Topotecan inhibits cancer cell migration by down-regulation of chemokine CC motif receptor 7 and matrix metalloproteinases

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Aim: The aim of this study was to investigate the effect of topotecan (TPT) on cancer cell migration.

Methods: Growth inhibition of TPT was analyzed by MTT assay, and cancer cell migration was measured by transwell double chamber assay. To verify the effect of TPT on the chemokine receptors CXCR4 and CCR7, quantitative PCR, semi-quantitative PCR and Western blot analysis were performed. The secretion of MMP-2 and MMP-9 was detected by enzyme-linked immunosorbent assay (ELISA) and gelatin zymography. To evaluate possible contributions of CCR7 to MMP secretion, the overexpression vectors pcDNA3.1+-CCR7 and CCR7 siRNA were transiently transfected into MDA-MB-435 cells.

Results: TPT inhibited cancer cell migration in a dose-dependent manner. Additionally, TPT significantly decreased the expression of CCR7 in both MDA-MB-435 and MDA-MB-231 cells and moderately reduced the expression of CXCR4 in MDA-MB-435 cells. The secretion of MMPs (MMP-2, MMP-9) was also inhibited by TPT. Overexpression of CCR7 increased the secretion of MMP-2/9 and cancer cell migration, whereas knockdown of CCR7 reduced active MMP-2/9 production and migration of MDA-MB-435 cells.

Conclusion: TPT inhibited cancer cell migration by down-regulation of CCR7 and MMPs (MMP-2 and MMP-9).

Keywords: topotecan; MDA-MB-435; MDA-MB-231; cancer metastasis; CXCR4; CCR7; MMP-2; MMP-9

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Introduction

Metastasis is a multistep process responsible for most cancer deaths[1-3]. Although malignant cancers can spread to a wide variety of body tissues, only a few tissues, such as lymph nodes, bone, liver and lung, offer favorable conditions for metastasized cancer cell survival[4]. It has recently been suggested that the chemokine and chemokine receptor family[1] play a significant role in this organ tropism.

Chemokines are a superfamily of chemotactic cytokines of 8- to 14-kDa molecular weight[5], initially described as regulators of leukocyte trafficking to sites of inflammation. During regulation, chemokines bind to their cognate receptors, most of which belong to the G-protein-coupled receptor family, and are expressed in endothelial cells and lymphocytes. Recent studies have revealed that cancer cells can respond to chemokine stimuli through expression of their respective receptors[6], altering cancer cell invasive and metastatic properties. Elevated levels of CXCR4 and CCR7 in breast carcinoma are significantly associated with increased malignancy, advanced disease and poor prognosis[7-11]. In breast cancer, CXCR4 expression increases progressively with increasing malignant potential[10], and CCR7 is associated with lymph node metastasis[11]. In melanoma, the recruitment of CCR7-positive cancer cells by CCL21 also leads to lymph node metastasis[12]. It appears that the normal physiology of CXCR4 and CCR7 has been usurped to promote the specific metastasis of neoplastic cells to distant organs, thereby making them prime targets for therapeutic intervention in the clinic.

Proteolytic enzymes play a fundamental role in cancer progression, providing access for tumor cells to the vascular and lymphatic systems, which support tumor growth and constitute an escape route for further dissemination. Among all of the proteolytic enzymes potentially associated with tumor invasion, MMP-2 and MMP-9 play fundamental roles in that they are responsible for degradation of extracellular matrix components such as collagen and proteoglycans.
Cell migration assay  Cell migration assays were performed using 12 mm diameter transwell double chambers with a 12 μm pore size (Costar, Cambridge, MA). The surface of the membrane was coated with 30 μg of Matrigel (Sigma) for 2 h at room temperature. Cancer cells were removed from the culture dishes using 0.25% EDTA in PBS and washed twice with physiologic PBS. Cells were resuspended in warmed fresh medium, and 3×10⁴ cells/well were seeded into the upper chamber. Different concentrations of TPT were loaded both in the upper and in the lower chambers, and 2% FCS was placed in the lower chamber as the chemoattractant. After incubation at 37 °C and 5% CO₂ 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 borate and 2% ethanol. The number of stained cells in five randomly selected fields was counted using bright-field microscopy at 40x magnification.

