Involvement of Matrix Metalloproteinases in the Inhibition of Cell Invasion and Migration Through the Inhibition of NF-κB by the New Synthesized Ethyl 2-[N-p-chlorobenzyl-(2'-methyl)anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOTO1007) in Human Cervical Cancer Ca Ski Cells

AN-CHENG HUANG1, SHU-CHUN HSU2, CHAO-LIN KUO2, CHING-LUNG LIAO3, KUANG-CHI LAI4,5, TSUNG-PING LIN6, SHIH-HWAR WU7,8, HSU-FENG LU9,10, NOU-YING TANG11, JAI-SING YANG12 and JING-GUNG CHUNG13,14,*

1Department of Nursing, St. Mary’s Medicine Nursing and Management College, Sansing Township, Yilan County 266;
2Institute of Chinese Pharmaceutical Sciences, Graduate Institutes of 3Chinese Medical Science, Departments of 12Pharmacology and 13Biological Science and Technology, 14Department of Biotechnology, Asia University, Wufeng, Taichung 413, Taiwan, R.O.C.

Abstract. JOTO1007 (ethyl 2-[N-p-chlorobenzyl-(2'-methyl)anilino-4-oxo-4,5-dihydrofuran-3-carboxylate) has anticancer effects in human cervical cancer Ca Ski cells. However, its mechanism of action on the cell migration and invasion of human cervical cancer Ca Ski cells is not fully understood. In this study, firstly, the effects of JOTO1007 on the migration and invasion of Ca Ski cells were examined by using matrigel counting. The results showed that JOTO1007 suppressed the migration and invasion of the Ca Ski cells. Secondly, the effect of JOTO1007 on the levels of proteins associated with cell metastasis was examined using Western blotting. The results indicated that JOTO1007 inhibited the levels of son of sevenless homolog 1 (SOS-1), growth factor receptor-bound protein 2 (GRB2), Ras homolog gene family, member A (RhoA), Rho-associated, coiled-coil containing protein kinase 1 (ROCK-1), focal adhesion kinase (FAK), phosphorylated-c-jun (p-c-jun), nuclear factor kappa B (NF-κB) p65, cyclooxygenase-2 (COX-2), extracellular signal-regulated kinases 1/2 (ERK1/2), matrix metalloproteinase-2 (MMP-2), MMP-7 and MMP-9, but promoted the levels of protein kinase C (PKC), phosphoinositide 3-kinases (PI3K), MAP kinase kinase kinase 3 (MEKK3), mitogen-activated protein kinase kinase 7 (MKK7), c-jun and inducible nitric oxide synthases (iNOS), while not affecting Ras, phosphorylated-ERK (p-ERK), p38 and c-jun N-terminal kinase 1/2 (JNK1/2), which finally led to the inhibition of migration and invasion of the Ca Ski cells in vitro. Overall, JOTO1007 inhibited NF-κB which then led to the inhibition of the MMP-2, -7 and -9 expression followed by the inhibition of migration and invasion in the Ca Ski cells.

Cervical cancer is one of the major causes of mortality in female cancer patients in the world. Patients with cervical cancer are often diagnosed at a late stage due to non-existent or inadequate screening. Therefore, improved treatment options for this type of malignancy are necessary (1, 2). Cervical cancer is a human papillomavirus (HPV)-induced disease which is associated with the expression of the viral oncopgenic proteins E6 and E7 that are capable of inactivating p53 and retinoblastoma protein (pRb), thus inducing continuous cell proliferation with the increasing risk of
accumulation of DNA damage and cancer development (3). One of the major methods for curing this disease is vaccination against HPV. However, cancer cell migration and invasion can lead to cancer metastasis, and therefore, it is very important to have better post-exposure treatment options.

Although ethyl 2-\{N-p-chlorobenzyl-(2’-methyl)anilinono-4-oxo-4,5-dihydrofuran-3-carboxylate (JOTO1007 / Figure 1) has been demonstrated to induce cytotoxicity in WEHI-3 murine leukemia cells (4) and to induce apoptosis via a mitochondria-dependent pathway closely related to the level of cytoplasmic Ca\(^{2+}\) in human cervical cancer Ca Ski cells (5), there is no available information to address whether or not JOTO1007 affects the migration and invasion of Ca Ski cells. Therefore, the focus of this study was the molecular mechanism of JOTO1007 in the migration and invasion of human cervical cancer Ca Ski cells.

