CXCR4 Protein Epitope Mimetic Antagonist POL5551 Disrupts Metastasis and Enhances Chemotherapy Effect in Triple-Negative Breast Cancer

Jingyu Xiang1, Michelle A. Hurchla1, Francesca Fontana1,2, Xinming Su1, Sarah R. Amend1, Alison K. Esser1, Garry J. Douglas3, Chidananda Mudalagiriyappa4, Kathryn E. Luker5,6,7, Timothy Pluard8, Foluso O. Ademuyiwa9, Barbara Romagnoli3, Gérald Tuffin5, Eric Chevalier5, Gary D. Luke5,6,7, Michael Bauer3, Johann Zimmermann9, Rebecca L. Aft4, Klaus Dembowski5, and Katherine N. Weilbaecher1

Abstract

The SDF-1 receptor CXCR4 has been associated with early metastasis and poorer prognosis in breast cancers, especially the most aggressive triple-negative subtype. In line with previous reports, we found that tumoral CXCR4 expression in patients with locally advanced breast cancer was associated with increased metastases and rapid tumor progression. Moreover, high CXCR4 expression identified a group of bone marrow–disseminated tumor cells (DTM–)–negative patients at high risk for metastasis and death. The protein epitope mimic (PEM) POL5551, a novel CXCR4 antagonist, inhibited binding of SDF-1 to CXCR4, had no direct effects on tumor cell viability, but reduced migration of breast cancer cells in vitro. In two orthotopic models of triple-negative breast cancer, POL5551 had little inhibitory effect on primary tumor growth, but significantly reduced distant metastasis. When combined with eribulin, a chemotherapeutic microtubule inhibitor, POL5551 additively reduced metastasis and prolonged survival in mice after resection of the primary tumor compared with single-agent eribulin. Hypothesizing that POL5551 may mobilize tumor cells from their microenvironment and sensitize them to chemotherapy, we used a “chemotherapy framing” dosing strategy. When administered shortly before and after eribulin treatment, three doses of POL5551 with eribulin reduced bone and liver tumor burden more effectively than chemotherapy alone. These data suggest that sequenced administration of CXCR4 antagonists with cytotoxic chemotherapy synergize to reduce distant metastases.

Introduction

The chemokine receptor CXCR4 plays an important role in the trafficking and homing of hematopoietic stem cells (HSC) and leukocytes. Its ligand SDF-1 (also called CXCL12) is produced by activated osteoblasts, bone marrow and lung stromal cells, and endothelial cells (1). During embryogenesis, the CXCR4–SDF-1 system is essential for HSC migration from fetal liver and home to the bone marrow (1–3). InCXCR4null mice, CXCR4 is required for the establishment of hematopoietic stem cells (HSC). Notably, HSCs from CXCR4null mice fail to engraft in lethally irradiated mice, indicating the critical role of CXCR4 in hematopoietic engraftment (4). Similarly, in bone marrow–disseminated tumor cell (DTC)–negative patients at high risk for metastasis and death, the establishment of these tumor–stromal interactions has shown to promote tumor cell-cycle arrest, survival, and resistance to chemotherapy in hematologic malignancies, such as multiple myeloma and chronic myelogenous leukemia (particularly with multiple myeloma; ref. 13), and in solid tumors (14). Similar to CXCR4 inhibition in HSC, CXCR4...
antagonists can mobilize tumor cells out of these protective stromal niches into the peripheral blood, which could enhance susceptibility to anoikis, and to conventional chemotherapy. In addition, CXCR4 antagonists may disrupt metastasis of tumors that use CXCR4 to home to bone and other SDF-1-rich distant organs. In contrast with their role in HSC and cancer cell mobilization, POL5551 and other CXCR4 inhibitors have also been shown to blunt mobilization of some other cell types, such as mesenchymal stem cells to sites of injury or inflammation, reducing for example neointimal formation (15, 16).

Breast cancer is the most prevalent cancer in women worldwide. More than 90% of the mortality of breast cancer patients is associated with metastasis and relapse. CXCR4 expression is detectable in breast cancer of various subtypes and has been associated with early metastasis and poorer prognosis, especially with triple-negative breast cancer (TNBC), a particularly aggressive subtype (17). The presence of microscopic disseminated tumor cells (DTC) in the bone marrow is also associated with early metastasis and poorer survival (18). As CXCR4 can be expressed by both primary tumors and circulating tumor cells, the interaction between tumor CXCR4 and the niche-attractant SDF-1 has been proposed to be an important player of early micrometastasis (19, 20).