Quantitative PCR  Cells (1×10⁴) were seeded onto a 9-cm dish. The relative gene expression was analyzed by quantitative PCR as described previously[22]. Quantification of the amplified product was performed on a cycle-by-cycle basis via the acquisition of a fluorescent signal generated by binding of the fluorophore Sybr Green I (Roche Diagnostics) to double stranded DNA. The primers used for PCR were as follows: CXCR4 forward 5′-ATCTTT-CCTGCCACCATCTACTCATC-3′; reverse 5′-ATCCAGACGCCAACATAGACCCACCTTTCA-3′; CCR7 forward 5′-GTGCCCGTCCTTCTCATCAG-3′; reverse 5′-GGCCAGGCCACCCCATTTTCA-3′; and β-actin as internal control: forward 5′-GAGC-GGGAAATCGTGCGTGACATT-3′; reverse 5′-GAAGGTAGTTTCGTGGGATGCC-3′. Changes in target gene mRNA were calculated using the ∆∆C_T method as follows: target gene mRNA (folder increase)=2−∆∆C_T, ∆C_T (sample)=C_T (target gene)−C_T (β-actin), ∆C_T (calibrator)=C_T (target gene)−C_T (β-actin), ∆ΔC_T=ΔC_T (sample)−ΔC_T (calibrator).

Gel electrophoresis and immunoblotting  Immuno-blotting analysis was performed as described previously[19]. Briefly, adherent cells were washed twice with PBS and 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 quantified with a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL), using bovine serum albumin (Pierce) as a standard. Equal amounts (40 μg) of protein were loaded in each lane of a 12% SDS-PAGE gel, followed by transfer to a PVDF membrane (Bio-Rad, Hercules, CA) on a semidry transfer appa-

Materials and methods  

Cell culture and reagents  MDA-MB-231 cells and MDA-MB-435 cells were obtained from the Shanghai Institute of Life Science, Chinese Academy of Sciences. MDA-MB-231 cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 100 U/mL penicillin and 100 mg/mL streptomycin. MDA-MB-435 cells were maintained in DMEM medium (Sigma) with 10% FCS. Monoclonal anti-CXCR4 and anti-CCR7 antibodies were purchased from Santa Cruz (Santa Cruz, CA). The monoclonal anti-β-actin antibody and horseradish peroxidase-linked anti-mouse IgG were obtained from Sigma. Validated CCR7 siRNA (ID s3217) and negative control siRNA (ID AM4611) were purchased from Ambion (Austin, TX). TPT was kindly provided by HenRui Co Ltd (Lianyungang, China).

Cell proliferation assay  To evaluate the effect of TPT on cancer cell proliferation, cell growth was studied using the MTT assay as described previously[22].

Cell migration assay  Cell migration assays were performed using 12 mm diameter transwell double chambers with a 12 μm pore size (Costar, Cambridge, MA). The surface of the membrane was coated with 30 μg of Matrigel (Sigma) for 2 h at room temperature. Cancer cells were removed from the culture dishes using 0.25% EDTA in PBS and washed twice with physiologic PBS. Cells were resuspended in warmed fresh medium, and 3×10⁴ cells/well were seeded into the upper chamber. Different concentrations of TPT were loaded both in the upper and in the lower chambers, and 2% FCS was placed in the lower chamber as the chemoattractant. After incubation at 37 °C and 5% CO₂ 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 borate and 2% ethanol. The number of stained cells in five randomly selected fields was counted using bright-field microscopy at 40x magnification.

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ratus (Bio-Rad). After being blocked with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with antibodies specific for CXCR4 (Santa Cruz) and CCR7 (Santa Cruz) with proper dilutions for 1 h. After washing, horseradish peroxidase-linked anti-mouse IgG (Sigma) was used as a secondary antibody and then incubated with the membrane for 45 min at room temperature. The signal was detected using ECL Western blotting detection reagents (Amersham Biosciences).

**Plasmids construction and transient transfection**

Full-length cDNA for CCR7 was amplified and cloned into T easy vector. Cloned fragments were recovered and ligated into pcDNA3.1⁺ (Invitrogen, Carlsbad, CA). Transfections were done using Lipofectamine 2000 transfection reagents (Invitrogen) according to manufacturer instructions. Briefly, MDA-MB-435 cells were seeded in 6-multiwell plates and were transfected at 70%–80% of confluence with 2.5 μg of pcDNA3.1⁺-CCR7. After 6 h, reagents were removed and cells were allowed to recover for 18 h. Cells transfected with empty vectors (pcDNA3.1⁺) were used as a control. PCR primers for CCR7 were as follows: forward, 5'-TTTGGA-TCCGCCCAGAGAGCGTCATG-3'; reverse: 5'-GGG-AAGGAGGTGTGTCGCCTATGGGGAGAAG-3'.

