Danthron inhibits the migration and invasion of human brain glioblastoma multiforme cells through the inhibition of mRNA expression of focal adhesion kinase, Rho kinases-1 and metalloproteinase-9

CHIN-CHUNG LIN[1,2], JIN-TANG CHEN[1], JAI-SING YANG[3], HSU-FENG LU[8], SHU-CHUN HSU[4], TZU-WEI TAN[3], YUH-TZY LIN[7], YI-SHIH MA[6,9], SIU-WAN IP[4], JIA-JIUAN WU[4], YU-CHINH LI[10] and JING-GUNG CHUNG[5]

[1]Fong-Yuan Hospital, Department of Health, Executive Yuan, Fong Yuan 402; [2]School of Medicine and Nursing, Hungkuang University, Taichung; Departments of [3]Pharmacology, [4]Nutrition and [5]Biological Science and Technology, [6]Graduate Institute of Chinese Medical Science China Medical University, Taichung 404; [7]Department of Clinical Pathology, Cheng Hsin Rehabilitation Medical Center, Taipei 112; [8]Department of Nursing, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli 356; [9]Department of Chinese Medicine, Chang-Hua Hospital, Department of Health, Executive Yuan, Changhua 513; [10]Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Science and Technology, Taichung 406, Taiwan, R.O.C.

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Abstract. In this study, we investigated the effect of danthron on the cell migration and invasion of human brain glioblastoma multiforme GBM 8401 cells *in vitro*. The changes of migration and invasion of GBM 8401 cells after treatment with danthron were detected by cell migration assay and cell invasion assay. The levels of mRNA gene expression associated with cell migration and invasion were detected by real-time PCR. Results indicated that human brain glioblastoma multiforme GBM 8401 cells treated with danthron *in vitro* migrated and invaded less than cells treated with phosphate-buffered saline (PBS) (control). Western blotting showed that danthron inhibited the protein levels of FAK, MMP-7, MMP-9 and uPA in GBM 8401 cells. Real-time PCR assay also showed that danthron inhibited the mRNA expression of matrix metalloproteinase-9 (MMP-9), FAK and ROCK-1 of GBM 8401 cells. These results showed that danthron inhibited invasion and migration of GBM 8401 cells by downregulating mRNA expression associated with these processes, resulting in reduced metastasis. Thus, danthron may be considered a therapeutic agent that can inhibit primary tumor growth and prevent metastasis.

Correspondence to: Dr Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C.
E-mail: jgchung@mail.cmu.edu.tw

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Introduction

Brain cancer is the eighteenth most common malignancy in Taiwan from the report of the People's Health Bureau of Taiwan. The chemotherapy of brain cancer is still unsatisfactory. Some of the cancer patients die not because of the tumor in the original location, but because of the development of metastasis (1). Therefore, it is important to suppress the spread of tumor cells through inhibiting the development of metastasis. Furthermore, the agents to regulate adhesion, invasion and motility of cancer cells may play a significant role in the development of new agents for treating cancer.

Danthron (1,8-dihydroxyanthraquinone), a component from *Rheum palmatum L.* (Polygonaceae) (2), has been shown to have several biological activities, however, the effects of danthron on cell migration and invasion has not been reported. It was reported that in guinea pig large intestine after induction of human melanosis coli and daily oral administration of the danthron led to a transient, dose-related wave of apoptosis of the colonic surface epithelial cells (3). A report also showed that danthron treatment may reduce neurotoxicity related to ß-amyloid protein by both dominant inhibitory effects on membrane lipid peroxidation and glutathione deprivation in primary cortical cultures (4). There are no reports to address danthron ihibited migration and invasion in human brain cancer cells. Therefore, in the present study, we focused on the inhibition of cell migration and invasion by danthron in human brain glioblastoma multi-

forme (GBM 8401) cells and also to find out the possible signaling pathways.

Materials and methods

Chemicals and reagents. The following reagents were purchased from the indicated suppliers. Matrigel invasion chambers were obtained from BD Biosciences (San Jose, CA, USA). Danthron, dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase, Tris-HCl, Triton X-100 and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco-BRL (Grand Island, NY, USA).

Cell culture. GBM 8401 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were maintained in 75-cm^2 tissue culture flasks with RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (100 Units/ml penicillin and 100 μg/ml streptomycin) and 1% glutamine and grown at 37˚C under a humidified 5% CO_2 and 95% air at one atmosphere. Subconfluent cells were passaged with a solution containing 0.25% trypsin and 0.02% EDTA (5).

