

Inhibition of Breast Cancer Metastasis by Selective Synthetic Polypeptide against CXCR4

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ABSTRACT

Metastasis shares many similarities with leukocyte trafficking. Among those chemokine receptors thought to be involved in hemopoietic cell homing, stromal cell-derived factor-1 and its receptor CXC chemokine receptor-4 (CXCR4) have received considerable attention. Like hemopoietic cell homing, levels of stromal cell-derived factor-1 are high at sites of breast cancer metastasis including lymph node, lung, liver, and the marrow. Moreover, CXCR4 expression is low in normal breast tissues and high in malignant tumors, suggesting that a blockade of CXCR4 might limit tumor metastasis. We therefore investigated the role of a synthetic antagonist 14-mer peptide (TN14003) in inhibiting metastasis in an animal model. Not only was TN14003 effective in limiting metastasis of breast cancer by inhibiting migration, but it may also prove useful as a diagnostic tool to identify CXCR4 receptor-positive tumor cells in culture and tumors in paraffin-embedded clinical samples.

INTRODUCTION

Metastasis plays a major role in the morbidity and mortality of breast cancer (1). Breast cancer metastasizes in a stereotypical pattern resulting in lesions found in the lymph node, lung, liver, and bone marrow. Drawing parallels with hemopoietic stem cell homing where attention has focused on the role that CXC chemokine receptor-4 (CXCR4) and its ligand stromal cell-derived factor-1 (SDF-1) in the process, recent advances in metastasis research have emerged suggesting that, like hematopoiesis, CXCR4 and SDF-1 may play a critical role in the organ-selective process of tumors including breast (2) and prostate cancer (3).

CXCR4 is a G-coupled heptahelical receptor that first drew attention as a major coreceptor for the entry of HIV. Activation of CXCR4 by SDF-1 results in activation of many downstream pathways including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and calcium mobilization (4–11). For hematopoietic stem cell activation, CXCR4 triggers migration to the marrow (12–19) and directs peripheral blood cells into the lymph nodes and spleen (20). Together these results suggest that SDF-1/CXCR4 may play a “lock and key” function for directing cells to a variety of target organs. Because CXCR4 is a major coreceptor for T-tropic HIV infection, a variety of compounds that target CXCR4 to prevent infection have been developed. In 1998, Tamamura *et al.* (21, 22) reported the identification of a specific CXCR4 inhibitor T140, a 14-residue peptide that possessed a high level of anti-HIV activity and antagonism of T cell line-tropic HIV-1 entry among all antagonists of CXCR4 (21–23) by mimicking SDF-1. Further improvements in the compound were achieved by amidating the COOH-terminal of T140 and by substituting basic

residues with non-basic polar amino acids to reduce the total-positive charges of the molecule. This resulted in the generation of a compound (TN14003) with properties that are far less cytotoxic and more stable in serum compared with T140 (24). The concentrations of T140 and TN14003 required for 50% protection of HIV-induced cytopathogenicity in MT-4 cells (EC₅₀) are 3.3 and 0.6 nM, respectively. The concentrations of T140 and TN14003 that induce a 50% reduction of the viability of MT-4 cells [50% cytotoxic concentration (CC₅₀)] are 59 and 410 μM, respectively. These results reflect the improved therapeutic index for TN14003 over T140 (SI_{TN14003} = 680,000; SI_{T140} = 17,879; selective index (SI) = CC₅₀/EC₅₀).

Similarly, if a means of limiting the activation of CXCR4 could be found, this mechanism may make it possible to limit the ability of the cancer cells to metastasize. In the present study, we investigated the potential usage of TN14003 for the diagnosis and inhibition of breast cancer metastasis.

MATERIALS AND METHODS

Cell Culture. Human breast carcinoma cell lines MDA-MB-231 (a gift of Z. Bhujwalla, Johns Hopkins University, Baltimore) and MDA-MB-435 (a gift of Lily Yang, Emory University, Atlanta) were cultured in 5% CO₂ at 37°C in RPMI 1600 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Human primary fibroblast 2091 cells (American Type Culture Collection) were cultured in DMEM (Sigma), supplemented with 10% fetal bovine serum and antibiotics.

Antagonist and Control Peptide Synthesis, and Biotin Labeling. The CXCR4 antagonist was synthesized by the Microchemical Core Facility at Emory University. We also created a control peptide by randomly scrambling the amino acid sequence of CXCR4 antagonist while maintaining the disulfide bond to preserve the U-type structure of the antagonist (NH₂-KY-Nal-YR-DK-Cit-RCRRP-Cit-C-amide). This control peptide does not bind to CXCR4 protein (Fig. 1A, a). The CXCR4 antagonist was biotinylated by using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockford, IL). A desalting column (Pierce) was used to remove unbound biotin and salts. The average number of biotins per CXCR4 antagonist was determined by 2-(4'-hydroxyazobenzene) benzoid acid tests (Pierce).

Tumor Cell Invasion Assay. For an *in vitro* model system for metastasis, we performed a Matrigel invasion assay by using a Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA). SDF-1α (400 ng/ml, R&D Systems, Minneapolis, MN) was added to the bottom chamber to induce the invasion of MDA-MB-231 cells through the Matrigel. CXCR4 antagonist (4 ng/ml) or anti-CXCR4 antibodies (MAB173; 25 μg/ml; R&D Systems) was added to the cells before the cells were seeded to the top chamber. The Matrigel invasion chamber was incubated for 22 h in a humidified tissue culture incubator. First, noninvading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells at the bottom of the Matrigel were fixed in methanol and stained with H&E. The invasion rate was determined by counting the H&E-stained cells.

Cytotoxicity. A cell proliferation assay (Promega, Madison, WI) was used to determine the cytotoxicity of the CXCR4 antagonist *in vitro*. The cell proliferation was measured by the CellTiter 96 AQ (Promega). Cells were seeded in 96-well clear plates (3000 cells/well in 100 μl of medium) with different concentrations of CXCR4 antagonist. Two days later, 20 μl of CellTiter 96AQ reagent was added into each well and incubated for an additional 2 h, and the absorbance at 490 nm was measured.

