The saponin DT-13 inhibits gastric cancer cell migration through down-regulation of CCR5-CCL5 axis

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[ABSTRACT]

AIM: To investigate the effect of DT-13 on gastric cancer cell migration, and to explore the possible mechanisms underlying the anti-metastasis activity of DT-13.

METHODS: Growth inhibition of DT-13 was analyzed by the MTT assay. Cell migration was measured by the scratch-wound assay and transwell double chamber assay. To investigate the possible mechanisms underlying the anti-metastasis activity of DT-13, chemokine receptors that are involved in cancer metastasis (CCR2, CCR5, CCR7, CXCR4, and CXCR6) were detected by conventional PCR. The effect of DT-13 on CCR5 and CXCR4 expression was further evaluated by quantitative PCR and Western blot, respectively. The secretion of CCL5 (ligand of CCR5) and SDF-1 (ligand of CXCR4) were detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: DT-13 inhibited BGC-823 and HGC-27 cell growth in a dose dependent manner, and the estimated IC50 value for 24 h treatment was 23.5 ± 5.1 μmol·L⁻¹ for BGC-823 cells and 35.6 ± 7.6 μmol·L⁻¹ for HGC-27 cells. DT-13 also significantly decreased gastric cancer cell migration. DT-13 significantly decreased the gene expression of CCR5 in both BGC-823 and HGC-27 gastric cancer cells, and moderately reduced the expression of CXCR4. Similar to the results of gene expression, significant down-regulation of CCR5 protein was observed, but CXCR4 protein levels were much less affected. CCL5 secretion, but not SDF-1 production, was inhibited by DT-13.

CONCLUSION: DT-13 inhibited gastric cancer cell migration by down-regulation of the CCR5-CCL5 axis.

[KEY WORDS] Liriope muscari; Saponin DT-13; BGC-823; HGC-27; Gastric cancer cell migration; CCR5; CCL5; CXCR4


Introduction

Cancer metastasis is often depicted as a multistage process in which malignant cells spread to distant organs [1-3]. Although malignant cells can spread to a wide variety of body tissues, tumors usually have a more restricted range of target tissues, e.g., lymph nodes, bone, liver, and lung [4]. It is recently suggested that this tropism is mediated by chemokines, and the tissue-specific expression of these chemokines might guide their cognate receptor-bearing tumor cells to specific destinations [5].

Chemokines are initially described as regulators of leukocyte trafficking for their ability to stimulate migration of leukocytes during inflammatory processes. Chemokines act together with their cell surface receptors to direct these immune cells to specific locations throughout the body [6-7]. Tumors may acquire the ability to subvert the chemokine system. Expressed by metastatic cancer cells, chemokine receptors, such as CXCR4, CCR7, CXCR6, CCR5, and CCR2, turn into important regulators that significantly enhance cancer metastatic potential [8]. CXCR4 is the most commonly over-expressed chemokine receptor in human
survival time of mice under normobaric hypoxia conditions, exerted promising anti-cancer effects. DT-13 can prolong the migration and angiogenesis [20-21]. However, the molecular prevent the hypoxic induction of breast cancer cell [22]. Dwarf lilyturf tuber were isolated and it was found that 25(\(\beta\)-D-glucopyranosyl (1\(\rightarrow\)3)]\(\beta\)-D-frucopyranoside (DT-13), exerted promising anti-cancer effects. DT-13 can prolong the survival time of mice under normobaric hypoxia conditions, inhibit the growth of S180 and ascites tumors [19], and prevent the hypoxic induction of breast cancer cell migration and angiogenesis [20-21]. However, the molecular mechanism of the anti-metastatic effect of DT-13 remains unclear. Here we explored a possible mechanism of the effect of DT-13 on gastric cancer cell migration. It was found that DT-13 significantly inhibited BGC-823 and HGC-27 gastric cancer cell migration in a dose-dependent manner. It was further shown that DT-13 decreased the expression of the CCR5-CCL5 axis, which might account for the anti-metastatic effects of DT-13.

Materials and Methods

Chemicals and reagents

DT-13 (Fig. 1) was kindly provided by Dr. Boyang Yu, China Pharmaceutical University. RPMI-1640 and MEM media were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum was obtained from Gibco (Grand Island, NY, USA). 3-(4, 5-dimethyl-2-thiazol)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was acquired from Ameresco (Solon, OH, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). SYBR Green realtime PCR Master Mix was obtained from TOYOBO (Osaka, Japan). The monoclonal anti-CCR5 and anti-CXCR4 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). The monoclonal anti-\(\beta\)-Actin antibody and horseradish peroxi-
dase-linked anti- mouse IgG were from Sigma. Human CCL5 and SDF-1 ELISA kits were purchased from R&D (Minneapolis, MN, USA).