Several CXCR4 antagonists have been developed for therapeutic applications, including HSC mobilization, and as antitumor therapies (21). In preclinical studies, small-molecule inhibitors of CXCR4 induced prostate cancer cell mobilization in vitro (22) and decreased pulmonary metastases in models of both osteosarcoma (23) and melanoma (24). POL5551 (Polyphor Ltd.) is a novel fully synthetic cyclic peptide antagonist of CXCR4 developed using protein epitope mimetic (PTEM) technology (25). A representative structure of a PTEM molecule incorporating a 8-hairpin is shown in Supplementary Fig. S1A. Like the FDA-approved CXCR4 inhibitor plerixafor, POL5551 competes with SDF-1 for the extracellular CXCR4 receptor domain, and an increased HSC mobilization activity compared with plerixafor (26). At high doses in mice, POL5551 mobilized HSC levels similar to that produced by G-CSF, a far greater mobilization than achieved with plerixafor, or that has been reported for other CXCR4 antagonists (26). In mouse models, POL5551 has been demonstrated to inhibit neointima hyperplasia in a model of atherosclerosis (27) and to prolong survival when added to anti-VEGF therapy in a model of glioblastoma (28).

In this study, we found that, in stage II/III breast cancer patients who did not have detectable bone marrow DTC, tumor CXCR4 expression could identify patients at risk for early mortality and metastasis. We hypothesized that antagonism of the CXCR4 receptor with POL5551 would reduce metastases and improve survival in CXCR4-expressing breast cancer, and addressed this hypothesis in preclinical models. We found that POL5551 inhibited tumor cell migration and decreased adhesion-independent survival in vitro, although it had no direct effect on tumor cell viability. In agreement, single-agent POL5551 reduced tumor cell metastasis to the distant sites outside the chest wall and lung, despite having no effect on the primary tumor growth, which suggests a disruption of the metastatic process rather than a direct antitumor effect. When combined with low-dose eribulin chemotherapy, POL5551 further decreased distant metastasis and prolonged survival in an orthotopic breast cancer model. We hypothesized that part of POL5551’s additive effects on chemotherapy could be explained by the displacement of cancer cells from chemoprotective niches in the tumor microenvironment. When administered shortly before and after eribulin, POL5551 treatment further reduced bone tumor burden. Our study suggests that combining CXCR4 antagonism to chemotherapy may be beneficial to treat TNBC patients, and provides supporting evidence to a newly initiated clinical trial with the CXCR4 antagonist POL6326 (ClinicalTrials.gov: NCT01837095).