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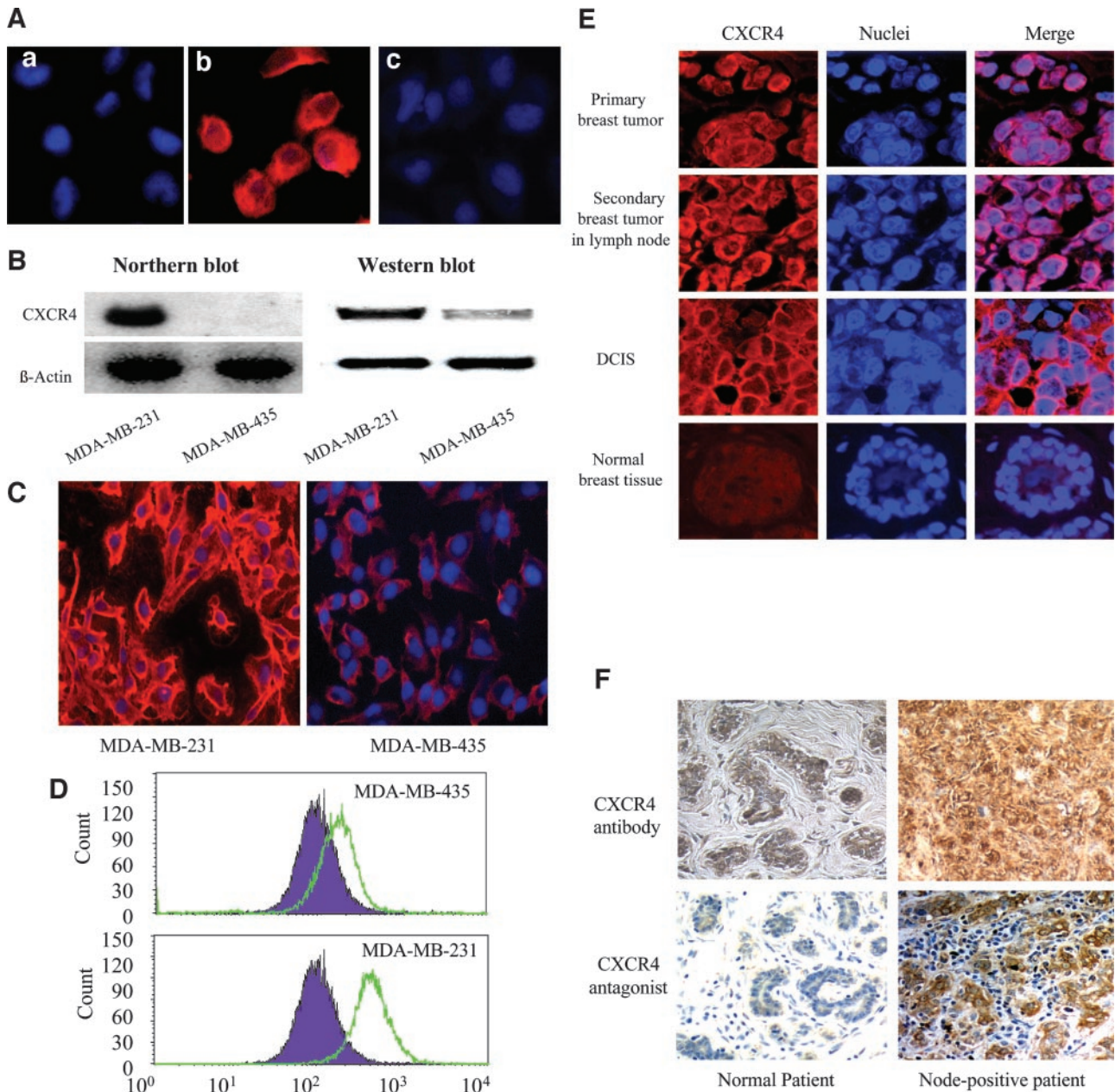


Fig. 1. Specificity of the CXCR4 antagonist. **A**, the binding of the antagonist to CXCR4 was blocked by preincubation of 400 ng/ml SDF-1. Cells were immunostained by using biotin-labeled control peptide (*a*) or biotin-labeled CXCR4 antagonist (*b* and *c*) and streptavidin-conjugated rhodamine (*red*). Cells were preincubated with SDF-1 for 10 min and then fixed in ice-cold acetone (*c*). Original magnification was $\times 200$. CXCR4, CXC chemokine receptor-4. **B**, Northern blot analysis and Western blot analysis results show the different expression levels of CXCR4 from breast cancer cell lines, MDA-MB-231 and MDA-MB-435. β -Actin was used as a loading control for both. **C**, immunofluorescence micrographs of CXCR4 protein on the surface of the cell from MDA-MB-231 and MDA-MB-435 cell lines by using biotinylated CXCR4 antagonist, TN14003, and streptavidin-conjugated R-phycoerythrin (*red*). Nuclei were counter-stained by cytox blue. Original magnification was $\times 100$. **D**, quantitation of CXCR4 expression on MDA-MB-231 and MDA-MB-435 cell lines by flow cytometer with the biotinylated CXCR4 antagonist. **E**, representative immunofluorescence staining of CXCR4 with the biotinylated CXCR4 antagonist on paraffin-embedded tissue sections of breast cancer patients and normal breast tissue. DCIS, ductal carcinoma *in situ*. **F**, representative immunostaining of CXCR4 by using biotin-labeled CXCR4 antagonist compared with anti-CXCR4 antibody (R&D Systems) on paraffin-embedded tissue sections from the same patients.