Cell culture

BGC-823 cells and HGC-27 cells were obtained from The Shanghai Institute of Life Science, Chinese Academy of Sciences. BGC-823 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. HGC-27 cells were maintained in MEM medium (Sigma) with 10% FCS.

Cell proliferation assay

To evaluate the effect of DT-13 on gastric cancer cell proliferation, cell growth was studied using the MTT assay. Briefly, BGC-823 and HGC-27 cells were plated at the density of 3 \(\times\) 10^4 in 96-well plates. After 24 h, cells were treated with different concentrations of DT-13 for 24 h. After treatment, MTT solution (20 \(\mu\)L, 5 mg/mL in PBS) was added to each well, and plates were incubated for 4 h at 37 \(^\circ\)C. The media were carefully removed, and 150 \(\mu\)L DMSO was added for another 15 min. The absorbance was recorded at 570 nm using an automated micro plate reader (Model 550, Bio-rad, Hercules, CA, USA).

Cell migration assay

The cell migration assay was performed using 12 mm diameter transwell double chamber with 12 \(\mu\)m pore size (Costar, Cambridge, MA, USA). Cancer cells were removed from the culture dishes using 0.25% EDTA in PBS and washed twice with physiological PBS. Cells were resuspended in warmed fresh medium, and 3 \(\times\) 10^4 cells/well was seeded into the upper chamber. A solution of 2% FCS was placed in the lower chamber as the chemotactrant. After incubation at 37 \(^\circ\)C in 5% CO_2 for 24 h, nonmigratory cells on the upper membrane were removed with a cotton swab; cells that migrated on the lower surface of the membrane were fixed in 100% ethanol and stained with 1% crystal violet (Sigma) in 0.1 mol L^{-1} borate and 2% ethanol. The number of stained cells in five, randomly selected fields was counted using bright-field microscopy at 40x magnification.

Scratch-wound assay

The scratch-wound assay was performed as previously described [22]. Briefly, BGC-823 and HGC-27 cells were seeded in equal numbers (1 \(\times\) 10^5) into wells of a 24-well plate and allowed to grow overnight. Cells were grown to...
confluency the day next, and the cell monolayer was scraped in straight lines (three lines per well) with a 200 μl pipette tip. Filling of the wound by migrating cells was monitored by photographs taken at different time points. The images were collated and the experiments were performed in triplicate.

**Quantitative and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from cultured cells using TRIzol reagent according to the manufacturer’s instructions, and the amount of RNA was quantified by NanoDrop (Thermo Scientific, Waltham, MA, USA). The first-strand cDNA was synthesized by 2 μg total RNA using the SuperScript-III First Strand cDNA Synthesis kit (Invitrogen). Conventional PCR was performed using GoTaq DNA polymerase (Promega, Madison, WI, USA) and the primers are listed in Table 1.

<table>
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<tr>
<th>Table 1</th>
<th>PCR primers designed in amplification of chemokine receptors</th>
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<tr>
<td>Targets</td>
<td>Primers</td>
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<tr>
<td>CCR2</td>
<td>5′-CATTCGTTCGTTCTCAGCTTATCA-3′</td>
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<tr>
<td></td>
<td>5′-ATTCCCAAGCACCACATCAT-3′</td>
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<tr>
<td>CCR5</td>
<td>5′-AAAATCTCTGGTTGGTCTG-3′</td>
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<td></td>
<td>5′-CAGGCCCCTTGGTGCCG-3′</td>
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<tr>
<td>CCR7</td>
<td>5′-AAGCTATGTCTCTCTTACT-3′</td>
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<tr>
<td></td>
<td>5′-GGGAACGTAGTGAGAAACG-3′</td>
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<tr>
<td>CXCR4</td>
<td>5′-AAACTTCCTCTGGCCACCAT-3′</td>
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<tr>
<td></td>
<td>5′-ACGGCAATAGGACCACTT-3′</td>
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<tr>
<td>CXCR6</td>
<td>5′-ACCTTTCCCTCCTCAT-3′</td>
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<td>5′-GTGCCACCATCAACAACA-3′</td>
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<tr>
<td>GAPDH</td>
<td>5′-ATCCCTACACCATCTTCAG-3′</td>
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<td></td>
<td>5′-GATGTCCTCCAGCTTACAAA-3′</td>
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Quantitative PCR was carried out on an iCycler Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR green as the detection dye. The primers used for quantitative PCR were: CXCR4 forward 5′-ATCTTCTCGCCACCATCATCTTCATCAT-3′; reverse 5′-ATCCAGACGCAACATAGGACACCTT-3′; CCR5 forward 5′-ACGGCATTGGCTCCTAAGCATC-3′; reverse 5′-ACCTTTGCGCCACGAGTAA-3′; 18S forward 5′-GTAAACCCTTGGGACCCATCT-3′; reverse 5′-CCATCTGGAGGTGTGTTG-3′. The amount of target gene mRNA relative to internal control was calculated using the ΔΔCT method: relative gene expression = 2^−ΔΔCT, ΔΔCT = C(Tgt gene) − C(T target gene) − C(T 18S).

