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Translational Research - From Lab to Clinic

New HIV-Drug Inhibits In Vitro Bladder Cancer Migration and Invasion

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Abstract

Objective: The CXCR4/CXCL12 axis appears crucial in the metastasis of bladder cancer. Our aim was to evaluate the potency of the CXCR4 antagonist, 4F-benzoyl-TE14011 (4F-bTE), as an anti-metastatic drug in this disease. In this study, we assessed the ability of 4F-bTE to inhibit tumor cell motility, invasion through extracellular matrix (ECM), matrix metalloproteinase (MMP) secretion and cytoskeletal responses to chemokine.

Methods: To assess the degree to which cells could migrate and invade ECM under various conditions, we used TCCSUP bladder cancer cells in a Boyden chamber system. To monitor actin polymerization, we stained cells on chamber slides with AlexaFluor 594 phalloidin. To measure matrix-metalloproteinase-2 and -9 (MMP) activity, we used gelatin zymography. To assess the effects of the CXCR4 antagonist 4F-bTE on each of the above parameters, we exposed bladder cancer cells either to chemokine CXCL12, alone, or to both CXCL12 and 4F-bTE. We also monitored cells for apoptotic and necrotic changes during drug treatment.

Results: The CXCR4 antagonist 4F-bTE markedly decreased CXCL12-induced bladder cancer cell migration and ECM invasion in Boyden chamber assays. The antagonist also blocked chemokine-induced actin polymerization as well as the induction of MMP-2 and MMP-9 in these cells.

Conclusion: The CXCR4 antagonist 4F-bTE has the potential to inhibit expression of the metastatic phenotype and may provide therapeutic value to patients.

Keywords: Chemokines; Bladder cancer; Migration; Invasion; CXCR4 antagonist; 4F-benzoyl-TE14011; MMP

1. Introduction

Pelvic lymph nodes are the primary sites for bladder cancer metastasis, followed by widespread metastasis to lung, liver and bone marrow [1]. Chemokines and their receptors have been shown to play pivotal roles in organ-specific metastasis [2]. They also control angiogenesis, B-cell lymphopoiesis and myelopoiesis [3,4]. The interaction of soluble chemokines with their specific, transmembrane G-protein-coupled receptors mediates their biological effects. To date, 18 chemokine receptors (CCR1-10, CXCR1-6, XCR1 and CX_3CR1) have been identified in humans [5]. Recent evidence indicates that tumor cells express distinct, tumor-specific, non-random patterns of chemokine receptors. Intriguingly, the specific chemokine ligands for these receptors exhibit peak expression in lymph nodes, lung, liver and bone marrow, the most common sites of cancer metastasis [2].

Previously, we identified CXCR4 as the only chemokine receptor in bladder tumor cells whose expression levels correlated with tumor progression [6]. Our

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studies also showed that the corresponding chemokine ligand CXCL12 had a strong chemoattractant effect on bladder cancer cells. Consistent with its role in invasive processes, the CXCR4/CXCL12 axis is also functionally linked to actin polymerization, which is responsible for lamellipodia formation, and secretion of collagenolytic matrix-metalloproteinases (MMPs), which are believed to facilitate movement through the ECM [6]. Our findings on bladder cancer cells are in agreement with other recent studies, which have shown that the CXCR4/CXCL12 axis is required for metastasis in a variety of tumor types [2,7,8]. In particular, Müller and coworkers observed that neutralizing antibodies directed against the chemokine receptor CXCR4 potently reduced the number of metastases deriving from mouse xenografts of human breast tumors [2]. Based on our results, the CXCR4/CXCL12 axis also appears to play a pivotal role in the metastasis of bladder cancer and is a potential therapeutic target.

Prior to the discovery of CXCR4's involvement in cancer metastasis, CXCR4 had been identified as an important co-receptor for the human immunodeficiency virus (HIV-1). CXCR4 is the major co-receptor for the entry of T-cell-tropic HIV-1 (X4-HIV-1) [9]. To date, several CXCR4 antagonists have been developed as HIV-entry inhibitors. Tamamura and co-workers synthesized the polyphemusin T140, a 14-residue peptide containing a disulfide bridge. Although T140 exhibited strong anti-HIV activity, it lacked serum stability. A new generation of T140 derivatives, including 4F-benzoyl-TE14011 (4F-bTE), were developed and characterized by both high anti-HIV activity and high serum-stability. The new compounds also exhibit low toxicity [10].