Materials and Methods
Patient clinical trial information
A total of 120 patients with newly diagnosed stage II–III breast cancer were enrolled on an open label randomized single-blind phase II trial (NCT00242203) to assess the efficacy of zoledronic acid (ZA) in reducing DTCs and improving bone health. Eighty-one patients who had evaluable data for both bone marrow DTC and primary tumor gene-expression profiling were chosen for this study (Supplementary Table S3). Exclusion criteria included previous history of malignancy, Easter Cooperative Oncology Group (ECOG) score of 2 or higher, and evidence of distant metastases by CT scan (of pelvis, abdomen, and chest) or 99mTc-MDP bone scan. The complete enrollment criteria and results of the study have been previously described (29, 30). Patients were monitored every 3 to 4 months for the first 2 years after enrollment, then every 6 months until year 5, and then annually thereafter. In the context of this trial, bone marrow aspirates were collected from each anterior iliac crest before treatment initiation, and DTC identified by cytokeratin staining as previously described (31). Patients were considered to be positive for DTC (DTC+), if at least one CK-positive cell was isolated from either BM aspirate (31). Gene-expression profiling was performed on the tumor biopsies of 81 of these patients (GEO accession #: GSE71258). A cutoff value for CXCR4 expression was selected using the dedicated Cutoff Finder application (http://molpath.charite.de/cutoff/; ref. 32) to analyze the entire set of 81 patients. On the basis of the distribution of primary tumor CXCR4 expression data, a cutoff value (10250) was determined: CXCR4 expression signal above 10,250 was defined as CXCR4High (10 patients), whereas below 10,250 was defined as CXCR4Low (28 patients). The overall survival (OS) by Kaplan–Meier curve and log-rank test was analyzed between CXCR4High and CXCR4Low patients. To investigate the association between CXCR4 expression and micrometastases at diagnosis, we then compared frequencies of DTCpos, if at least one CK-positive cell was isolated from either BM aspirate (31). Gene-expression profiling was performed on the tumor biopsies of 81 of these patients (GEO accession #: GSE71258). A cutoff value for CXCR4 expression was selected using the dedicated Cutoff Finder application (http://molpath.charite.de/cutoff/; ref. 32) to analyze the entire set of 81 patients. On the basis of the distribution of primary tumor CXCR4 expression data, a cutoff value (10250) was determined: CXCR4 expression signal above 10,250 was defined as CXCR4High (10 patients), whereas below 10,250 was defined as CXCR4Low (28 patients). The overall survival (OS) by Kaplan–Meier curve and log-rank test was analyzed between CXCR4High and CXCR4Low patients. To investigate the association between CXCR4 expression and micrometastases at diagnosis, we then compared frequencies of DTCpos in CXCR4High versus CXCR4Low patients, and tested the association by Fisher exact test and OR. To analyze the effect of CXCR4 on survival, we considered the gene-expression profiling and DTC status of the 38 of 81 patients that were assigned to the placebo arm of the trial. Of these, 16 patients had bone marrow micrometastases. DTCpos (20 patients) were divided into CXCR4High (n = 5) and CXCR4Low (n = 15) and analyzed for OS by Kaplan–Meier curve analysis and log-rank test.

Cell lines and reagents
The 4T1-FL-GFP BALB/c murine breast cancer cell line was generously provided by Dr. David Piwnica-Worms (The University of Texas MD Anderson Cancer Center, Houston, TX) and modified to coexpress firefly luciferase and GFP as previously described (33).
The bone metastatic MDA-MB-231 variant (34) was a kind gift of Dr. T. Guise (Indiana University, Bloomington, IN). CXCL12-CGLuc, NGLuc-CXCR4, and NGLuc-CXCR7 MDA-MB-231 cells were generated as previously described (35).

Cells were cultured at subconfluence in DMEM + 10% FBS + 0.5% penicillin–streptomycin (pen/strep). A disulfide bond was installed on the resin, and macrocyclization was performed in solution. The peptide was purified by preparative reverse-phase HPLC. Conversion into acetate salt was performed using an ion-exchange resin, and the final preparation of POL5551 was recovered by lyophilization. In mice, POL5551 was well tolerated when exposed at intravenous bolus doses up to 120 mg/kg, with little effect on blood pressure, autonomic nervous, hepatic or cardiac tissues, and no effect on mortality during subsequent 3 days after dosing was observed (27). The plasma half-life of POL5551 in mice after subcutaneous administration is about 30 minutes (26), the exposure profile following subcutaneous administration of a single 30-mg/kg dose to mice is provided in Supplementary Fig. S1B. For in vitro experiments, POL5551 was dissolved in PBS to desired concentration. For in vivo studies, POL5551 (20 mg/kg) was diluted in saline and administered by subcutaneous injection.

Eribulin (trade name: HALAVEN) was purchased from Eisai Co. Eribulin was diluted in PBS in vitro to desired concentration. For in vivo studies, eribulin was diluted in saline and administered by i.v. injection once a week at 0.1 mg/kg for primary mammary fat pad therapy and 0.2 mg/kg for metastatic therapy.

**Split luciferase assay**

For the split-luciferase assay, CXCL12-CGLuc or unfused CGLuc MDA-MB-231 cells (2 × 10^4 cells per well in the 96-well plate) were cocultivated overnight with NGLuc-CXCR4 or NGLuc-CXCR7 MDA-MB-231 cells in DMEM with 0.5% FBS/0.3% penicillin/streptomycin, followed by incubation with indicated concentrations of POL5551 for 6 hours. Bioluminescence from Gausia luciferase complementation was measured 4 hours later using the Biolux Gaussia Luciferase Assay Kit (New England Biolabs) according to the manufacturer’s protocol.

