

Full-length article

Effect of 7-hydroxystaurosporine on glioblastoma cell invasion and migration¹

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Key words

7-hydroxystaurosporine; protein kinase C; cell movement; cadherins; BRCA1 protein

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Abstract

Aim: To investigate the effect of 7-hydroxystaurosporine (UCN-01), a selective protein kinase C (PKC) inhibitor, on cell growth, migration, and invasion in invasive human glioblastoma U-87MG cells. **Methods:** PKC activity was determined based on the PKC-catalyzed transfer of the ³²P-phosphate group from [g-³²P]ATP into a PKC-specific peptide substrate. Cell viability was measured by MTT assay. Cell invasion and migration were evaluated by a Boyden chamber assay and scratch wound assay, respectively. Protein expression was analyzed using Western blot assay. The formation of 3-dimensional cellular aggregates was examined by a cell-cell aggregation assay. **Results:** UCN-01 treatment resulted in concentration- and time-dependent inhibition of U-87MG cell growth at higher doses (> 100 nmol/L), and reduced cell invasion and migration capability at less cytotoxic doses (<100 nmol/L). UCN-01 significantly repressed PKC activity. Consistent with this result, UCN-01 blocked cell invasion stimulated by phorbol 12-myristate-13-acetate (PMA) and ethanol (EtOH), 2 PKC activators. Enforced expression of the tumor suppressor genes BRCA1 and PTEN increased the anti-invasion potential of UCN-01. Exposure to UCN-01 caused a dose-dependent increase in cell adhesion molecule E-cadherin. The effect of UCN-01 on the formation of cell-cell aggregation was significantly reduced by the addition of an anti-E-cadherin antibody. **Conclusion:** UCN-01 inhibits the invasion and migration of human glioma cells. Accordingly, UCN-01 can have potential clinical applications for the treatment of human glioma metastasis.

Introduction

Protein kinase C (PKC) is a family of serine/threonine protein kinases that transduce signals for tumorigenesis, tumor cell invasion, and metastasis, and has thus been newly targeted for use in cancer treatment^[1]. Activation of PKC augments tumor cell metastatic potential, whereas suppression of PKC activity through PKC inhibitors reduces tumor cell invasion and migration^[1,2]. 7-Hydroxystaurosporine (UCN 01) is a selective PKC inhibitor derived from the non-selective protein kinase inhibitor staurosporine^[3]. A number of studies, including some conducted in our own laboratories, have revealed that UCN-01 holds promise for use as a

single agent or in combination with other chemotherapeutic agents, such as camptothecin, 5-fluorouracil, tamoxifen, and ionizing radiation, in inhibiting tumor cell growth *in vitro* and *in vivo*^[3–9].

Our previous studies corroborate other studies, which indicate that the anti-tumor activities of UCN-01 are associated with arrest of cell cycle progression, including G1/S and/or G2/M^[4,5], apoptosis induction^[5–7,10], and the inhibition of DNA repair^[11,12]. Most notably, UCN-01 abrogates the S or G2 arrest caused by chemotherapeutic agents^[4]. It has also been found that UCN-01 inhibits microvessel formation (angiogenesis), which is required for tumor formation and growth^[13,14]. Taken together, these studies indicate

that UCN-01 is a profound anti-tumor agent in several different tumors, including glioma. Moreover, UCN-01 has entered Phase I trials as a single agent in the USA and Japan^[3,15].

In the present study, the effects of UCN-01 on cell growth, invasion, and migration were investigated in U-87MG, an invasive human glioblastoma cell line. The purpose of this study is to explore whether UCN-01 can be used as a new therapeutic agent in the treatment of glioblastoma growth and invasion.

Materials and methods

Cell culture and chemicals The human glioblastoma cell line U-87MG was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained as monolayer cultures in D-MEM supplemented with 10% fetal calf serum (FCS), glutamine 2 mmol/L, streptomycin 100 mg/L, and 100 kU/L benzylpenicillin G (BioWhittaker, Walkersville, MD, USA). Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue saline solution and examining the cells using a haemocytometer. The doubling time of U-87MG cell was approximately 30 h under our culture conditions. UCN-01 was obtained from the Laboratory of Molecular Pharmacology, Division of Basic Science, NCI, NIH (Bethesda, MD, USA) and stored at -20 °C as a 10 mmol/L stock solution in 20% Me₂SO. Phorbol 12-myristate-13-acetate (PMA) and ethanol (EtOH) were purchased from Sigma Chemical Company (St Louis, MO, USA). PMA was dissolved in 20% Me₂SO and frozen at -20 °C for storage purposes. UCN-01, PMA, and EtOH were further diluted in 2% medium prior to use.

