Abstract. The cytotoxic effects of a new compound, ethyl 2-[N-p-chlorobenzyl-(2'-methyl)] anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01007) have been tested in mouse leukemia WEHI-3 cells. In this study, the mechanisms by which JOT01007 acts on a human cervical cancer cell line (Ca Ski) to bring about an increase in the ratio of Bax/Bcl-2, reduction of the mitochondrial membrane potential (MMP), increase in the levels of cytoplasmic Ca2+, activation of caspases and fragmentation of DNA, and apoptosis were investigated. Flow cytometric analysis demonstrated that JOT01007 induced a decrease of MMP in Ca Ski cells. JOT01007 induced an increase in the level of cytoplasmic Ca2+, which was inhibited by BAPTA (calcium chelator), and BAPTA accelerated the MMP reduction, and significantly blocked JOT01007-induced apoptosis. Western blotting demonstrated that JOT01007 induced an increase in the levels of p53, p21, cytochrome-c, caspase-3 and Bax, but decreased the level of Bcl-2. In conclusion, our data demonstrate that JOT01007-induced apoptosis occurs via a mitochondria-dependent pathway closely related to the level of cytoplasmic Ca2+ in Ca Ski cells.

Cancer of the uterine cervix is currently among the six most common cancers among females in Taiwan. Based on reports from the "People’s Health Bureau of Taiwan", it was demonstrated that about 8.33 per 100,000 women die annually from cancer of the uterine cervix. Currently therapeutic approaches for human uterine cervix cancer in Taiwan include chemotherapy, surgery and radiotherapy. However, conventional strategies for treatment of this cancer are not yet satisfactory.

Cancer chemotherapy has used traditional agents, which are cytotoxic to DNA, to block DNA synthesis, induce cell cycle arrest and cell death. Cell death can be divided into apoptosis and necrosis. Apoptosis is a well regulated and organized death process that plays an important role in controlling the development and homeostasis of multi cellular organisms (1). The apoptotic effects include characteristics, such as the cellular morphological change, chromatin condensation, membrane blebbing, oligonucleosomal DNA cleavage, translocation of phosphatidyl serine of the plasma membrane from the inner to the outer leaflet and activation of a family of caspases – a key hallmark of apoptosis (2-4). The characteristic changes that occur during apoptosis serve as useful markers which can be exploited when determining the effectiveness and mode of action of new compounds.

Evidence has shown that most cancer therapy drugs kill tumor cells through induced apoptosis (5, 6). A widely known fact is that the expression of the anti-apoptotic protein (Bcl-2) decreases as pro-apoptotic proteins (Bax, Bad) increase during apoptosis (7).

Although ethyl 2-[N-p-chlorobenzyl-(2'-methyl)]anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01007/ Figure 1) has been demonstrated to induce cytotoxicity in WEHI-3 leukemia cells, there is no available information to address whether JOT01007 affects human cervical cancer Ca Ski cells. Therefore, in this study the molecular mechanism and the role Ca2+ may play in the induction of apoptosis in Ca Ski cells were examined.
Materials and Methods

Chemicals and reagents. JOT01007 was synthesized in our laboratory as described previously (8). Propidium iodide (PI), RNase, trypsin 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, Germany). RPMI-1640, fetal bovine serum (FBS), glutamine, penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from OncoImmunin Inc (Gaithersburg, MD, USA).

Human cervical epidermoid carcinoma cell line (Ca Ski). Ca Ski cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Ca Ski cells were placed into 75 cm² tissue culture flasks and grown at 37°C under a humidified 5% CO₂ and 95% air at one atmosphere in RPMI-1640 medium supplemented with 10% FBS, penicillin-streptomycin and 1% L-glutamine. Ca Ski cells were cultured for several generations and were checked for viability for each generation as described previously (9, 10).