Migration and invasion assays. Transwell migration assay. GBM 8401 cells were serum deprived in RPMI-1640 supplemented with 1% charcoal-stripped FBS for 24 h. The lower side of the Transwell filter was precoated with 10 μg type IV collagen. The lower chamber of the each well was filled with RPMI-1640 containing 1% charcoal-stripped FBS with supplements as described above. The filter in 6.5-mm Transwell was inserted in 24-well plates then about 2.5x10^4 cells/filter of GBM 8401 cells were placed on the filter then cells were treated with 0, 50 and 100 μM danthron for 24 and 48 h. Migrated cells were stained with hematoxylin and eosin (H&E) then were examined and photographed under a microscope (6,7).

Invasion assay. The same protocols were performed as described in the migration assay except that cells were placed on a matrigel-coated Transwell filter (Matrigel invasion chamber, BD Biosciences) then were examined and photographed under a microscope (6,7).

Western blotting assay. GBM 8401 cells (1x10^6 cells/well) in 6-well plates were treated with 100 μM danthron then incubated for 0, 6, 12, 24 and 48 h, or were treated with 50 and 100 μM danthron for 48 h, and all cells from each treatment were harvested and washed with cold PBS for detecting the changes of protein which correlated with apoptosis. Briefly, about 50 μg protein from each sample was resolved over 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (blot). The blot was soaked in blocking buffer (5% non-fat dry mik/0.05% Tween-20 in 20 mM TBS at pH 7.6) at room temperature for 1 h then were incubated with anti-FAK, anti-MMP-7, anti-MMP-9 and anti-uPA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer at 4˚C for overnight. Then followed by

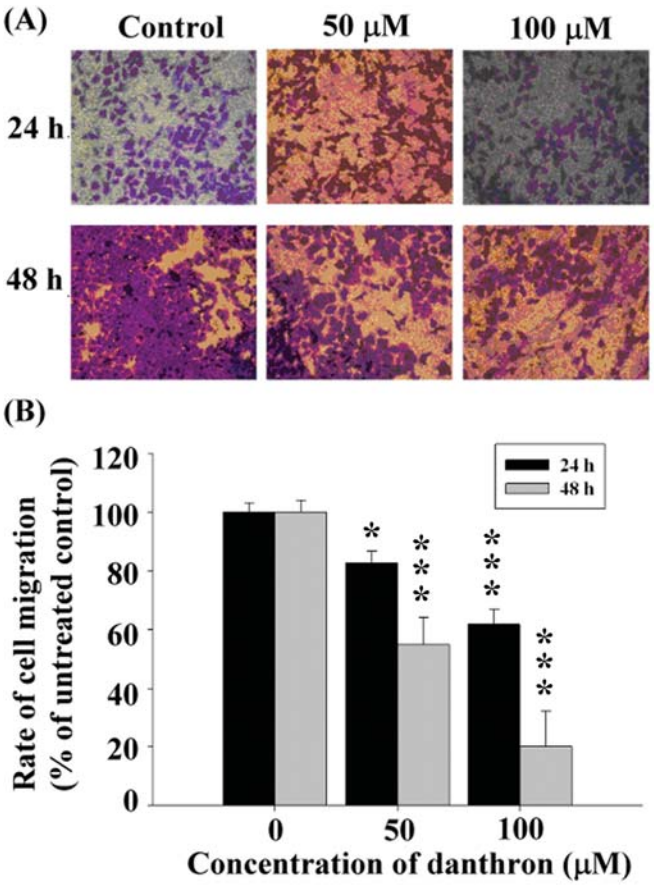

Figure 1. Danthron inhibited the migration of GBM 8401 cells *in vitro*. BGM 8401 cells (1x10^4 cells/well) in 12-well plates were treated with 0, 50 and 100 μM danthron for 24 and 48 h. Cells were placed on the Transwell filter to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at x200 (A). Quantification of cells from the lower chamber, were performed by counting cells at x200 (B). ***P<0.001, significant difference between danthron-treated groups and the control.

secondary antibody horseradish peroxidase conjugate and were detected by chemiluminescence and autoradiography using X-ray film (8,9). For equal protein loading, each membrane was stripped and re-probed with anti-ß-actin antibody (8,9). The relative amounts of specific proteins from each treatment were quantified by densitometry scanning of X-ray films and analyzed by Eagle Eye Image System (Stratagene, La Jolla, CA).