**Western blot analysis**

Western blot analysis was performed as described previously [17]. Briefly, adherent cells were washed twice with PBS then lysed in RIPA buffer. Cell lysates were incubated at 4 °C for 15 min, and cellular debris was pelleted by centrifugation at 15 000 × g for 15 min at 4 °C. Total protein was quantitated with the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), using bovine serum albumin (Pierce) as a standard. Equal amounts (50 μg) of protein loaded in each lane of a 12% SDS-PAGE gel followed by transfer to a PVDF membrane (Bio-Rad) on a semi-dry transfer apparatus (Bio-Rad). After blocking with 5% non-fat milk for 1 h at room temperature, the membrane was incubated with antibodies specific for CCR5 or CXCR4 with proper dilutions for 1 h. After washing, horseradish peroxidase-linked anti-mouse IgG was used as a secondary antibody, and incubated with the membrane for 45 min at room temperature. Signal was detected by ECL Western blotting detection reagents (Amersham Biosciences).

**ELISA**

Cells were incubated with serum-deprived medium for 24 h and the supernatants were harvested. Each supernatant was centrifuged at 2 000 × g and stored at −70 °C. Enzyme-linked immunosorbent assay was performed with human CCL5 (or SDF-1) ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the instructions of the manufacturer.

**Statistical Analysis**

Results were expressed as x ± s. Data shown were representatives of three independent experiments. Statistical analysis of the data was performed using the unpaired Student’s t test. P values were two-sided at which a value of 0.05 was considered statistically significant.

**Results**

**Effect of DT-13 on gastric cancer cell proliferation**

To evaluate the effect of DT-13 on the viability of BGC-823 and HGC-27 cells, cell proliferation was investigated using the MTT assay performed with logarithmically growing cells. Cancer cells were cultured in the absence or presence of various concentrations of DT-13 for 24 h. As shown in Fig. 2, DT-13 dose-dependently inhibited the growth of BGC-823 and HGC-27 cells. Linear regression of the data in Fig. 2 allowed the prediction of the IC50 (23.5 ± 5.1 μmol·L−1) of DT-13 for BGC-823 cells. For HGC-27 cells, the IC50 value for 24 h treatment was determined to be 35.6 ± 7.6 μmol·L−1. The concentrations (1, 5, and 10 μmol·L−1) which did not cause significant cell death (≤ 20%) were then employed in subsequent studies.

Fig. 2 Evaluation of cell viability of BGC-823 and HGC-27 cells treated with different concentrations of DT-13 for 24 h. Proportions of survival cells were determined by MTT assay.
Effect of DT-13 on gastric cancer cell migration

The effect of DT-13 on gastric cancer cell migration was explored. The migratory and invasive capacities of gastric cancer cells treated with different concentrations of DT-13 were assessed by in vitro wound scratch and chamber invasion assays. The images from the cell scratch assay showed that the number of migrating cells decreased markedly in the presence of 5 or 10 μmol·L⁻¹ DT-13, as shown in Fig. 3A. In the chamber invasion assay, BGC-823 or HGC-27 cells were allowed to migrate for 24 h, and a great number of cells moved to the lower side of the membrane were observed in the control group. BGC-823 cell invasion rate was inhibited 45% by 5 μmol·L⁻¹ DT-13 and 79% by 10 μmol·L⁻¹ DT-13. For HGC-27 cells, the inhibition rates were 50% and 69%, respectively (Figs. 3B and 3C). Collectively, these data showed that treatment with DT-13 significantly decreased BGC-823 and HGC-27 cell migration in a dose-dependent manner.