In Vitro Hemopoietic Progenitor Cell Colony Formation. We evaluated the CXCR4 antagonist for toxicity on hemopoietic progenitor cell colony formation (method described in Ref. 25). Human bone marrow cells were obtained from healthy adult volunteers by iliac crest puncture and aspiration into preservative-free heparin under a protocol approved by the University of Michigan's Investigational Review Board. Mononuclear cells were isolated by density separation on Ficoll-Hypaque (specific gravity 1.077). After two rounds of plastic adherence at 37°C for 1 h each in Iscove's Modified Delbecco's Medium (IMDM) with 10% fetal bovine serum, 10% equine serum, and 1 μ M hydrocortisone (Invitrogen), the nonadherent cells were recovered. CD34⁺ bone marrow cells were isolated by positive immunoselection from the low-density nonadherent cell frac-

tions (Miltenyi Biotec Inc., Auburn, CA). Thereafter, the cells were cultured in 35-mm Petri dishes (Stem Cell Technologies) in a 1.1-ml mixture of 0.8% methylcellulose in α medium (Invitrogen) supplemented with 30% FCS, 1% BSA (Stem Cell Technologies), 10^{-4} β -Mercaptoethanol, (β -ME), 5 units/ml human erythropoietin (Janssen-Cilag), and 2% spleen cell-conditioned medium (Stem Cell Technologies) in the presence or absence of 1.4 mg/ml G418 and 2 μ g/ml doxycycline (Sigma). Colonies were scored on day 14 of incubation as derived from colony-forming units-granulocyte/macrophage, and burst-forming units-erythroid (26–33). The identification of colonies was confirmed by Wright-Giemsa staining of cytopsin preparations of colonies. CXCR4 antagonist was added every day at half the dose after the initial dose at day 0. The drug is stable for at least

36 h and decays with a half-life of 20 h in RPMI with 10% fetal bovine serum inside a CO₂ tissue culture incubator (data not shown).

Fluorescence-Activated Cell Sorter Analysis. MDA-MB-231 and MDA-MB-435 cells grown on a 60-mm dish were incubated with 0.5 μg/ml biotinylated CXCR4 antagonist for 20 min on ice. After the incubation, cells were collected and washed with a PBS solution. Streptavidin-conjugated phycoerythrin or FITC was applied at 1:100 dilution to the cells. The cells were incubated for 30 min at room temperature in the dark, followed by three washes of PBS. The cells were resuspended in 600 μl of PBS, filtered through 30-μm pore size filter (VWR, Willard, OH), and analyzed by using Becton Dickinson (Franklin Lakes, NJ) FACScan equipped with Cell Quest software. Phycoerythrin or FITC fluorescence was detected in FL2 channel (excitation 488 nm/emission 575 nm) or in FL1 channel (ex 488 nm/em 530 nm), respectively.

Animal Experiments. Animal experiments were performed on 6 to 8-weeks-old CB-17 severe combined immunodeficient (SCID) female mice (Taconic Farms, Germantown, NY) with seven animals per group. 17β-Estradiol (60 day release, 0.72 mg; Innovative Research, Sarasota, FL) was inserted s.c. into all animals 1 day before the tumor cell injection. All animals received two injections of MDA-MB-231 tumor cells (2×10^6) delivered through the tail vein, 6 days apart (day 0 and day 6). For the treatment, animals were given i.v. injection of either CXCR4 antagonist or its control peptide that does not recognize CXCR4 twice weekly (100 ng/g body weight) from day 0, immediately before the first injection of tumor cells. Mice were sacrificed 55 days after tumor cell injections. Major organs were harvested in optimum cutting temperature (Fisher Scientific, Suwanee, GA) compound and frozen in liquid nitrogen. The collected tissue sections were subjected to H&E histostaining, reverse transcription (RT)-PCR, and real-time RT-PCR of human CXCR4.

For *in vivo* toxicity studies, mice were given an injection of the CXCR4 antagonist at 100 ng/g body weight twice weekly for 45 days. The control mice were treated with vehicle by the same protocol. After the 45-day treatment, the mice were sacrificed. Their liver and kidney sections were subjected to the evaluation of toxicity by H&E staining. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University.

Histostaining and Immunofluorescence. Animal organs were snap-frozen in optimum cutting temperature in liquid nitrogen, sectioned, fixed in ice-cold acetone, and maintained at -80°C. The tissues were stained with H&E to evaluate the presence/absence of tumors. For immunofluorescence detection of CXCR4, the sections were washed in water and PBS and blocked to eliminate nonspecific binding (Avidin and Biotin Blocking Solution; Zymed Laboratories, Inc., San Francisco, CA). The slides were subsequently incubated for 45 min at room temperature with 0.05 μg/ml biotinylated CXCR4 antagonist. The slides were washed three times with PBS and incubated in streptavidin-R-phycoerythrin (1:150 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature. Finally, the slides were washed with PBS and mounted in an anti-fade mounting solution (Molecular Probes, Eugene, OR).

Formalin-fixed paraffin-embedded tissue sections were heated at 58°C for 30 min. These specimens were washed with xylene three times for 5 min each, followed by washes with 100%, 95%, and 75% ethanol and rinsed with PBS. To block nonspecific binding, the samples were incubated in avidin-block and biotin-block sequentially. The biotinylated CXCR4 antagonist (0.05 μg/ml) was applied to tissue sections and the samples were further incubated for 45 min in a humidified chamber at room temperature. The slides were washed three times with PBS and incubated in streptavidin-Rhodamine (1:150 dilution; Jackson ImmunoResearch Laboratories) for 30 min at room temperature. After washing and mounting in an anti-fade mounting solution (Molecular Probes), the samples were analyzed on a Nikon Eclipse E800 microscope. All protocols for human tissue studies were reviewed and approved by the Institutional Review Board at Emory University. The grading system was according to the following common histological grading systems: 0 (<20% of cells are positive), 1 (21–50%), 2 (50–75%), and 3 (>75%). CXCR4 staining was considered as positive if its histological grade is 2 or 3.

