Adhesion and migration of tumor cells are crucial steps in tumor invasion and metastasis. In the present study, we investigated the effects of saponin monomer 13 of dwarf lilyturf tuber (DT-13) on metastasis of human breast cancer cells (MDA-MB-435) during hypoxia. The effects and molecular mechanisms of DT-13 on MDA-MB-435 cells metastatic phenotype in vitro and in vivo were evaluated by RNA interference; quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot assays. DT-13 had no significant effects on cell adhesion and migration under normoxia conditions. Under hypoxic conditions, MDA-MB-435 adhesion to vitronectin was inhibited by about 43.5% or 60.8% after exposure of the cells to DT-13 at 1 μM or 10 μM, respectively. DT-13 decreased the migratory response by hypoxia at 1 or 10 μM, and inhibition ratios were 20% and 30%, respectively. DT-13 inhibited hypoxia-induced expression of αVβ3 integrin, tissue factor (TF) and early growth response gene-1 (Egr-1) and decreased excretion of matrix metalloproteinase 9 (MMP-9) of MDA-MB-435 cells under hypoxic conditions. After Egr-1 short interference RNA (siRNA) treatment, DT-13 could still inhibit the up-regulation of TF mRNA and protein levels and its pro-coagulant activity (PCA) under hypoxia. In nude mice, DT-13 decreased extravasation of MDA-MB-435 cells in the lung after tail vein injection. Our data suggest that DT-13 inhibits MDA-MB-435 cells metastasis during hypoxia via regulation of TF, and the effect of DT-13 on TF is partly mediated by Egr-1.

Key words DT-13; hypoxia; tissue factor; early growth response gene-1; tumor metastasis

Materials and Methods

Cell Lines and Cell Culture Human breast carcinoma MDA-MB-435 cells were cultured as monolayers in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin. To create hypoxic conditions, 70—80% confluent cells were placed in an anaerobic chamber (HERAcell 150, Thermo) with 1% O2 and 5% CO2, at 37 °C.

Cytotoxicity Assays Inhibitory effects were determined using the Cell Counting Kit (CCK-8 kit, Dojindo Laboratories). Briefly, MDA-MB-435 cells were harvested, washed with phosphate buffered solution (PBS) and re-suspended in culture medium at 5×104 cells/well. Twenty-four hours later, cells were treated with DT-13 (provided by Professor Bo-
measured by a microplate reader, and the optical density was assayed in Transwell chambers (8 μm pore size; Corning, Lindfield, NSW, Australia). The surface of the filter membrane was coated with 30 μg of Matrigel (Sigma) for 2 h; 3 × 10° cells/well were seeded into the upper chamber and were allowed to invade toward a serum gradient (2% FCS) in the bottom well for 12 h at 37 °C in 1% O2 or 24% O2. Subsequently, the cells were fixed in 10% buffered formalin, permeabilized in 0.1% Triton-X 100 and stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (Sigma). Cells remaining on the upper side of the insert were removed by wiping with cotton wool, and the membrane was mounted on a glass slide. Adhesion Assays Adhesion assays were performed in 96-well culture plates. Wells were pre-coated overnight at 4 °C with bovine serum albumin (BSA, 2% w/v, Sigma) or vitronectin (10 μg/ml, Sigma). The cells were seeded at 2 × 10^4/100 μl/well in serum-free DMEM supplemented with 0.1% BSA. The cells pre-treated with DT-13 (12 h) were allowed to adhere for 60 min with 1% O2 or 24% O2 at 37 °C. Non-adherent cells were removed by gentle washing with PBS, and adherent cells were measured with the CCK-8 kit.

**Invasion Assays** Human breast cancer cell invasion was assayed in Transwell chambers (8 μm pore size; Corning, Lindfield, NSW, Australia). The surface of the filter membrane was coated with 30 μg of Matrigel (Sigma) for 2 h; 3 × 10° cells/well were seeded into the upper chamber and were allowed to invade toward a serum gradient (2% FCS) in the bottom well for 12 h at 37 °C in 1% O2 or 24% O2. Subsequently, the cells were fixed in 10% buffered formalin, permeabilized in 0.1% Triton-X 100 and stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (Sigma). Cells remaining on the upper side of the insert were removed by wiping with cotton wool, and the membrane was mounted on a glass slide. Cells that had migrated to the underside of the membrane were counted under a fluorescence microscope at 400 × magnification, and the average number of cells in five microscope fields/membrane was determined.