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**Fig. 3** DT-13 decreased gastric cancer cell migration. (A) Images of the cell scratch assay were taken 24 h after scratching. Movement of cells resulted in a filling of the surface area of the scar. (B) BGC-823 and HGC-27 cells were cultured with increasing concentrations of DT-13 for 24 h, and representative pictures of migrated cells were shown. Microscopy images showed cells that migrated into the lower chamber. (C) The cell migration was quantified by counting migrated cells in five randomly selected fields 24 h after seeding. Control cells remained untreated. *P < 0.05 and **P < 0.01 vs the control cells, respectively.
DT-13 reduced CCR5 expression in gastric cancer cells

Previous studies from this group demonstrated that malignant cells highly express chemokine receptors and (or) their ligands, which contribute significantly to cancer metastasis [13, 17]. To investigate the possible molecular mechanisms of DT-13, chemokine receptor expression profiles in gastric cancer cells were investigated. BGC-823 and HGC-27 cells were treated with 10 μmol·L⁻¹ DT-13 for 24 h, then total RNA was isolated and subjected to specific primers of CCR2, CCR5, CCR7, CXCR4, and CXCR6. It was shown that CXCR6, which is highly expressed in breast cancer cells, was not detected in BGC-823 gastric cancer cells. The gene expression of CCR2 and CCR7 was unaffected. Treatment with 10 μmol·L⁻¹ DT-13 on BGC-823 and HGC-27 cells significantly decreased CCR5 gene expression, and modestly reduced CXCR4 gene expression (Fig. 4A). The levels of CCR5 and CXCR4 expression after DT-13 treatment were then investigated. As shown in Fig. 4B, BGC-823 and HGC-27 cells, cultured with different concentrations of DT-13, showed greatly decreased CCR5 mRNA expression, as compared with control cells. CXCR4 mRNA levels were much less efficiently down-regulated by DT-13. Similar to the results of gene expression, significant down-regulation of the CCR5 protein was observed. As shown in Fig. 4C, DT-13 was more potent at inhibiting CCR5 expression than CXCR4. Although the CXCR4 protein was much less affected, treatment with 10 μmol·L⁻¹ DT-13 resulted in a 40% reduction of CXCR4 protein levels in BGC-823 cells, indicating that CXCR4 was also responsible for the anti-metastatic effect of DT-13 in BGC-823 cells. Taken together, these results indicated that CCR5 and CXCR4 might be associated with the anti-tumor effect of DT-13.

DT-13 inhibited CCL5 secretion in gastric cancer cells

The predominant ligand of CCR5 is CCL5 (RANTES), and the CCR5-CCL5 axis has been demonstrated to be essential in cancer cell migration [17]. CXCR4 acts through SDF-1 (CXCL12) to stimulate cancer cell metastasis. The effect of DT-13 on the secretion of CCL5 and SDF-1 in BGC-823 and HGC-27 cells was examined. Gastric cancer cells were incubated with different concentrations of DT-13 for 24 h, and supernatants were collected for analysis by enzyme-linked immunosorbent assay (ELISA). As shown in Fig 5A, treatment with increasing concentrations of DT-13 resulted in a decrease of CCL5 production in both cell lines. In contrast, no significant differences in the secretion of SDF-1 were found (Fig. 5B). These data showed that CCL5 was inhibited by DT-13, and indicated that the inhibitory effect of DT-13 on gastric cancer cell migration was likely caused, at least in part, by changes of the CCR5-CCL5 axis.

Discussion

It was reported that DT-13 exerts a wide spectrum of physiological and pharmacological activities, such as anti-inflammatory, anti-tumor, and cardiovascular protective effects. Previous studies indicated that DT-13 inhibits breast cancer MDA-MB-435 cell migration and adhesion under hypoxia [20]. In addition, DT-13 is reported to decrease angiogenesis induced by vascular endothelial growth factor (VEGF) [21]. In this study, it was shown that in normal oxygen conditions, DT-13 decreased the expression of the CCR5-CCL5 axis, which might contribute substantially to the anti-metastatic effect of DT-13 in gastric cancer cells.