Expression vectors and transfection A full-length BRCA1 or PTEN cDNA was expressed in a pCMV-Tag2B vector (Stratagene, La Jolla, CA, USA), which allows for the expression of proteins with an N-terminal FLAG sequence, as described previously^[16]. Transfection of BRCA1 or PTEN was performed using a transfection reagent, Lipofect-Amin 2000, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD, USA) as described previously^[16]. The efficiency of gene transfer was determined by a β -galactosidase assay using co-transfection with plasmid pRSV- β -gal.

Measurement of PKC activity Aliquots of 4×10^6 untreated and treated cells were washed with ice-cold Dulbecco's phosphate-buffered saline (PBS), suspended in a homogenization buffer (0.05 mol/L Tris-HCl, pH 7.5, containing EDTA 5 mmol/L, EGTA 10 mmol/L, β -mercaptoethanol 0.6 g/L, leupeptin 10 mmol/L, PMSF 1 mmol/L), and broken

by probe sonication. The nuclei and unbroken cells were removed using low-speed spin, and the resulting supernatant was spun at $100\,000 \times g$ for 30 min. The nuclear pellet was resuspended in HB and was referred to as a whole cell lysate. The $100\,000 \times g$ supernatant was termed the soluble or cytosolic fraction. The $100\,000 \times g$ pellet was washed with homogenization buffer, suspended by probe sonication in homogenization buffer containing 0.1% Triton X-100, incubated on ice for 1 h, and spun again at $100\,000 \times g$. The resulting supernatant was termed the particulate or membrane fraction.

Total PKC activity in the three fractions was estimated using a commercially available assay system (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's instructions. This assay is based on the PKC-catalyzed transfer of the ³²P-phosphate group from [γ -³²P] ATP into a PKC-specific peptide substrate (amino acids 65-658 of the EGF receptor with the phosphorylation site on Thr-654) in the presence of Ca²⁺ phosphatidylserine, and phorbol 12-myristate 13-acetate. All 3 fractions were diluted with 0.05 mol/L Tris-HCl (pH 7.5), and 25 μ g of each were added to equal volumes of reaction solution (consisting of Tris-HCl 0.05 mol/L, CaCl₂ 1.5 mmol/L, dithiothreitol 7.5 mmol/L, PKC peptide substrate 45 mmol/L, and dioleoyl-glycerol 82 mmol/L) as provided by the manufacturer. Reactions were carried out at 37 °C for 15 min. After 15 min, the reactions were stopped using a stop reagent (provided by the manufacturer); one hundred microlitre of each were blotted on glass fiber filters and extensively washed with 0.1% orthophosphoric acid. The bound radioactivity was counted using liquid scintillation spectroscopy. The protein content in each of the 3 fractions was measured. Radioactivity values resulting from the phosphorylation of endogenous substrates were subtracted from all determinations. Reactions were performed in triplicate and the values were averaged. Each assay was performed at least 3 times.

Measurement of cell viability Cell viability, an indicator of cytotoxicity, was evaluated using an MTT assay as described previously^[17]. Four sets of experiments were performed in 10 wells for each treatment.

In vitro invasion assay *In vitro* invasion assay was performed with a modified Boyden chamber^[17]. The surfaces of filter (0.8 μ m pore size) were coated with 25 μ g Matrigel of uniform thickness for 1 h at room temperature. The uniformity of the coating was checked by Coomassie blue staining and low-power microscope observation. The lower chamber was filled with 10% FCS medium containing fibronectin (16 μ g per chamber) as the chemoattractant. Cells (1×10^8 cells/L) re-suspended in the medium containing 2% FCS and 50 or

to UCN-01 resulted in a loss of cell viability in a concentration- and time-dependent manner. ID_{50} , a dose at which 50% of the cells lost their viability, was approximately 400 nmol/L and 260 nmol/L after the 24-h and 48-h treatments, respectively ($P < 0.01$). At UCN-01 < 100 nmol/L doses, no significant effect on cell viability was observed at any exposure time points ($P > 0.01$). UCN-01 doses of < 100 nmol/L were thus selected for use in further studies. We also determine the cytotoxicity of UCN-01.

