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Regorafenib Induces Apoptosis and Inhibits Metastatic Potential of Human Bladder Carcinoma Cells

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Abstract. *The aim of the present study was to verify the effects of regorafenib on apoptosis and metastatic potential in TSGH 8301 human bladder carcinoma cells in vitro. Cells were treated with different concentration of regorafenib for different periods of time. Effects of regorafenib on cell viability, apoptosis pathways, metastatic potential, and expression of metastatic and anti-apoptotic proteins were evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, flow cytometry, cell migration and invasion assay, and western blotting. We found regorafenib significantly reduced cell viability, cell migration and invasion, and expression of metastatic and anti-apoptotic proteins. In addition, regorafenib significantly induced accumulation of sub-G_1 phase cells, loss of mitochondrial membrane potential, and expression of active caspase-3 and caspase-8. These results show that regorafenib not only induces apoptosis, but also inhibits metastatic potential in bladder cancer TSGH 8301 cells in vitro.*

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Bladder cancer, the most common malignancy found in the urinary tract, is classified into non-muscle-invasive and muscle-invasive (MIBC) disease (1). The majority of patient mortality is due to metastasis (2). Current treatment strategies for MIBC include radical cystectomy, chemotherapy, and radiotherapy. However, the survival rates of patients with MIBC have not significantly improved in the past decades (3). Therefore, development of new anticancer agents for patients with MIBC is needed.

Several receptor tyrosine kinases are overexpressed in bladder cancer and activate intracellular signaling transduction, resulting in tumor progression. Many tyrosine kinase inhibitors have been shown to reduce cell proliferation and metastasis through blockage of epithelial growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR) signaling pathways in bladder cancer (4). Sorafenib (Nexavar) is a multi-tyrosine kinase inhibitor approved for treatment of advanced renal cell and advanced hepatocellular carcinoma (5, 6). Rose *et al.* found that sorafenib induces apoptosis and inhibits cell migration in bladder cancer cells (6). Regorafenib (Stivarga) is a sorafenib analog approved for treatment of colorectal cancer and gastrointestinal stromal tumors. In a previous study, we found both sorafenib and regorafenib induce apoptosis *via* suppression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in hepatocellular carcinoma (HCC) cells (7, 8). However, whether regorafenib has the potential to be used in the treatment of bladder

cancer is unknown. The aim of the present study was to investigate the effect of regorafenib on cell growth and metastatic potential of TSGH-8301 human bladder carcinoma cells *in vitro* by using MTT assay, flow cytometry, cell migration and invasion assay, and western blotting.

Materials and Methods

Chemicals and agents. Regorafenib was kindly provided by Bayer Corporation (Whippany, NJ, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, and penicillin streptomycin (PS) were purchased from Gibco/Life Technologies (Carlsbad, CA, USA). 3,3'-Dihexyloxacarbocyanine Iodide ($DiOC_6$) was bought from Enzo Life Sciences (Farmingdale, NY, USA). RNase was bought from Fermentas (St. Leon-Rot, Baden-Wurttemberg, Germany). Propidium iodide (PI), CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit, and CaspGLOW™ Red Active Caspase-8 Staining Kit were obtained from Biovision (Mountain View, CA, USA). MTT was purchased from Sigma-Aldrich (St. Louis, MO, USA). Matrigel and Transwell (8-μm pore size) were purchased from Selleck Chemicals (Houston, TX, USA) and Corning Life Sciences (Tewksbury, MA, USA), respectively. Primary antibody to X-linked inhibitor of apoptosis protein (XIAP) was bought from Thermo Fisher Scientific (Fremont, CA, USA). Primary antibody to β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody to induced myeloid leukemia cell differentiation protein (MCL1) was bought from BioVision (Milpitas, CA, USA). Primary antibody to matrix metallopeptidase 9 (MMP9) was obtained from EMD Millipore (Billerica, MA, USA). Cellular FLICE (FADD-like IL1β-converting enzyme)-inhibitory protein (C-FLIP) were bought from Cell Signaling Technology, Inc. (Danvers, MA, USA). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Cell culture. TSGH 8301 human bladder carcinoma cells were used for this study and obtained from Professor Jing-Gung Chung at the Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, ROC. TSGH 8301 cells were incubated in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37˚C in a humidified incubator containing 5% CO_2 (9).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. TSGH 8301 cells were seeded into 96-well plates at 1×10^4 cells/well, incubated overnight, and then treated with different concentration of regorafenib (0-50 μM in 0.1% dimethylsulfoxide) for 24 and 48 h. The effect of regorafenib on cell viability of TSGH 8301 cells was evaluated with MTT assay as previously described (5).

