

Ethyl 2-[N-m-chlorobenzyl-(2'-methyl)]anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01006) Induces Apoptosis in Human Cervical Cancer HeLa Cells

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Abstract. Human cervical cancer is potentially lethal, and therefore the development of effective and tolerable therapeutic options is vital. In the present study, the *in vitro* effect of the synthesized compound JOT01006 ($C_{21}H_{20}ClNO_4$) on human cervical epithelioid carcinoma cell line (HeLa) was examined. The results demonstrated that JOT01006 induced morphological changes and cytotoxicity (decreased the percentage of viable cells) in a dose-dependent manner. JOT01006 induced apoptosis which was analyzed by flow cytometric methods and confirmed by DAPI staining and DNA fragmentation analyzed by DNA gel electrophoresis. JOT01006 also induced reactive oxygen species (ROS) overproduction before causing endoplasmic reticulum (ER) stress which was also confirmed by the increased levels of Grp78 and Gadd153. Western blotting was selected to demonstrate that JOT01006 promoted p53, Bak, PARP, caspase-3 levels and decreased the levels of Bcl-2 and Bcl-xL. Our results also showed that JOT01006 also promoted caspase-12 production followed by apoptosis. The results also showed that JOT01006 inhibited the migration of HeLa cells potentially through inhibition of MMP-2 and -9.

Induction of apoptosis is one of the mechanisms of action of anticancer agents. It has been demonstrated that apoptosis can be induced through mitochondria-dependent and -independent pathways (1). It is well-known that many chemotherapeutic agents can trigger the mitochondrial apoptotic pathway by means of various stress signals (1). These signals can induce mitochondrial membrane permeabilization leading to cytochrome C release, caspase-3 activation and then apoptosis. Endoplasmic reticulum (ER) stress also causes apoptosis. Many agents have been shown to induce apoptosis *via* the overproduction of reactive oxygen species (ROS) before leading to the mitochondria-dependent pathway for apoptosis (2, 3). ROS have also been shown to cause ER stress (4). ER stress results in damage to most of the principal biological macromolecules giving the potential for apoptotic cell death (5).

Our previous studies have shown that JOT01006 (formula: $C_{21}H_{20}ClNO_4$) induced cytotoxicity in mice leukemia WEHI-3 cells (6). In this study, human cervical epithelioid carcinoma HeLa cells were treated with JOT01006 and analyzed for the correlation between ROS and apoptosis. The effects of JOT01006 on the cell cycle and the signal pathway for apoptosis associated with different levels of proteins were also investigated.

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Materials and Methods

Materials. The compound JOT01006 ($C_{21}H_{20}ClNO_4$) was synthesized in our laboratory as described previously (Figure 1) (6). The following chemicals and reagents were obtained from the indicated companies: Propidium iodide (PI), RNase, trypan blue, Tris-HCl and triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA); BAPTA, dimethyl sulfoxide (DMSO) and potassium phosphates were obtained from Merck Co. (Darmstadt,

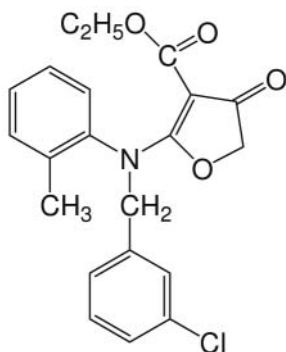


Figure 1. Ethyl 2-[N-m-chlorobenzyl-(2'-methyl)]anilino-4-oxo-4,5-dihydrofuran-3- carboxylate (JOT01006).

Germany); minimum essential medium (MEM), fetal bovine serum (FBS), glutamine, penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA) and Caspase-3 activity assay kit was purchased from OncoImmunit, Inc. (Gaithersburg, MD, USA).

Cell culture. The HeLa cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The HeLa cells were placed into 75 cm³ tissue culture flasks and grown at 37°C, under humidified 5% CO₂ and 95% air at one atmosphere in MEM medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate. The HeLa cells were cultured for several generations and were sub-cultivated and checked for viability each generation as described previously (7, 8).

Cytotoxic activity assay. The HeLa cells were plated in 12-well plates at a density of 2x10⁵ cells/well and grown for 24 h. Different concentrations of JOT01006 were then added to the cells to give a concentration of 0, 50, 100, 150 and 200 µM, while only DMSO (solvent) was added to the control group. Morphological changes of the cells were photographed under a phase-contrast light microscope. Flow cytometric analysis was used to determine cell viability as described previously (7, 8).

Cell cycle and sub-G1 analysis. Approximately 2x10⁵ cells/well of HeLa cells in 12-well plates with various concentrations (0, 50, 100, 150 and 200 µM) of JOT01006 were incubated for 48 h. The cells were harvested by centrifugation and were fixed gently (drop by drop) with 70% ethanol phosphate-buffered saline (PBS) they were stored at 4°C overnight and then re-suspended in PBS containing 40 µg/mL PI, 0.1 mg/mL RNase and 0.1% Triton X-100 in a dark room. After 30 minutes at 37°C, the cells were analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA) with an argon ion laser at 488 nm wavelength. Then the cell cycle was examined and analyzed (7, 8). Annexin V-FITC and a PI double staining kit from PharMingen (San Diego, CA, USA) were used for apoptotic cell quantification (7, 8).