Morphological changes and cell viability of Ca Ski cells. The Ca Ski cells were plated in 12-well plates at a density of 2x10⁵ cells/well and grown for 24 h. JOT01007 was then added to cells at a final concentration of 0, 10, 50, 100, 150 and 200 μM, while DMSO alone was added to the control group. The cells were grown at 37°C, 5% CO₂ and 95% air for up to 72 h. Morphological changes of cells were photographed under phase-contrast light microscopy. Flow cytometric assay was used to determine cell viability as described previously (9, 10).

DAPI staining. Approximately 2x10⁵ cells/well of Ca Ski cells were grown in 6-well plates at a density of 2x10⁵ cells/well and grown for 24 h. JOT01007 was then added to cells at a final concentration of 0, 10, 50, 100, 150 and 200 μM. The cells were washed twice using phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Fixed cells were washed twice with PBS and stained with 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxyl]-2-(2-amino-5-methylphenoxyl) ethane-N,N',N’-tetra acetic acid penta-acetoxymethyl ester (Indo 1/AM) (3 μg/ml), incubated at 37°C for 30 min and then analyzed to detect the changes in MMP by flow cytometry (9, 10).

DNA laddering fragmentation. About 1x10⁶ cells/well of Ca Ski cells were grown in 6-well plates and treated with JOT01007 at 0, 10, 50, 100, 150 and 200 μM for 48 h prior to staining. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Fixed cells were washed twice with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma) for 30 min. Stained cells were examined under fluorescence microscopy, photographed and apoptotic cells identified (9, 10).

Flow cytometry
(i) Reactive oxygen species (ROS). Approximately 2x10⁵ cells/well of Ca Ski cells in 12-well plates were treated with 100 μM JOT01007 for 0.5, 1, 2, 4, 6, 12 and 24 h. The cells were harvested and washed twice, re-suspended in 500 μl of a solution of 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) (10 μM) and incubated at 37°C for 30 min then analyzed to detect the changes of ROS by flow cytometry (12).
(ii) Mitochondrial membrane potential. Approximately 2x10⁵ cells/well of Ca Ski cells in 12-well plates were treated with 100 μM JOT01007 for 1, 2, 4, 6 and 12 h. The cells were harvested and washed twice using PBS, re-suspended in 500 μl of a solution of 3,3'-dihexyloxacarbocyanine (DiOC₃) (4 mol/L) and incubated at 37°C for 30 min and then analyzed to detect the changes in MMP by flow cytometry (9, 10).

Western blotting. The effects of JOT01007 on p53, Bax, Bcl-2, cytochrome-c, AIF, caspase-3 and -8, Fas, GRP78, GADD153 and caspase-12 of Ca Ski cells were examined by Western blotting. The total protein was collected from Ca Ski cells treated with 0, 10, 50, 100, 150 and 200 μM of JOT01007 for 48 h, and the p53, Bax, Bcl-2, cytochrome-c, AIF, caspase-3 and -8, Fas, GRP78, GADD153 and caspase-12 were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously (9,10).

Statistical analysis. The Student’s t-test was used for the statistical analysis between the JOT01007 treated and control groups.

Results

Effects of JOT01007 on the morphology and cell viability of human cervical cancer Ca Ski cells. The results of microscopical examination and PI staining in flow cytometric experiments indicated that JOT01007 induced morphological changes (Figure 2) and decreased the percentage of viable cells is significantly compared to the control. These effects were dose- and time-dependent (Figure 3A and B).

Effects of JOT01007 on cell cycle and apoptosis in Ca Ski cells. The cell cycle and apoptosis were also analyzed by...
flow cytometry and the results demonstrated that a dose of 0-100 μM of JOT01007 led to G0/G1 arrest but a dose of 150-200 μM led to G2/M arrest (Figure 4A and B). JOT01007 induced apoptosis in Ca Ski cells and this effects was dose-dependent (Figure 4C).