Detection of the sub-G_1 cell population. TSGH 8301 cells were seeded into 6-well plates at 5×10^5 cells/well, incubated overnight, and then treated with 30 μM regorafenib for 24 and 48 h. Propidium iodide (PI) buffer (40 μg/ml PI, 100 μg/ml RNase and 1% Triton X-100 in PBS) was used to identify cell-cycle distribution. The effect of regorafenib on sub-G_1 cell population was analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA) as previously described (8).

Detection of active caspase-3. TSGH 8301 cells were seeded into 6-well plates at 5×10^5 cells/well incubated overnight, and then treated with 30 μM regorafenib for 24 and 48 h. Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fluoromethyl ketone (DEVD-FMK) was conjugated by fluorescein isothiocyanate (FITC) to make a working solution (1 μl FITC-DEVD-FMK in 300 μl PBS), which was used to monitor expression of active caspase-3. The effect of regorafenib on active caspase-3 was analyzed by using flow cytometry (FACSCalibur; Becton-Dickinson) as described by Chiang *et al*. (10).

Detection of active caspase-8. TSGH 8301 cells were seeded into 6-well plates at 5×10^5 cells/well, incubated overnight, and then treated with 30 μM regorafenib for 24 and 48 h. Z-Ile-Glu(OMe)-Thr-Asp(OMe)-FMK (IETD-FMK) was conjugated by sulfo-rhodamine (Red-IETD-FMK) to make a working solution (1 μl Red-IETD-FMK in 300 μl PBS), which was used to monitor expression of active caspase-8. Effect of regorafenib on active caspase-8 was analyzed by using flow cytometry (FACSCalibur; Becton-Dickinson) as described by Chen *et al*. (11).

Detection of mitochondrial membrane potential (Ψ_m). TSGH 8301 cells were seeded into 6-well plates at 5×10^5 cells/well, incubated overnight, and then treated with 30 μM regorafenib for 24 and 48 h. $DiOC_6$ solution (4 μM $DiOC_6$ in 500 μl PBS) was used to detect $\Delta\Psi_m$. The effect of regorafenib on $\Delta\Psi_m$ was evaluated by using flow cytometry (FACSCalibur; Becton-Dickinson) as described by Wang *et al*. (12).

Migration assay. Transwell insert with 8 μm pore size was purchased from Corning (Tewksbury, MA, USA). TSGH 8301 cells were seeded into 10 cm diameter dishes at 3×10^6 cells, incubated overnight, and then treated with different concentration of regorafenib for 48 h. After treatment, 1×10^6 cells were collected by centrifugation and resuspended in 1 ml serum free RPMI-1640 then 100 μl cell suspension was put into the apical chamber of the transwell insert, and then incubated for 48 h. One hundred microliters of RPMI-1640 with 10% serum was added to the basolateral chamber. After 24-h incubation, the migrated cells on the transwell membrane were fixed with a mixture of methanol and acetic acid (3:1) for 15 min and then stained by 0.5% crystal violet solution. Migrated cells were photographed under a light Nikon ECLIPSE T*i*-U microscope at ×100 and then quantified using ImageJ software version 1.50 (National Institutes of Health, Bethesda, MD, USA) (13).

Invasion assay. Transwell insert with 8 μm pore size was coated with 50 μl matrigel solution (25 μl matrigel in 25 μl serum-free RPMI-1640) and incubated overnight at 37˚C. TSGH 8301 cells were seeded into 10 cm diameter dishes with 3×10^6 cells, incubated overnight, and then treated with different concentration of regorafenib for 48 h. After treatment, 1×10^6 cells were collected by centrifugation and resuspended in 1 ml serum-free RPMI-1640 then 100 μl of cell suspension was put into the apical chamber of the transwell insert and incubated for 48 h. One hundred microliters of RPMI-1640 with 10% serum was added to the basolateral chamber. After the incubation period, matrigel on the transwell membrane was removed by sterile cotton swab. The invaded cells on the transwell membrane were fixed with mixture of methanol and acetic acid (3:1) for 15 min and then stained by