Northern and Western Blot Analyses. For Northern blot analysis, total RNA (15 μg) was prepared with Trizol (Invitrogen) according to manufacturer's instruction and loaded on a 1.4% agarose-formaldehyde gel. After transferring to nitrocellulose, the blot was probed with ³²P-labeled CXCR4 frag-

ments (GenBank accession number AI920946) and later washed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)/0.5% SDS for 30 min at room temperature and three times in 0.2× SSC/0.5% SDS for 30 min at 50°C. For Western blot analysis, equivalent concentrations of total cellular proteins were resolved by SDS/PAGE (10% gel) and subjected to immunoblot analysis using polyclonal rabbit anti-CXCR4 antibody (Ab-2; Oncogene) and a monoclonal mouse anti-β-actin (Sigma).

Real-Time RT-PCR Analyses. For RT-PCR, total RNA was prepared from three slices (20 μm of each slice) of frozen mouse lung with Trizol (Invitrogen), according to manufacturer's instruction. The human CXCR4-specific primers for 149 bp are 5'-GAACCCTGTTCCGTGAAGA and 5'-CTTGCCGTCATGCTTCTCA (GenBank accession number NM_003467), and the primers for β-actin are 5'-GACAGGATGCAGAAGGAGAT and 3'-TGCTTGCTGATCCACATCTG (GenBank accession number X00351). First strand cDNA synthesis was done using a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA). The 20 μl of volume included 0.5 μg of RNA, 200 μM dNTPs, 2.5 mM MgCl₂, 10 mM DTT, 8 units RNase inhibitor, 30 units of reverse transcriptase, and 1.25 μM random hexamers in 1× RT buffer. The reaction was performed at 42°C for 30 min followed by 25°C for 10 min, and heating the samples to 95°C for 10 min stopped the reaction. The RT reaction was stored at -20°C until usage or immediately used as a template for the PCR. SYBR Green quantitative PCR amplifications were performed in an iCycler with a multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). The reactions were carried out in a 15-μl reaction volume containing 7.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM each forward and reverse primer, and 1 μl of cDNA from RT-reaction described above. The thermal profile for the real-time PCR was 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 54°C for 20 s, and 72°C for 30 s. We amplified and sequenced a 341 bp-PCR fragment as standard cDNA template with a pair of CXCR4 primers described previously (3). This fragment contained the human CXCR4 target fragment (141 bp) and primers as described above. In each run, a dilution series of the standard cDNA template (copy number in each reaction could be calculated) for CXCR4 gene and β-actin gene were run along with the unknown samples of the lung tissues. The automatic data acquisition and subsequent data analysis were performed by using the iCycler program after PCR amplification. The average copy number of CXCR4 gene was calculated per microgram of total RNA.

Statistical Analysis. All statistical significances were determined by Student's *t* test.

RESULTS

Specificity of CXCR4 Antagonist. Initially experiments were performed to verify that the CXCR4 antagonist binds to the predicted SDF-1 binding sites on the CXCR4 receptor. For these studies, MDA-MB-231 cells were incubated in the absence (Fig. 1A, *b*) and presence (Fig. 1A, *c*) of 400 ng/ml SDF-1α for 10 min and then fixed in ice-cold acetone. The immunofluorescence of the biotin-labeled CXCR4 antagonist was negative in both membrane and cytosol in the cells pretreated with SDF-1α for 10 min (Fig. 1A, *c*). We further explored the utility of the biotinylated CXCR4 antagonist as a probe of CXCR4 coupled with immunofluorescence staining of cultured breast cancer cells and paraffin-embedded tissues from breast cancer patients. MDA-MB-231 had high levels of mRNA and protein for CXCR4 as shown in Northern blots and Western blots compared with MDA-MB-435 (Fig. 1B). When the biotinylated CXCR4 antagonist was used to stain the two cell types, the high expressing MDA-MD-231 cells were brightly stained (Fig. 1C, *left*), consistent with the high protein levels of CXCR4. On the other hand, binding of biotinylated CXCR4 antagonist to MDA-MB-435 was dramatically less (Fig. 1C, *right*) consistent with the low surface CXCR4 expression in these cells. Flow cytometry was used to confirm these results and demonstrated as expected that the MDA-MD-435 cells had limited binding of the biotinylated CXCR4 antagonist (Fig. 1D, *top*) in contrast to the results with MDA-MD-231 (Fig. 1D, *bottom*). Immunofluorescence

staining with the biotinylated CXCR4 antagonist on the paraffin-embedded tissue sections of cancer patients demonstrated that CXCR4 antagonist can be used to detect CXCR4 receptors of tumor cells from the archived paraffin-embedded tissue sections (Fig. 1E). Fig. 1E shows that CXCR4 expression levels are low in normal tissues (no red rhodamine staining) whereas primary tumors and the lymph node metastasis from the same patient showed elevated CXCR4 protein levels. We stained a total of 41 patient tissues provided by Avon breast cancer tissue bank at Grady Memorial Hospital (Atlanta, GA) and found that 0 of 4 normal, 9 of 12 ductal carcinoma *in situ*, and 23 of 25 node-positive cases are positive of CXCR4. Importantly, we noted that many samples carrying the diagnoses of ductal carcinoma *in situ* already acquired CXCR4 overexpression (Fig. 1E). Fig. 1F shows the immunostaining of CXCR4 on paraffin-embedded tissue sections from the same patient by using either anti-CXCR4 antibody (R&D Systems) or biotinylated CXCR4 antagonist. The CXCR4 staining by antibody appears to be positive on tissue sections from normal patients (Fig. 1F, top left), and it was not localized to membrane. In addition, the connecting tissues are also stained, and cell boundary was not identifiable in malignant patient tissues (Fig. 1F, top right). On the other hand, the CXCR4 staining by biotinylated antagonist was localized on membrane, and the difference between normal and malignant tissues was clear (Fig. 1F, bottom).

