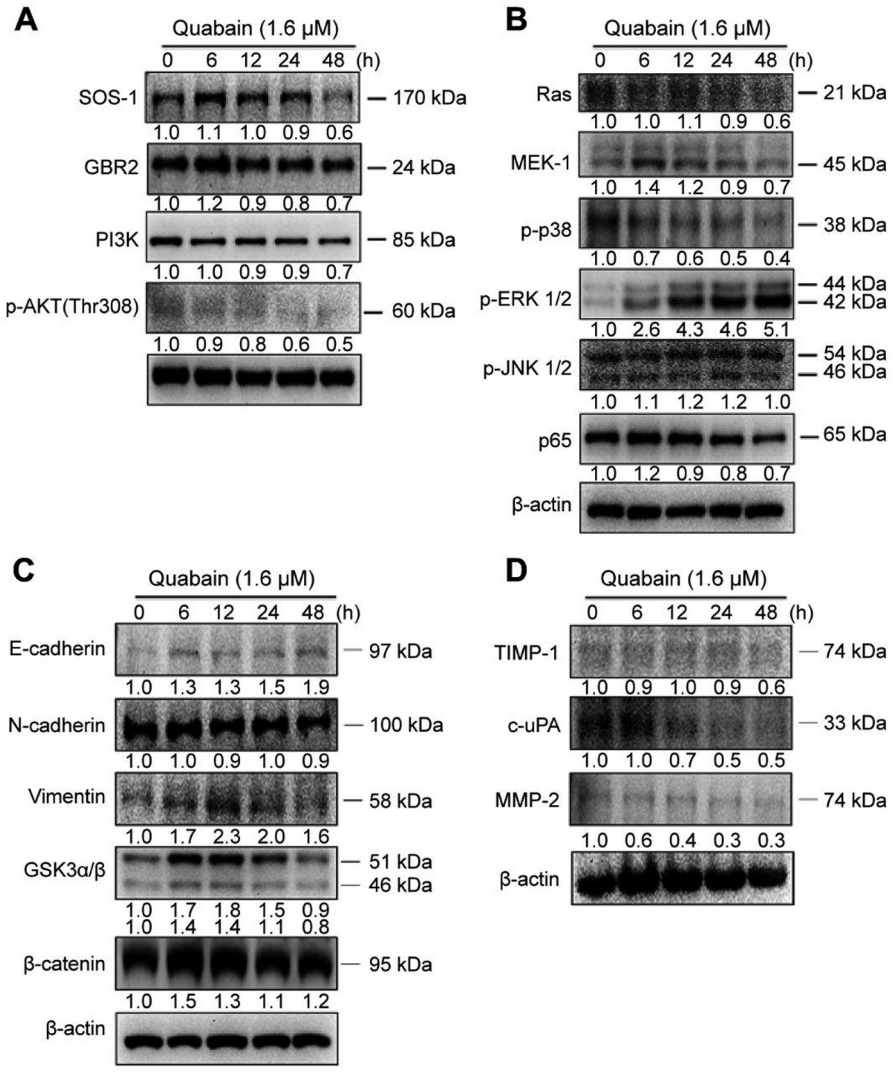

Figure 6. *Ouabain affected the levels of migration- and invasion-associated proteins in AGS cells. Cells (1×10^6 cells/dish) were treated with 0 and 1.6 μM ouabain for 0, 6, 12, 24, and 48 h. Cells were harvested for total protein quantitation and SDS-PAGE was performed as described in the Materials and Methods. The levels of SOS1, GRB2, PI3K, and p-AKT (A); Ras, MEK1, p-p-38, p-ERK 1/2, p-JNK1/2, and NF-kB (p65) (B); N-cadherin, E-cadherin, Vimentin, GSKα/β and β-catenin (C); TIMP-1, c-uPA, and MMP-2 (D), were analyzed by western blotting as described in Materials and Methods. β-actin was used as an internal control.*

GSK3α/β, and β-catenin levels (Figure 6C) in AGS cells. According to our results, ouabain suppresses cell migration and invasion of AGS cells by inhibiting SOS1, GRB2, PI3K, and p-AKT expressions. Activated PI3K/AKT signaling could promote cancer cell invasion and oncogenesis (47). Activated Akt and cell division cycle 42 (Cdc42) are critical for cell motility (48); thus, inhibition of these proteins is a potential way to inhibit cancer cell migration.