Real-time polymerase chain reaction (PCR). BGM 8401 cells (1x10^6 cells/well) were plated in 6-well tissue culture plates and 100 μM danthron was added to cells for 24 h. Cells from each sample was collected, the total RNA was extracted using the Qiagen RNeasy Mini Kit as described previously (10-12). All RNA samples were reverse-transcribed for 30 min at 42˚C with High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems). Quantitative PCR was performed by the condition: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 sec at 95˚C, 1 min at 60˚C using 1 μl of the cDNA reverse-transcribed as described above, 2X SYBR-Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers: MMP-7-F-G

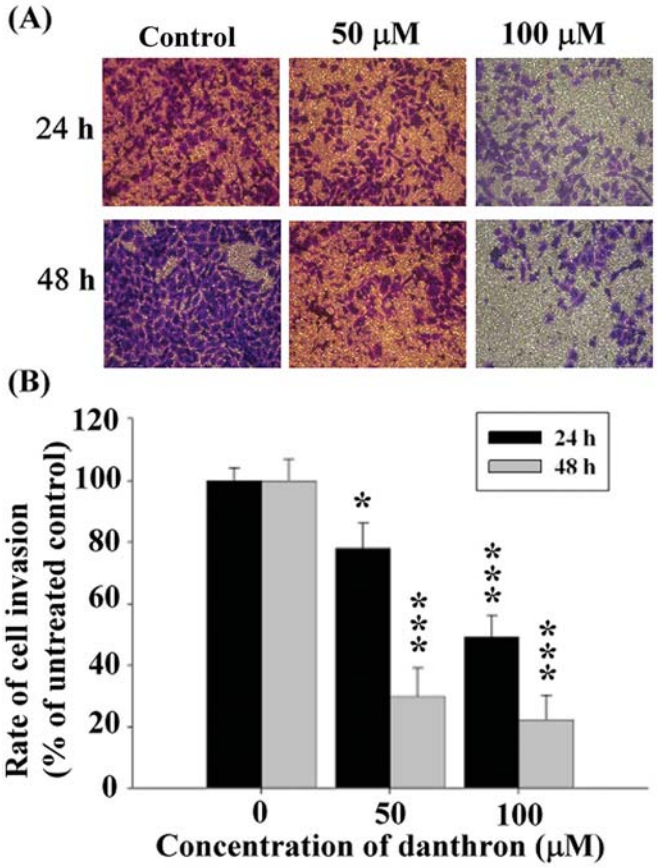

Figure 2. Danthron inhibited the invasion of GBM 8401 cells *in vitro*. BGM 8401 cells ($1x10^4$ cells/well) in 12-well plates were treated with 0, 50 and 100 μM danthron for 24 and 48 h. Cells were placed and cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at x200 (A). Quantification of cells in the lower chamber, were performed by counting cells at x200 (B). ***P<0.001, significant difference between danthron-treated groups and the control.

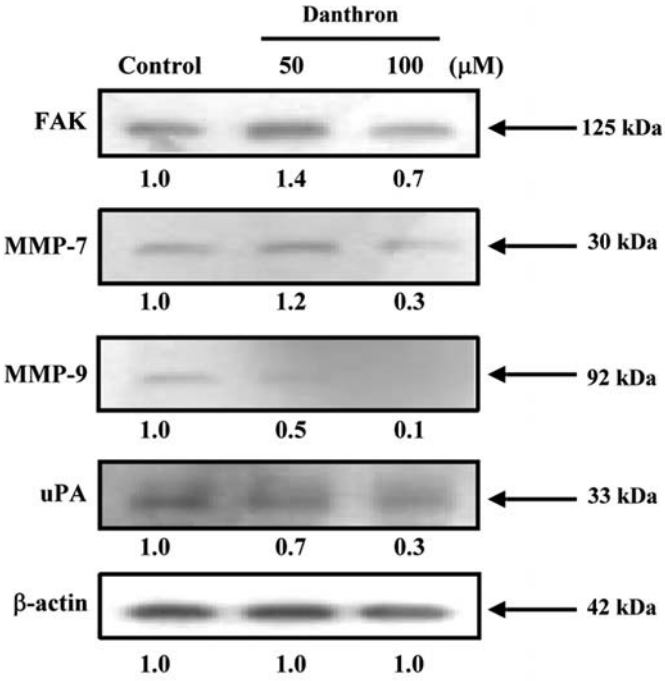

Figure 3. Danthron inhibited the levels of proteins associated with apoptosis in GBM 8401 cells. Cells ($1x10^6$/ml) were treated with 50 and 100 μM danthron for 48 h then the cytosolic fraction and total protein were determined. Evaluations of the associated protein levels were then carried out by Western blotting as described in Materials and methods.