Inhibition of Breast Cancer Cell Invasion *in Vitro* by CXCR4 Antagonist. For an *in vitro* model system for metastasis, we used a Matrigel invasion chamber (Becton and Dickinson). SDF-1 α was added to the lower chamber to induce CXCR4-positive breast cancer cells to invade through the Matrigel. In the absence of SDF-1, the invasion was very low. With 400 ng/ml SDF-1 α in the bottom chamber, significantly greater numbers of MDA-MB-231 cells responded to the chemo-attractant and migrated into the bottom chamber. This SDF-1-mediated invasion was suppressed by the addition of 25 μ g/ml antibody (167 nM) directed against the CXCR4 receptor

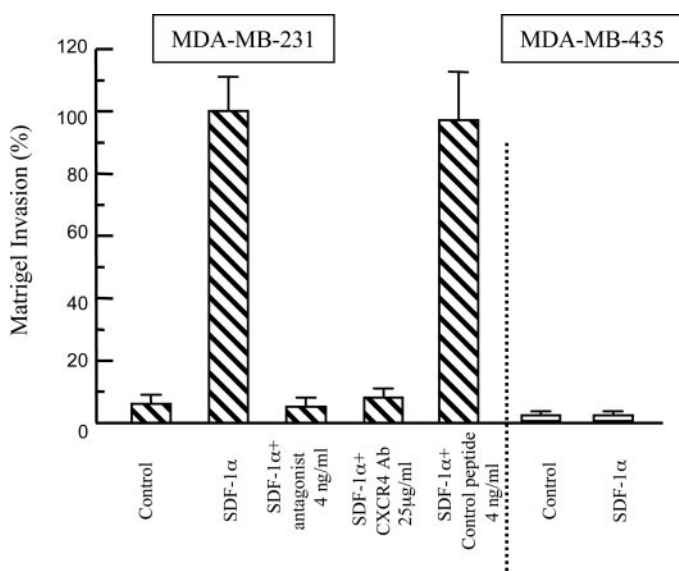


Fig. 2. Inhibition of CXCR4/SDF-1 mediated invasion of MDA-MB-231 *in vitro* by the CXCR4 antagonist. Matrigel invasion assay results show that CXCR4/SDF-1-mediated invasion of MDA-MB-231 was blocked either by 25 μ g/ml CXCR4 antibody (MAB173; R&D Systems) or by 4 ng/ml CXCR4 antagonist. We seeded cells on top of the Matrigel and added SDF-1 to the lower chamber. Invasive cells penetrate Matrigel and end up on the other side of the Matrigel. We estimated invasion by counting the number of invading cells stained by H&E at the bottom side of the Matrigel chamber and setting the average of invading cell numbers of MDA-MB-231 with SDF-1 added to the lower chamber as 100%. MDA-MB-435 cells with low CXCR4 expression levels did not invade through Matrigel even in the presence of SDF-1 at the bottom chamber. SDF-1, stromal cell-derived factor-1; CXCR4, CXCR4 chemokine receptor-4.

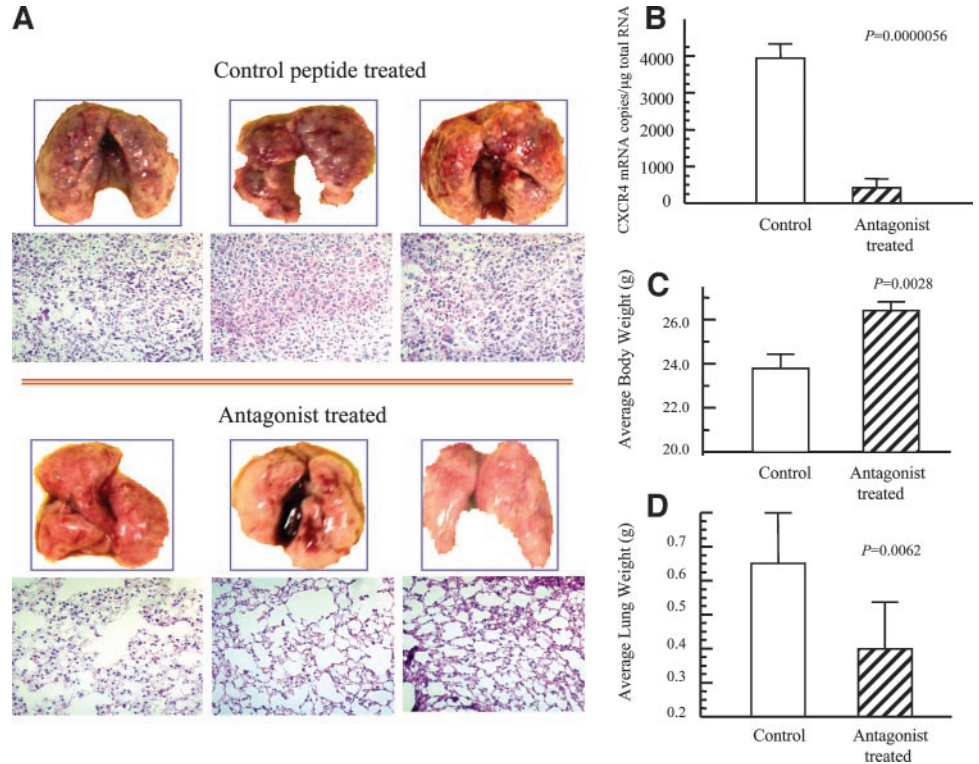
(MAB173; R&D; Fig. 2). Like the CXCR4 antibody, CXCR4 antagonist also inhibited invasion of the tumor cells *in vitro* but did so more effectively at 4 ng/ml (2 nM) concentration. Increasing antagonist concentration to 10 nM did not increase the blocking efficiency. The scrambled control peptide did not inhibit Matrigel invasion. On the other hand, CXCR4-negative MDA-MB-435 cells failed to invade through Matrigel even with SDF-1 α in the bottom chamber (Fig. 2, right).

