A novel synthetic 2-(3-methoxyphenyl)-6,7-methylenedioxoquinolin-4-one arrests the G2/M phase arrest via Cdc25c and induces apoptosis through caspase- and mitochondria-dependent pathways in TSGH8301 human bladder cancer cells

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Abstract. 2-(3-methoxyphenyl)-6,7-methylenedioxoquinolin-4-one (MMEQ) is a novel synthesized compound, and this study investigated the effects of MMEQ on molecular signal pathways of the induction of apoptosis in TSGH8301 human bladder cancer cells. The studies included examining the effects of morphological changes by contrast-phase microscope, the percentage of viable cells, cell cycle distribution mitochondria membrane potential ($\Delta\Psi_m$), ROS and caspase activities were examined by flow cytometry, apoptotic cells were examined by DAPI staining and the changes of associated apoptosis proteins levels were examined by Western blotting. Release of apoptotic factors from mitochondria was examined by confocal laser microscope. Our results showed that MMEQ caused morphological changes and inhibited the cell growth of TSGH8301 cells in a time- and dose-dependent manner. MMEQ induced G2/M arrest through the promotion of chk1, chk2 and cdc25c in TSGH8301 cells. MMEQ caused a marked increase in the percentage of DNA damage and apoptosis as characterized by DAPI and DNA fragmentation. The specific inhibitors of caspase-8, -9, and -3 blocked MMEQ-induced growth inhibition action. A remarkable loss of $\Delta\Psi_m$ and increase in ROS production were observed after a 24-h treatment. MMEQ promoted the levels of caspase-3, caspase-8, caspase-9, Bax, Bcl-xs, decreased the levels of Bcl-2 and Bid and then led to dysfunction of $\Delta\Psi_m$, following the releases of cytochrome c, AIF and Endo G from mitochondria to cytosol and nuclei, and finally caused cell apoptosis. In conclusions, these molecular mechanisms provide insight into MMEQ-caused growth inhibition, G2/M arrest and apoptotic cell death in TSGH8301 cells.

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Introduction

Cell cycle control is the major regulatory mechanism of cell growth (1-3) and if the agents cause arrest of the cycle at one of the G0/G1, S or G2/M phase they may induce apoptosis (2-4). The roles of cyclins and cyclin-dependent kinases (Cdks) in cell cycle progression which is regulated by the coordinated action of Cdks in association with their specific regulatory

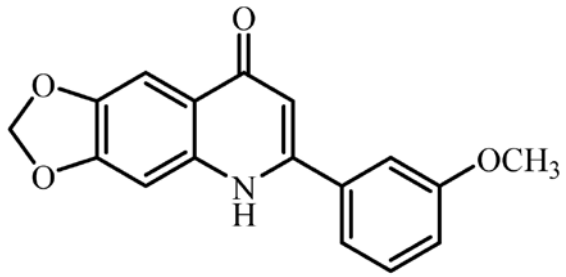


Figure 1. The structure of MMEQ (2-(3-methoxyphenyl)-6,7- methylenedioxo-quinolin-4-one).

cyclin proteins (4). Some of cytotoxic drugs such as taxol can induce G2/M phase arrest in cancer cells (5-7). Anticancer drugs also can eliminate cancerous cells through the induction of apoptosis.

Apoptosis is an intracellular suicide program through the activation of caspases, a family of cytoplasmic cysteine proteases (8). Caspase-3, -8 and -9 are the key components of the apoptotic machinery, playing an important role in apoptosis before finally leading to DNA fragmentation. Therefore, the best strategy for killing cancer cells is to induce cell cycle arrest and apoptosis.

Families of molecules consisting of unique quinolin cores, derived from either natural isolation or synthetic route, have been shown to have some biological activities. A previous report showed that the derivatives of quinoline exhibited cytotoxicity toward cancer cells in low range of micromolar concentrations (9). In our laboratory, we synthesized 2-(3-methoxyphenyl)-6,7-methylenedioxoquinolin-4-one (MMEQ), which exhibited cytotoxic activity in bacteria (9). However, there is no available information to address MMEQ induced apoptosis in human cancer cells. Therefore, this new investigation will be helpful in further elucidation of undiscovered biological properties of this novel antitumor MMEQ to TSGH8301 human bladder cancer cells.

Materials and methods

Chemicals and reagents. MMEQ (2-(3-methoxyphenyl)-6,7-methylenedioxoquinolin-4-one; Fig. 1) was obtained from Professor Sheng-Chu Kuo (Graduate Institute of Pharmaceutical Chemistry, College of Pharmacy, China Medical University). Potassium phosphates, dimethyl sulfoxide (DMSO), propidium iodide, Triton X-100, trypan blue and ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Invitrogen Corp. (Carlsbad, CA, USA). The primary antibodies were obtained as follows: antibodies for caspase-8, caspase-9 and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA); antibodies for cytochrome c, TRAIL, Bax, Bcl-Xs, Bid, β-actin, AIF, Endo G and horseradish peroxidase (HRP)-linked goat anti-mouse IgG, goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK), caspase-3 inhibitor (Z-DEVD-FMK) (R&D Systems, Minneapolis, MN, USA) were dissolved in DMSO and diluted in cell culture medium before use.

Cell culture. The human bladder cancer cell line (TSGH8301) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm^2 tissue culture flasks with 90% RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 μg/l sodium bicarbonate and supplemented with 10% FBS, and 1% penicillin-streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin) (10) and grown at 37˚C under a humidified 5% CO_2 atmosphere.