Effect of UCN-01 on PKC activity of U-87MG cells

Subconfluent cells were left untreated or treated with 100 nmol/L UCN-01 for 4 h and then harvested for assay of PKC activity. The membranous fractions (404 ± 23 pmol ^{32}P /min,

$P < 0.01$) and cytosolic fractions (114 ± 46 pmol ^{32}P /min, $P < 0.01$) were significantly inhibited in the cells treated with UCN-01 compared to the untreated control cells (885 ± 42 pmol ^{32}P /min for the membranous fractions, 289 ± 65 pmol ^{32}P /min for the cytosolic fractions). Similarly, reduced PKC activity was also observed after treatment with 50 nmol/L UCN-01 (data not shown).

Effect of UCN-01 on invasion and motility of U-87MG cells

Cell invasion and migration are crucial processes in tumor metastasis. A modified Boyden chamber assay was performed in order to determine the ability of U-87MG cells to invade through biological matrices *in vitro*, based on the percentage of cells able to penetrate the reconstituted base-

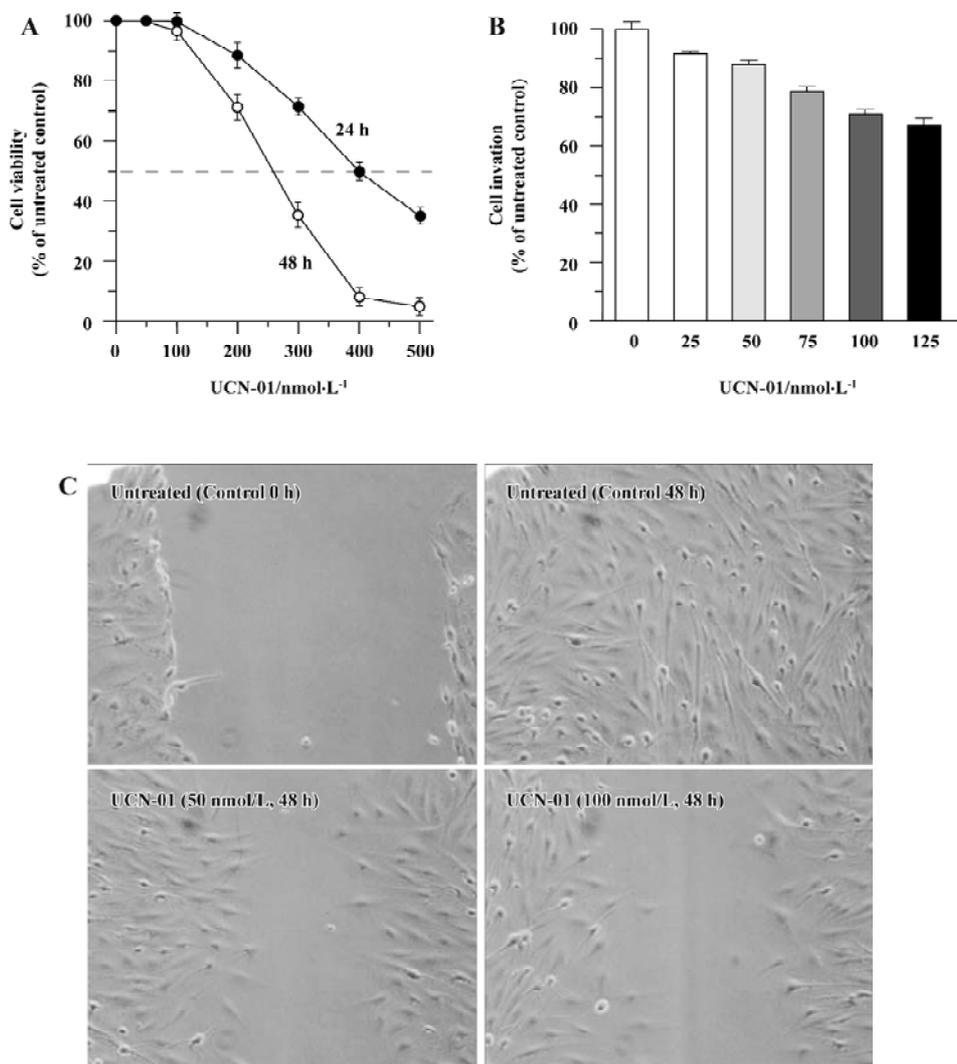


Figure 1. Effect of UCN-01 on U-87MG cell viability, invasion, and migration. (A) MTT assays of cell viability. Mean \pm SEM. $n = 3$. (B) *In vitro* invasion assay. Data (mean \pm SEM) are expressed as the percentage of the control response from 3 independent experiment assays that yielded similar results. (C) Scratch wound assay

ment membrane-coated filters and attach to the lower surface of filter. As shown in Figure 1B, the invasion capacity of U-87MG cells was markedly inhibited when UCN-01 was present in the upper chamber; a reduction of approximately 12% and 25% ($P < 0.01$) was observed for 50 and 100 nmol/L UCN-01 after 48-h treatment, respectively. A similar inhibition of cell invasion activity by UCN-01 was also observed in U-373, another glioblastoma cell line (data not shown).