**MTT assay**

The MTT assay was performed as described previously (37).

**Scratch wound assay**

MDA-MB-231 cells (10^5 cells/well in 24-well plate) were seeded to form a confluent monolayer. After overnight serum starvation (0.5% FBS), a wound gap was created by scratch with a pipette tip and POL5551 (0.1–5 µmol/L) was added. Images of cells were taken with a Nikon Eclipse TE300 inverted microscope connected to a MagnaFire camera model 59802 (Optronics), as previously described (38). The extent of gap closure was measured after 24 hours using ImageJ (NIH).

**Survival assay**

To test for survival, MDA-MB-231 cells were plated to 6-well ultra-low attachment plates at a cell density of 5 × 10^5 per well in 0.5% FBS DMEM. After 48 hours of incubation with SDF-1 (12.5 ng/mL and 50 ng/mL) and in the presence or absence of POL5551 (8 µmol/L), 1:10,000 aliquot of the cells were plated to 6-well plates and grown in 10% FBS DMEM for a week. Cells were fixed in 10% buffered formalin and stained with 0.5% crystal violet dissolved in 1% SDS. Cell density was quantified by measuring the absorbance at 570 and 630 nm by a plate reader (BioTek; ref. 39).

**Animal studies**

BALB/c and NOD-scid-IL2R gammadnull (NSG) mice were obtained from The Jackson Laboratory. Animals were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University (St. Louis, MO). All animal experiments were approved by the Washington University Animal Studies Committee.

For xenograft experiments, 6- to 8-week-old female NSG mice were inoculated with 5 × 10^5 MDA-MB-231 cells in Matrigel (BD Biosciences) in the #9 mammary fat pad to generate orthotopic breast tumors. As an experimental model of bone metastasis, 1 × 10^5 4T1 or MDA-MB-231 cells were injected into the left cardiac ventricle, as previously described (40).

In neoadjuvant–adjuvant regimens, POL5551 was administered at a dose of 20 mg/kg s.c. twice a day from day 7 as a monotherapy, or from day 10 in combination with eribulin. Eribulin was administered on days 10, 17, and 24 (0.1 mg/kg, i.v.). For the “framing dosing” experiment, on day 10, POL5551 (20 mg/kg) was administered s.c. 4 hours before, 4 hours after, and again 18 hours after chemotherapy with eribulin (0.2 mg/kg, i.v.). A simulated exposure profile following administration of POL5551 using this dose regimen is shown in Supplementary Fig. S1C. Vehicle-treated controls received saline solution (i.v. or s.c. as appropriate, see individual experiments for details).

**Bioluminescence imaging**

Bioluminescence imaging was performed using an IVIS 100 device (Caliper Life Sciences), as previously described (40).

**Microcomputed tomography**

Tibiae and femurs were removed post-mortem, scanned in a 17-mm holder using microcomputed tomography (µCT-40; Scanco Medical), and evaluated as described previously (38).

**Radiography**

Osteolytic lesion was imaged by an X-ray imaging system (Faxitron). Quantification of the osteolytic lesion area was completed using Image-Pro Plus (MediaCybernetics).

**Complete blood count**

Whole blood was analyzed on a Hemavet Automated Coulter Counter (CBC Tech).

**Immunohistochemistry**

Immunohistochemistry staining was completed by a routine ABC method according to protocol (available online). Antigen retrieval was by the microwave heating method with 10 mmol/L sodium citrate buffer (pH, 6.0). The anti-human keratin 18
Statistical analysis

Experiments were analyzed using a two-tailed Student's t test (2 groups), one-way ANOVA (>2 groups or repeated measures), or two-way ANOVA (two variables, P value refers to the interaction) using Prism® (GraphPad Software, Inc.). Results were considered to reach significance at \( P \leq 0.05 \) and are indicated with asterisks (**, \( P \leq 0.01 \); ***, \( P \leq 0.001 \)). Data are presented as mean values; error bars represent SEM. Mouse weight changes are presented as mean and range.