Materials and Methods

**Chemicals and reagents.** The compound JOTO1007 was synthesized in our laboratory as described previously (4) and dissolved in dimethyl sulfoxide (DMSO). DMSO, propidium iodide (PI), RNase, trypsin blue and Triton X-100 were obtained from Sigma (Sigma, St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). Polycarbonate filters (8 \(\mu\)m pores) were obtained from Millipore, Co. (Billerica, MA, USA).

**Human cervical epidermoid carcinoma cell line (Ca Ski).** The Ca Ski cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75 cm\(^2\) tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS containing 1% penicillin-streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin) with 1% glutamine and grown at 37°C under humidified 5% CO\(_2\) and 95% air. The cells were cultured for several generations and checked for viability at each generation (4, 5).

**In vitro migration assay.** The chemotactic directional migration was evaluated using a 24-well Transwell insert as described previously (6, 7). Firstly, the filters were coated with 30 μg type I collagen (Millipore, MA, USA) for 1 h. Secondly, the Ca Ski cells (10\(^4\) cells/0.4 ml RPMI-1640 medium) were plated onto the upper chamber of 24-well plates with DMSO (1 μl) or JOTO1007 (50 or 100 μM) and allowed to undergo migration for 24/48 h. Third, the non-migrated cells remaining in the upper chamber were removed with a cotton swab. Finally, the cells were stained with 0.1% crystal violet and photographed under a light microscope at \(\times 200\). Each treatment was assayed in duplicate in three independent experiments (7, 8).

**In vitro invasion assay.** The in vitro invasion assay was carried out by the method of Huang et al. (6). Briefly, at first, the 24-well Transwell filter inserts were each coated with 30 μg Englebreth-Holm-Swarm sarcoma tumor extract (EHS Matrigel Basement Membrane Matrix, BD, San Jose, CA USA) and incubated at 25°C for 1 h to form a genuine reconstituted basement membrane. Then, the Ca Ski cells (10\(^4\) cells/0.4 ml RPMI-1640) were placed onto the upper compartment of 24-well plates and incubated with DMSO (1 μl) or JOTO1007 (50 or 100 μM) at 37°C for 24/48 h in a humidified atmosphere with 95% air and 5% CO\(_2\). The cells on the upper and lower surfaces of the filter were then fixed with 4% formaldehyde in PBS and stained with 2% crystal violet. The cells on the upper surface of the filter were removed by wiping with a cotton swab, and the cells that had penetrated through the matrigel to the lower surface of the filter were counted and photographed under a light microscope at \(\times 200\). Each treatment was assayed in duplicate in three independent experiments (7, 8).

**Preparation of cell protein extracts and Western blotting analysis.** About 1×10\(^6\) Ca Ski cells/well were plated onto 6-well tissue culture plates and incubated for 24 h. JOTO1007 was added to the wells at a final concentration of 100 μM followed by incubation 0, 6, 12, 24 or 48 h at 37°C. DMSO (1 μl) alone was added to the control group. After incubation the cells were collected by ultracentrifugation, followed by centrifugation at 13,000 g for 10 min at 4°C to remove cell debris and the supernatants from each sample were collected for determination of protein concentrations using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. Each sample was analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (8) in order to examine the effect of JOTO1007 on son of sevenless homolog 1 (SOS-1), Ras, growth factor receptor-bound protein 2 (GRB2), Ras homolog gene family, member A (RhoA), Rho-associated, coiled-coil containing protein kinase 1 (ROCK-1), protein kinase C (PKC), phosphoinositide 3-kinases (PI3K), MAP kinase kinase kinase 3 (MEKK3) as described previously (8) in order to examine the effect of JOTO1007 on son of sevenless homolog 1 (SOS-1), Ras, growth factor receptor-bound protein 2 (GRB2), Ras homolog gene family, member A (RhoA), Rho-associated, coiled-coil containing protein kinase 1 (ROCK-1), protein kinase C (PKC), phosphoinositide 3-kinases (PI3K), MAP kinase kinase kinase 3 (MEKK3), and 4-phosphatidyl inositol-3 kinase (PI3K).
Statistical analysis. All data are presented as mean±S.D of three experiments. Statistical differences were evaluated using the Student’s t-test and considered significant at *p<0.05.