To examine whether UCN-01 anti-invasion potential is associated with its ability to suppress cell spreading and migration, the effect of UCN-01 on U-87MG cell motility was also analyzed using a scratch wound assay. As demonstrated by the representative fields shown in Figure 1C, 50 nmol/L UCN-01 markedly inhibited the flattening and spread of both cell lines along the edges of the wound compared to the untreated control cells. When this experiment was repeated using a UCN-01 dosage of 100 nmol/L instead of 50 nmol/L, the anti-migration observed was more significant.

Effect of UCN-01 on PMA- and EtOH-stimulated cell invasion We determined the effect of UCN-01 on the stimulation of cell invasion caused by PMA and EtOH, two PKC activators. U-87MG cells spontaneously penetrated through an artificial basement membrane, and the number of invading cells significantly increased when PMA or EtOH was included in the cell suspension [invasion index 58 ± 4 (PMA), 62 ± 6 (EtOH) versus the untreated control, 46 ± 3 ; $P < 0.01$]. However, both spontaneous invasion and PMA- or EtOH-promoted cell invasion were markedly inhibited by UCN-01 (Figure 2A). The ability of UCN-01 to suppress PMA- and EtOH-promoted cell invasion could not be attributed to increased cytotoxicity, since UCN-01 did not significantly affect the cytotoxicity of PMA or EtOH (Figure 2B).

Effect of BRCA1 and PTEN on anti-invasion activity of UCN-01 To determine the effect of the tumor suppressor genes BRCA1 and PTEN on the anti-invasion potential of UCN-01, we compared the invasion behavior of U-87MG cells that had been transiently transfected with the BRCA1 or PTEN gene in the presence and absence of UCN-01 (100 nmol/L). The full-length BRCA1 or PTEN cDNA was expressed in a pCMV-Tag2B vector, which allows for the expression of proteins with an N-terminal FLAG sequence. Exponentially growing cells (3×10^5 cell/well) in 6-well tissue culture dishes were transfected with either the BRCA1 or PTEN vector (5 μg /well) overnight, washed, and then incubated for 48 h. Overexpression of the BRCA1 or PTEN transgene was confirmed by the determination of BRCA1 or PTEN protein using an anti-FLAG antibody (M2). Significant expression levels of the BRCA1 (220 kDa) and PTEN (67 kDa) proteins were observed in U-87MG cells 48 h after trans-