DAPI staining for apoptosis. Approximately 2x10⁵ cells/well of HeLa cells were grown in 6-well plates and incubated with various

concentrations (0, 50, 100, 150 and 200 µM) of JOT01006 for 48 hours prior to staining. The cells were washed three times with PBS and fixed with 4% paraformaldehyde. The fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml, Sigma) for 30 min. DAPI binds the adenine/thymine clusters in double stranded DNA which lead to increase in its fluorescence intensity. The stained cells were examined by fluorescence microscopy to identify apoptotic cells (8, 9).

DNA fragmentation assay. Approximately 1x10⁶ cells/well of HeLa cells were grown in 6-well plates and incubated with 150 µM JOT01006 for 0, 24, 48 and 72 hours. The DNA from treated and non-treated cells was isolated and determined by in 0.8% agarose gel electrophoresis and photographed by fluorescence microscopy as described previously (7).

Reactive oxygen species (ROS) assay. Approximately 2x10⁵ cells/well of HeLa cells in 12-well plates were incubated with 150 µM JOT01006 for 0, 0.5, 1, 3 and 6 hours. The cells were harvested and washed twice, re-suspended in 500 µl of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) and incubated at 37°C for 30 min and then analyzed to detect the changes of ROS by flow cytometry (9).

Cytoplasmic Ca²⁺ levels assay. Approximately 2x10⁵ cells/well of HeLa cells in 12-well plates were incubated with 150 µM JOT01006 for 0.25, 1, 2, 3, 6, and 8 hours. The cells were harvested and washed twice using PBS, and re-suspended in Indo 1/AM (1-[2-amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane- N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester) (3 µg/ml), incubated at 37°C for 30 min and then analyzed to detect the changes of cytoplasmic Ca²⁺ level by flow cytometry (9).

Mitochondrial membrane potential (ΔΨ_m) assay. Approximately 2x10⁵ cells/well of HeLa cells in 12-well plates were incubated with 100 µM JOT01006 for 1, 3, 6 and 10 hours. The cells were harvested and washed twice by using PBS, re-suspended in 500 µl of 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) (4 mol/L) and incubated at 37°C for 30 min and then analyzed to detect the changes of ΔΨ_m by flow cytometry (9).

Cell migration assay. Approximately HeLa 2x10⁴ cells/ml in 10 cm plates were incubated with 50 /or 75 µM JOT01006 for 18, 24 and 36 hours. Then cells in the plate were photographed with a phase-contrast microscopy.

Western blotting. Thirty µg of protein were collected from HeLa cells treated with 0, 50, 100, 150, 200 µM JOT01006 for 48 h, and were separated on 12% sodium dodecylsulfate polyacrylamide gel. After electrophoresis, the proteins were blotted and stained by primary antibodies for p53, PARP, Bax, Bcl-2, caspase-3, -7, -9, -12, AIF, Bcl-xL, Akt, MMP-2, -9, Grp78 and Gadd153, then followed by second antibodies to bind each specific primary antibodies and fluorogenic substrate to react with second antibodies (enzyme-conjugated antibodies that react with a fluorogenic substrate to yield a highly fluorescent product) (9, 10). Densitometry analysis was performed using a GS 670 Imaging Densitometer with software Molecular Analyst (BioRad, Hercules, USA) after exposure on a Kodak OMAT X-ray film (9-11).

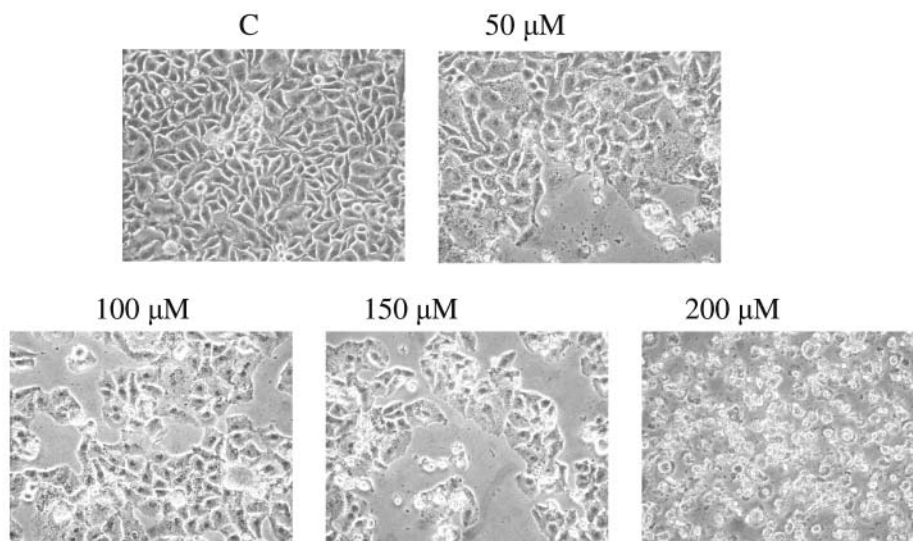


Figure 2. The morphological changes in HeLa cells after JOT01006 treatment. The HeLa cells were incubated with 50, 100, 150, and 200 μM JOT01006 for 24 h and photographed under a phase-contrast microscopy. C: Control, DMSO (solvent) alone. The viable cells were determined by flow cytometry. Each point is the mean \pm S.D. of three experiments. * $p < 0.05$.

Statistical analysis. The Student's *t*-test was used for the statistical analysis between the JOT01006 treated and control groups. $p < 0.05$ = significant.