Results

High CXCR4 expression associated with high rate of metastases even in patients without detectable bone marrow DTCs

The presence of microscopic DTC in bone marrow is an independent poor prognostic factor for patients with localized breast cancer (18). As CXCR4 also correlates with metastatic potential (41, 42), we investigated the prognostic implications of CXCR4 expression in the primary tumor and the presence of bone marrow DTC, using gene-expression profiling of primary breast tumors from patients enrolled in our previous phase II trial (38 evaluable patients). The ZA treatment arm was not used because it has been previously reported that treatment of ZA can increase disease-free survival in patients with breast cancer (44–46). Eighteen of 38 (47.3%) of these patients had detectable DTCs in the bone marrow at diagnosis. We first evaluated the impact of CXCR4 expression in the primary tumor (Fig. 1A and Supplementary Fig. S2) on survival, and found that high CXCR4 expression was associated with poor survival, consistent with previous reports (47), and with a higher incidence of metastases (Table 1) to bone, lung, and liver (60%), than CXCR4low patients (25%). Importantly, metastases took over five times longer to develop in the CXCR4low expression patients (median of 55 months from diagnosis) compared with those in the CXCR4high patients (median of 10 months). We next evaluated CXCR4 expression in the DTC-negative population. We found that DTCpos/CXCR4high patients had a significantly lower survival (**, \( P = 0.005 \); Fig. 1B) than DTCneg/CXCR4low. These data suggest that low CXCR4 expression and the absence of bone marrow DTC define a highly favorable prognosis group in patients with locally advanced, stage II and III breast cancer. Because we found that high CXCR4 expression was associated with metastasis and poor prognosis even in patients with no detectable bone marrow DTC, we hypothesized that blocking CXCR4 activity may reduce both visceral and bone metastases.

CXCX4-selective antagonist POL5551 decreases breast cancer cell migration and promotes anoikis

We evaluated a novel fully synthetic cyclic peptide CXCR4 antagonist, POL5551, for effects on breast cancer cell viability and migration. Breast cancer cells can express both of the CXCL12/SDF-1 receptors, CXCR4 and the alternative inhibitory receptor CXCR7 (48). To confirm POL5551’s specificity for CXCR4 over CXCR7, we used a complementation-imaging model (35), in which the N-terminal portion of the Gaussia luciferase (GLuc) is fused to CXCR4 or CXCR7 whereas the C-terminal portion of GLuc is fused to the ligand SDF-1. In the presence of coelenterazine, reconstitution of GLuc and subsequent light production serves as a quantitative measure of receptor-ligand binding. POL5551 bioactivity (Supplementary Fig. S3A). POL5551 inhibited luciferase activity at low nanomolar concentration in human MDA-MB-231 breast cancer cells expressing GLuc-engineered CXCR4, but not GLuc-CXCR7, demonstrating specific bioactivity against CXCR4 and not CXCR7 (Fig. 2A).

We investigated the bioactivity of POL5551 on breast cancer cell viability and migration, because the activation of the CXCR4 receptor with ligand SDF-1 mediates many cellular functions, including chemotaxis and survival (1). Under standard culture conditions, neither the chemokine SDF-1 nor the CXCR4
antagonist POL5551 had significant effects on MDA-MB-231 viability (Fig. 2B). However, when cultured in anoikis-inducing suspension conditions (0.5% FBS and ultra-low attachment plates), SDF-1 promoted the survival of MDA-MB-231 cells in a concentration-dependent manner, and POL5551 significantly decreased the survival of tumor cells in suspension (Fig. 2E and F). Others and our own data have shown that CXCR4 antagonists, such as AMD3100, can block SDF-1–mediated migration of breast cancer cells (Supplementary Fig. S3B; ref. 49). In a scratch migration assay, POL5551 decreased chemotactic migration of SDF-1–expressing MDA-MB-231 cells in a concentration-dependent manner (Fig. 2C and D). These data show that POL5551 had little direct bioactivity on breast cancer cell viability, but inhibited SDF-1–dependent migration and resistance to anoikis.