Morphological changes. Approximately $2x10^5$ cells/well of TSGH8301 cells were grown in 12-well plates for 24 h before they were treated with MMEQ at 0, 0.375, 0.75, 1.5, 3 and 6 μM and cells were incubated at 37˚C, 5% CO_2 and 95% air for 48 h. Then cells were examined and photographed under phase-contrast microscope as described previously (11,12).

Determinations of cell viability, cell cycle and apoptosis by PI and Annexin V staining. Approximately $2x10^5$ cells/well of TSGH8301 cells were grown in 12-well plates for 24 h before they were treated with MMEQ at 0, 0.375, 0.75, 1.5, 3 and 6 μM and cells were incubated at 37˚C, 5% CO_2 and 95% air for 48 h. Cells were harvested by centrifugation. For viability determination, cells were stained with PI (5 μg/ml) then were analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) as previously described (12,13). For cell cycle and sub-G1 (apoptosis) determinations, the harvested cells were fixed gently by putting 70% ethanol in 4˚C overnight and then re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in dark room for 30 min at 37˚C. Those cells were analyzed with a flow cytometer equipped with an argon ion laser at 488 nm wavelength (5,14). Annexin V staining was conducted by a FITC Annexin V Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA).

DAPI staining and DNA gel electrophoresis. Approximately $5x10^4$ cells/ml of TSGH8301 cells were treated with 0, 0.375, 0.75, 1.5, 3 and 6 μM of MMEQ for 24 h. Cells on the culture wells were stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride, Invitrogen Corp.) then photographed using a fluorescence microscope as previously described (12,15). For DNA fragmentation assay, $5x10^6$ of TSGH8301 cells/ml were treated with 0, 0.375, 0.75, 1.5, 3 and 6 μM MMEQ for 24 h before isolating the cells to extract DNA to be used in DNA gel electrophoresis, as described previously (15).

Detections of reactive oxygen species (ROS), cytosolic Ca^{2+} levels and mitochondria membrane potential ($\Delta\Psi_m$). Approximately $2x10^5$ cells/ml of TSGH8301 cells were treated with 3 μM of MMEQ for 0, 1, 3, 6, 12 and 24 h. The cells were harvested and re-suspended in 500 μl of 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM; Invitrogen Corp.) for ROS production, Indo 1/AM (3 μg/ml, Invitrogen Corp.) for cytosolic Ca^{2+} and in 500 μl of $DiOC_6$ (1 μmol/l, Invitrogen Corp.) for $\Delta\Psi_m$. Cells were then incubated at 37˚C for 30 min and then were analyzed by flow cytometry (Becton-Dickinson FACS Calibur) as previously described (11,15).

Caspase-8, -9, -3 and a pan-caspase inhibitors inhibit MMEQ-induced growth inhibition action in TSGH8301 cells. Approximately $2x10^5$ cells/ml of TSGH8301 cells were pretreated with caspase-3 inhibitor (Z-IETD-FMK), a caspase-8 inhibitor (Z-LEHD-FMK), a caspase-9 inhibitor (Z-DEVD-FMK) and pan-caspase inhibitor (Z-VAD-FMK) before cells were treated with 3 μM of MMEQ for 48 h (16). TSGH8301 cells were harvested for determination of cell viability as described above.

Western blotting of cell cycle and apoptosis associated proteins. Approximately $1x10^7$ cells of TSGH8301 cells were treated with 3 μM MMEQ for 0, 6, 12, 24 and 48 h. Cells were collected by centrifugation and the total proteins were quantitated from each treatment. Equal amounts of lysate protein were run on 12% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA). After blocking, the blots were incubated with specific primary antibody against caspase-3, caspase-8, caspase-9, cytochrome c and TRIAL, Bax, Bcl-X, Bid, AIF, Endo G, Chk2, Chk1, Wee 1, Cdc25c, Cdc2, cyclin A and B1 overnight and further incubated for 1 h with HRP conjugated secondary antibody (Santa Cruz). Bound antibodies were detected by ECL kit (Millipore) as described previously (17-20).

Immuno-staining and confocal laser microscopy. The TSGH8301 cells ($5x10^4$ cells/well) were placed on 4-well chamber slides, then were treated without (control) or with 3 μM MMEQ for 24 h, cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton-X 100 in PBS for 1 h with blocking of non-specific binding sites using 2% BSA. The fixed cells were then incubated with anti-human AIF, Endo G and cytochrome c primary antibody (1:100 dilution) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution), followed by DNA staining with PI. The cells in each slide were then photomicrographed by using a Leica TCS SP2 Confocal Spectral Microscope as described previously (19-21).

Statistical analysis. Student's t-test was used to analyze differences between treated and control groups. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Results

Growth inhibition of MMEQ on TSGH8301 human bladder cancer cells. We investigated the growth inhibition effects of MMEQ on TSGH8301 cells. The results in Fig. 2A show that MMEQ induced morphological changes in TSGH8301 cells in a dose-dependent manner. Fig. 2B and C show that MMEQ decreased the percentage of viable cells and the IC_{50} of the MMEQ was 0.82 μM in TSGH8301 cells. We therefore investigated whether MMEQ could induce cell cycle arrest and apoptosis in TSGH8301 cells. The results from flow cytometric assay showed that MMEQ induced G2/M arrest (Fig. 3A). Sub-G1 group (apoptosis) occurs in a time-dependent manner in TSGH8301 cells and Annexin V-positive cells (Fig. 3C) were counted by flow cytometric assay.