Results

Effect of JOT01006 on morphological changes and cell viability of HeLa cells. The results from morphological examinations under phase-contrast microscopy and PI staining experiments indicated that the JOT01006 induced morphological changes and decreased the percentage of viable cells in HeLa cells significantly. These effects were dose-dependent (Figures 2 and 3).

Effect of JOT01006 on cell cycle and sub-G1 group HeLa cells. The results of the cell cycle and sub-G1 determinations under flow cytometric analysis indicated that the JOT01006 induced G0/G1 arrest and increased the percentage of sub-G1 (apoptosis) in the HeLa cells. These effects were dose-dependent (Figures 4A, B and C).

Effect of JOT01006 on apoptosis in HeLa cells. The results of DAPI staining experiments under fluorescence microscopy indicated that the JOT01006 induced apoptosis in the HeLa cells. These effects were dose-dependent (Figure 5).

The results from DNA gel electrophoresis experiments under fluorescence microscopy indicated that the JOT01006 induced apoptosis in the HeLa cells based on the occurrence of a DNA ladder. These effects were time-dependent (Figure 6).

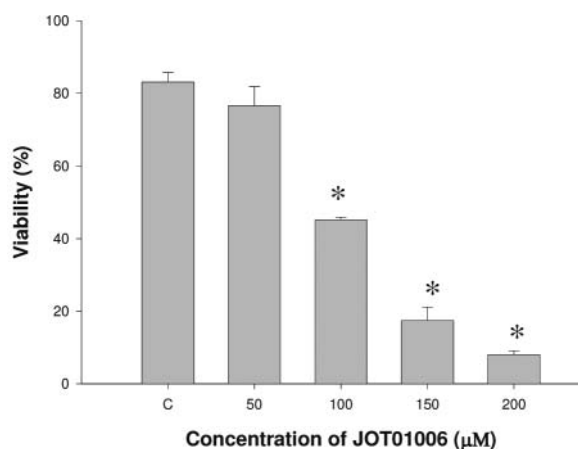


Figure 3. Percentage of viable HeLa cells after JOT01006 treatment.

Effect of JOT01006 on reactive oxygen species (ROS) in HeLa cells. The levels of ROS increased in the JOT01006-treated group compared to that in the control group as shown by flow cytometric analysis. The ROS level was found to reach a peak but maximum time was 6 hours of exposure to JOT01006 at 150 μM (Figure 7). The effects were time-dependent.

Effect of JOT01006 on cytoplasmic Ca^{2+} levels in HeLa cells. The levels of Ca^{2+} increased in the JOT01006-treated group compared to that in the control group as shown by flow