Our aim was to evaluate the potency of the small molecule CXCR4 antagonist 4F-bTE as an anti-metastatic drug in bladder cancer. As described below, we assessed the ability of this compound to inhibit tumor cell motility, invasion through extracellular matrix, MMP secretion and chemokine-induced cytoskeletal reorganization.

2. Materials and methods

2.1. CXCR4 antagonist

The polyphemusin II peptide derivative 4F-bTE was synthesized as described previously [10].

2.2. Cell line

The undifferentiated bladder cancer cell line TCCSUP was originally obtained from the American Type Culture Collection (Manassas, VA). TCCSUP was cultured in 75 cm^2 flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 U/ml penicillin and 10 mg/ml streptavidin (Sigma, St. Louis, MO).

2.3. In vitro cell migration and invasion assays

Migration and invasion assays were performed in a Boyden chamber system as described previously [6]. Briefly, chemotactic migration of bladder cancer cells (TCCSUP) was investigated using 24-well cell-culture chambers containing inserts with 8-μm pores (BD PharMingen, San Diego, CA). Invasion assay chambers also contained a reconstituted extracellular matrix membrane overlying the insert (Matrigel with 8-μm pore membrane, BD PharMingen). Cells (5×10^4 cells/500 μl) were suspended in chemotaxis buffer (DMEM/0.1% BSA/ 12 mM HEPES) and were added to the upper chamber of the assay wells. Migration and invasion assays were performed in presence of a chemokine CXCL12 gradient (R&D Systems, Minneapolis, MN) established by placing chemokine (100 ng/ml) in the lower chamber. Control wells contained buffer alone. To investigate the inhibitory effect of the CXCR4 antagonist 4F-bTE, some bladder cancer cells were pre-incubated with 100 nM 4F-bTE for 1 h at 37 °C prior to placing them in the upper chamber. All experiments were repeated three times with triplicate samples.

2.4. Apoptosis/cell death assay

Apoptotic cells and necrotic cells were quantified using the Annexin V-FITC and propidium iodide (PI) detection kit (BD PharMingen, San Diego, CA) following standard protocols. Annexin V-FITC is a marker of apoptotic cells and the entry of PI into un-permeabilized cells acts as a marker for cells undergoing necrosis. TCCSUP cells were treated with 100 nM 4F-bTE for 24 h at 37 °C. Untreated TCCSUP cells served as negative control. Some cells were incubated with 100 μM etoposide as a positive control to induce apoptosis. Cells were adjusted to a concentration of 1×10^5 cells/100 ul in PBS. Each sample was stained with 5 μl Annexin V-FITC and 5 μl propidium iodide. Cells were analysed on a FACSscan (Becton Dickinson, Mountain View, CA) and flow cytometry data were analysed using Cellquest software (Becton Dickinson, Mountain View, CA).

2.5. Actin polymerization assay

To assess the effect of 4F-bTE on actin polymerization, TCCSUP cells were seeded on Labtech-eight-well-chamber slides pre-coated with 0.01% poly-L-lysine solution (Sigma, St. Louis, MO). Cells were serum-starved overnight and then incubated with 100 ng/ml CXCL12 (R&D Systems, Minneapolis, MN) ± 100 nM 4F-bTE for 30 min at 37 °C to stimulate actin polymerization. After 30 min, cells were fixed for 2 min (4 °C) in 3% paraformaldehyde in PBS, permeabilized for 5 min with 0.2% Triton X-100, incubated with 5 mU/ml AlexaFluor 594 phalloidin (Molecular Probes, INC., Eugene, OR) for 1 h, washed with PBS and mounted in Vectashield aqueous mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Fluorescence microscopy was performed using a Nikon Eclipse, E600 microscope.

2.6. Gelatin zymography

To investigate the effects of 4F-bTE on MMP activity, TCCSUP bladder cancer cells were cultured with 100 ng/ml CXCL12 (R&D Systems, Minneapolis, MN) ± 100 nM 4F-bTE under serum-free conditions. After 24 h at 37 °C, cell supernatants were collected and samples were resolved on 10% SDS-PAGE containing 0.3% gelatin. Following electrophoresis, gels were incubated in buffer containing 2.5% Triton X-100, 50 mM Tris-HCl, 6.5 mM $CaCl_2$, 5 μM $ZnCl_2$