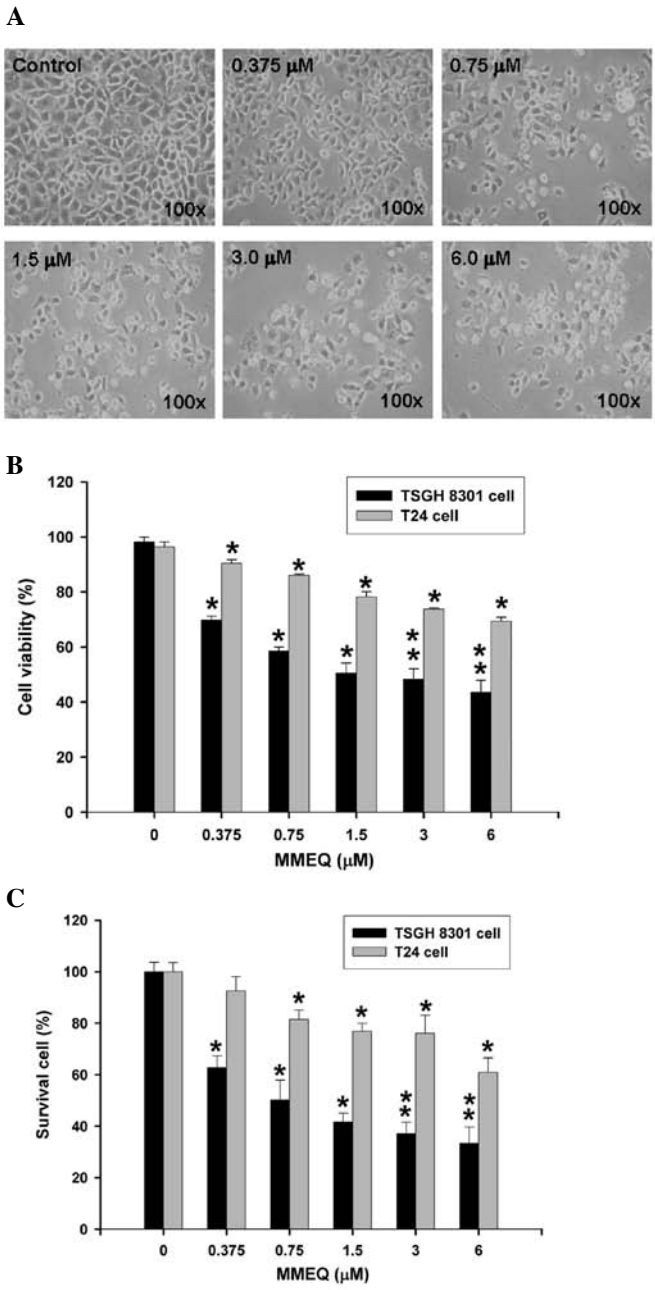


Figure 2. MMEQ induces morphological changes and decreases viable TSGH8301 and T24 cells. Approximately $5x10^5$ cells/well of TSGH8301 and T24 cells were grown in a 12-well plate for 24 h then treated with various concentrations of MMEQ for 48 h. Then cells were photographed (A). Viable cells (B) and cytotoxicity (C) were counted as described in Materials and methods. Each point is mean ± SD of three experiments. Significantly different from the control ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

MMEQ induces DNA damage and apoptosis in TSGH8301 cells. In order to make sure MMEQ induced apoptosis through DNA damage, the cells were treated with MMEQ then isolated for DAPI staining, Comet assay and DNA gel electrophoresis. The results in Fig. 4, show that the cells exposed to MMEQ display chromatin condensation (Fig. 4A) and DNA fragmentation (Fig. 4B). These results indicated that MMEQ induced DNA damage and DNA fragmentation in a dose-dependent manner.