GATGGTAGCAGTCTAGGGATTAACT; MMP-7-R-AG GTTGGATACATCACTGCATTAGG; MMP-9, MMP-9-F-CGCTGGGCTTAGATCATTCC; Rock-1-F-ATGAGTT TATTCCTACACTCTACCACTTTC; R-GTGCCGGATGC CATTCAC Rock-1-R-TAACATGGCATCTTCGACACTC TAG; Fak-F-TGAATGGAACCTCGCAGTCA, Fak-R-TC CGCATGCCTTGCTTTT. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates and expression fold-changes were derived using the comparative C_T method (13).

Statistical analysis. The data are presented as mean ± SD of three experiments. Statistical differences between experimental and control groups were evaluated using the Student's t-test and considered significant at P<0.05.

Results

Danthron inhibits the migration of GBM 8401 cells in vitro. In order to examine whether danthron could inhibit the migration of GBM 8401 cells were examined by using a Transwell migration assay and results are shown in Fig. 1. At high dose (100 μM) of danthron and longer (48 h) incubation time period led to greater inhibition of cell migration in GBM 8401 cells. These effects were dose- and time-dependent.

Danthron inhibited the invasion of GBM 8401 cells in vitro. To examine whether danthron could inhibit the invasion of GBM 8401 cells they were examined by using a Transwell penetrate migration assay and the results are shown in Fig. 2. Fig. 2A shows that GBM 8401 cells in majority invaded from the upper to the lower chamber in the control group, but the penetration of the EHS-coated filter by GBM 8401 cells was inhibited in the presence of danthron. This inhibitory effect was higher at 100 μM danthron than that of 50 μM. The quantification of cells in the lower chamber (Fig. 2B) indicated that danthron significantly inhibited GBM 8401 cell invasion, and these effects were dose- and time-dependent.

Danthron inhibits the levels of proteins associated with migration and invasion in GBM 8401 cells. In order to verify whether danthron inhibited the migration and invasion of GBM 8401 cells are associated with the protein levels, the Western blotting method was conducted. As shown in Fig. 3, the proteins levels of FAK, MMP-7, MMP-9 and uPA were decreased in GBM 8401 cells after treated with danthron. The decreased levels of those proteins correlate with the inhibition of migration and invasion.

Danthron inhibited the levels of mRNA expression in GBM 8401 cells. To confirm that the danthron inhibition of the

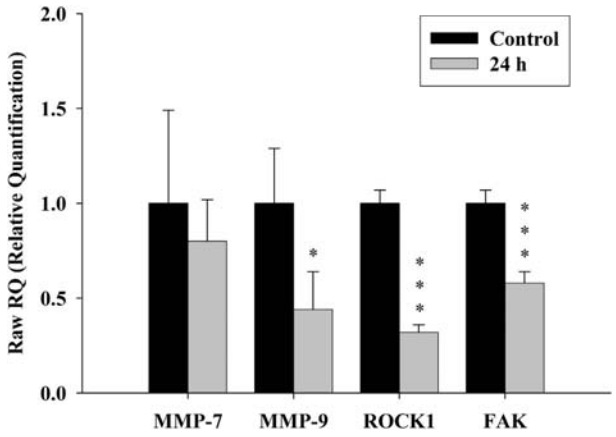

Figure 4. Danthron inhibits the levels of mRNA expression in GBM 8401 cells. Cells were treated with 100 μM danthron for 0 and 24 h then the total mRNA were prepared and were carried out by real-time PCR for examining the mRNA expression levels of MMP-9, ROCK-1 and FAK. Significantly different between danthron and control treatment groups (*P<0.05; **P<0.01; ***P<0.001).