**Enzyme-Linked Immunosorbent Assay (ELISA)** Cells were incubated under hypoxic conditions for 12 h, and then conditioned medium was collected and stored at −80°C until analysis. The matrix metalloproteinase 9 (MMP-9) concentrations were measured at 405 nm in a microplate reader.

**Short Interference RNA (siRNA) Transfection** The chemically-modified siRNA oligonucleotides specific for early growth response gene-1 (Egr-1) were purchased from Ambion (Austin, TX; Genbank accession nos. NM_001964). Transfections (50 nM Egr-1 siRNA) were accomplished using the Silencer siRNA Transfection II kit according to the supplier’s instructions (Ambion). Twenty-four hours after transfection, MDA-MB-435 cells were placed in hypoxia (1% O2) for 12 h.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)** RNA was isolated using TRIzol Solution (Roche) following the manufacturer’s instructions. Primers were designed using the Primer Premier 5 software, and cDNA was prepared using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, U.S.A.) with oligo-dT and random hexamers under standard conditions. The primers used for qRT-PCR of Egr-1, TF, αv, β3 and β-actin were: Egr-1 forward 5′-TGGTTGTTGTCATGCTACA-3′ and reverse 5′-TGGTTGTTGTCATGCTACA-3′; TF forward 5′-GCCAGGAAAGGGAAT-3′ and reverse 5′-CAGTGTAATGAGTCTTTTCCT-3′; αv integrin forward 5′-GCCTCATCCGATGGTCATGCTCACT-3′ and reverse 5′-CTTGAGTCACTCCGATGGTCATGCTCACT-3′; β3 integrin forward 5′-CAGTGTAATGAGTCTTTTCCT-3′ and reverse 5′-CAGTGTAATGAGTCTTTTCCT-3′; and β-actin forward 5′-TACCAGGGCGAAGAG-3′ and reverse 5′-CGTGGGGCTGGTCTTTATG-3′.

**Western Blot Analysis** Cellular protein extraction and Western blot analyses were performed as previously described. Briefly, 40 μg of protein was fractionated on 10—12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Enhanced Chemiluminescence (ECL) nitrocellulose membranes under semi-dry conditions. Immunodetection was performed using the ECL system (Amersham Pharmacia Biotech). Blots were probed with murine anti-human antibodies against Egr-1 (R&D Systems), TF (R&D Systems), αv integrin (Santa Cruz), β3 integrin (Santa Cruz) and actin (Sigma). Horseradish peroxidase (HRP) linked anti-mouse immunoglobulin G (Sigma) was used as a secondary antibody. Immunoreactive proteins on the membrane were visualized by ECL Western blotting detection reagents (Amersham, U.K.).

**Fluorescent Labeling** Cells were incubated for 30 min at 37°C with serum free Opti-Eagle’s Minimum Essential Media (Opti-MEM, Invitrogen) containing the fluorescent cell tracker dye rhodol-based fluorophore (CMRA, red fluorescent) or a 5-chloromethyl-fluorescein diacetate (CMFDA, green fluorescent) at 2 μM (Molecular Probes, Eugene, Oregon). After washing, cells were incubated for an additional 30 min with dye-free medium, washed, and trypsinized.

**Tumor Cell Extravasation in Nude Mice** MDA-MB-435 cells treated with DT-13 (10 μM) for 12 h were labeled in vitro with CMRA (red fluorescent), and normal MDA-MB-435 cells were labeled in vitro with CMFDA (green fluorescent) as described earlier. Cells labeled with the red and
green fluorescent dyes were mixed at a ratio of 1:1, and 2.5×10^6 cells suspended in PBS were introduced by tail vein injection. The ratio of green-to-red fluorescent cells in the injected suspension was measured by counting in a fluorescence microscope. Lungs were harvested 15 min or 7 h after injection, and the tissues were sectioned in a cryostat. The fluorescent cells in five random fields (200× magnification) of each section were counted.15)

**Statistical Analysis**  Quantitative data are expressed as mean±S.D. Comparisons were analyzed by the Student’s *t* test. Significance was defined as *p*<0.05.

**RESULTS**

**Effects of DT-13 on Cell Proliferation in Hypoxia**  Exponentially growing MDA-MB-435 cells were cultured continuously in the absence or presence of different concentrations of DT-13 or HCPT in hypoxia. The effects of DT-13 on cell growth were assessed by the commonly used CCK-8 kit assay at different concentrations (10^-7—10^-4 M). As shown in Fig. 2, DT-13 treatment for 12 h had not significantly inhibited the growth of MDA-MB-435 cells. The inhibitory effect of DT-13 was less than 15% whereas HCPT (10 μM) was 61.41%. The concentrations which did not cause cell death were then employed in subsequent studies.