CXCR4 Antagonist Blocked Breast Cancer Metastasis in Animal Model. To extend our *in vitro* findings, we established an experimental metastasis animal model of breast cancer metastasis. MDA-MB-231 cells in conjunction with the control peptide or CXCR4 antagonist were administered twice i.v. to female SCID mice supplemented with 17 β -estradiol. The CXCR4 antagonist or control peptide treatment was continued twice weekly for 55 days. All seven mice of the control group receiving an injection of MDA-MB-231 cells and treated with control peptide developed lung metastases. Three representative pictures of lungs in Fig. 3A exhibit bubble-looking lung metastases in the control group (top panel). The crude estimation of the area of metastases on the lung surface from seven animals was $41.2 \pm 6.9\%$. On the other hand, three representative pictures of lungs in Fig. 3A show significantly fewer lung metastases in the group treated with CXCR4 antagonist (bottom panel). The crude estimation of the area of metastases on the lung surface from seven animals treated with CXCR4 antagonist was $6.2 \pm 1.7\%$. The *P* of these estimations was 0.0000044 (*t* test). The tissues from the lung of these animals were processed for H&E staining. Whereas the lung tissues from the CXCR4 antagonist-treated animals maintained the morphology of normal lung tissues, those from the control group were filled with human tumor cells with big nuclei. The results were further confirmed by semi-quantitative real-time RT-PCR using CXCR4 primers that are specific for human CXCR4 (Fig. 3B). These results demonstrated that there was significant expression of human CXCR4 mRNA in the metastasis-infiltrated lungs of those animals that received an injection of MDA-MB-231 cells and that were treated with the control peptide (Fig. 3B). In contrast, the RT-PCR analyses confirmed that there were significantly fewer metastases in the lungs of CXCR4 antagonist-treated SCID mice that were given an injection of MDA-MB-231 cells. These results agreed well with H&E staining of lung tissues (Fig. 3A). Further analysis revealed that the average mRNA copy number for human CXCR4 per μ g of total RNA of CXCR4 antagonist treated animals' lung was 10.6% of those in the control peptide-treated lungs. Paralleling these findings were the observations that the average body weight was higher in antagonist-treated animals compared with control peptide-treated animals (Fig. 3C). Lung weight reflected the tumor burden of the animal (Fig. 3D).

Cytotoxicity of CXCR4 Antagonist. Decreased metastasis to the lung in CXCR4 antagonist-treated animals could be attributable to the failure to metastasize or to the cytotoxicity of the treatment. To determine the cytotoxicity of the CXCR4 antagonist, CXCR4-positive MDA-MB-231 cells were treated with different concentrations of the antagonist, and the effects on proliferation were determined. The CXCR4 antagonist did not affect cell proliferation even at 10 nM concentration (Fig. 4A). Thus, it is unlikely that CXCR4 antagonist-treated animals could not form extensive lung metastasis because of the cytotoxic effect of CXCR4 antagonist on MDA-MB-231 cells.

Systemic Toxicity. To evaluate the possibility for systemic toxicity of the CXCR4 antagonist, several organs were examined microscopically. Fig. 4B shows representative H&E staining of liver and kidney tissues from mice treated either with PBS injection or CXCR4 antagonist. In particular, no central necrosis was observed in the liver and no tubular necrosis in the kidney. H&E staining results demonstrate that there was no damage in these organs of the representative

Fig. 3. CXCR4 antagonist blocks metastasis in an experimental metastasis animal model. We injected MDA-MB-231 tumor cells (2×10^6) into the tail vein of female mice twice (day 0 and day 6) and divided them into two groups, one with antagonist treatment, TN14003, and the other with control peptide injection. We injected 100 ng of CXCR4 antagonist/g of mouse twice weekly intravenously. Tumor cells were injected on the first day of treatment. **A**, pictures of lungs and their H&E stainings (original magnification, $\times 20$) from the three antagonist-treated mice and three control peptide-treated mice. **B**, quantitative real-time RT-PCR of CXCR4 using primers that only recognize human CXCR4 confirms that animals receiving an injection of MDA-MB-231 cells and treated with control peptide developed lung metastasis (average of seven animals in each group). On the other hand, animals receiving an injection of MDA-MB-231 cells and treated with CXCR4 antagonist developed significantly fewer metastases. **C**, average body weight of animals treated with CXCR4 antagonist compared with that of animals treated with control peptide. **D**, average lung weight of animals treated with CXCR4 antagonist compared with that of animals treated with control peptide. Human CXCR4 receptors on MDA-MB-231 cells responded to murine SDF-1. Mouse SDF-1 α is highly conserved, with $>95\%$ identity to human, feline, and rat (47, 48). Conversely, human CXCR4 receptor only has $<85\%$ identity to murine CXCR4 (49, 50). *SDF-1*, stromal cell-derived factor-1.