Effects of JOT01007 on apoptosis in Ca Ski cells using DAPI staining. Apoptosis was detected by the DAPI staining method after 48 h of continuous exposure to JOT01007. As shown in Figure 5, JOT01007 induced apoptosis in a concentration-dependent manner.

Effects of JOT01007 on DNA fragmentation in Ca Ski cells using DNA gel electrophoresis. We studied the occurrence of DNA fragmentation from Ca Ski cells treated with various concentrations (0, 10, 50, 100, 150 and 200 μM) of JOT01007 by DNA gel electrophoresis. The DNA was isolated from cells after 48 h of continuous exposure to
JOT01007 and subjected to electrophoresis as described previously (13). As shown in Figure 6, JOT01007 induced DNA fragmentation in a concentration-dependent manner.

Effects of JOT01007 on reactive oxygen species (ROS) in Ca Ski cells using flow cytometry. Levels of ROS slightly decreased in the JOT01007-treated group compared to that in the control group. ROS levels did not show a significant difference between JOT01007 treated and control groups (Figure 7). These results indicate that compound did not induce ROS production.

Effects of JOT01007 on the cytoplasmic Ca^{2+} levels in Ca Ski cells using flow cytometry. Cytoplasmic Ca^{2+} levels increased...
in the JOT01007-treated group compared to that in the control group. On exposure to JOT01007 at 100 μM, cytoplasmic Ca\(^{2+}\) increased by approximately 27~48% in 24~48 h, while earlier treatment times did not show a significant difference (Figure 8).

**Effects of JOT01007 on MMP in Ca Ski cells using flow cytometry.** The MMP decreased in the JOT01007-treated group compared to that in control group. The decrease of the MMP was proportional to the duration of JOT01007 treatment from 1-24 h at 100 μM, revealing a time-dependent effect (Figure 9).

**Effects of JOT01007 on p53, Bax, Bel-2, cytochrome-c, AIF, caspase-3 and -8, Fas, GRP78, GADD153 and caspase-12 of Ca Ski cells using Western blotting.** The results of the Western blotting experiments are shown in (Figure 10). JOT01007 induced increased expressions of p53, Bax, cytochrome-c, AIF, active caspase-3, Fas, caspase-8 and -12, GRP78 and GADD153, but decreased the expression of Bcl-2. This may have caused to the occurrence of apoptosis in the cells examined.

**Discussion**

JOT01007 synthesized in our laboratory exhibited cytotoxic activity against mouse leukemia WEHI-3 cells (8). In this study, we explored the mechanisms by which JOT01007 acts on a human cervical cancer cell line (Ca Ski) to induce apoptosis through the mitochondria-dependent pathway. We showed that this compound decreased the percentage of viable cells (Ca Ski) in both a dose- and time-dependent manner. JOT01007 may induce cell death through either necrosis or apoptosis in Ca Ski cells. The IC\(_{50}\) of JOT01007 on Ca Ski cells was 100 μM in our study. We then demonstrated that incubation of cells with various concentrations of JOT01007 triggered apoptosis (sub-G\(_1\) group\(^2\)), which appeared to be mediated by a caspase-dependent pathway in a dose- and time-dependent fashion.

![Figure 5. Effects of JOT01007 on apoptosis in Ca Ski cells using DAPI staining.](image)

![Figure 6. Effects of JOT01007 on DNA fragmentation in Ca Ski cells using DNA gel electrophoresis.](image)
JOT01007-induced apoptosis in Ca Ski cells was supported by the: (i) occurrence of sub-G₁ group using flow cytometry, (ii) demonstration of DAPI staining of cells under fluorescence microscopy, (iii) fragmentation of DNA in gel electrophoresis. In addition, morphological examination revealed cell shrinkage, chromatin condensation and loss of cell-to-cell contact in JOT01007-treated Ca Ski cells. It has been reported that the loss of substrate adherence is an early event in apoptosis of colon adenoma cells after treatment with an apoptosis-inducing agent (14). The DNA damage caused by JOT01007 in these studies was also confirmed by Comet assay that showed the dose-dependent effect (data not show).