**The Inhibitory Effect of DT-13 on Tumor Cell Adhesion to Vitronectin**  During the 60 min adhesion assay, few of the cells adhered to the control BSA coated wells. In normoxic conditions, MDA-MB-435 cells adhered only weakly to vitronectin, and DT-13 had no significant effect on cell adhesion (Fig. 3A). As shown in Fig. 3B, MDA-MB-435 cell adhesion to vitronectin increased by 68.8% in hypoxic conditions (*p*<0.01 compared with normoxia). DT-13 treatment decreased the tumor cell adhesion to vitronectin in hypoxia at both concentrations of 1 and 10 μM, and the inhibitory rates were 43.5% (*p*<0.05, compared with hypoxia) and 60.8% (*p*<0.01, compared with hypoxia), respectively.

![Fig. 2. Inhibitory Effect of DT-13 on the Proliferation of MDA-MB-435 Cells in Hypoxia](image)

The results shown were the mean of three parallel experiments (triplicate wells) for each concentration point.

![Fig. 3. Effects of DT-13 on Cell Adhesion](image)

(A) Cells adhesion assay under normoxia. (B) Cells adhesion assay under hypoxia. (C) The expression of αv mRNA assay by qRT-PCR. (D) The expression of β3 mRNA by qRT-PCR. (E) Effect of DT-13 on protein expression of αvβ3 under normoxia. (F) Effect of DT-13 on protein expression of αvβ3 under hypoxia. **p**<0.01 compared with normoxia. ***p***<0.01 compared with hypoxia. *p*<0.05 compared with hypoxia.
Tumor αβ3 integrin contributes to the spontaneous metastasis of breast tumors. Our previous findings showed that hypoxia-induced adhesion to vitronectin was mediated specifically via αβ3 integrin. As shown in Figs. 3C and D, α or β3 mRNA expressions were up-regulated by 737% and 496% under hypoxia, respectively, and DT-13 (1 or 10 μM) treatment of MDA-MB-435 cells significantly inhibited αβ3 mRNA expression. DT-13 at 10 μM inhibited α by 58.2% and β by 48.6%. These results corresponded to those found at the protein level (Fig. 3F), indicating that adhesion to vitronectin was mediated specifically via αβ3 integrin, and DT-13 inhibited hypoxia-induced cell adhesion partially through the αβ3 pathway. DT-13 had no significant effect on the protein expression of αβ3 (Fig. 3E) under normoxia conditions.

Effects of DT-13 on MDA-MB-435 Cell Migration

Site-specific metastasis has been proposed to be regulated, in part, by adhesion to and invasion towards the extracellular matrix (ECM) at metastatic sites. Therefore, to explore the role of DT-13 in these processes, we firstly explored the effect of DT-13 on cell migration under normoxia. A transwell assay was done while cells were cultured during normoxia overnight. DT-13 had no significant effect on cell migration under normoxia conditions (Fig. 4A). As shown in Fig. 4C, the migratory response was more than 48.4% higher with the cells under hypoxia than the normoxic control (p<0.01). When 1 or 10 μM of DT-13 diluted with serum-free medium were placed in the lower chamber of the transwell in which MDA-MB-435 cells were seeded in the upper chamber for 12 h, the migratory response under hypoxia were inhibited by 20% and 30%, respectively, as compared to the control cells cultured under normoxic conditions.

Tumor cells secrete matrix metalloproteinases (MMPs), which degrade ECM and facilitate tumor cell invasion. In our study, DT-13 had no significant effect on MMP-9 secretion under normoxia conditions (Fig. 4B). As shown in Fig. 4D, hypoxia induced MMP-9 secretion in MDA-MB-435 cells and addition of DT-13 (10 μM) significantly attenuated the MMP-9 secretion during hypoxia. These results demonstrated that DT-13 reduced MMP-9 secretion during hypoxia and decreased cell invasion in breast cancer cells.