Results

Effect of JOTO1007 on the migration of Ca Ski cells in vitro. The underside of the collagen type I coated filters was observed and photographed under a light microscope. Representative photographs of the collagen type I are presented in Figure 2A and indicated that fewer cells were present in the JOTO1007 treated groups. The number of migrated cells are shown in Figure 2B and indicated that JOTO1007 inhibited the migration of the Ca Ski cells by
38.4% or 72.6% after 24 h treatment with 50 or 100 μM JOTO1007 and by 37.5% or 73.4% after 48 h treatment with 50 or 100 μM JOTO1007. The effects compared to the control were dose- and time-dependent.

**Figure 3.** Effects of JOTO1007 on the invasion of Ca Ski cells in vitro. Invading cells in representative lower transwell chambers after treatment with JOTO1007 (50 or 100 μM) ×200 (A). Mean number of invading cells from three independent experiments (B). *p<0.05, significant difference between JOTO1007-treated groups and the control.

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**Effect of JOTO1007 on the invasion of Ca Ski cells in vitro.** Invading cells in the matrigel control filters are shown in Figure 3A and indicated that fewer cells invaded to the lower chamber in the JOTO1007 treated groups. The quantification of the cells in the lower chambers shown in Figure 3B indicated that JOTO1007 inhibited the invasion of the Ca Ski cells by 54.1% or 66.7% after 24 h treatment with 50 or 100 μM JOTO1007 and form 42.8% to 71.4% after 48 h treatment with 50 or 100 μM JOTO1006. The effects were dose- and time-dependent.
Figure 4. Western blotting of proteins associated with invasion and migration after incubation with 100 μM JOTO1007. Veh: vehicle (1 μl of DMSO) alone.
Effect of JOTO1007 on the protein levels associated with migration and invasion. The results from Western blotting are shown in Figure 4 and indicated that JOTO1007 inhibited the expression of SOS-1 and GRB2 (Figure 4A), RhoA and ROCK-1 (Figure 4B), MEKK3 and MKK7 (Figure 4C), FAK and p-c-jun (Figure 4D), NF-κB p65 and COX2 (Figure 4E), JNK1/2, ERK1/2 and p38 (Figure 4F), and MMP-2 and MMP-9 (Figure 4G), but increased the levels of PKC and PI3K (Figure 4C), c-jun (Figure 4D) and iNOS (Figure 4E). However, there was no effect on Ras, p-ERK, MMP-7.

Discussion

Cell migration involves several growth factors through the binding to receptors on the cell surface and the stimulation of downstream signaling pathways resulting in cytoskeletal reorganization and stimulation of the motility machinery of the cell (11). This cellular process provides a variety of molecular targets for the development of therapeutic agents inhibiting cancer invasion and metastasis (12, 13).

In the present study, the results indicated that JOTO1007 inhibited the migration and invasion of Ca Ski cells in a time-dependent manner (Figures 2 and 3). Western blotting showed that JOTO1007 decreased the levels of NF-κB p65 and MMP-2, -7 and -9 (Figure 4), and decreased the protein levels of FAK, ROCK-1 and Rho A (Figure 4), which are also involved in cell migration. NF-κB lies dormant in the cytoplasm via the binding of I-kappa B (IκB) inhibitory proteins (14, 15), however, after the dissociation of inactive NF-κB/IκB complexes by stimulating cytokines, NF-κB can enter the nucleus and bind to cis-acting κB sites in the promoters and enhancers of key cellular genes.

It is well known that MMP expression plays an important role in the invasion of malignant cancer cells into the surrounding and far distant normal tissue and the inhibition of MMP transcription may be a useful strategy for controlling the MMP activity even in the early tumor stages (16-18). Other investigators have also demonstrated that increased levels of MMP-2 or MMP-9 correlate with the invasive properties of several tumor cells. In this study, JOTO1007 reduced the levels of ERK1/2 in the Ca Ski cells. It has been reported that ERK1/2 are members of the mitogen-activated protein kinase (MAPK) family, which are the key factors of transmitting cell proliferation signals (19). ERK1/2 are also known to be important for the activation of NF-κB (20). NF-κB is critically involved in the regulation
of tumor cell proliferation, apoptosis and oncogenesis (21). Therefore, the present results suggested that inhibition by JOTO1007 of migration and invasion in Ca Ski cells may involve both ERK1/2 MAPK and NF-κB signaling pathways.

Taken together, these findings suggested that JOTO1007 may inhibit the invasion and migration in Ca Ski cells through inhibition of NF-κB leading to down-regulation of MMP-2 and MMP-9 levels. The possible pathways of JOTO1007 inhibition of migration and invasion are shown in Figure 5. JOTO1007 may be a novel anti-metastatic agent and it maybe considered for further investigation in vivo.

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References