It has also been well documented that the abnormal expression of MMP-9, MMP-2, vimentin, N-cadherin, and E-cadherin in cancer cells leads to decreased adhesion, and enhanced migration and invasion. Thus, we suggested that ouabain blocks the metastatic process of AGS cells by inhibiting metastasis-related protein expression. Increased activity and levels of MMPs, in particular MMP-2 and MMP-9, are involved in cancer cell migration, invasion, and metastasis (49). Thus, their inhibition may lead to decreased AGS cell metastasis. Herein, our western blot results indicated that ouabain decreased the levels of MMP-2 in AGS cells. In addition, we showed that ouabain increased p-ERK1/2 and p-JNK in AGS cells. Extracellular signal-regulated kinase (ERK)/MAPK signaling pathways are also involved in cancer cell metastasis (50).

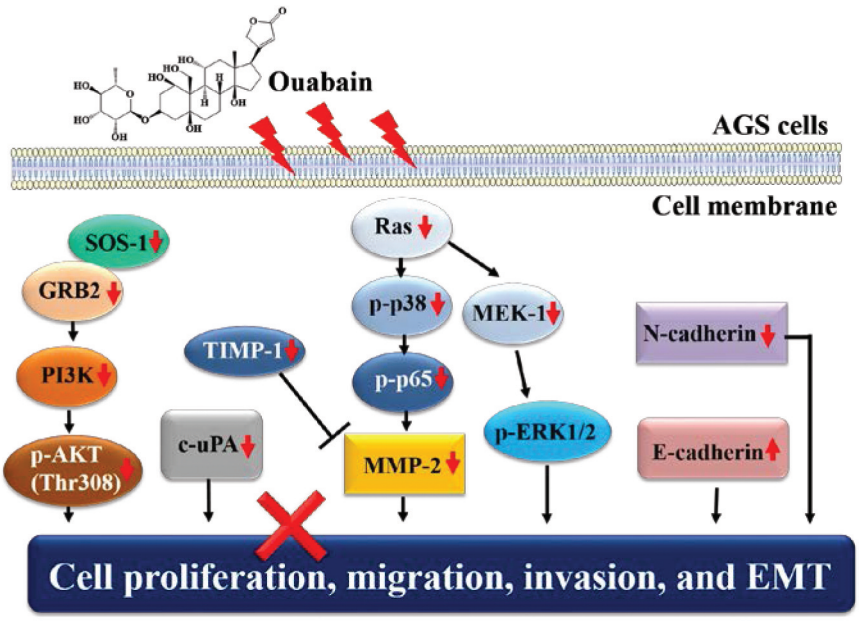

Figure 7. *The possible signaling pathways involved in the ouabain-induced suppression of cell migration and invasion of AGS cells.*

Furthermore, ouabain was found to decrease the expression of Son of sevenless 1 (SOS1) and Ras in AGS cells. The aberrant activation of Ras protein plays a causal role in human cancer (51). SOS1, a guanine nucleotide exchange factor (GEF), catalyzes the formation of active Ras (GTP-bound Ras), leading to activation of the various downstream signaling pathways (52). It has been reported that inhibition of SOS1 could reduce the levels of active Ras in Ras-driven tumors (53, 54). Other studies have already demonstrated that in K562 cells (*KRAS* wild-type), SOS1 inhibitors result in complete inhibition of the Ras/ERK pathway, whereas in Calu-1 cells (KRAS mutants) they result in 50% inhibition (55). The principal effectors of Ras are ERK and PI3K (56) and the Ras/ERK and Ras/PI3K/AKT pathways have been shown to be aberrantly activated in gastric cancer (57-59). Therefore, the actual roles of Ras/ERK, ERK/MAPK, and Ras/PI3K/AKT pathways in ouabain-treated GC need further investigation.

In conclusion, ouabain significantly inhibited cell migration and invasion of AGS cells *in vitro* by affecting N-cadherin and E-cadherin expression and by inhibiting AKT, PI3K, Ras, and the p38/MAPK signaling pathway. Furthermore, expression of p-p38, SOS1, MEK1, and NF-kB p65 was decreased, leading to decreased MMP-2 activity *in vitro* and inhibition of cell migration and invasion. The signaling pathways modulated by ouabain suppressed cell migration and invasion of AGS cells, as outlined in Figure 7.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Study conception and design: HYC, MDY, JCL, and CHC; Acquisition of data: HYC, MDY, YCC, YSM, and SFP; Analysis and interpretation of data: HYC, CLL, PYC, TCH; Drafting of manuscript: JCL, and CHC; Critical revision: JCL, and CHC. All Authors discussed the results and commented on the article.

Acknowledgements

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, ROC. This study was supported by the Research Funds of China Medical University (CMU107-ASIA-17) and Chang Bing Show Chwan Memorial Hospital (RD106061).

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Received June 28, 2021
Revised July 16, 2021
Accepted July 21, 2021