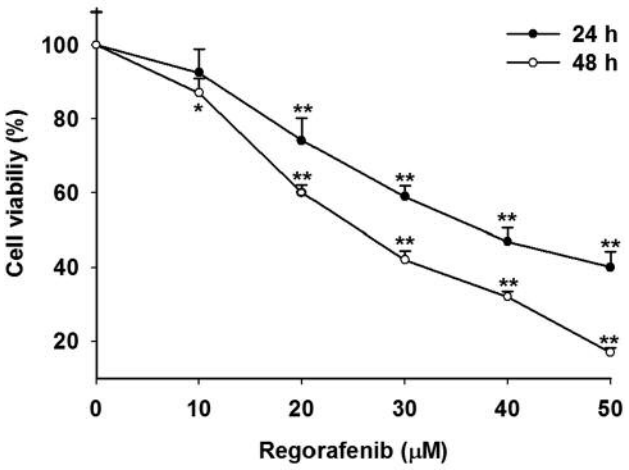

Figure 1. *Effect of regorafenib on cell viability of bladder cancer TSGH 8301 cells. Cells were treated with different concentration of regorafenib for 24 and 48 h. Cell viability was evaluated with MTT assay. Significantly different at *p<0.05 and **p<0.01 as compared to the control group. Results are presented as the mean±standard error.*

0.5% crystal violet solution. The invaded cells were photographed under a light Nikon ECLIPSE T*i*-U microscope at ×100 and then quantified by using ImageJ software version 1.50 (National Institutes of Health) (14).

Western blotting assay. TSGH 8301 cells (3×10^6) were seeded into 10 cm diameter dishes, incubated overnight, and then treated with 30 μM regorafenib for 24 and 48 h. Total cell proteins from each group were extracted by lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, and 1 mM phenylmethanesulfonyl fluoride). The effect of regorafenib on protein expression levels of MMP-9, XIAP, MCL1, and c-FLIP was determined with western blotting assay as described by Chen *et al*. (15). Protein bands were quantified by using ImageJ software version 1.50 (National Institutes of Health).

Statistical analysis. Student's *t*-test was used to test significance of difference of means between treatment group and control. Results are presented as mean±standard error. Statistical significance was achieved if $p<0.05$. Three independent repeats of each experiment were performed.

Results

Regorafenib induces cytotoxicity in TSGH 8301 cells. We used the MTT assay to evaluate the effect of regorafenib on cell viability in TSGH 8301 cells. We found that regorafenib significantly inhibited tumor cell growth in dose- and time-dependent manners. Regorafenib (10-50 μM) significantly reduced cell viability by 5-60% and 10-80% at 24 and 48 h after treatment, respectively, compared to the control (Figure 1).

Regorafenib triggers intrinsic and extrinsic apoptotic pathways in TSGH 8301 cells. We used flow cytometric apoptosis assay to investigate regorafenib-induced apoptotic pathways. We found the regorafenib significantly induced accumulation of cells in the sub-G_1 phase and expression of active caspase-3 in TSGH 8301 cells (Figure 2A and B). Regorafenib also significantly elicited expression of active caspase-8 and loss of Ψ_m by 26-53% and 28-50%, respectively, as compared to the control (Figure 2C and D).

Regorafenib suppresses cell migration and invasion in TSGH 8301 cells. Cell migration and invasion assays were used to evaluate effect of regorafenib on cell migration and invasion in TSGH 8301 cells. Figure 3A and B indicate regorafenib significantly reduced cell migration and invasion by 95-98% and 94-98%, respectively, compared to the control.

Regorafenib reduces expression of metastatic and anti-apoptotic proteins in TSGH 8301 cells. Western blotting assay was used to verify effect of regorafenib on metastatic and anti-apoptotic proteins expression in TSGH 8301 cells. We found regorafenib significantly reduced protein expression levels of MMP9, XIAP, MCL1, and c-FLIP by 30-90% compared to the control (Figure 4).

Discussion

Sorafenib, an anti-HCC agent, has been indicated to induce apoptosis and repress cell migration of bladder cancer cells (6). In a previous study, we found regorafenib, a novel sorafenib derivative, inhibited cell growth and triggered apoptosis in HCC cells *in vitro* (8). However, whether regorafenib elicits apoptosis and reduces metastatic potential of bladder cancer cells has not been elucidated. Therefore, we investigated the effect of regorafenib on apoptosis and metastatic potential of TSGH 8301 bladder cancer cells.