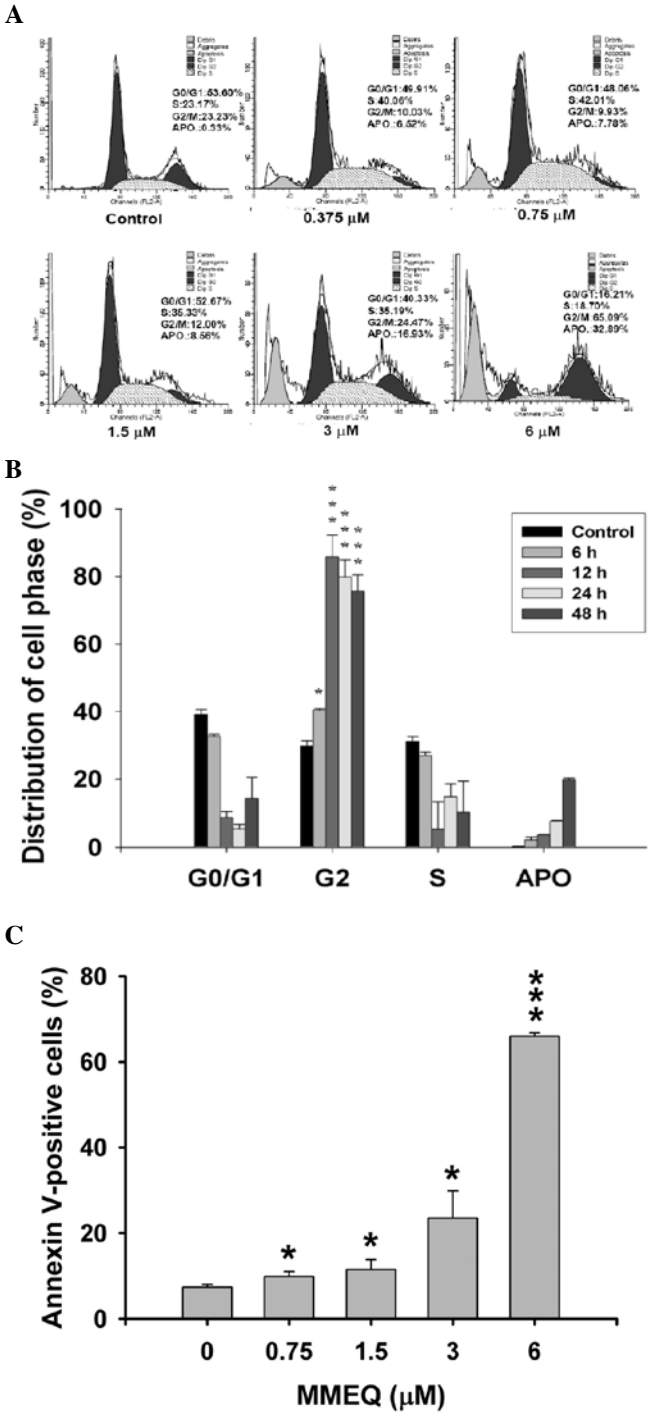


Figure 3. MMEQ induces G2/M arrest and apoptosis (sub-G1) in TSGH8301 cells. Approximately $5x10^5$ cells/well of TSGH8301 cells were treated with 0, 0.375, 0.75, 1.5, 3 and 6 μM MMEQ for 48 h. Then cells were harvested for assaying the distribution of cell cycle (A), the percentage of cell cycle (B) and Annexin V-positive cells (C) were counted by flow cytometric assay as described in Materials and methods. The percentage of apoptosis was calculated. Each point is the mean ± SD of three experiments. Significantly different from the control ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

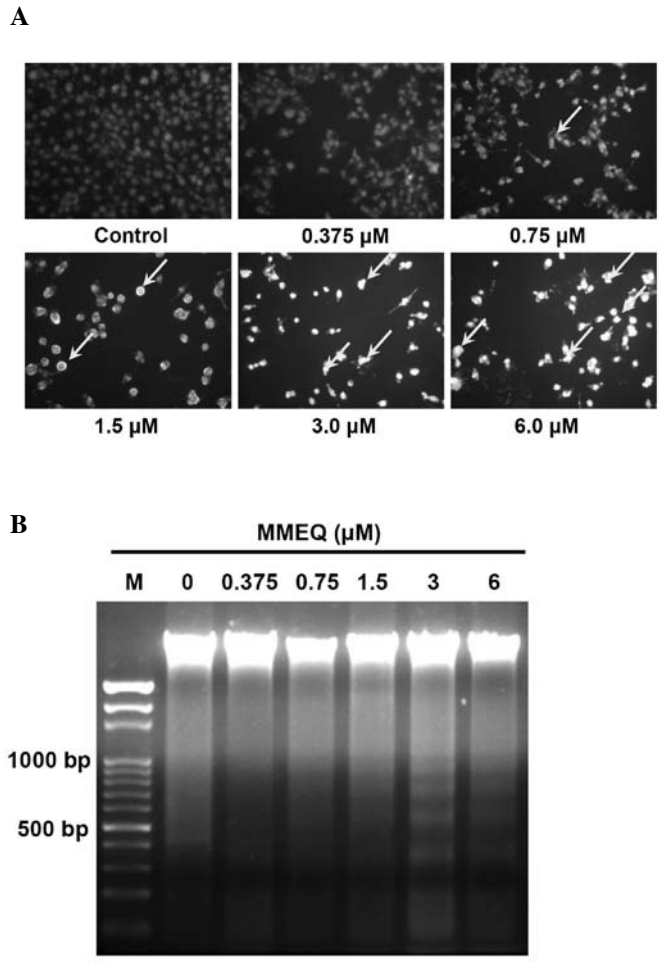


Figure 4. MMEQ induces DNA condensation, DNA damage and DNA fragmentation in TSGH8301 cells. Approximately $1x10^6$ of TSGH8301 cells were treated with 0, 0.375, 0.75, 1.5, 3 and 6 μM MMEQ for 48 h. Cells from each sample were harvested for DAPI staining (A) and DNA gel electrophoresis (B) as described in Materials and methods. Each point is the mean ± SD of three experiments.

MMEQ induces reactive oxygen species (ROS) production and loss of mitochondrial membrane potential ($\Delta\Psi_m$) in TSGH8301 cells. In order to investigate whether or not MMEQ induced DNA damage due to the production of ROS, the cells were treated with MMEQ for various time periods. Cells were harvested for examining the ROS production and for examining the levels of $\Delta\Psi_m$. Our results in Fig. 5 show that MMEQ increased the amounts of ROS (Fig. 5A) and Ca^{2+} (Fig. 5C) but decreased the levels of $\Delta\Psi_m$ (Fig. 5B) in TSGH8301 cells. The high amounts of ROS production was observed at MMEQ exposure for 24 h in TSGH8301 cells.