Effects of DT-13 on TF in Human Breast Cancer Cells under Hypoxic Conditions

DT-13 was shown above to decrease the expression of αβ3 integrin, which is known to be regulated by the cytoplasmic tail of TF. Therefore, we detected the effect of DT-13 on TF. The PCA in cultures of human cancer cells was previously shown to be associated with TF. We confirmed that the human breast carcinoma MDA-MB-435 cells exhibited PCA and demonstrated that it depended on the presence of factors VII and X in the substrate plasma. As shown in Fig. 5A, MDA-MB-435 (1×10⁵ cells/well) expressed TF mildly under normoxic conditions and PCA of TF increased by 40.5% after 12 h of hypoxia. DT-13 treatment decreased hypoxia induced up-regulation of TF activity in a dose-dependent manner, and the inhibition of DT-13 was 40.8% at 1 μM and 87.9% at 10 μM. Hypoxia also resulted in marked up-regulation of TF in metastatic mammary carcinoma cell lines. Expression of TF mRNA in hypoxia was about 75-fold higher than that in normoxia (Fig. 5B). The expressions of TF mRNA were 33% and 68% lower than in hypoxia after DT-13 treatment 1 μM or 10 μM, respectively (Fig. 5B). Meanwhile, we also showed that the DT-13 correspondingly attenuated hypoxia-induced up-regulation of the TF protein (Fig. 5C). These data demonstrated that DT-13 could directly regulate TF expression in cancer cells under hypoxia, but the mechanism remains unknown.

Effects of DT-13 on Egr-1 in Human Breast Cancer Cells under Hypoxic Conditions

Because the binding sequences for the Egr-1 transcription factor are found in three GC-rich regions of the TF promoter that is critical for maintaining basal expression of the gene, it is reasonable to hypothesize that the inhibitory effect of DT-13 on TF may be
related to Egr-1 activity. As shown in Fig. 6A, the expression of Egr-1 mRNA in MDA-MB-435 under hypoxic conditions was increased by 27-fold compared with that under normoxia. DT-13 significantly inhibited hypoxia induced up-regulation of Egr-1 mRNA by 50% or 62% at 1 or 10 μM, respectively. siRNA Egr-1 as a positive control decreased the expression of Egr-1 mRNA by 86%. DT-13 also decreased the protein levels of Egr-1 induced by hypoxia (Fig. 6B) in human breast cancer cells. Taken together, these experiments suggested that the inhibition of cancer cell migration induced by DT-13 was caused, at least in part, by affecting TF through regulation of Egr-1.

**Effects of DT-13 on Egr-1/TF Signaling Pathway under Hypoxic Conditions**

Hypoxia markedly up-regulated TF and Egr-1 in the metastatic mammary carcinoma cell line. Egr-1 siRNA dramatically attenuated but did not fully inhibit this hypoxia-induced up-regulation of TF expression, suggesting that this phenomenon cannot be attributed only to the regulation of Egr-1.

Interestingly, DT-13 could still inhibit the up-regulation of TF mRNA, protein, and PCA levels in hypoxia after Egr-1 siRNA treatment (Fig. 7). These results showed that the DT-13-mediated decrease of TF expression during hypoxia in breast cancer cells was not only due to its inhibitory effect on Egr-1. Taken together, the findings suggest that DT-13 regulates hypoxia-induced expression of TF in human breast cancers through both Egr-1 dependent and independent mechanisms.

**DT-13 Decreases Extravasation of MDA-MB-435 Cells in Vivo**

MDA-MB-435 cells were labeled with fixable fluorescent dyes in order to identify them after intravenous (i.v.) injection in nude mice. Cells were stained with the red fluorescent dye CMRA or the green fluorescent dye CMFDA, each of which is cell permeable and, within cells, becomes entrapped by covalent reaction with cytoplasmic proteins. This approach was used in nude mice to study breast cancer cell extravasation, by CMRA (red fluorescent) labeling of DT-13 treated MDA-MB-435 cells and CMFDA (green fluorescent) labeling of normal MDA-MB-435 cells. Figure 8A showed DT-13 treated cells and normal MDA-MB-435 were mixed at a ratio of 1:1. In the control studies, lungs harvested at 15 min (instead of 7 h) showed relatively few fluorescent cells which mostly cells were adhering to the lung endothelium that have not yet migrated (Fig. 8B). Figure 8C showed DT-13 treated cells than normal MDA-MB-435 cells labeled MDA-MB-435 cells in the lung at 7 h after tail vein injection, and less DT-13 treated cells than normal MDA-MB-435 cells were observed. The data are summarized in

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**Fig. 5. Effects of DT-13 on Hypoxia-Induced Expression of TF**

(A) TF activity assay. (B) The expression of TF mRNA by qRT-PCR. (C) TF protein level assay. **p<0.01 compared with normoxia. *p<0.01 compared with hypoxia. *p<0.05 compared with hypoxia.