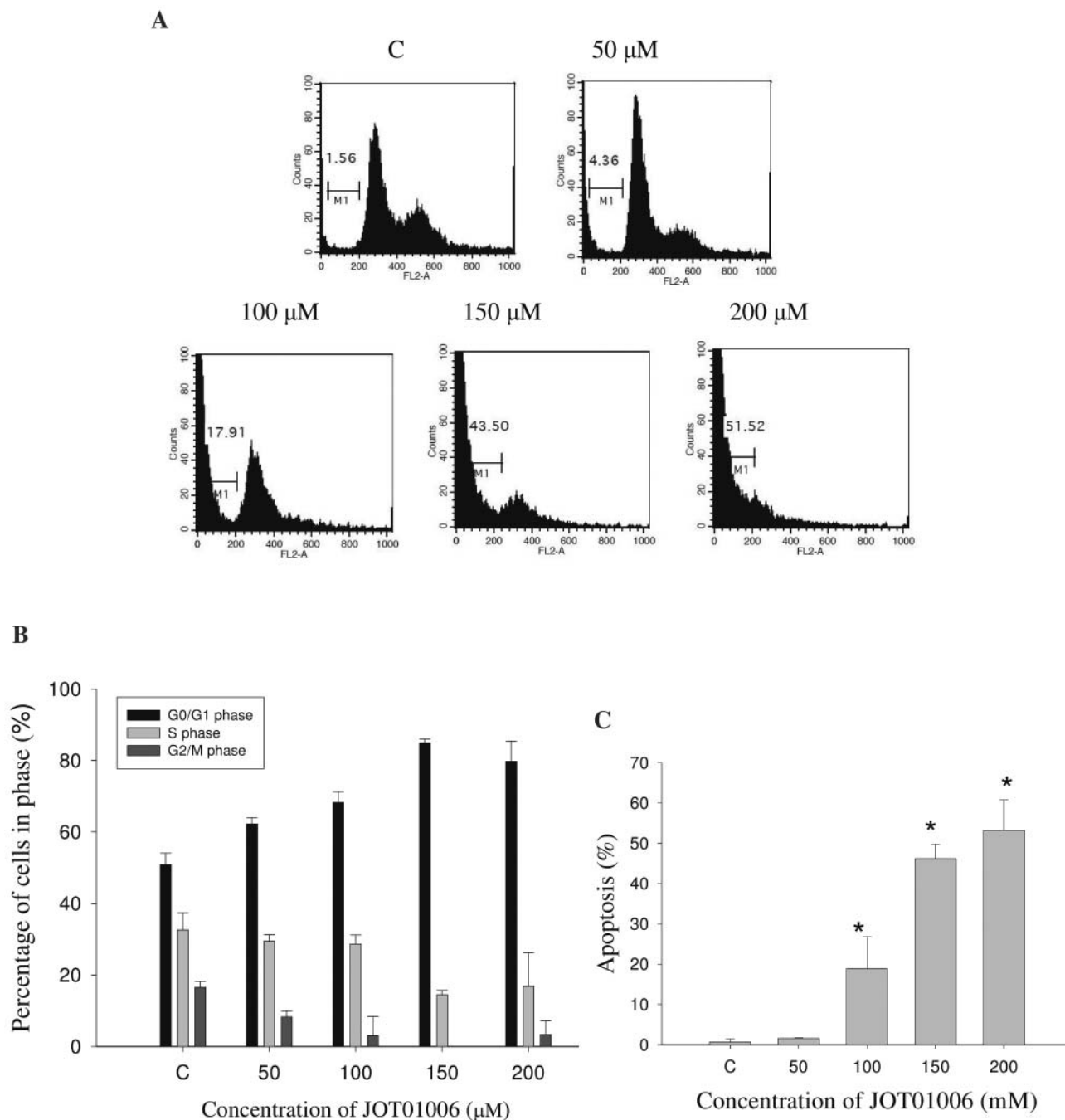


Figure 4. The cell cycle arrest and sub-G1 of HeLa cells after JOT01006 treatment. The HeLa cells were exposed to 0, 50, 100, 150 and 200 μM JOT01006 for 48 h and the cells were harvested for cell cycle analysis by flow cytometry, panel A: representative profiles; panel B: the percent of cells in each phase and panel C: the percent of cells in apoptosis. Data represents the mean ± S.D. of three experiments. * $p < 0.05$.

cytometric analysis. On exposure to JOT01006 at 150 μM, the cytoplasmic Ca^{2+} concentration increased gradually 1 to 8 h (Figure 8).

Effect of JOT01006 on mitochondrial membrane potential ($\Delta\Psi_m$) levels in HeLa cells. The levels of $\Delta\Psi_m$ decreased in the JOT01006-treated (150 μM) group compared to that in

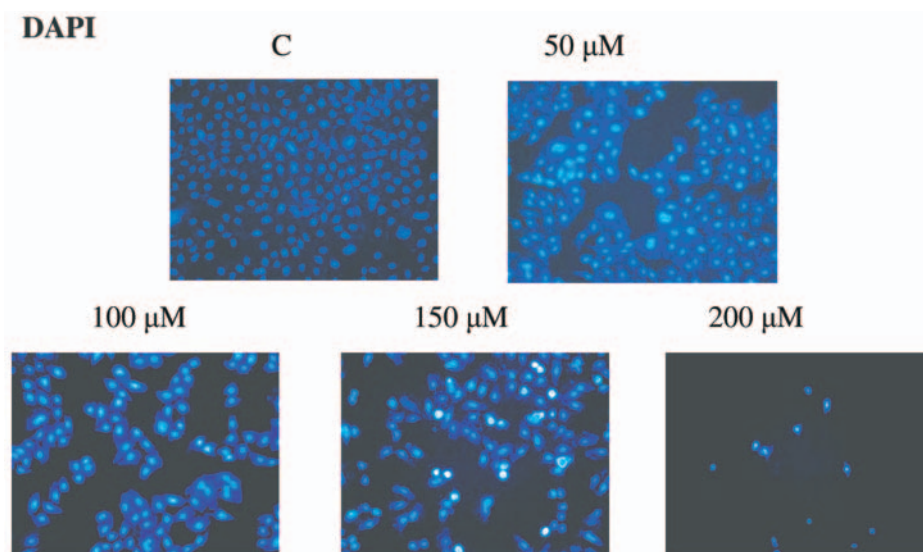


Figure 5. Apoptosis of HeLa cells treated with JOT01006 examined by DAPI staining. The HeLa cells were incubated with 0, 50, 100 and 200 μM JOT01006 for 48 h and apoptosis was determined by using DAPI staining before being photographed under fluorescence microscopy. C: Control, DMSO (solvent) alone.

the control group as shown by flow cytometric analysis gradually from 1 to 6 hours of exposure (Figure 9).

Effect of JOT01006 on cell migration HeLa cells. Cell migration decreased in the JOT01006-treated group compare to that in the control group as shown by photographs. On exposure to JOT01006 at 75 μM , the migration of cells decreased after 24 h in the 75 μM treatment (Figure 10).

Western blotting to examine the effects of JOT01006 on p53, PARP, Bax, Bcl-2, caspase-3, -7, -9, -12, AIF, Bcl-xL, Fas, Akt, MMP-2, -9, Grp78 and Gadd153 in HeLa cells. The results of Western blot are shown in Figure 11 (panel A: p53, PARP, Bax, Bcl-2, caspase-3 and -7; panel B: AIF, Bcl-xL, caspase-9 and Fas; panel C: MMP-2, -9, caspase -12, Grp78 and Gadd153). An increase in the expression of p53, PARP, Bax, caspases-9, 3, 7, and -12, Grp78 and Gadd153 and decrease of the expression of Bcl-2, Bcl-xL, Akt, MMP-2 and -9 which may contribute to the occurrence of apoptosis of the cells were examined (Figure 11).