MMEQ decreases percentage of viable cells via a caspase-dependent pathway. In order to investigate the MMEQ-induced apoptosis in TSGH8301 cells through the caspases-dependent pathways, we pretreated cells with caspase-3, -8, -9 and pan-caspase inhibitor individually, then treated the cells with 3 μM of MMEQ for 48 h. Cells were harvested for determining the percentage of viable cells. The results are shown in Fig. 6A-D, caspase-3, -8, -9 and pan-caspase inhibitor significantly increased the percentage of viable cells in MMEQ-treated cells. These results suggested that caspase-8, -9 and -3 were mediated a caspase-dependent pathway in MMEQ-induced apoptosis in TSGH8301 cells.

MMEQ affects associated protein levels of apoptosis and G2/M phase arrest in TSGH8301 cells. In order to investigate the possible signaling pathways for MMEQ induced apoptosis and

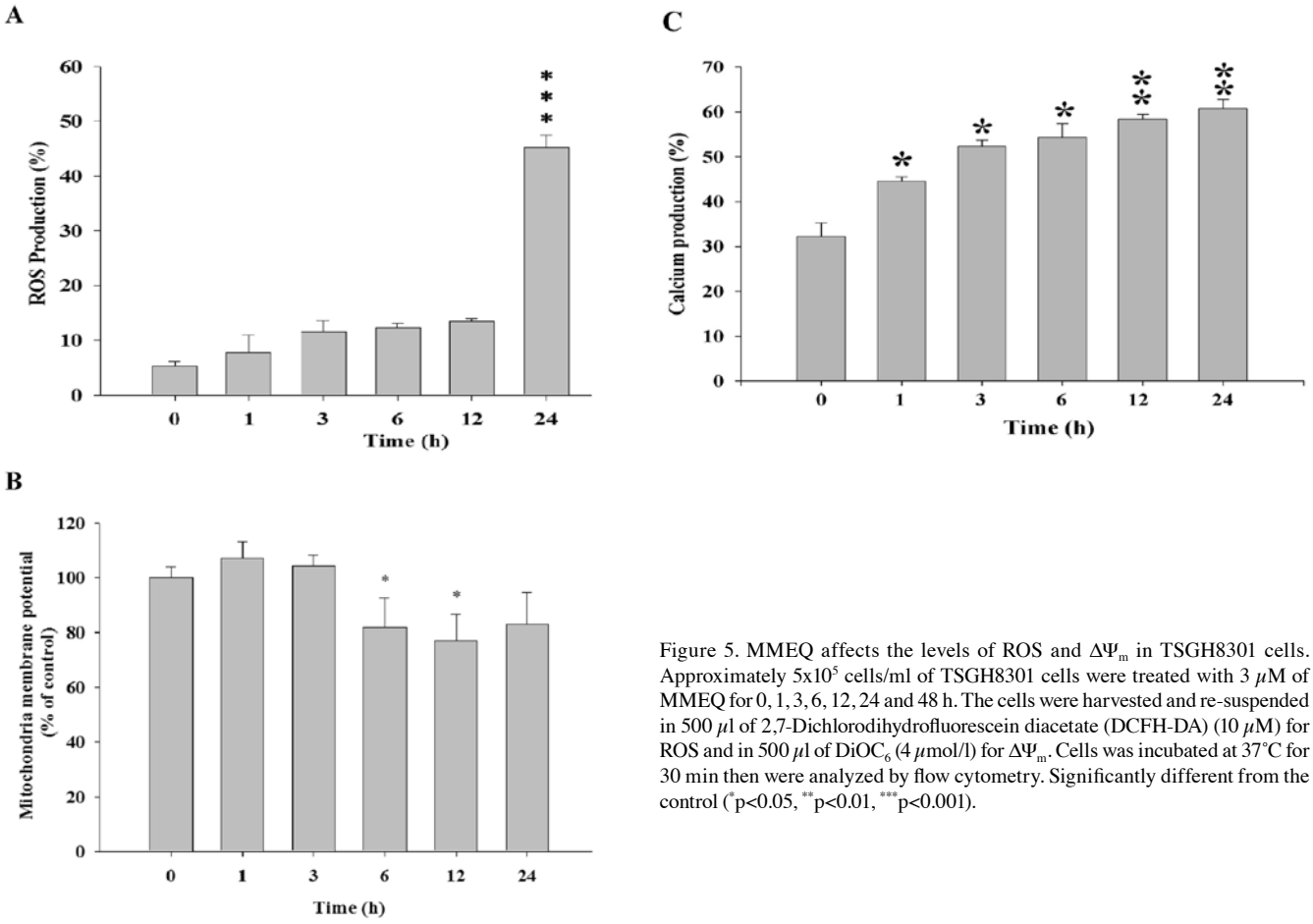


Figure 5. MMEQ affects the levels of ROS and $\Delta\Psi_m$ in TSGH8301 cells. Approximately $5x10^5$ cells/ml of TSGH8301 cells were treated with 3 μM of MMEQ for 0, 1, 3, 6, 12, 24 and 48 h. The cells were harvested and re-suspended in 500 μl of 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) for ROS and in 500 μl of $DiOC_6$ (4 μmol/l) for $\Delta\Psi_m$. Cells was incubated at 37˚C for 30 min then were analyzed by flow cytometry. Significantly different from the control ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

