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RESEARCH ARTICLE

BIOLOGICAL CHARACTERIZATION OF A SYNTHETIC CXCR4 ANTAGONIST WITH DUAL ANTIMETASTASIS AND ANTIANGIOGENESIS ACTIVITIES

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ABSTRACT

Metastasis and angiogenesis are two of the major obstacles that hinder the successful treatment of human cancers. C-X-C chemokine receptor type 4 (CXCR4), an important regulator of cancer metastasis and angiogenesis, is predominantly expressed in many metastatic cancers. We evaluated the antimetastasis and antiangiogenesis effects DV1, our recently developed potent CXCR4 antagonist, both *in vitro* and *in vivo*. DV1, a mimetic of the essential molecular moieties of a naturally existing CXCR4 ligand, consists of all D amino acids. At low nanomolar concentrations, DV1 strongly inhibited the in vitro tubule formation activity of HUVECs, the transwell migration of CXCR4+ cancer cells, and *in vivo* cancer metastasis and growth. These results support the continued development of this compound as a potential CXCR4 antagonist and a therapeutic drug that targets angiogenesis and cancer metastasis.

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INTRODUCTION

The principal cause of death from cancer is the metastasis of tumor cells from the primary neoplasm to different distant vital organs and cancer cell proliferation at those sites (Wang and Huang, 2017). Nevertheless, current therapies are aimed at the destruction and removal of cancer cells per se, whereas no effective therapeutic approaches are specifically directly at controlling the processes that allow cancer metastasis to occur. Metastasis of cancer cells is associated with the expression of many proteins, such as adhesion receptors and proteases, as well as other homing and angiogenic factors. Among the letter, chemokine ligands (e.g., stromal cell-derived factor-1 (SDF-1α), also known as CXCL12)) and receptors (e.g., CXCR4), are of particular interest in studies on metastasis (Müller *et al*., 2001; Hinton *et al*., 2010). The activation or inhibition of the CXCR4-dependent signaling pathway is mediated by the binding of its ligands − either endogenous (SDF-1α) or exogenous (e.g., viral macrophage inflammatory protein-II (vMIP-II) or synthetic molecules). CXCR4-overexpressing cancer cells favor metastasis to regional lymph nodes, lung, bone marrow, liver, and the central nervous system, where the CXCR4 ligand, SDF-1α is highly expressed (Burger and Kipps, 2006; Muller *et al*., 2001; Murphy, 2001; Zhao *et al*., 2015). CXCR4 over expressing cancers metastasize to the bone marrow from the blood stream in an SDF-1α–dependent manner (Geminder *et al*., 2001). Neutralization of the activity of CXCR4 with an anti-CXCR4 monoclonal antibody (mAb) or CXCR4 antagonists, such as T22, T140 or TN14003 polypeptides, or AMD3100 has been shown to inhibit the metastasis of cancer cells to lungs and prolong survival of xenograft *SCID* mice (Muller *et al*., 2001; Tamamura and Fujii, 2005; Liang *et al*., 2004), indicating that CXCR4 is involved in the directional migration of cancer cells to their target organs. In addition to metastasis, SDF-1α/CXCR4 engagement with the membrane of targeted cells causes a number of other biological changes, including chemokine production and angiogenesis (Wang *et al*., 2005). SDF-1α is secreted by cancers and by associated stromal myofibroblasts and is able to recruit endothelial cells into cancers to promote or enhance angiogenesis (Orimo *et al*., 2005; Darash-Yahana *et al*., 2004). Angiogenesis is a critical step for cancer growth beyond a size of 1-2 mm in diameter and for metastasis to distant organs. The increase in calcium flux, protein kinase phosphorylation, mitogen-activated protein

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kinase (MAPK), matrix metalloproteinase-2 (MMP-2), and MMP-9 activations are involved in SDF-1α/CXCR4 interaction-mediated signaling (Ganju *et al*., 1998; Vila-Coro *et al*., 1999; Bleul *et al*., 1996; Son *et al*., 2006). In the past several years, several groups, including ours, have demonstrated that the N-terminus of CXCR4 and its first extracellular loop (ECL1) are critical for SDF-1α binding and that the ECL2 of CXCR4 is involved in receptor signaling (Lu *et al*., 1997; Doranz *et al*., 1999; Zhou *et al*., 2001; Crump *et al*., 1997; Heveker *et al*., 1998; Choi *et al*., 2011; Choi *et al*., 2014). The structures of several chemokines and synthetic lignads that bind CXCR4 have been determined by either NMR or X-ray techniques (Crump *et al*., 1997; Dealwis *et al*., 1998; Liwang *et al*., 1999; Wu *et al*., 2010; Qin *et al*., 2015). We have used the structures of these ligands to design and develop an unique class of CXCR4 inhibitors and have identifiedDV1 as an initial lead (Yang *et al*., 2016; Zhou *et al*., 2002). DV1 significantly inhibited signals induced by SDF-1α, thereby acting as a potent CXCR4 antagonist. DV1 suppressed the *in vitro* tubule formation by HUVECs, the migration of CXCR4+ cancer cells, and *in vivo* cancer metastasis and growth, especially when administered in combination with paclitaxol. DV1 therefore show significant therapeutic potential for further development and clinical application.

MATERILAS AND METHODS

Synthesis of DV1

The DV1was synthesized according to the methods published previously (29). The purity (>95%) of final DV1(LGASWHRPDKCCLGYQKRPLP) was checked by analytical HPLC (Waters 1525) through a C18 reverse-phase column, while the molecular weight of DV1 was determined by MALDI-TOF mass spectrometry (Autoflex, Bruker).