**RNA interference**

Validated siRNA molecules targeted against human CCR7 (NM_001838) and a negative control siRNA were purchased from Ambion. Cells were plated the day before the transfection procedure was started. siRNA was transfected at a final concentration of 1, 10, and 50 nmol/L, respectively, according to the manufacturer’s protocol. Twenty-four hours after transfection, reagents were removed and cells were harvested. CCR7 knockdown was confirmed by quantitative PCR and Western blotting.

**ELISA**

Cells were incubated with serum-deprived medium for 24 h and the supernatants were harvested. Each supernatant was centrifuged at 2000×g and stored at -70 °C. Enzyme-linked immunosorbent assays were performed with human MMP-2 and MMP-9 kits (R&D Systems) according to the manufacturer’s instructions.

**Gelatin zymography**

Cells were incubated with serum-free medium for 24 h, and then conditioned medium was collected and stored at -70 °C until analysis. MMP activity was assessed using 10% zymogram gels (Bio-Rad, Richmond, CA). Gels were renatured in buffer containing 2.5% Triton X-100 for 30 min at room temperature. After being washed in developing buffer (100 mmol/L Tris, 5 mmol/L CaCl₂ and 0.04% NaN₃) for 30 min, gels were incubated with developing buffer overnight at 37 °C. Gels were stained with Coomassie brilliant blue R-250, and enzymatic activity was characterized by the presence of clear zones in a background of blue staining.

**Statistical analysis**

Statistical analysis of the data was performed using the unpaired Student’s t test, Mann-Whitney U test and Spearman correlation coefficient analysis. *P* values were two-sided and a value of 0.05 was considered statistically significant.

**Results**

**TPT inhibits the proliferation and migration of cancer cells**

TPT has been reported to induce cell death in human breast cancer cells[17]. To evaluate the effect of TPT on the viability of MDA-MB-231 and MDA-MB-435 cells, cell proliferation was investigated using the MTT assay performed with logarithmically growing cells. As shown in Figure 1A, TPT significantly inhibited the growth of MDA-MB-435 cells and MDA-MB-231 cells. The proliferation of MDA-MB-435 cells challenged with 0.1 μmol/L TPT for 24 h was reduced to 71.9% compared with non-treated cells, whereas the ratio was 78.3% in MDA-MB-231 cells challenged with 1 μmol/L TPT.

We next explored the effect of TPT on cancer cell migration. To address this issue, we performed invasion studies using the classic transwell in vitro assay. Cells in serum-free medium were seeded in the upper chamber and different concentrations of TPT were loaded in both the upper and the lower chambers. As such, 2% FCS was used as the chemoattractant in the lower chamber. As shown in Figure 1B and 1C, TPT significantly inhibited cancer cell migration in both MDA-MB-231 and MDA-MB-435 cells.

**TPT reduces the expression of chemokine receptors**

CXC chemokine receptor 4 (CXCR4) and CC chemokine receptor 7 (CCR7) are membrane proteins that are highly expressed in malignant cancer cells. Cells expressing CXCR4 and CCR7 usually metastasize to organs and tissues and secrete respective chemokines, such as SDF-1 and CCL21[10,11]. Subsequently, we examined the effect of TPT on CXCR4 and CCR7 expression. We found that TPT significantly decreased CCR7 gene expression and modestly reduced CXCR4 gene expression in MDA-MB-435 cells (Figure 2A and 2B). MDA-MB-231 cells treated with TPT showed down-regulation of CCR7 expression; however, CXCR4 expression was unaffected. Similar to the results of gene expression, a significant down-regulation of CCR7 protein was observed in both cell lines, but CXCR4 protein levels were decreased only in MDA-MB-435 cells (Figure 2C). These data probably ruled out a critical role of CXCR4 in TPT-induced inhibition on cancer cell migration and indicated that CCR7 might be associated with the inhibitory
Tumor cells are known to secrete MMPs, which are thought to degrade the extracellular matrix (ECM) and facilitate tumor cell invasion in tissues. Here, we examined the effect of TPT on the secretion of MMP-2 and MMP-9 in MDA-MB-435 and MDA-MB-231 cells. Cells were incubated with different concentrations of TPT for 24 h, after which supernatants were collected for analysis by enzyme-linked immunosorbent assay (ELISA) and gelatin zymography. As shown in Figure 3A, treatment with increasing concentrations of TPT resulted in a decrease in active MMP-9 (86 kDa band) and MMP-2 secretion (72 kDa band) in MDA-MB-435 cells. A significant reduction of active MMP-2 secretion was also observed when MDA-MB-231 cells were treated with 1 μmol/L TPT for 24 h (Figure 3B). However, in contrast to MDA-MB-435 cells, no significant changes of active MMP-9 secretion were detected in MDA-MB-231 cells, as measured by gelatin zymography.
zymography (Figure 3B). Only a slight decrease in optical density values (about 15%) was found when active MMP-9 secretion was analyzed by ELISA, reflecting a weak effect of 1 μmol/L TPT. Our data showed a more potent inhibition by TPT on active MMP-2 secretion. As such, these experiments indicated that TPT inhibited MMPs secretion (especially MMP-2) and therefore impaired ECM degradation.