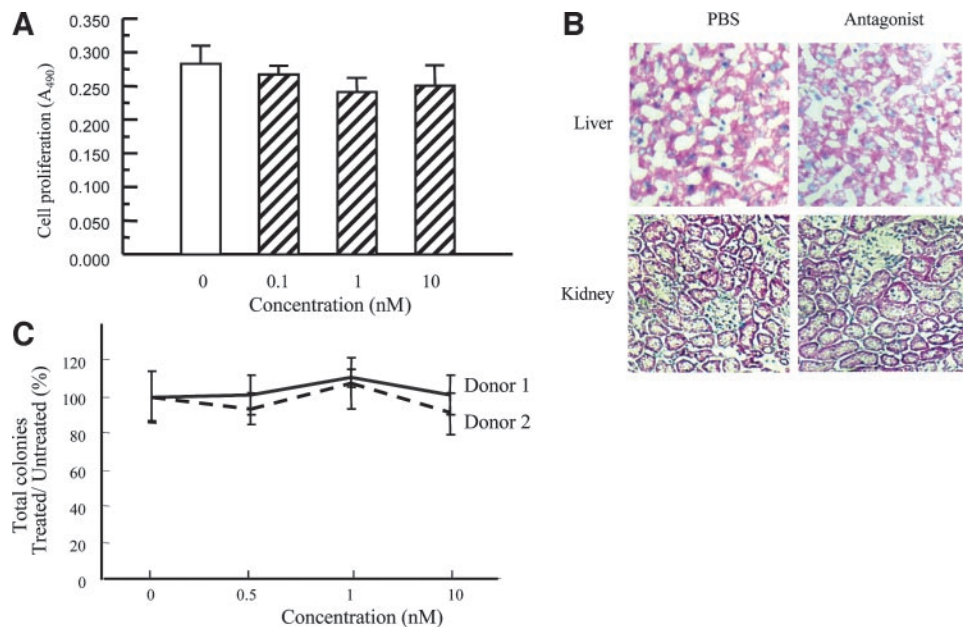


mice of each group. We next evaluated the CXCR4 antagonist for the toxicity on hemopoietic progenitor cell colony formation. For these studies, at 10 nM, the highest concentration tested, there was no discernable effect on hemopoietic progenitor cell colony formation (Fig. 4C). The number of colonies per well was not significantly different with treatment for colony-forming units-granulocyte/macrophage and burst-forming units-erythroid. Nor was the total number of colonies altered by addition of CXCR4 antagonist. The cytotoxicity of the CXCR4 antagonist was also tested on human CXCR4-negative 2091 human primary fibroblast cells and found that CXCR4 antagonist did not affect cell growth rate of 2091 cells, at concentrations ranging from 1 to 10 μ M (data not shown).

DISCUSSION

The present study investigated the antimetastatic activity of a small synthetic peptide against CXCR4. The pretreatment of cells with SDF-1 α may induce endocytosis of CXCR4 receptors. Because cells were only treated with SDF-1 α for short time, some CXCR4 proteins should be in a process of endocytosis (being internalized) whereas the others still remained on cell surface. The immunofluorescence of the biotin-labeled CXCR4 antagonist was negative in both membrane and cytosol in the cells pretreated with SDF-1 α for 10 min (Fig. 1A, c). The fact that the immunofluorescence of the biotinylated CXCR4 antagonist was negative in both membrane and cytosol of cells pre-

Fig. 4. CXCR4 antagonist is safe. **A**, cell proliferation assay on MDA-MB-231 cells show that CXCR4 antagonist did not affect cell growth of MDA-MB-231 cells up to 10 nM. **B**, animals treated with CXCR4 antagonist for 45 days twice weekly at 100 ng/g body weight (three mice in each group) remained healthy. Representative H&E staining of their liver and kidney tissue sections from the treated mice compared with those receiving an injection of PBS. Original magnification was $\times 100$. **C**, we evaluated CXCR4 antagonist for the toxicity on hemopoietic progenitor cell colony formation. Colony formation from CD34 $^{+}$ cells was determined and scored on day 14 of incubation as a total of burst-forming units-erythroid (BFU-E) and colony-forming units-granulocyte/macrophage (CFU-GM). CXCR4 antagonist was added every day at half of a loading dose after the initial dose at day 0. At the highest concentration tested, 10 nM CXCR4 antagonist did not affect hemopoietic progenitor cell colony formation. *CXCR4*, CXC chemokine receptor-4.



treated with SDF-1 α for a short time suggested that CXCR4 antagonist likely competes with SDF-1 for binding to CXCR4 protein. In addition, we further investigated the utility of the biotinylated CXCR4 antagonist as a probe of CXCR4 coupled with immunofluorescence staining and fluorescence-activated cell sorter analysis of two types of cultured breast cancer cells with different CXCR4 levels. We found that biotinylated CXCR4 antagonist can be useful as a quantitative diagnostic tool to identify CXCR4 receptor-positive tumors in culture and clinical samples (Fig. 1). *In vitro* invasion assays showed that CXCR4-negative MDA-MB-435 cells could not invade through Matrigel whereas CXCR4-positive MDA-MB-231 cells did in the presence of SDF-1 α at the bottom chamber (Fig. 2). Furthermore, MDA-MB-435 cells could not invade through Matrigel even in the presence of 1% fetal bovine serum at the bottom chamber whereas MDA-MB-231 cells did (data not shown). These results suggest that CXCR4 expression may impact on Matrigel invasion. Of note, Ellison *et al.* (34) reported that breast-specific genes were not detectable in MDA-MB-435 whereas melanocyte-specific genes were expressed in this cell line. Thus MDA-MB-435, originally considered as a breast cell line, may, in fact, be a melanoma cell line (34, 35). To increase the probability to form metastasis in our animal model, 2×10^6 tumor cells were i.v. administered twice (day 0 and day 6) to female SCID mice supplemented with 17 β -estradiol (60-day release pill). All animals treated with the control peptide twice weekly for 55 days developed lung metastases. On the other hand, CXCR4 antagonist treated animals had 85% reduction in lung metastases compared with the control group based on the estimations of the metastatic area on lung surface ($P = 0.0000044$). Our semiquantitative Real-time RT-PCR revealed that CXCR4 antagonist-treated animals contained only 6.3% of human tumor cells in their lungs relative to control group (Fig. 3). This decreased metastasis to lung in CXCR4 antagonist-treated animals was not because of a cytotoxicity of antagonist because the CXCR4 antagonist did not affect cell proliferation even at 10 nM concentration (Fig. 4). Because the CXCR4 antagonist worked at 1–2 nM concentrations and the estimated average *in vivo* concentrations with twice weekly injection of 2 μ g of CXCR4 antagonist was ≥ 1 nM, we determined whether CXCR4 antagonist affected proliferation of MDA-MB-231 cells up to 10 nM that was 10-fold higher than the working concentration. In addition, H&E staining of liver and kidney tissues from mice treated with CXCR4 antagonist did not exhibit any central necrosis in the liver or tubular necrosis in the kidney. We also evaluated the CXCR4 antagonist for the toxicity on hemopoietic progenitor cell colony formation and, at 10 nM, the highest concentration tested, there was no discernable effect on hemopoietic progenitor cell colony formation. Therefore, CXCR4 antagonist described here can be used as an excellent therapeutic agent to inhibit breast cancer metastasis. In parallel with our study, Tamamura *et al.* (36) reported recently that slow-release administration by using an Alzet osmotic pump to deliver T140 analog, 4F-benzoyl TN14003 gave a statistically significant reduction in pulmonary metastasis of MDA-MB-231 in SCID mice.