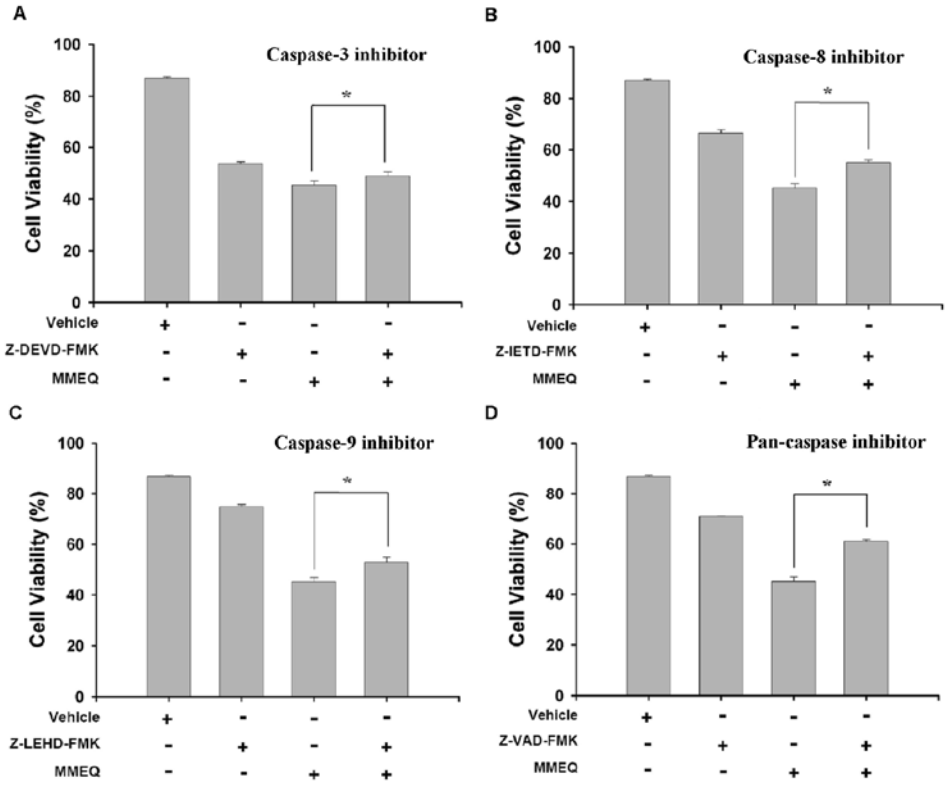


Figure 6. Caspase-8, -9, -3 and pan-caspase inhibitors inhibit MMEQ-induced growth inhibition action in TSGH8301 cells. TSGH8301 cells were pretreated with caspase-3 inhibitor (Z-IETD-FMK) (A), a caspase-8 inhibitor (Z-LEHD-FMK) (B), a caspase-9 inhibitor (Z-DEVD- FMK) (C) and pan-caspase inhibitor (Z-VAD-FMK) (D) then cells were treated with 3 μM of MMEQ for 48 h. TSGH8301 cells were harvested for determination of cell viability as described in Materials and methods. Significantly different from the control ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