**Effect of CCR7 on MMPs and cancer cell migration**
Recent studies have demonstrated that the CXCR4/SDF-1 axis induces MMP activity in cancer cells and then pro-
motes cancer cell migration and metastatic potential\textsuperscript{15,16}. However, studies on the relationship between CCR7 and MMPs are limited. To investigate the possible contributions of CCR7 to MMP secretion, we transfected MDA-MB-435 cells with the CCR7 gene. As shown in Figure 4A and 4B, gelatin zymography and ELISAs for MMP-2 and MMP-9 showed strong up-regulation of active MMP-2 and MMP-9 secretion in transfected MDA-MB-435 cells (435/CCR7), but not in cells transfected with empty vector (435/EV). To further explore the role of CCR7 in MMP production, MDA-MB-435 cells were transfected with siRNA specifically designed against CCR7 (siCCR7). Both 10 and 50 nmol/L of the double-strand oligonucleotides significantly inhibited (>80%) CCR7 expression (Figure S1). Silencing of CCR7 with 50 nmol/L siRNA significantly reduced MMP-2 and MMP-9 secretion, as measured by ELISA (Figure 4A) and gelatin zymography (Figure 4B). Overexpression of CCR7 also increased MDA-MB-435 cell migration, and knockdown of CCR7 attenuated cell migration (Figure 4C and 4D). These data demonstrated a critical role of CCR7 in regulat-
ing cancer cell migration as well as MMP-2/9 secretion, and indicated that the inhibitory effect of TPT was likely caused, at least in part, by changes in CCR7.

**Discussion**

TPT has been established as a second line anti-cancer agent for solid tumors (eg, SCLC). Previous clinical reports have highlighted the potential activity of TPT against tumor metastasis\cite{20, 21}. In this study, we showed that TPT decreased cancer cell migration via down-regulation of CCR7 and MMPs (MMP-2 and MMP-9).

The chemokine and chemokine receptor family have important roles in cancer metastasis\cite{23–25}. One of the predominant chemokines that associates with the migration, adhesion, and invasion of cancer cells is SDF-1/CXCR4. CXCR4 is one of the few genes that are up-regulated in bone-metastasized breast cancer cells. Cells that metastasize to lung also highly express CXCR4. CCR7, which mediates the survival and migration of immune cells to lymph nodes, has recently been associated with nodal metastasis of squamous cell carcinomas of the head and neck (SCCHN)\cite{26}. These findings suggest that inhibition of CXCR4 and CCR7 may be a strategy for the prevention of cancer metastasis. We examined the effect of TPT on CXCR4 and CCR7 expression. We found that TPT strongly inhibited CCR7 expression and modestly decreased CXCR4 expression in MDA-MB-435 cells, whereas only CCR7 expression was reduced in MDA-MB-231 cells. We next showed that TPT significantly reduced active MMP secretion. Compared with MMP-2, MMP-9 was less efficiently inhibited. Finally, we demonstrated that overexpression of CCR7 elevated MMP-2/9 secretion and cancer cell migration, whereas knockdown of CCR7 substantially reduced active MMP-2/9 production and migration of MDA-MB-435 cells. These results suggested that CCR7 played a crucial role in TPT-induced inhibition in cancer cell migration.