Our data, combined with other published results, demonstrates that CXCR4/SDF-1 interaction is one of the major requirements for breast cancer metastasis. The elevated level of CXCR4 in primary tumors correlates with the metastatic potential of tumors. Kang *et al.* (37) demonstrated that CXCR4 is one of the genes implicated in metastasis in breast cancers. If the high-CXCR4 levels in the primary tumor are the prerequisite of lymph node involvement, it may be possible to prevent cancer spread in CXCR4-positive patients by using CXCR4 antagonist. However, further investigation will be necessary to understand the key regulators of CXCR4/SDF-1 activation and their contribution to tumor formation and metastasis, such as the interaction of

VEGF with CXCR4 (8, 38). Furthermore, CXCR4 overexpression has been found in other tumors besides breast cancer, such as brain tumors (Refs. 39–41), pancreatic cancer (42), ovarian epithelial tumors (43), prostate cancer (3), kidney cancer (44), and nonsmall cell lung cancer (45). Neutralizing CXCR4/SDF-1 activation with the CXCR4 antibody impaired breast cancer metastasis to the lymph node and lung in animal models for breast cancer metastasis (2), and similar results have been observed in prostate cancer bone metastasis.⁵ We identified a synthetic 14-mer peptide that blocked the CXCR4 receptor binding to its ligand SDF-1 and inhibited CXCR4/SDF-1-mediated invasion *in vitro* at much lower drug concentration (2 nM, 4 ng/ml) than anti-CXCR4 antibodies (167 nM, 25 μ g/ml; R & D Systems). Furthermore, this antagonist blocked lung metastasis in an animal model by using a far lesser amount (twice weekly injection of 2 μ g for 20 g of mouse) than anti-CXCR4 antibody (twice weekly injection of 1 mg for 20 g of mouse; Ref. 2). The anti-invasion and antimetastasis activity of this peptide correlated well with its inhibitory activity on SDF-1 α binding to CXCR4. This antagonist is proven safe when accessed by proliferation assay, animal histology, and hemopoietic progenitor cell colony formation. Thus, the CXCR4 antagonist TN14003 is an effective therapeutic agent of breast cancer metastasis as well as inhibitor of T-tropic HIV infection.

Anti-CXCR4 antibody is capable of decreasing breast cancer metastasis at high concentrations *in vivo* (50 mg/kg; Ref. 2). However, antibody therapy may be limited by the following: (a) difficulty or expense of commercial-scale production; (b) delivery problem and slow diffusion because of a large mass; and (c) exclusion of monoclonal antibody from compartments like the blood/brain barrier (46). Certain antibodies like Herceptin have proven their efficiency in clinical trials. However, Herceptin is clearly a superior antibody over the currently available CXCR4 antibodies we have tested. The special aspect of the CXCR4 antagonist described here is its potency. Because this antagonist works at a very low concentration, it has potential as a drug. The amount of CXCR4 antagonist we used to block lung metastasis in the animal model was a twice weekly injection of 2 μ g whereas the amount of CXCR4 antibody Muller *et al.* (2) used was 1 mg/injection. Based upon the current cost of manufacture and purchase, the CXCR4 inhibitor is nearly 500 times less expensive than monoclonal antibodies when comparing equal molarities. Thus this clearly overcomes the cost problem of using antibodies for therapy or experiments. Furthermore, it has been theorized that in a compact tumor mass, large molecules such as antibodies with a molecular weight of M_r 150,000 may not easily diffuse between cells inside of the solid tumor. Therefore, antibodies may be inefficient molecules to target cells deep within the tumor mass. Especially, the use of antibody (M_r 150,000) or antibody fragment [F(ab')₂, M_r 30,000] as an imaging probe for positron emission tomography or single photon emission computed tomography to detect CXCR4-positive tumors is not practical. This is because positron emission tomography or single photon emission computed tomography nuclides have short half-lives (20–109 min) whereas antibody or antibody fragments will take a long time (at least 24 h) to reach the target site (tumor) and become clear of the blood and tissues. In part for these reasons, we are in the process of developing fluorine-18 labeling of CXCR4 antagonist for noninvasive positron emission tomography imaging of CXCR4-positive cells in our animal model.

The biotinylated CXCR4 antagonist is a potent reagent for detecting CXCR4 receptors from cultured cancer cells and paraffinized tissues on breast cancer patients through the use of immunofluores-

⁵ YX Sun, A Schneider, Y Jung, JH Wang, J Dai, J Wang, K Cook, NI Ishag-Osman, Z Liang, AJ Koh-Paige, H Shim, K Pienta, ET Keller, LK McCauley, and RS Taichman, manuscript in preparation.

cence and flow cytometry. The combined usage of the CXCR4 antagonist and other antibodies of known metastatic markers (e.g., Her-2) may be a specific and accurate prediction for breast cancer metastasis. Additional research and more samples from breast cancer patients will be needed to confirm whether the increase in CXCR4 expression level is a prerequisite of metastasis and if the elevated CXCR4 expression levels can be a predicting marker for metastasis. The antagonist to CXCR4 may be useful in interfering with tumor invasion and metastasis of other cancers in addition to breast cancer.

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