Many anticancer agents induce extrinsic and intrinsic apoptosis in bladder cancer (16). Expression of active caspase-8 and loss of Ψ_m are essential for induction of extrinsic and intrinsic apoptotic pathways, respectively (10). Caspase-3, a death protease, is activated in both extrinsic and intrinsic apoptotic pathways. Active caspase-3 modulates cleavage of crucial cellular proteins and formation of apoptotic DNA fragmentation (17). Karamitopoulou *et al*. suggested active caspase-3 expression in patients with bladder cancer to be a positive prognostic factor with expected 1-year longer survival than those without expression (18). We found that regorafenib triggered the expression of both active caspase-3 and caspase-8 together with loss of Ψ_m in TSGH 8301 cells (Figure 2). Anti-apoptotic proteins including XIAP, MCL-1, and c-FLIP are disruptors of anticancer agent-induced apoptosis (8). High protein levels of XIAP and c-FLIP are also linked to

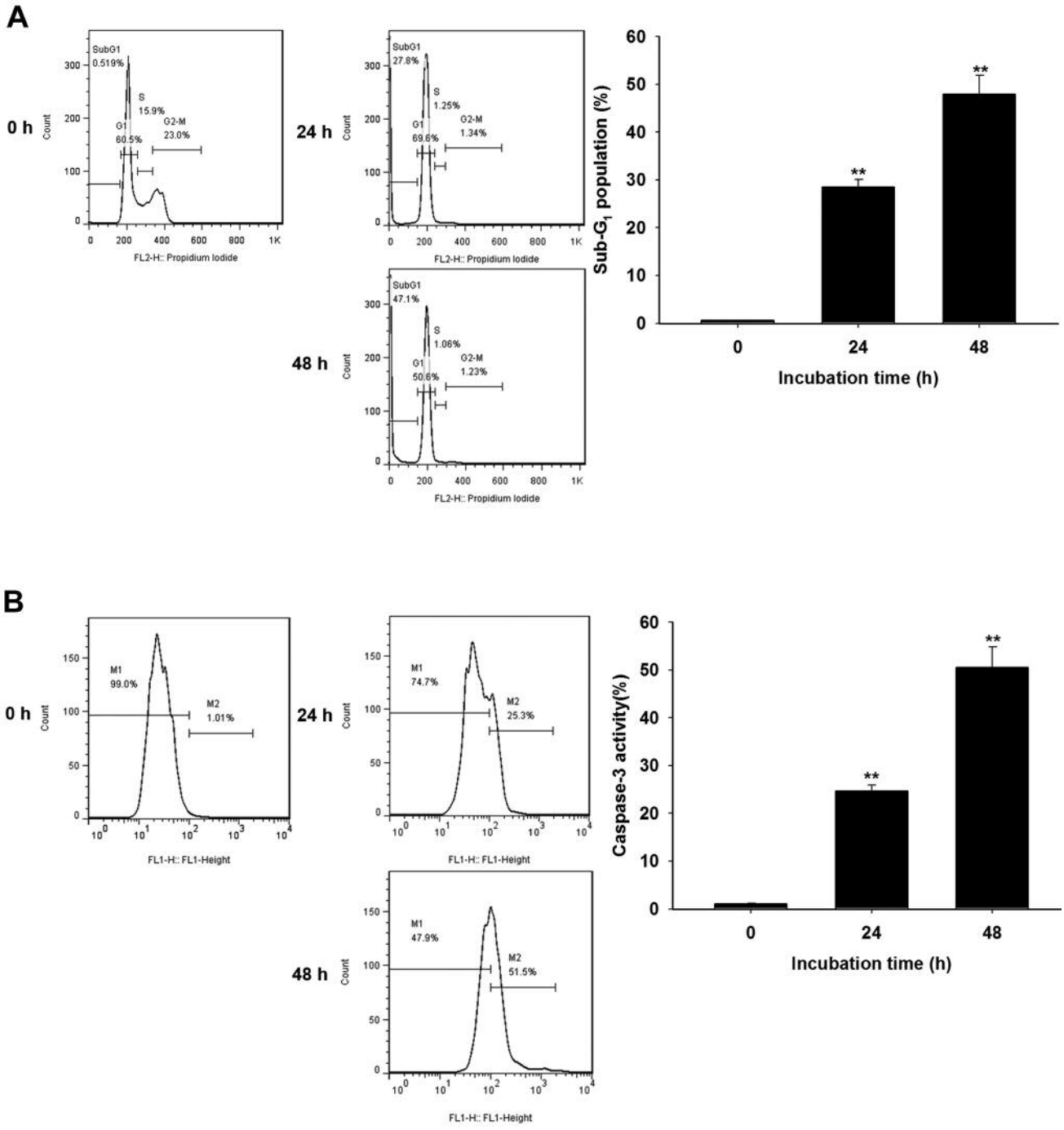

Figure 2. *Continued*

poor prognosis in patients with bladder cancer (19-20). Our data indicate that regorafenib inhibited expression of XIAP, MCL-1, and c-FLIP in in TSGH 8301 cells (Figure 4).

Mortality of patients with MIBC, which comprises 10-20% of all patients with bladder cancer at diagnosis, is significantly attributed to tumor metastasis, accounting for 50% of patients who die from bladder cancer (21). Degradation of extracellular matrix by MMPs is associated with tumor invasion and metastasis. MMP9, 92 kDa gelatinase B, is overexpressed in invasive bladder cancer and related to poor prognosis (22). Our data show that regorafenib reduced the expression of MMP9 (Figure 4) and