Cancer cells may express various functional chemokine receptors, and different chemokine receptors play different roles in cancer metastasis. Although TPT decreased the expression of CXCR4 and CCR7 in MDA-MB-435 cells, the inhibition of CXCR4 was less efficient. In MDA-MB-231 cells, TPT reduced CCR7 expression, whereas the expression of CXCR4 was unaffected. These results probably reflected significant differences between CXCR4 and CCR7. Clinical studies\cite{27} indicate that both CXCR4 and CCR7 are predictors of cancer metastasis, patient outcome and overall survival, but multivariate analysis demonstrates that CXCR4 and CCR7 are independent factors, and there are no associations between the two. Furthermore, MDA-MB-435 cells express low levels of CXCR4 and do not invade through Matrigel in the presence of SDF-1\cite{28}. MDA-MB-231 but not MDA-MB-435 cell migration is stimulated by SDF-1alpha\cite{29}. These observations probably indicate that CXCR4/SDF-1 do not substantially contribute to MDA-MB-435 cell migration. Our study also confirmed that CCR7, but not CXCR4, played a critical role in the effect of TPT on cancer cell migration. However, because our experiments showed that 0.1 μmol/L TPT resulted in a moderate decrease in CXCR4 expression in MDA-MB-435 cells, we could not completely rule out the involvement of CXCR4 in TPT-induced inhibition on MDA-MB-435 cells.

Using MDA-MB-435 cells as a model, our results also demonstrated that CCR7 mediated active MMP-2/9 secretion. CXCR4 induces MMP activity. Singh et al showed that the CXCR4/SDF-1 axis induced high levels of MMPs in prostate cancer cells, including MMP-2, MMP-3, MMP-9, MMP-11, and MMP-14\cite{30}. Chinni et al demonstrated that SDF-1 elevated MMP-9 expression and then facilitated prostate cancer cell bone metastasis. The PI3/AKT pathway contributed significantly to the process with which SDF-1 increased MMP-9 expression\cite{31}. However, compared with CXCR4, less is known about the role of CCR7 in regulating the secretion of MMPs. The first research on the relationship between CCR7 and MMPs was performed by Redondo and colleagues\cite{32}. Redondo showed that MMP-9 was regulated by CCL21, the ligand of CCR7, and MMP-9 was involved in B-cell chronic lymphocytic leukemia nodal infiltration. In this study, we showed that overexpression of CCR7 promoted the secretion of MMP-2/9 as well as migration of MDA-MB-435 cells and that silencing of CCR7 resulted in decreased MMP-2/9 secretion and attenuated cancer cell migration. These results suggested a possible role of CCR7 in mediating MMP-2/9 secretion. Interestingly, TPT induced down-regulation of CCR7 expression, but did not dramatically alter the secretion of MMP-9 in MDA-MB-231 cells. Only a less significant reduction (about 15%) of active MMP-9 secretion was observed by ELISA, probably caused by the incomplete inhibition of CCR7 expression. As such, 1 μmol/L TPT resulted in an approximate 50% decrease in the expression of CCR7 in MDA-MB-231 cells, whereas 0.1 μmol/L TPT achieved a 90% decrease in MDA-MB-435 cells. The apparent inability of TPT to eliminate CCR7 expression in MDA-MB-231 cells could reflect an inability to significantly decrease active MMP-9 secretion.

Physiologically, CCR7 is expressed in immune cells, eg, naive T cells, memory T cells, B cells, and mature dendritic cells. The expression of CCR7 is associated with lymph node...
metastases, stromal invasion and overall survival[27]. In breast cancer patients, lymph nodes are the first site of regional nodal metastasis and the presence of metastasis in lymph nodes is important for staging and prognosis. CCL21, the ligand of CCR7, is highly expressed in lymph nodes. Therefore, functional CCR7 may facilitate cancer cells to metastasize to lymph nodes in response to CXCL21. Increasing emphasis has been placed on developing CXCR4 antagonists in cancer therapy. Given this, our data also show the possibility of making anti-CCR7 treatment a new strategy for the treatment of cancer metastasis.

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Author contribution

Sen-sen LIN, Sheng-tao YUAN and Lu-yong ZHANG designed the research; Sen-sen LIN, Ren-ping ZHAO and Wen-lu LIANG performed the research; Li SUN and Yan-kai ZHANG contributed new reagents or analytical tools; Sen-sen LIN, Sheng-tao YUAN and Lu-yong ZHANG analyzed the data; and Sen-sen LIN prepared the manuscript.

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