In this study, apoptosis was observed at 6h and up to 72 h after treatment with JOT01007. We also showed that JOT01007 induced sub-G₁ arrest, DNA fragmentation and DAPI staining, as indications of apoptosis, activation of caspase-3 and promotion of Bax expression, but a decreased

Figure 7. Effects of JOT01007 on reactive oxygen species (ROS) in Ca Ski cells using flow cytometry.
Bcl-2 expression in Ca Ski cells. Our studies also showed that pretreatment of Ca Ski cells with Ac-DEVD-CHO (a caspase-3 inhibitor) inhibited JOT01007-induced caspase-3 activity that led to a decrease in apoptosis in the examined cells (data not shown).

Evidence shows that chemotherapeutic agents act primarily via inducing cancer cell death through the mechanism of apoptosis (6). However, many cancer cells carry or develop defects in the regulation of genes that control apoptosis rendering them resistant to the induction of apoptosis through a wide variety of stimuli (15, 16). Many chemotherapeutic agents can trigger the mitochondrial apoptotic pathway via various stress signals (17). These signals can indirectly induce mitochondrial membrane permeabilization or may have a direct action on mitochondrial proteins and lipids. In the present study, we demonstrated that JOT01007 may induce apoptosis via Ca\textsuperscript{2+} production and induce the loss of the mitochondrial membrane potential, in turn leading to the release of cytochrome-c and caspase-3 activation.

The mitochondrion plays an important role in the regulation of apoptosis (18, 19). Mitochondrial dysfunctions, including loss of MMP, permeability transition, and release of cytochrome-c from the mitochondrion into the cytosol, are associated with apoptosis (20).
Our results indicated that JOT01007 induced a rapid loss of MMP and release of cytochrome-c in Ca Ski cells. Western blots showed that JOT01007 decreased Bcl-2 levels and promoted Bax levels that led to a decrease in the MMP then activating mitochondria-mediated cytochrome-c release and the sequential activation of caspase-3. The results of the present study suggest that apoptosis induced by this compound occurs through a mitochondria-dependent, ROS-independent pathway.

We also found that JOT0100 induced an early increase in cytoplasmic Ca^{2+}, usually ascribed to endoplasmic reticulum (ER) stress, resulting in Ca^{+2} release from ER to cytosol (21). It was reported that cellular Ca^{2+} overload promotes mitochondrial Ca^{2+} uptake, subsequently contributing to a transition in mitochondrial permeability and the release of mitochondrial apoptogenic factors into the cytosol (21). Therefore, Ca^{2+} plays a critical role in compound-induced mitochondria-dependent apoptosis in Ca Ski cells.

**Conclusion**

These are the first findings regarding JOT01007-induced apoptosis in human cervical cancer Ca Ski cells. JOT01007-induced apoptosis in Ca Ski cells occurs via a caspase-3 and
Lin et al: Ethyl 2-[N-p-chlorobenzyl-(2'-methyl)]anilino-4-oxo-4,5-dihydrofuran-3-carboxylate Induces Apoptosis in Ca Ski Cells

Figure 10. Effects of JOT01007 on p53, Bax, Bcl-2, cytochrome-c, AIF, caspase-3 and -8, Fas, GRP78, GADD153 and caspase-12 of Ca Ski cells using Western blotting. Panel A: p53, bax, Bcl-2, cytochrome-c, AIF, caspase-3 and -8; Panel B: Fas, GRP78, GADD153 and caspase-12.

Figure 11. Proposed model of JOT01007 mechanism of action for induction of apoptosis in Ca Ski cells. JOT01007 increased the production of Ca^{2+} and decreased MMP levels leading to caspase-3 activation before causing apoptosis.
mitochondria-dependent pathway which also involves Ca^{2+} levels (Figure 11). These findings may offer some insights into the therapeutic value of JOT01007 in human cervical cancer.

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References


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