Discussion

The sensitivity and resistance of tumor cells to chemotherapeutic agents depend on the activation of various signal pathways to apoptosis (12). In this study, the effects of JOT01006 on cell cycle arrest and apoptosis were investigated and the results clearly demonstrated that JOT01006 induced G0/G1-phase arrest (Figure 4) and

DNA fragmentation

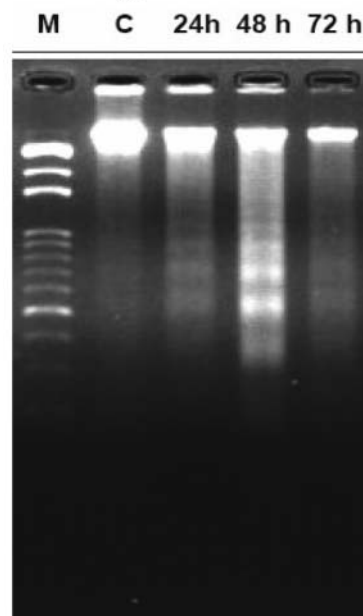


Figure 6. Apoptosis of HeLa cells treated with 150 μM JOT01006 were examined by DNA gel electrophoresis. The HeLa cells were incubated with with 150 μM JOT01006 for 24, 48 h and 72 h. The DNA was extracted and subjected to electrophoresis and then photographed under fluorescence microscopy. M: DNA size marker. C: Control, DMSO (solvent) alone.

apoptosis (Figures 4, 5 and 6) in the HeLa cells. The JOT01006 induced cell death was characteristic of apoptosis, as evidenced by DNA cleavage analysis by three

ROS

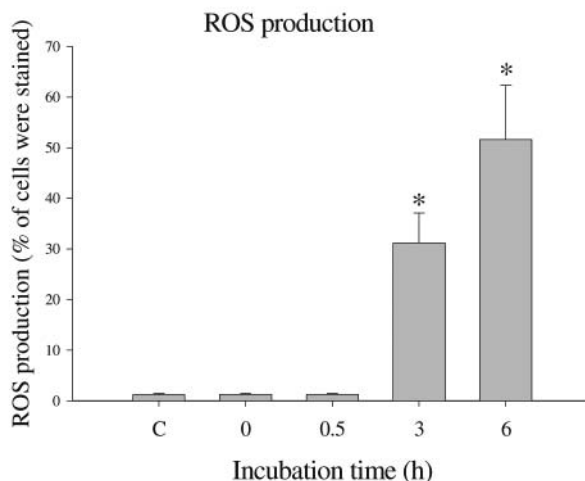
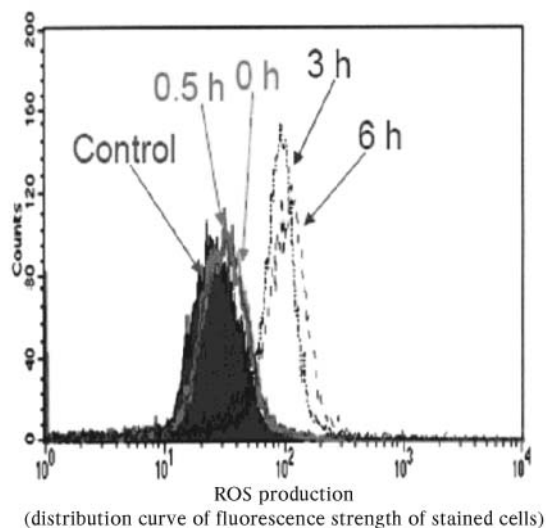


Figure 7. Flow cytometric analysis of reactive oxygen species (ROS) in HeLa cells with 150 μ M JOT01006 for various time periods. The HeLa cells (5×10^5 cells/ml) were treated with 150 μ M JOT01006 for 1, 3, 6 h to detect the changes of ROS. The cells that were stained by the DCFH-DA dye were determined by flow cytometry. *significant differences between JOT01006 and control (solvent alone) $p < 0.05$.

Calcium release

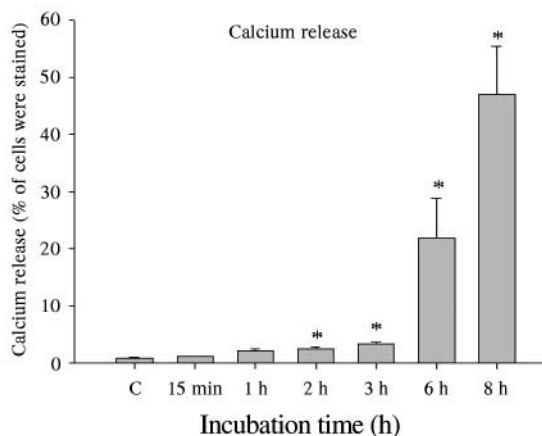
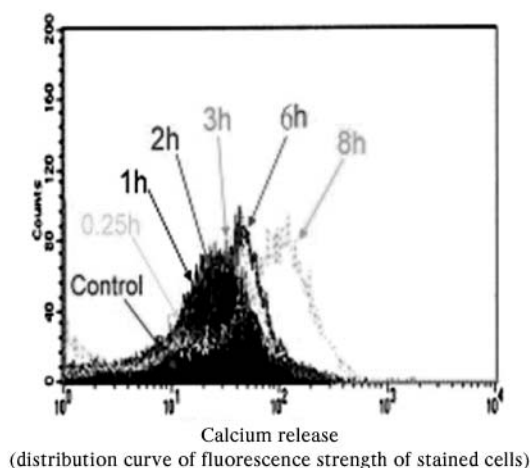


Figure 8. Flow cytometric analysis of Ca^{2+} concentration in HeLa cells treated with 150 μ M JOT01006 for various time periods. The HeLa cells (5×10^5 cells/ml) were treated with 150 μ M JOT01006 for 1, 2, 3, 6, and 8 h. The cells that were stained by Indo-1/AM dye were determined by flow cytometry as described. *significant differences between JOT01006 and control (solvent alone) $p < 0.05$.

different methods: sub-G1 group analysis by flow cytometry; DNA gel electrophoresis of DNA fragmentation and DAPI assay technique confirming the presence of fragmented oligonucleosomal DNA within the apoptotic cells.