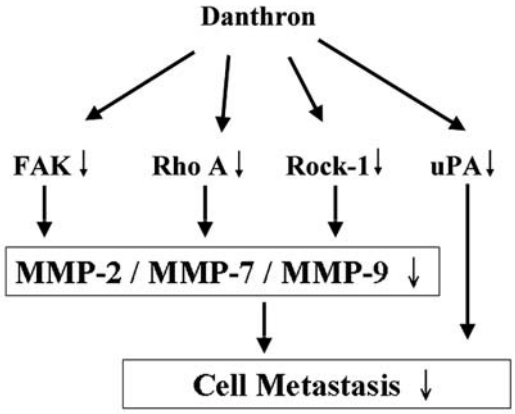

Figure 5. Molecular signaling pathways for danthron-inhibited invasion and migration in human brain glioblastoma multiforme GBM 8401 cells *in vitro*.

migration and invasion of GBM 8401 cells, ultimately result in the alterations of the expression of these genes at the mRNA level, we conducted a series of real-time PCR analyses for examining the levels of MMP-9, ROCK-1 and FAK and the results are shown in Fig. 4 indicating that expression levels of MMP-9, ROCK-1 and FAK mRNA were decreased.

Summary of the molecular signaling pathways for danthron-inhibited invasion and migration in human brain glioblastoma multiforme GBM 8401 cells *in vitro* is shown in Fig. 5.

Discussion

There is no any information regarding to the effects of danthon on the migration and invasion of human cancer cell lines. Therefore, in the present study we investigated whether danthron inhibited migration and invasion of human brain glioblastoma multiforme GBM 8401 cells *in vitro* and the results showed that danthron inhibits migration and invasion of cancer cells. The major feature of cellular invasion mechanisms is via mediation by cell surface receptors which exist in transformed cells, and they are often overexpressed and upregulated. So far the receptor of danthron is unknown. Evidence shows that epidermal growth factor receptor (EGFR) is the most frequently upregulated in tumors (14,15). Tumor invasion requires degradation of basement membranes and proteolysis of extracellular matrix (ECM) (14). Many proteolytic enzymes such as matrix metalloproteinase (MMPs) and serine proteinases are involved in tumor host interactions for degradation of the underlying basement membrane (16,17). MMPs are produced by cancer cells and several reports have shown that elevated expression of MMP appears to be related to the invasion and aggressiveness (18,19). MMP-2 and MMP-9 both are recognized to play an important role in cancer cell invasion which may due to their ability to cleave the type IV collagen (20).

Our results showed that danthron inhibited the migration (Fig. 1) and invasion (Fig. 2) in GBM 8401 cells and those effects are dose- and time-dependent. Furthermore, we also showed that danthron inhibited the expression of mRNA of MMP-9, Rho kinases - ROCK-1 (ROCK-1) and focal adhesion kinase (FAK) in GBM 8401 cells (Fig. 3).

Rho kinase (ROCK) belongs to a family of serine/threonine kinases, mediates some of the downstream signalling of RhoA (21), is the Rho GTPase effector, and is involved in regulation of cell adhesion and migration. ROCK-1 also contributes to pancreatic cancer cell invasion and/or metastasis by facilitating cancer cell migration (22). Another report also showed that ROCK-1 could be of potential therapeutic value in lung cancer (23).

It was reported that FAK is a non-receptor protein tyrosine kinase associated with cell adhesion, cell cycle and migration (24). Evidence indicates that overexpression of FAK is correlated with tumor progression (25). Cell migration involves assembly and disassembly of focal adhesion and FAK is activated mainly in focal adhesion and it plays an important role in cell-ECM interactions that affect cell migration, proliferation and survival (25-27). It was also reported that apigenin inhibited adhesion, migration and invasion of ovarian cancer A2780 cells through attenuated FAK expression and through reducing its protein stability (28). Therefore, FAK may be a therapeutic target for cell migration and invasion because it is a key convergence point for many growth factor pathways of cancer cells.

A major role in the decomposition of basement membranes is also played by 52-kDa uPA. In solid tumors the expression of uPA is increased, the activation of the uPA/uPAR/plasmin proteolytic network also plays key roles in tumor invasion and dissemination of various malignancies (29,30). Thus, we examined the association between danthron and expressions of MMP and uPA in GBM 8401 cells and RT-PCR revealed a marked decrease in the level of MMP-9 mRNA after danthron treatment for 24 h. Western blotting also showed that danthron inhibited the levels of uPA in a dose-dependent manner. These results indicated that danthron suppressed the expression of MMP-9 and uPA in human brain glioblastoma multiforme GBM 8401 cells *in vitro*.

In summary, our study showed that danthron inhibited the levels of MMP-9, ROCK-1, FAK and uPA leading to decreased proliferation, migration and invasion of human brain glioblastoma multiforme GBM 8401 cells *in vitro* (Fig. 4). Thus, these findings indicate that danthron may serve as a therapeutic agent to inhibit migration and invasion in cancer cells of patients, and future study on its clinical application seems to be worthwhile.

Acknowledgements

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