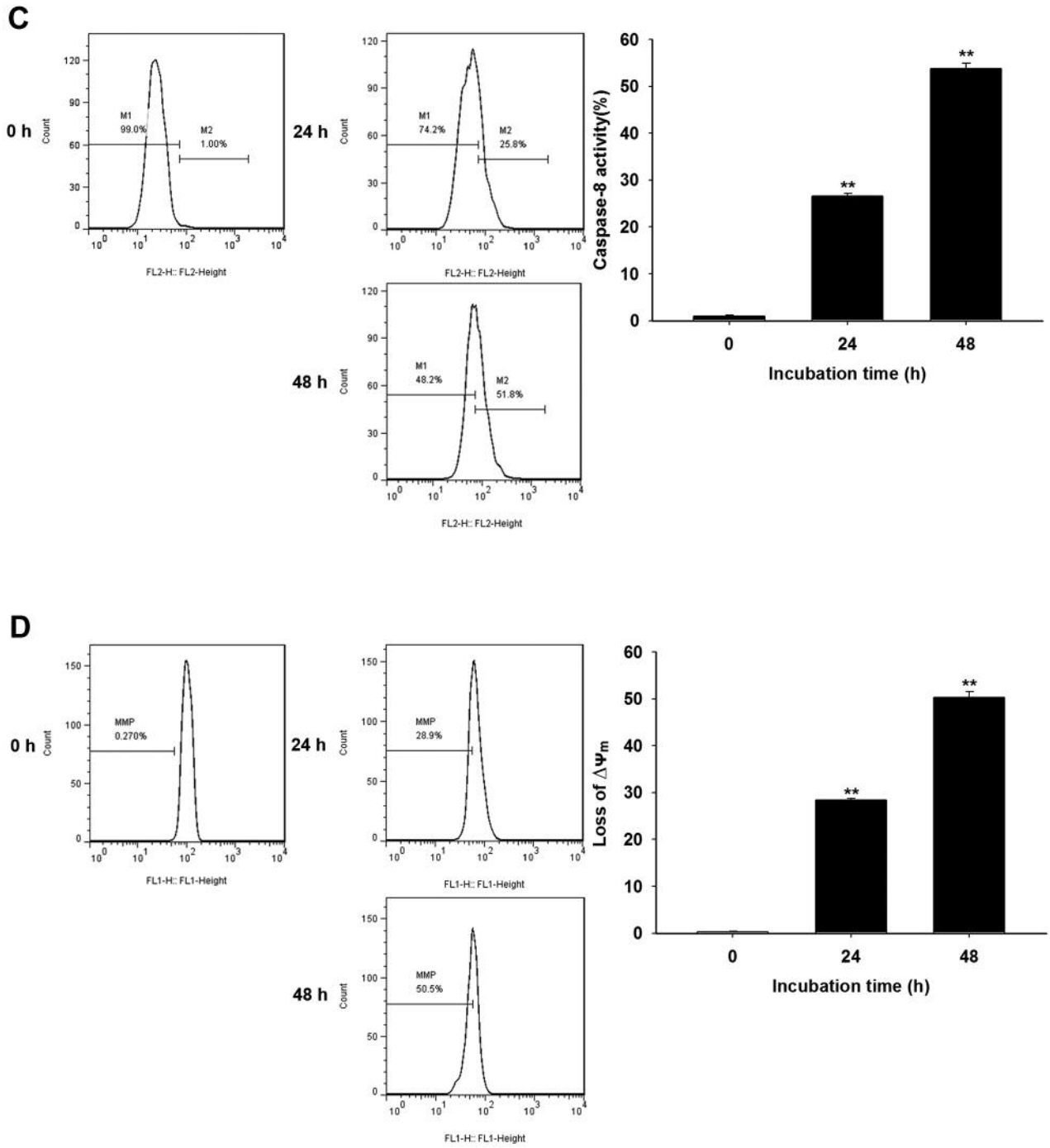

Figure 2. *Effect of regorafenib on apoptosis pathways in TSGH 8301 cells. Cells were treated with 30 μM regorafenib for 24 and 48 h. Change of sub-G_1 population (A), expression of active caspase-3 (B) and caspase-8 (C), and mitochondrial membrane potential (Ψ_m) (D) were determined by flow cytometry. **Significantly different at p<0.01 compared to the control group. Results are presented as the mean±standard error.*

cell migration and invasion (Figure 3) in TSGH 8301 cells.

In conclusion, this study revealed that regorafenib not only induced apoptosis, but also inhibited metastatic potential of bladder cancer cells. We suggest that regorafenib may be a potential therapeutic agent for patients with bladder cancer.