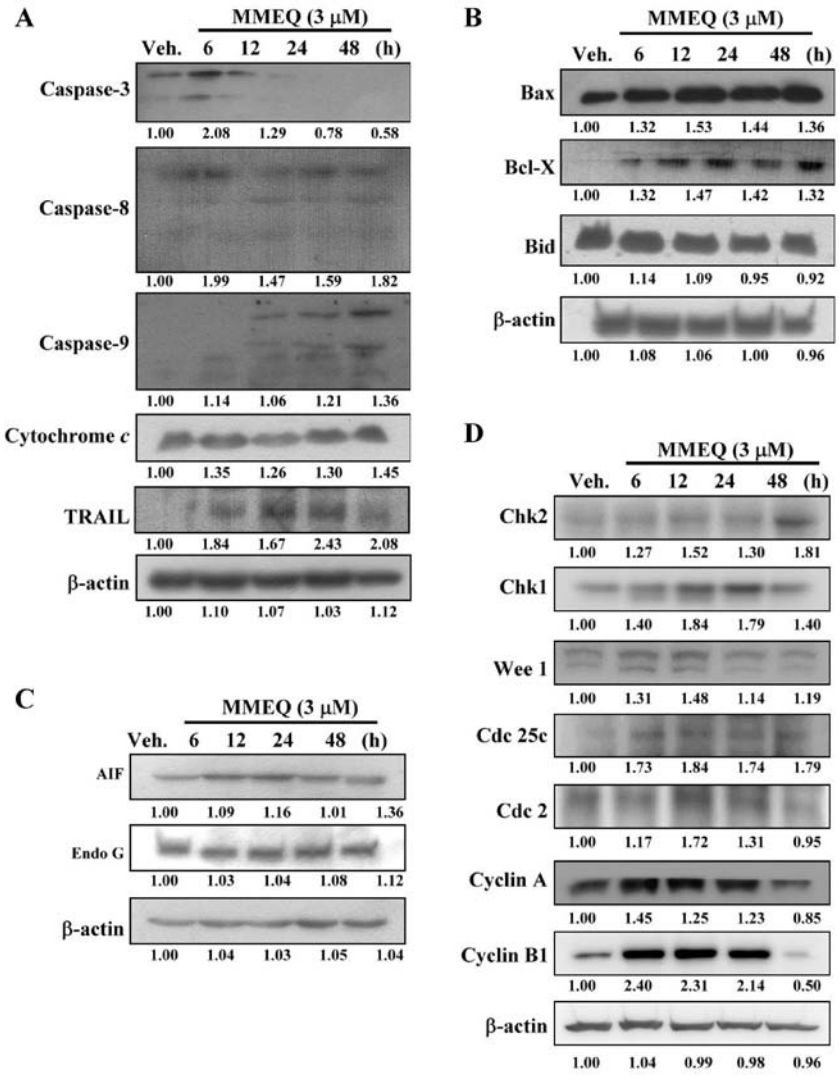


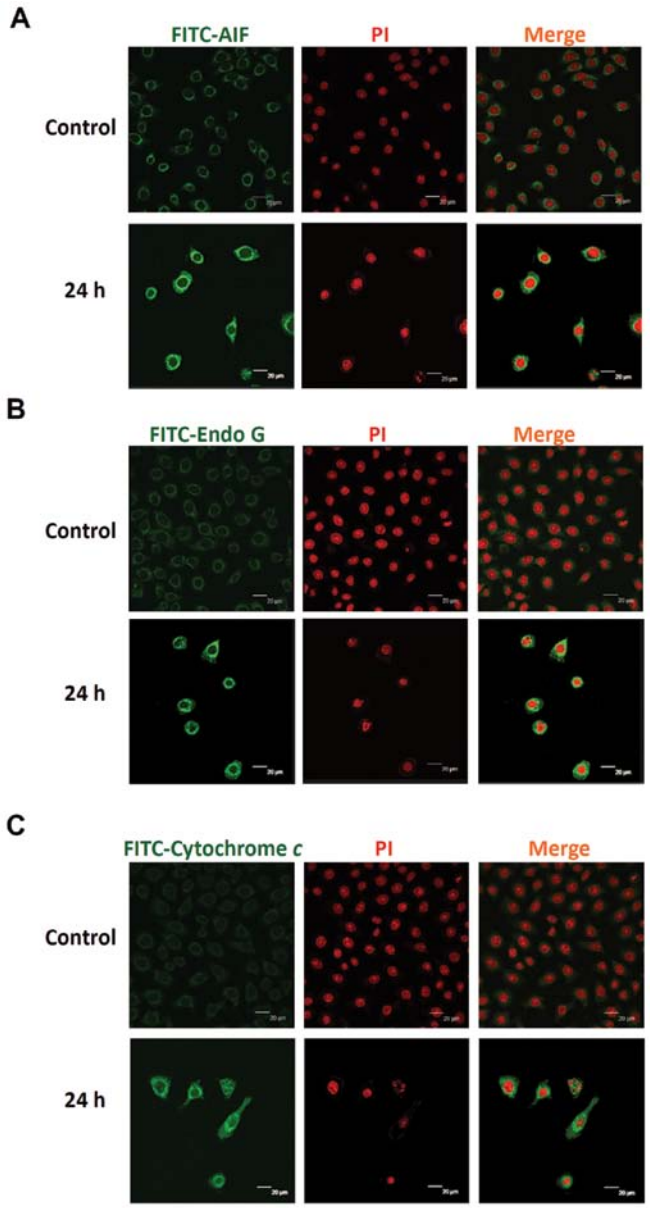
Figure 7. MMEQ affects the protein levels of apoptosis and G2/M phase arrest in TSGH8301 cells. The TSGH8301 cells were treated with 3 μM MMEQ for 0, 6, 12, 24 and 48 h and the total proteins were prepared then detected by Western blotting. The primary antibodies for caspase-3, caspase-8, caspase-9, cytochrome c and TRIAL (A), Bax, Bcl-X and Bid (B), AIF and Endo G (C), Chk2, Chk1, Wee 1, Cdc25c, Cdc2, cyclin A and B1 (D) then each sample as stained by secondary antibody as described in Materials and methods.

G2/M phase arrest in TSGH8301 cells, the cells were treated with 3 μM of MMEQ for various time periods before analysis of the protein level change by Western blotting. The results are shown in Fig. 7, where MMEQ increased caspase-3, caspase-8, caspase-9, cytochrome c and TRIAL (Fig. 7A) and Bax and Bcl-X (Fig. 7B), AIF and Endo G (Fig. 7C) protein levels in TSGH8301 cells. We suggest that MMEQ-induced apoptosis is mediated by induction of death receptor, mitochondrial and caspase-8, -9 and -3 pathways. Furthermore, the results in Fig. 7D indicate that MMEQ decreased the levels of Cdc25c, Cdc2, cyclin A and B1 and increased the levels of Chk2, Chk1 and Wee 1 that led to G2/M arrest in TSGH8301 cells.

MMEQ promotes AIF, Endo G and cytochrome c translocation in TSGH8301 cells. As illustrated in Fig. 8, MMEQ-treated TSGH8301 cells reacted with AIF, Endo G and cytochrome c primary antibodies and PI staining results indicated that MMEQ treatment for 24 h increased the levels of AIF and Endo G (Fig. 8A and B); it was also translocated from mitochondria to nuclei. Moreover, cytochrome c translocated from mitochondria to cytosol (Fig. 8C).

Discussion

It is well-documented that anti-cancer agents and DNA-damaging agents arrest the cell cycle at one of the G0/G1, S, or G2/M phase and then cause cell death through apoptosis (3,19,22). Checkpoint enzymes of cell cycle ensure that cells have time for DNA repair, whereas apoptotic cell death eliminate irreparable or unrepaired damaged cells. Our results showed that MMEQ induced a time- and dose-dependent cytotoxic effect on the TSGH8301 cells; however, the exact mechanism is still unknown. The purpose of this study was to elucidate the molecular mechanism of MMEQ decreasing the percentage of viable TSGH8301 cells. MMEQ induced G2/M arrest and apoptosis of TSGH8301 cells in a dose- and time-dependent (Fig. 2A and B). This is the first report describing the G2/M phase arrest and apoptosis of MMEQ on TSGH8301 cells. Therefore, the inhibition of cell viability



observed in MMEQ-treated TSGH8301 cells may go through apoptotic cell death in addition to cell cycle arrest (Figs. 2 and 7D). Based on the results from Western blotting (Fig. 7D) indicated that MMEQ promoted the levels of Chk1 and Chk2, which led to an increase in the levels of Cdc25c and cyclin B1, but decreased the levels of Wee1 and cyclin A causing G2/M arrest.