JOT01006 may exert its pro-apoptotic effects by increasing the levels of ROS. It has been reported that emodin enhanced arsenic trioxide-induced apoptosis

through the generation of ROS and inhibition of survival signaling in human cancer cell lines (13), however it has also been reported that emodin induced apoptosis in human promyeloleukemic HL-60 cell was independent of ROS production (14). It is well known that ROS can cause endoplasmic reticulum (ER) stress leading to Ca^{2+} release from the ER. The use of Western blot demonstrated that

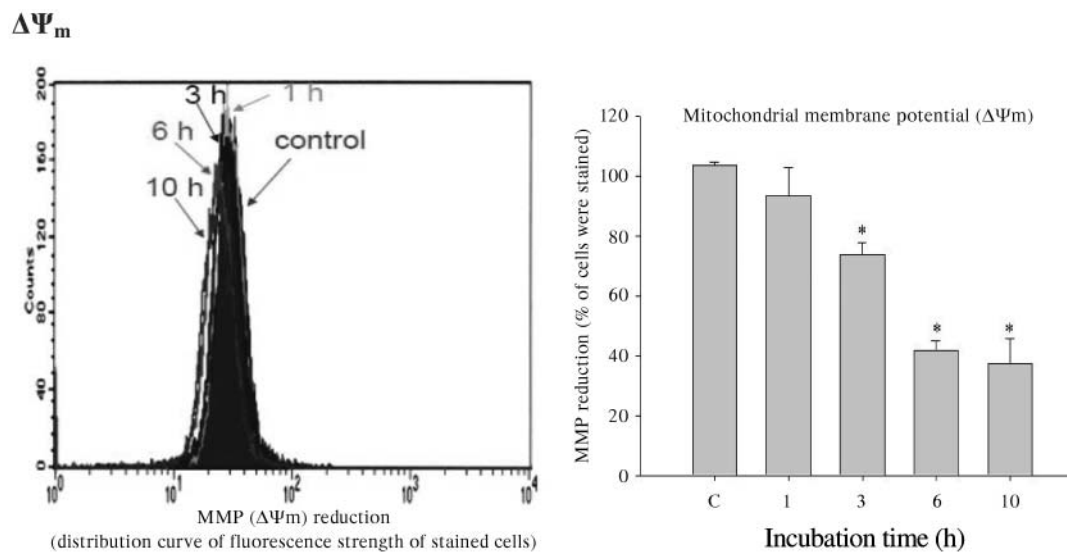


Figure 9. Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in HeLa cells treat with $150 \mu\text{M}$ JOT01006 for various time periods. The cells that were stained by DiOL6 dye were determined by flow cytometry. *significant differences between JOT01006 and control (solvent alone) $p < 0.05$.

Cell migration

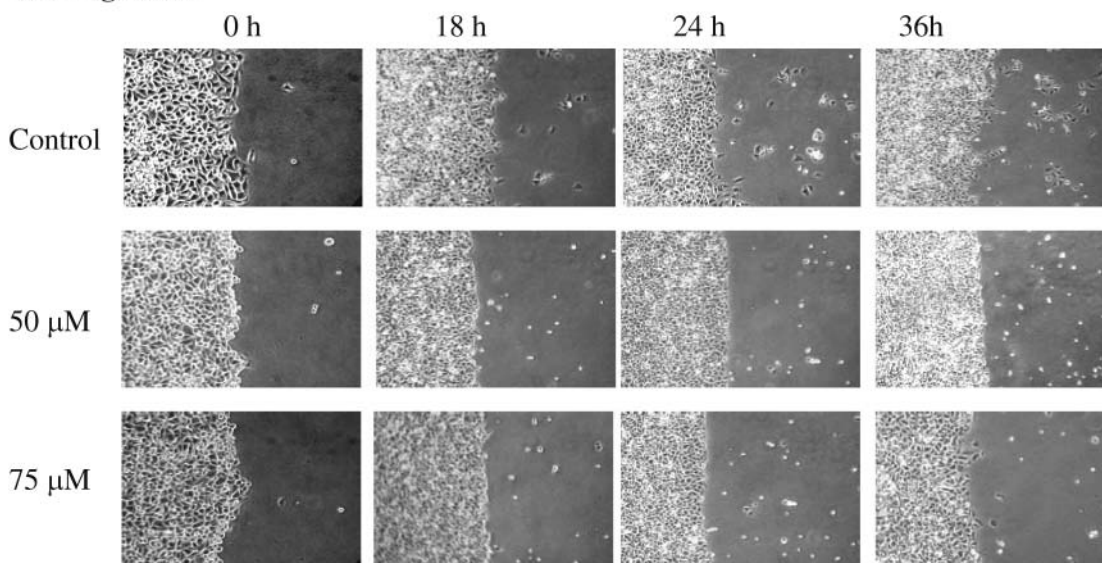


Figure 10. Effects of JOT01006 on the migration of HeLa cells. The HeLa cells (2×10^4 cells/ml) were treated with 0, 50 or $75 \mu\text{M}$ JOT01006 for 18, 24 and 36 h before being photographed.