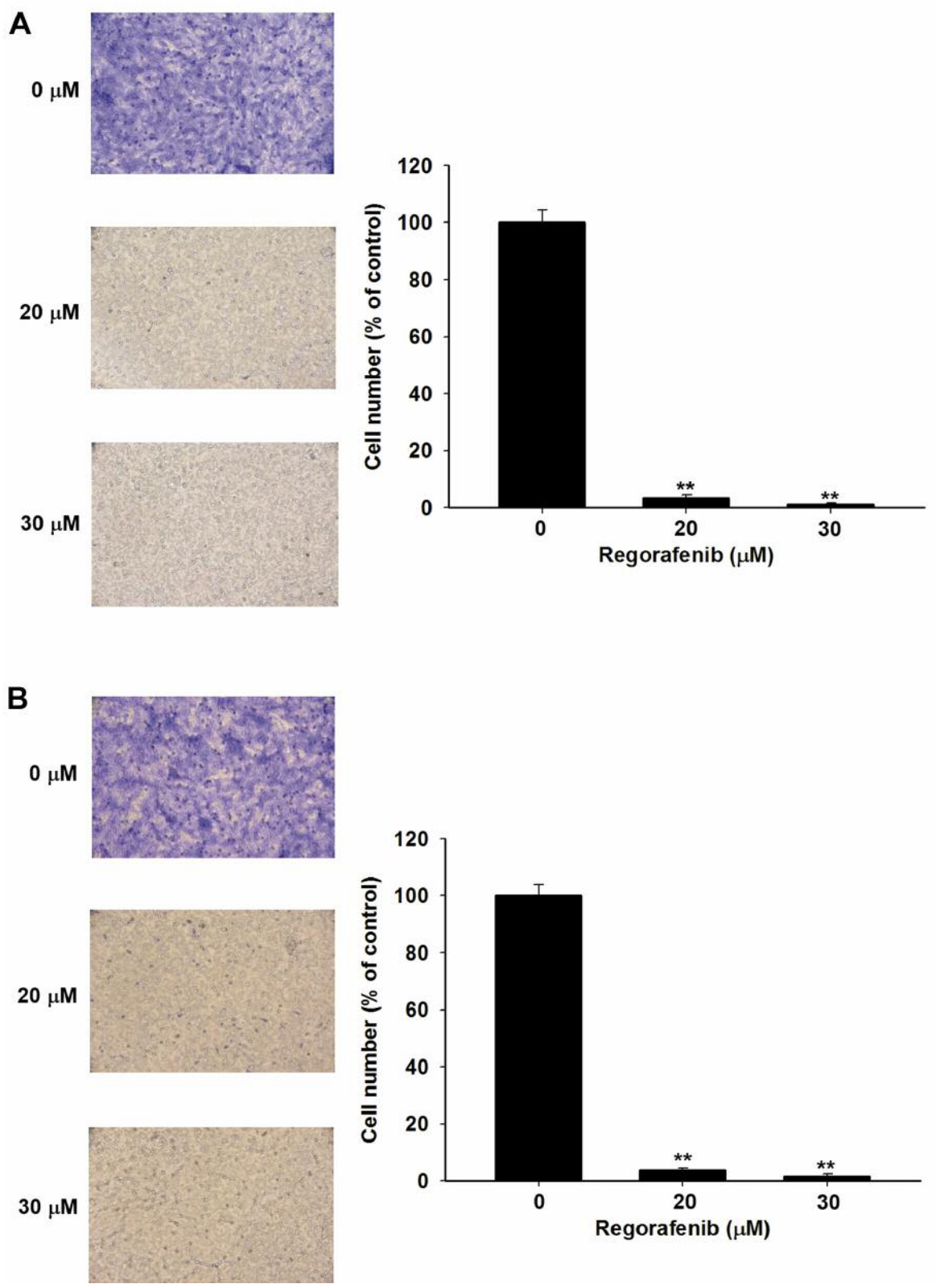

Figure 3. *Effect of regorafenib on cell migration and invasion of TSGH 8301 cells. Cells were treated with different concentration of regorafenib for 48 h. Procedures after treatment were in detail described in materials and methods. (A) Cell migration. (B) Cell invasion. **Significantly different at p<0.01 compared to the control group. Results are presented as the mean±standard error.*

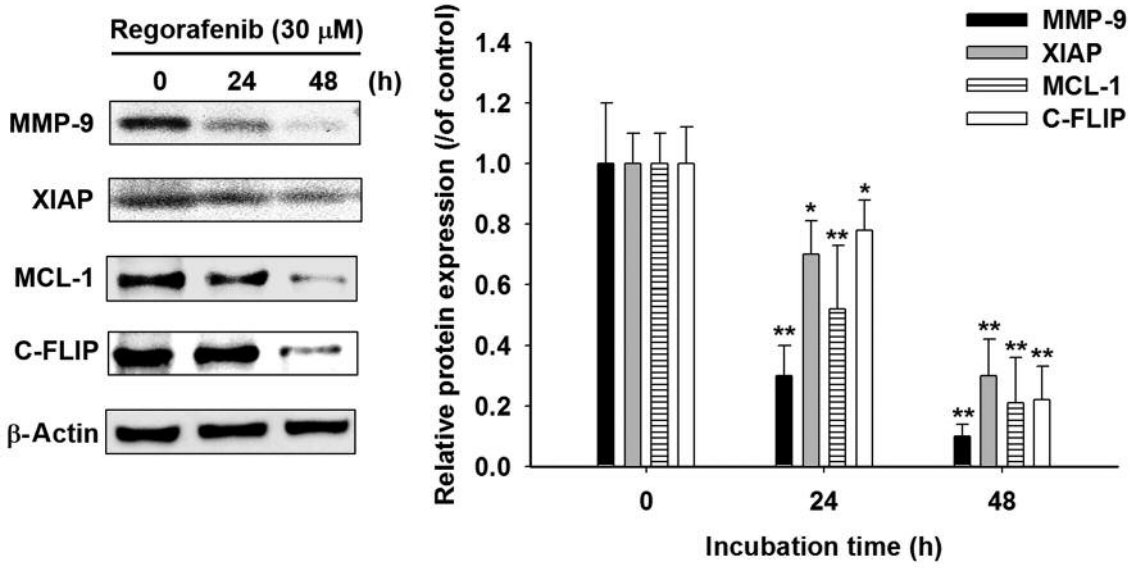

Figure 4. *Effect of regorafenib on protein levels of matrix metallopeptidase 9 (MMP9), X-linked inhibitor of apoptosis protein (XIAP), induced myeloid leukemia cell differentiation protein (MCL-1), and cellular FLICE (FADD-like IL1β-converting enzyme)-inhibitory protein (c-FLIP) in TSGH 8301 cells. Cells were treated with 30 μM regorafenib for 24 and 48 h. Change in protein levels was evaluated using western blotting. Significantly different at *p<0.05 and **p<0.01 compared to the control group. Results are presented as the mean±standard error.*

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