Our data demonstrated that MMEQ resulted in apoptosis of TSGH8301 cells based on the observations of DNA condensation by DAPI staining, DNA ladder by DNA gel electrophoresis and sub-G1 phase occurrence by flow cytometric analysis (Figs. 3 and 4). Many studies have shown that chemotherapeutic and chemo-preventive agents cause apoptotic cell death through the mediation of caspases (23). Caspase-3, the an executioner caspase for apoptosis, can be activated by a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8 (8,24,25). In this study, the activation of caspase-3, -8 and -9 were observed in MMEQ-treated TSGH8301 cells (Figs. 6 and 7A). Moreover, MMEQ-induced apoptosis was significantly attenuated in the presence of specific inhibitors of caspase-3, caspase-8 and caspase-9 (Fig. 6). Flow cytometric analysis also showed that MMEQ decreased the levels of $\Delta\Psi_m$ in TSGH8301 cells which may be due to the changes of ratio of Bax and Bcl-2 because Western blotting already showed that MMEQ promoted the levels of Bax (Fig. 7B). Results from Western blotting also indicated that MMEQ promoted the protein levels of AIF (Fig. 7C), Endo G (Fig. 7C) and cytochrome c (Fig. 7A). These results were confirmed by confocal laser microscopy

Figure 8. MMEQ promotes AIF, Endo G and cytochrome c release from mitochondria in TSGH8301 cells. The TSGH8301 cells were incubated with 3 μM MMEQ for 24 h and they were fixed and stained with primary antibodies for AIF (A), Endo G (B) and cytochrome c (C), and then they were stained by second antibodies (green fluorescence) and the proteins were detected by a confocal laser microscopic system as described in Materials and methods. The nuclei were stained by PI (red fluorescence). Areas of colocalization between protein expressions and nuclei in the merged panels are yellow. Scale bar, 20 μm.

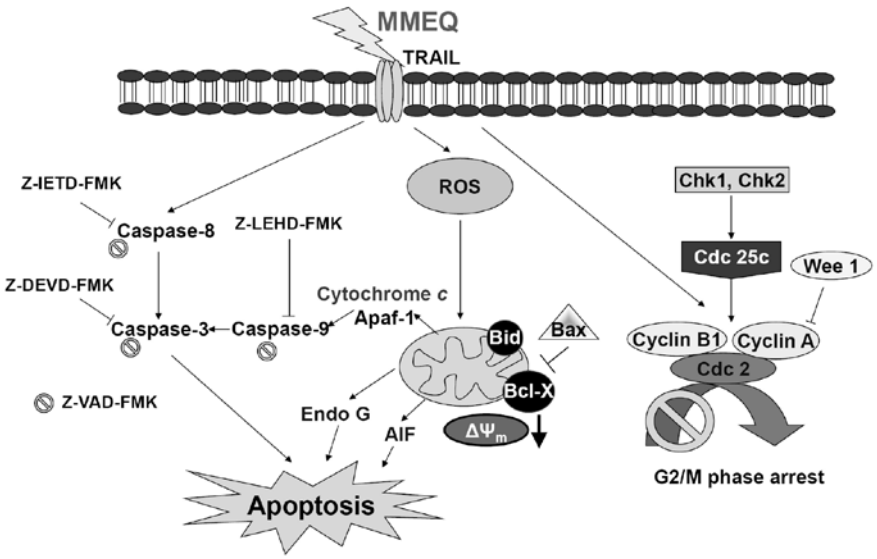


Figure 9. The proposed signaling pathways of MMEQ-induced apoptosis in human bladder cancer TSGH8301 cells.

(Fig. 8). Results also showed that MMEQ induced the levels of TRAIL which is one of the death receptor for agents (Fig. 7A). Flow cytometric analysis showed that MMEQ promoted the levels of ROS and Ca^{2+} (Fig. 5A and C) which may cause DNA damage based on the Comet assay which showed that MMEQ induced DNA damage in TSGH8301 cells (data not shown). These results suggest involvement of both mitochondrial and death receptor pathways in MMEQ-induced apoptosis. Further characterizations of the mitochondrial and death receptor pathways in MMEQ-treated TSGH8301 cells show that they help to establish the relative contributions of caspase-9 and caspase-8 cascades to MMEQ-induced apoptosis (Fig. 9).

In summary, this investigation shows that MMEQ arrested the cell cycle at the G2/M phase and induced apoptosis of TSGH8301 cells. MMEQ also caused DNA damage and induced ROS productions which may relate to DNA damage. MMEQ also caused a marked increase in apoptosis, which was accompanied by activated caspase- 8, -9, and -3 and release of AIF, Endo G and Cytochrome c from mitochondria of TSGH8301 cells. Taken together, these findings provide important new insights into the possible molecular mechanisms of the anti-cancer activity of MMEQ in TSGH8301 human bladder cancer cells.

Acknowledgements

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