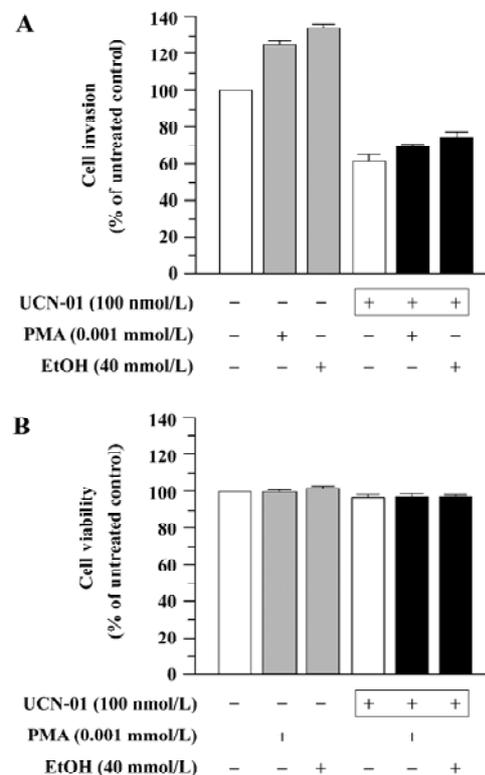


Figure 2. Effect of UCN-01 on PMA- and EtOH-induced promotion of cell invasion. (A) Subconfluent U-87MG cells were trypsinized and counted. Cell viability was also assessed using the MTT assay 48 h after treatment with UCN-01 (100 nmol/L) and/or PMA (1 $\mu\text{mol/L}$) or EtOH (40 mmol/L) in 96-well tissue culture plates (B) Mean \pm SEM. $n = 3$ independent experiments.

fections (Figure 3A). The transfected cells were collected and subjected to the *in vitro* invasion assay. As shown in Figure 3B, the transfection with the “empty” pCMV-Tag2B control vector had no effect on UCN-01 modulation of cell invasion, whereas overexpression of the BRCA1 or PTEN transgene significantly enhanced the anti-invasion potential of UCN-01. BRCA1 or PTEN transfection alone caused a 20% reduction in cell invasion; 100 nmol/L UCN-01 inhibited 30% of cell invasion. However, UCN-01 caused an 87% and a 90% inhibition in invasion in cells transfected with BRCA1 and PTEN, respectively. These results suggest that a combination of UCN-01 and BRCA1 or PTEN transgene synergistically inhibits the invasive behavior of glioblastoma cells.

UCN-01 increases E-cadherin expression To explore the mechanism(s) underlying the effect of UCN-01 on U-87MG cell invasiveness and migration, we analyzed the expression of E-cadherin protein, an adhesion molecule, by an immunoblot assay, following treatment with UCN-01. As illustrated in Figure 4A, U-87MG cells contained a low basal

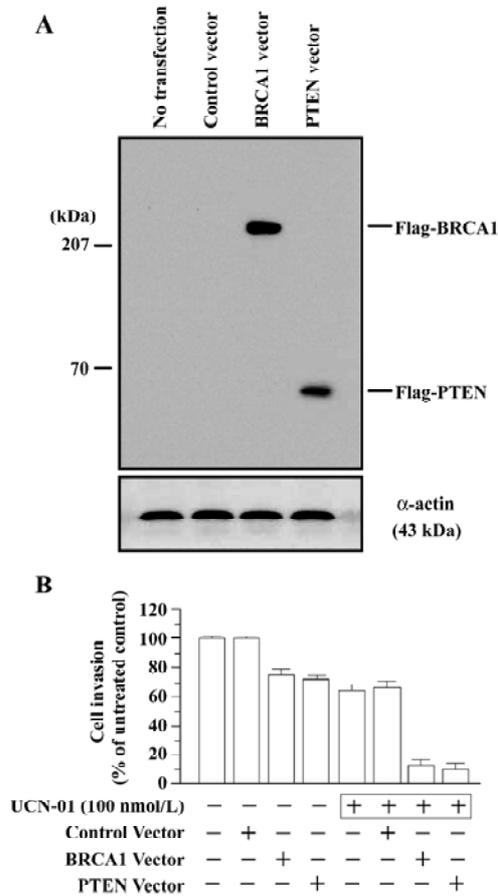


Figure 3. Effect of BRCA1 or PTEN on anti-invasion potential of UCN-01. Cells harvested and subjected to the immunoblot assay (A) and the *in vitro* invasion assay (B) 48 h after transfections. A representative set of experiments from 3 individual transfections is shown.

level of the endogenous E-cadherin protein. Forty-eight hours exposure to UCN-01 resulted in a significant dose-dependent increase. UCN-01(50 and 100 nmol/L resulted in approximate 2.2- and 6.3-fold increases in the E-cadherin protein. Similar results were obtained for E-cadherin mRNA expression in response to UCN-01 treatment (Figure 4C).