**Fig. 6. Effects of DT-13 on Hypoxia-Induced Expression of Egr-1**

(A) The expression of Egr-1 mRNA by qRT-PCR. (B) Western blotting for protein assay of Egr-1. **p<0.01 compared with normoxia. *p<0.01 compared with hypoxia. *p<0.05 compared with hypoxia.
Fig. 8D as cell count ratios per 200× microscope field. The cell count ratio in the lung at 15 min was not significantly different from that in the cell suspension used for tail vein injection. The cell count ratio in the lung at 7 h was significantly higher than that in the cell mixture. These data provide evidence that DT-13 decreased lung extravasation of MDA-MB-435 cells after tail vein injection.

DISCUSSION

DT-13 exerts a wide spectrum of physiological and pharmacological activities.20) The beneficial effects of DT-13 on tumors11) and other systems, such as in the cardiovascular21) and immune systems22) have been extensively investigated. However, studies on the effect of DT-13 on cancer metastases are rare. Here, we first report that DT-13 decreased breast cancer cells metastasis in vivo and in vitro, potentially through the down-regulation of TF expression under hypoxia, and this effect may be derived from its ability to decrease Egr-1 expression. These results suggest that DT-13 could be a potential agent against tumor metastasis, like many other plant-derived natural compounds.23)

Metastatic disease is the primary cause of death for most cancer patients. Complex and redundant pathways involving the tumor cell and the microenvironment mediate tumor invasion at the primary site, survival and arrest in the bloodstream, and progressive outgrowth at a distant site. Previous research reported that tumor αvβ3 integrin contributes to the spontaneous metastasis of breast tumors from the mammary gland to the bone.24) MMPs have been shown to control the processes of tumor invasion and metastasis.25) Platelets facilitated the invasiveness of tumor cells due to improvement of MMP-9 secretion.26,27) Our approach addressed specifically the role of DT-13 on the expression of αvβ3 in MDA-MB-435 cells. During hypoxia, DT-13 reduced both MDA-MB-435 adhesion to vitronectin and the protein expression of αvβ3. Hypoxia induced MMP-9 expression but decreased amounts of MMP-9 secreted in response to DT-13 (10 μM). Therefore, DT-13 may inhibit the invasion of tumor cells by decreasing MMP-9 levels in the microenvironment with
tumor cells. Further in vivo studies showed that DT-13 had anti-tumor effects, specifically on metastasis. Our combined in vivo and in vitro results suggest that DT-13 can inhibit human mammary adenocarcinoma metastasis by inhibiting tumor cells adhesion and invasion.

Our further experiments determined whether DT-13 had any effect on TF in MDA-MB-435 cells under hypoxia. The effect of TF on metastasis may result from the production of growth factors or adhesion proteins.28 The expression of several cell adhesion molecules, notably the integrin αvβ3, has been associated with the metastatic potential of tumor cells.29 TF influences αvβ3 function by regulation of thrombin.30 TF is also a high-affinity transmembrane receptor and co-factor for cellular initiation of the plasma coagulation cascade in vivo. The anti-tumor effects, specifically on metastasis. Our combined in vivo and in vitro results suggested that DT-13 affected hypoxia-induced expression of TF.

In this study, we also examined the effect of DT-13 on Egr-1 in MDA-MB-435 cells under hypoxic conditions. Egr-1 is a zinc finger transcription factor with binding sequences found in three GC-rich regions in the TF promoter.31 The results clearly showed that hypoxia significantly increased expression of Egr-1 proteins, while DT-13 down-regulated the expression of Egr-1, which regulated the activation of the TF pathway. After Egr-1 siRNA treatment, DT-13 could still inhibit TF RNA and protein levels and its activity. These results suggested that DT-13 affected hypoxia-induced expression of TF in MDA-MB-435 cells partly by inhibiting Egr-1 expression and consequently decreasing its binding to the TF promoter.

In conclusion, this is the first report of the inhibitory effects of DT-13 on tumor cell adhesion and migration during hypoxia in breast cancer cells. Our results provide evidence of a possible link between the anti-tumor metastasis effects of DT-13 and TF. Recent encouraging observations of anti-tumor effects of heparinoids34–36 suggest that TF-directed agents could prove to have effective results. Thus, DT-13 may be a component of TCM with potential anti-tumor metastasis activity.

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