JOT01006 induced Grp 78 and Gadd153 expression (based on the increased levels of both proteins) and both these proteins play an important role in ER stress (15). Our results also demonstrated that JOT01006 may cause a decrease in the mitochondrial membrane potential (loss of $\Delta\Psi_m$) and increased PARP levels and activation of caspase-3 leading to apoptosis. It thus appears that JOT01006 induces apoptosis through the mitochondria-dependant

pathway. JOT01006 promoted the levels of Bax which is a pro-apoptotic protein and decreased Bcl-2 and Bcl-xL which are anti-apoptotic proteins. Both of these effects contributed to the loss of $\Delta\Psi_m$ in the mitochondria which led to the increase in the caspase-3. Caspase-3 has been demonstrated to be one of the down-stream effector caspases (16), and has been shown to be involved in cleaving a number of substrates that induce cells death (17). We

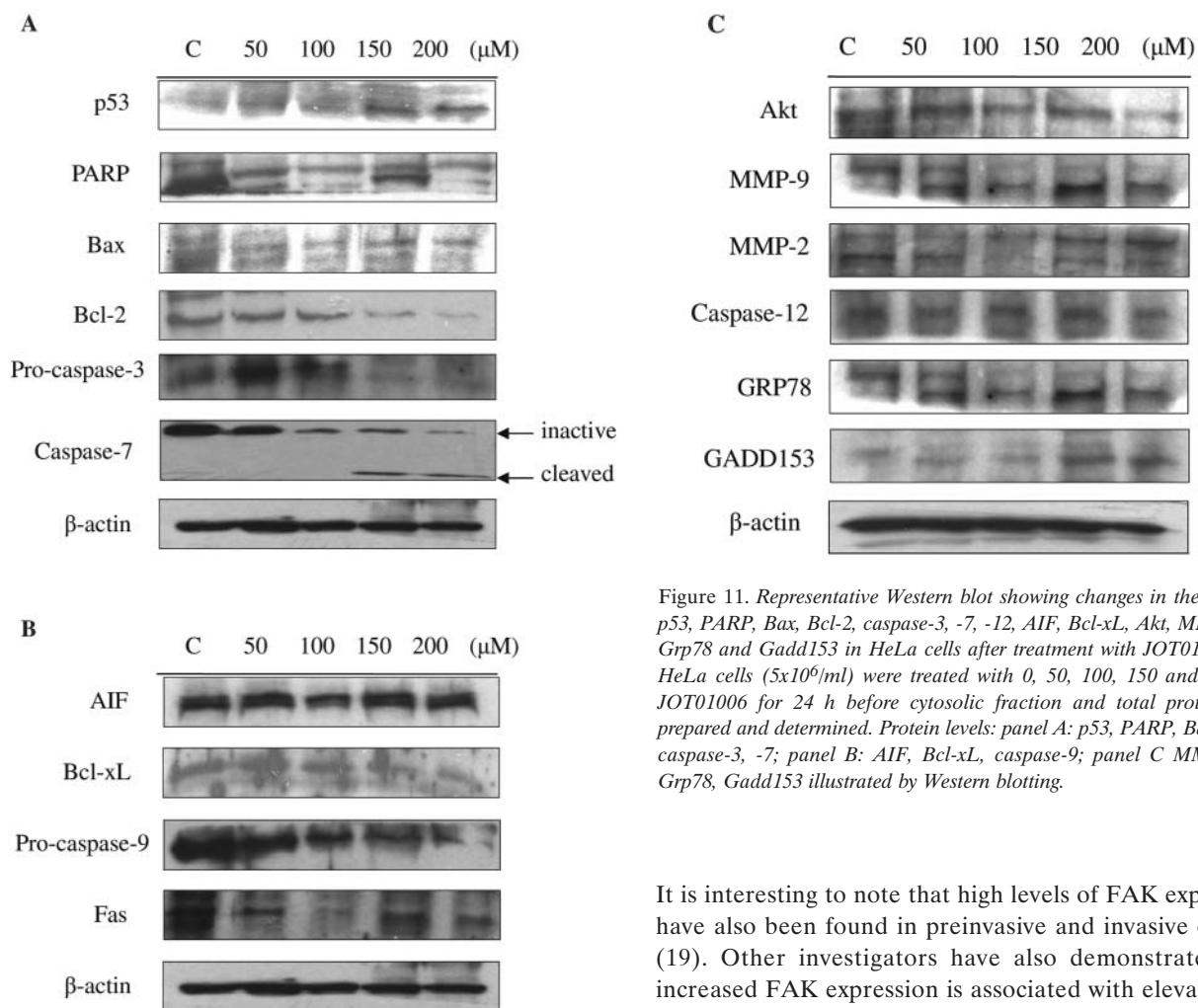


Figure 11. Representative Western blot showing changes in the levels of p53, PARP, Bax, Bcl-2, caspase-3, -7, -12, AIF, Bcl-xL, Akt, MMP-2, -9, Grp78 and Gadd153 in HeLa cells after treatment with JOT01006. The HeLa cells ($5 \times 10^6/ml$) were treated with 0, 50, 100, 150 and 200 μM JOT01006 for 24 h before cytosolic fraction and total protein were prepared and determined. Protein levels: panel A: p53, PARP, Bax, Bcl-2, caspase-3, -7; panel B: AIF, Bcl-xL, caspase-9; panel C: MMP-2, -9, Grp78, Gadd153 illustrated by Western blotting.

proceeded to examine caspase-3 activity, and we found that JOT01006 induced an increase in caspase-3 activity, dependent on the incubation period (data not shown).