Effect of UCN-01 and anti-E-cadherin antibody on formation of cell-cell aggregates To ascertain whether E-cadherin plays an important role in the protection of UCN-01 against the migration and invasion activity of U-87MG cells, we examined the effect of UCN-01 on the formation of cell-cell aggregates. UCN-01 100 nmol/L significantly inhibited the formation of 3-dimensional cellular aggregates (aggregation index=1.3, $P<0.01$) compared to the untreated control U-87MG cells, which showed a significant degree of cell-cell aggregation (aggregation index=0.65). However, in the presence of the E-cadherin antibody, the protective ability of UCN-01 against the formation of cell-cell aggregates was

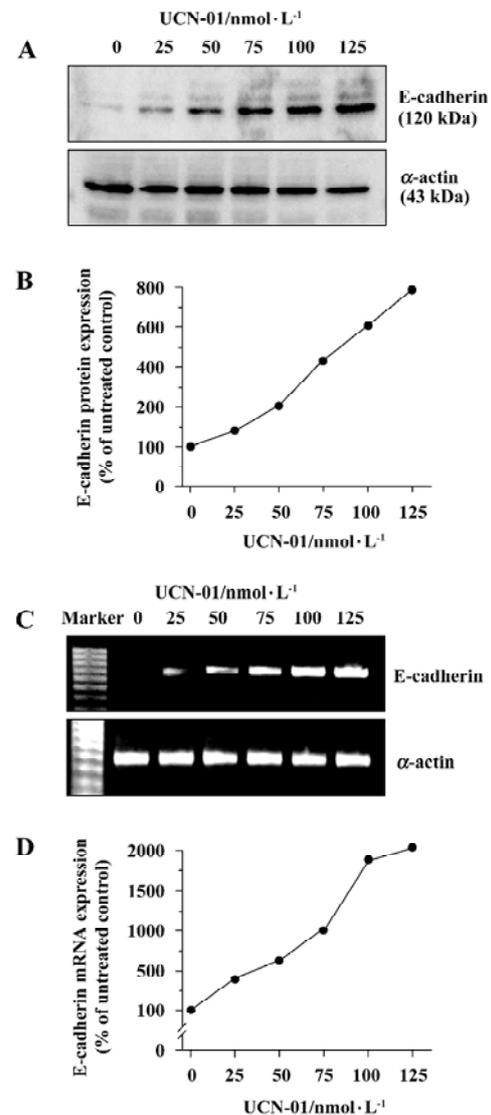


Figure 4. Effect of UCN-01 on E-cadherin expression. Cells were treated with indicated concentration of UCN-01 for 24 h and subjected to Western blotting for protein expression (A) and RT-PCR (C) for mRNA expression. Protein expression (B) and mRNA (D) expression bands were quantitated by densitometry and expressed relative to the α-actin bands and the untreated control band from 2 experiments, respectively. Standard errors were less than 5%.

markedly reduced (aggregation index=0.8). Similar results were observed in another human glioblastoma cell line, U-373 (data not shown). These results suggest that E-cadherin can play a critical role in the anti-invasion potential of UCN-01.

Discussion

PKC is viewed as a new target for suppressing tumor cell

invasion and migration, and therefore metastasis^[1,2]. In the present study we found that in human gliocarcinoma U-87MG cells, UCN-01 inhibits cell proliferation in a concentration- and time-dependent fashion at higher doses (>100 nmol/L). Furthermore, at less cytotoxic doses (<100 nmol/L), UCN-01 reduces the PKC activity and suppresses the cell invasion and migration capabilities. The anti-invasion and anti-migration potential of UCN-01 was confirmed by further experiments in which the PMA- or EtOH-promoted activities of cell invasion were blocked by UCN-01. Both PMA and EtOH are potent activators of PKC^[1,18]. These findings corroborate previous observations that PKC plays a central role in the invasion of glioblastoma-derived cell lines^[19]. Therefore, the profound inhibition of tumor cell invasion and migration are novel features that contribute to the anti-tumor properties of UCN-01.