CXCR4 mediated migration assay

MDA-MB-231bo cells were cultured in RPMI1640 medium with 10% fetal bovine serum, 100 IU penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine. On the day of experiment, the cells were collected following the standard procedure of trypsinization, washing and centrifugation. A sample containing 10^4 cells were incubated with 0 or 100 nM DV1 for an hour, and then were seeded at 100 μL per well in the upper chambers (8 μm pore size) of HTS transwell 96-well plates (Corning USA). The upper chambers were placed into the lower chambers, which contained 100 μL assay buffer and 0 or 10 ng/mL SDF-1α. After culturing the transwell plate in a 37 °C cell culture incubator for 24 h, the upper chambers were removed and cells that had migrated to the lower chambers and the bottom of the transwell inserts were collected and counted.

HUVEC tubule formation assay

Matrigel (BD Biosciences) was dissolved overnight at 4°C. Aliquots of 250 μL matrigel were added to each well of a 24-well plate, and incubated for 45 min at 37°C to allow the matrigel to solidify. Human umbilical vein epithelial cells (HUVECs, 5×10^4) in 500 μL endothelial cell growth medium were added to each well and incubated for 4 h at 37°C to allow the cells to form tube-like structures. The DV1 (100 nM) and vehicle control were added to the cells, followed by incubation for 24 h at 37°C in a 5% CO_2 atmosphere. Results were examined and photographed under a fluorescence microscope (Eclipse TE2000-U, Nikon).

Statistical Analysis

A statistical analysis was performed using the One-way ANOVA (Prism 5.0, GraphPad Software). *P* values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We have previously carried out extensive studies to characterize the structure-function relationship and mechanism of action for DV1 and its analogs in the context of its CXCR4 binding and signaling (Zhou *et al*., 2001; Yang *et al*., 2016; Zhou *et al*., 2002; Choi *et al*., 2011; Choi *et al*., 2005; Tian *et al*., 2005). We have shown that the N-terminal module of the DV1 is very important. Our objective in this paper is to evaluate a highly potent and target-selective lead product for the inhibition of migration/metastasis of CXCR4-overexpressing MDA-MB-231bo cells and of tube formation activity by HUVECs.

DV1 inhibits the migration of breast cancer cells toward the SDF-1α gradient

To determine whether DV1 blocks the cancer cell mobilization/metastasis, we have chosen a bone marrow metastasis cell line, MDA-MB-231bo (gifted by Dr. Michael Y Li) for our studies. These cells express CXCR4 but not estrogen-receptor (ER). The ability of DV1 to inhibit the migration of CXCR4+ MDA-MB-231bo toward the SDF-1α gradient was studied using type I collagen (Sigma) coated transwell plates (Corning, 5 μm pore size). Cells ($1x10^6$) were incubated in the presence or absence of 100 nMDV1 for an hour at 37 °C, and introduced into the upper chambers (100 μl per well) that were placed into the lower chambers which contained 100 μL medium and different concentrations of SDF-1α (0 or 10 ng/mL). After 2 h at 37°C in 95% air/5% CO_2, the breast cell migration caused by SDF-1α was significantly inhibited by preincubation of the cells with DV1 ($p<0.01$)

DV1 inhibits the HUVEC tubule formation activity

In addition to mediate migration/metastasis of tumor cells, the CXCR4–SDF-1α axis also promotes and enhances tumor-associated neoangiogenesis. The human umbilical vein epithelial cells (HUVECs) can form tubular structures and meshes of capillary-like vessels after plating on a Matrigel matrix for 24 h. The inhibitory activity of DV1 on HUVEC tube formation was first evident at 50 nM and complete inhibition was observed at 500 nM. Our HUVEC viability assay (CellTiter-Blue assay kit, Promega) has confirmed that DV1 at concentrations up to 50 μM has no cytotoxic effects in the HUVECs. These results confirmed that the anti-angiogenesis activity of DV1 was not caused by cell death. Therefore, DV1 is a desirable new candidate inhibitor of CXCR4 that targets angiogenesis.

DV1 exhibits in vivo anti-metastasis and anticancer activity in a xenograft model of breast cancer

We explored whether DV1 exhibits any in vivo anti-metastatic and antitumor growth (angiogenesis) activity after breast cancer cells are inoculated subcutaneously (s.c.). Mice treated

by i.v. injection with (1) saline, (2) 0.1 mg/kg control peptide DV1, (3) 0.1 mg/kg DV1 (i.v.), (4) 5 mg/kg paclitaxel (i.p.), (5) 0.1 mg/kg DV1 (i.v.) plus 5 mg/kg paclitaxel (i.p.); and (6) 0.1 mg/kg DV1 (i.v.) plus 5 mg/kg paclitaxel (i.p.), at days 0, +1, +2, +3, +4, and +5 post injection of $2x10^6$MDA-MB-231-Luc-GFP (bone metastasis) cells. At 1–3 weeks post treatment, mice sacrificed and lungs are excised and weighted or monitored using IVIS-200. DV1 exhibits single-agent activity that significantly inhibits both metastasis and growth of xenograft MDA-MB-231 tumors either used alone, or in combination with paclitaxel. It exhibited strong synergize anticancer effects with paclitaxel. We conclude that DV1 is a potent anti-metastasis and anticancer growth agent that is worth further developing.

Conclusion

In summary, we have employed a dual-labeled marrow metastasis cell line to evaluate and characterize DV1, a new potent CXCR4 antagonist. This compound has dual action, as it inhibits neovascularization as well as migration/metastasis of CXCR4+ cancer cells. These notable activities of DV1 were not due to cell death. DV1 therefore represents a valuable lead candidate for developing effective therapeutics that target CXCR4, which is widely expressed in cancer cells and tumor vasculatures and especially theSDF-1α-CXCR4 interaction and function.

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