The results of Western blotting, showed that JOT01006 promoted the levels of caspase-12 followed by apoptosis which is another signal pathway for agent-induced apoptosis (18). A proposed model for the JOT01006 mechanism of action for G0/G1 arrest and apoptosis in HeLa cells is shown in Figure 12.

The results also showed that JOT01006 inhibited cell migration of HeLa cells on culture plates. Furthermore, Western blotting was also used to demonstrate that JOT01006 inhibited the levels of MMP-2 and -9 which may be responsible for the inhibition of cell migration. It is well-known that the process of tumor cell invasion relies on several cell properties, including proteolysis, actin dynamics, adhesion and motility. We also found that JOT01006 inhibited epidermal growth factor receptor (EGFR) and FAK expression by PCR (data not shown).

It is interesting to note that high levels of FAK expression have also been found in preinvasive and invasive cancers (19). Other investigators have also demonstrated that increased FAK expression is associated with elevated cell motility (20).

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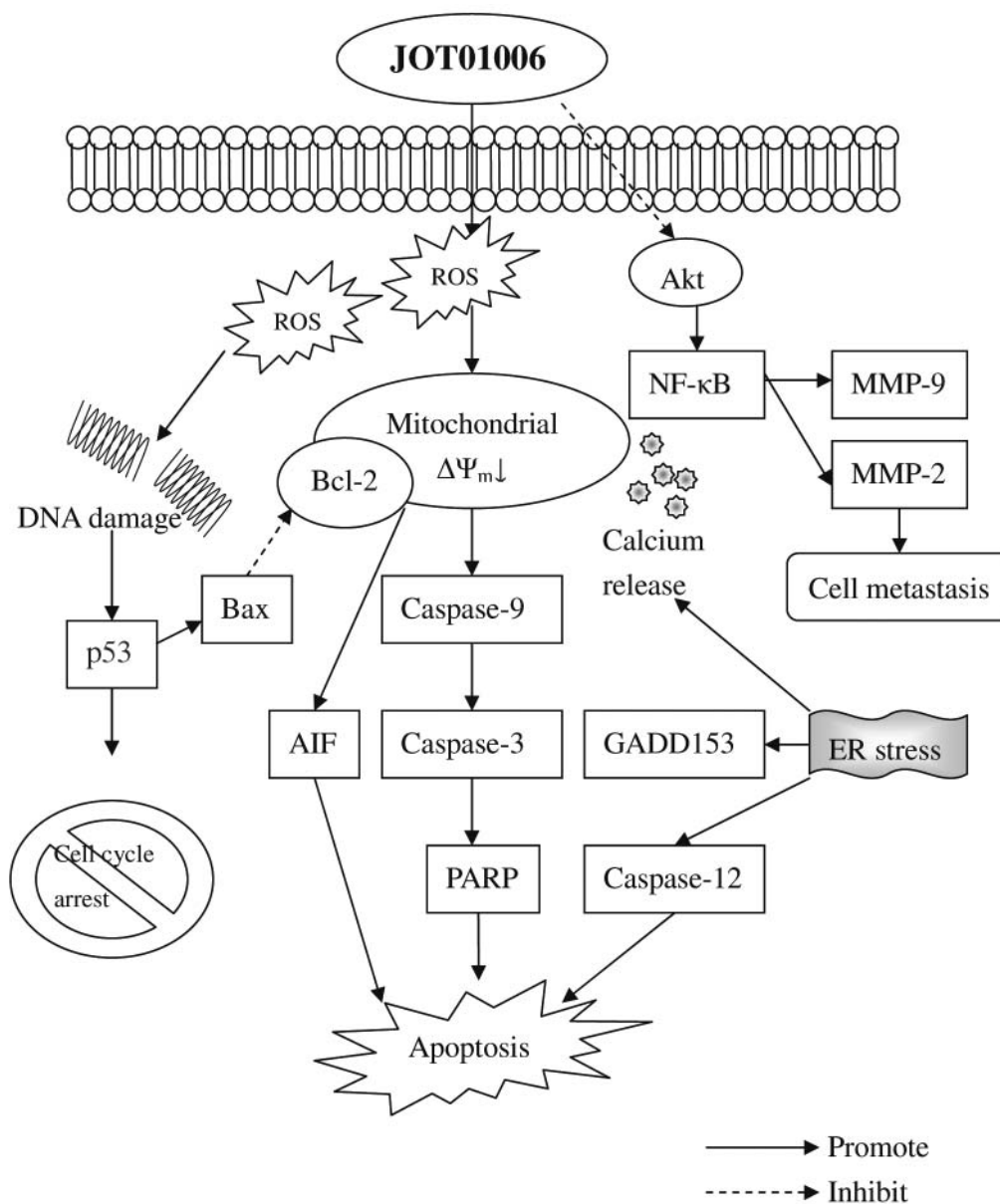


Figure 12. Proposed model of JOT01006 mechanism of action for G0/G1 arrest and apoptosis in HeLa cells. JOT01006 induced p53 expressions leading to G0/G1 arrest. JOT01006 increases the production of ROS and Ca²⁺ production and Bax but decreased Bcl-2, MMP($\Delta\Psi_m$) levels that leads to caspase-3 activity before causing apoptosis in HeLa cells.

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