Although the mechanisms by which suppression of cell invasion and migration by UCN-01 have yet to be discovered, our study has revealed an important finding: despite the inhibition of PKC activity, up-regulated expression of the cell adhesion E-cadherin protein accompanies UCN-01-modulated cell invasion and migration activity. Moreover, a significant reduction of UCN-01 resulted in increased formation of cell-cell aggregates by the E-cadherin antibody. E-cadherin, a transmembrane glycoprotein, is a key mediator of cell-cell adhesion, which acts via the formation of a complex with 3 major cytoplasmic catenins (α , β , and γ), and reportedly plays a key role in controlling the invasive and metastatic progression of a variety of human carcinoma cells, including glioblastoma^[20]. Disruption of the E-cadherin/catenin complex, due primarily to decreased or lost expression of E-cadherin, is shown to be correlated with increased cell proliferation, motility, and invasiveness associated with the progression of tumors^[21]. Moreover, it is also found that tamoxifen, a PKC inhibitor, blocks the invasion and migration of tumor cells by increasing the expression and functions of E-cadherin/catenin complexes^[1]. Therefore, E-cadherin is believed to be a critical mediator of the anti-tumor invasion and migration potential of UCN-01, although further experiments are needed for clarification of the relationship between E-cadherin expression and PKC activity.

In addition, we also found that the enforced expression of two tumor suppressor genes, BRCA1 or PTEN, by a transient transfection assay significantly affects the inhibition potential of UCN-01 on U-87MG glioblastoma cell invasion in a synergistic manner. BRCA1 (Breast Cancer susceptibility gene 1) is located at human chromosome 17q21, and mutations of BRCA1 are known to confer an added risk for breast and ovarian cancers in women, and for prostate

cancer in men^[22]. Increasingly, evidence indicates that invasive breast tumors show decreased BRCA1 mRNA expression and a loss of BRCA1 immunochemical staining relative to non-invasive tumors and benign tissues^[23]. A clinical observation suggests that E-cadherin expression is potentially correlated with BRCA1-associated breast cancer^[24]. Our recent studies also found that indole-3-carbinol, a promising phytochemical produced by cruciferous vegetables, results in up-regulation of BRCA1 and E-cadherin/catenin complex expression, which results in the suppression of tumor invasion and migration^[17]. In addition, we also found that alcohol promotes tumor cell invasion and migration and is associated with down-regulation of BRCA1^[24].

Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) was identified in the 10q23 chromosome region and is often found in mutated forms in a wide range of human malignancies, including glioblastoma^[25]. The restoration of the PTEN gene into U-87MG cells has been found to markedly suppress cell migration and invasion by negative regulation of the signals generated at the focal adhesions, and by the direct dephosphorylation and inhibition of FAK, which is involved in the cell's interactions with extracellular matrix proteins responsible for the migration and enhancement of cell spreading through phosphorylation of its tyrosine^[26]. Loss of PTEN expression and inactivation of wild-type PTEN function can often be observed in advanced or invasive tumors. Accordingly, these findings suggest that both BRCA1 and PTEN can function as inhibitors of tumor invasion and metastasis. Thus, our present findings provide *in vitro* evidence to suggest that a combination of UCN-01 function along with BRCA1 or PTEN gene therapy could be potentially useful in the clinical prevention and treatment of glioblastoma, although it is evident that for this to occur, further studies are required.

In conclusion, we discovered that UCN-01, as a selective PKC inhibitor, suppressed the cell migration and invasion and inhibits the PMA- and EtOH-promoted cell invasion in human glioblastoma U-87MG cells. Moreover, this inhibition of glioblastoma cell invasion and migration by UCN-01 is characterized by an enhanced expression of cell adhesion molecule E-cadherin. Therefore, UCN-01 can be a potent anti-tumor agent in the therapy of glioblastoma, not only via the suppression of proliferation of glioblastoma cells, but also via the inhibition of cell invasion and metastasis. Our results, which support previous studies^[19], also indicate that the PKC signal transduction pathway can play an important role in glioblastoma cell invasion and metastasis, and that the suppression of this pathway may significantly impair the malignant progression of human glioblastoma.

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