The chemokine and chemokine receptor family have important roles in cancer metastasis [23-24]. One of the predominant chemokine receptors that has been demonstrated to be associated with cancer metastasis is CCR5. A previous study underscored the critical importance of the CCR5-CCL5 axis in cancer cell migration [17]. The overexpression of CCR5, or CCL5 administration, stimulated cancer cell migration. Inhibition of CCR5 expression through shRNA knockdown, or neutralization of CCL5, greatly inhibited the metastasis of cancer cells. Furthermore, CCR5 and CCL5 were found to be highly expressed in cancer lymphatic metastases. Given the prominent role of the CCR5-CCL5 interaction in cancer metastasis, the CCR5-CCL5 axis represents a significant therapeutic target, and potential anti-cancer candidates targeting this axis should have great therapeutic potential. The effect of DT-13 on the expression of chemokine receptors was examined, and it was found that DT-13 strongly inhibited CCR5 expression in both BGC-823 and HGC-27 gastric cancer cells. It was further shown that DT-13 significantly reduced CCL5 secretion. These results suggested that the CCR5-CCL5 axis played a crucial part in the anti-metastatic effect of DT-13.

Chemokine receptors belong to the family of seven-transmembrane G-protein-coupled receptors (GPCRs). Binding of chemokines to their cognate receptors activates heterotrimeric G proteins, which in turn triggers diverse signaling cascades that give rise to cell migration. It has been demonstrated that the binding of CCL5 to CCR5 induces the dissociation of heterotrimeric G-proteins into Gα and Gβγ subunits. Free Gαp activates the small G-protein Ras, which in turn activates other core components like PI3K-Akt, MAPK, ERK, FAK, and Rho GTPase [25-27]. CCR5 antagonists are suggested to mediate the activation of p38 (MAPK), ERK ½, and Akt kinases in human hepatic stellate cells (HSCs) [28] and human acute monocyctic leukemia THP-1 cells [29]. Similarly, DT-13 is reported to reduce phosphorylated ERK 1/2 and Akt levels in human umbilical vein endothelial cells [29] and phosphorylated p38 in MDA-MB-435 cells [30]. These results suggest that DT-13 might inhibit the activation of p38 and Akt by down-regulation of the CCR5-CCL5 axis, and indicate that DT-13 might act as an effective inhibitor of CCR5-CCL5 signaling.

Recently, intensive research has indicated that CXCR4 is involved in increasing the metastatic potential of many cancer cell lines, including breast cancer [31], colon cancer [32], gastric cancer [33] and lung cancer [34]. Suppression of CXCR4 expression is found to correlate with the inhibition of gastric cancer cell migration and invasion [35]. CXCR4-
Fig. 4  Effect of DT-13 on the expression of chemokine receptors in HGC-27 and BGC-823 cells. Cells were incubated with different concentrations of DT-13 for 24 h, and the expression of chemokine receptors was analyzed by quantitative PCR, semi-quantitative PCR and western blot. (A) Gene expression of CCR2, CCR5, CCR7, CXCR4, and CXCR6 in gastric cancer cells challenged with 10 μmol·L⁻¹ DT-13. Total cellular RNA was extracted and subjected to RT-PCR using specific primers. (B) Relative gene expression of CXCR4 and CCR5. Total RNA was tested for CXCR4 and CCR5 mRNA levels by quantitative PCR. (C) CXCR4 and CCR5 proteins were detected by Western blot, and relative protein levels were quantified. *P < 0.05, **P < 0.01 vs the control group, respectively
targeting therapy may be applied as a new strategy for the treatment of a broad spectrum of cancers. Unexpectedly, although DT-13 decreased the expression of CCR5 and CXCR4 in gastric cancer cells, the inhibition on CXCR4 was less efficient. Meanwhile, DT-13 inhibited CCL5 secretion in a dose-dependent manner, but had no effect on SDF-1 production. This probably reflected that the concentrations of DT-13 employed in this study was not enough to inhibit the CXCR4- SDF-1 axis, because 10 μmol L⁻¹ DT-13 employed in this study was not enough to inhibit the assay. However, these experiments showed that 10 μmol L⁻¹ DT-13 produced a moderate decrease in CXCR4 protein in HGC-27 and BGC-823 cells, and it was not possible to completely rule out the involvement of CXCR4 in DT-13-induced inhibition on gastric cancer cell migration.

To summarize, it was clearly shown that DT-13 significantly inhibited the migration of gastric cancer cells in vitro. Molecular analysis revealed that DT-13 strongly repressed the expression of CCR5-CCL5 axis, which might play a crucial role in cancer metastasis. Further animal studies are required to confirm the effect of DT-13 on gastric cancer metastasis.

References