CXCR4 antagonist POL5551 decreases metastasis but has little effect on primary tumor growth

Because POL5551 decreased CXCR4-mediated migration and tumor cell survival specifically in suspension, we evaluated the effect of POL5551 on local recurrence and distant metastasis. Orthotopic mammary fat pad (MFP) MDA-MB-231 tumors were established in immunocompromised NSG mice. POL5551 monotherapy was initiated when tumors were palpable (day 7) and continued until study termination. Primary MFP tumors were surgically resected by mastectomy on day 21 and mice were followed for the development of local recurrence and distant metastasis by bioluminescent imaging (BLI; Fig. 3A). We confirmed that the POL5551 dosing used in this study was sufficient to mobilize hematopoietic cells into the blood (22) and increase WBC counts (Supplementary Fig. S4). Although POL5551 did not reduce primary tumor growth (Fig. 3B and C and Supplementary Fig. S4), distant metastases to chest and leg bones, as assessed by BLI, were significantly decreased in POL5551-treated mice (Fig. 3D, E, and H). Metastasis to the liver was examined by both ex vivo BLI and histology; however, no significant differences were found between the treatment groups (Fig. 3I; Supplementary Fig. S4B and S4C). Micrometastatic tumors in the lung, as quantified

Table 1. Metastasis profiling of CXCR4High and CXCR4Low patient groups

<table>
<thead>
<tr>
<th>Percentage of patients with distant metastasis</th>
<th>First metastasis detected (median months; range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4High</td>
<td>60%; 6/10</td>
</tr>
<tr>
<td>CXCR4Low</td>
<td>25%; 7/28</td>
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*P < 0.005; metastases detected in bone, liver, lung, and other organs.

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**Figure 2.** CXCR4-selective antagonist POL5551 decreases breast cancer cell migration and promotes anoikis in vitro. A, complementation imaging. Binding of SDF-1-CGLuc to NGLuc-CXCR4 or NGLuc-CXCR7 reconstitutes Gluc, producing light as a quantitative measure of ligand/receptor binding. SDF-1-CGLuc MDA-MB-231 cells were coincubated overnight with NGLuc-CXCR4 or NGLuc-CXCR7 MDA-231 cells, followed by incubation with various concentrations of POL5551 for 6 hours (* * *; P < 0.001). B, MDA-231 cells were incubated with various concentration of POL5551 for 48 hours and cell viability was assessed by an MTT assay. C and D, a wound gap was created by scratch in confluent monolayers of SDF-1–secreting MDA-231 cells. Gap closure was measured after 24 hours of treatment with various concentrations of POL5551 (*; P < 0.05; ***; P < 0.001). E and F, cell survival after forced suspension culture with SDF-1 (12.5 and 50 ng/mL) and POL5551 (8 μmol/L). Cell colonies were stained with crystal violet. The absorbance of crystal violet dye dissolved with 1% SDS was measured by plate reader at 570 to 630 nm (*; P < 0.05; **; P < 0.01; ***; P < 0.001).
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histologically by measurement of human cytokeratin-18 (CK-18)–positive tumor cells, were significantly decreased in POL5551-treated mice compared with vehicle controls ($P = 0.001$; Fig. 3F and G).

To confirm POL5551 inhibition of metastasis in the context of a fully competent immune system and in a metastasis model, 4T1 murine breast cancer cells were inoculated into the left cardiac ventricle of BALB/c mice to establish disseminated metastatic disease. Mice were treated with either POL5551 or saline from day 3 to the end of the study (Fig. 4A). Histomorphometric analyses showed that POL5551 treatment significantly decreased tumor metastases into the kidney ($P = 0.03$; Fig. 4D and E), and micro-CT analysis showed a significant reduction in tumor-associated bone loss ($P = 0.03$; Fig. 4F and G). These findings were associated with a nonsignificant trend toward reduction of bone and whole-body tumor burden when measured by BLI (Fig. 4B and C).

Together, these data show that POL5551 as a single agent had little direct effect on primary tumor burden, but decreased metastases and metastatic tumor burden in two breast cancer models.

**Combination of eribulin with POL5551 in the neoadjuvant and metastatic settings improves survival compared with eribulin alone**

In addition to investigating the effect of POL5551 administered as a monotherapy, we also evaluated the effect of POL5551 in combination with cytotoxic chemotherapy. Eribulin is a microtubule-inhibiting chemotherapeutic agent, with strong activity in patients with metastatic breast cancer that have failed both

Figure 3.

**CXCR4 antagonist POL5551 decreases metastasis, but has little effect on primary MFP tumor growth in a xenograft NSG model.** A, MDA-MB-231 mammary fat pad orthotopic model schema: MDA-MB-231 tumor cells were injected to the mammary fat pad of mice. Treatment with POL5551 (20 mg/kg, s.c., twice daily, $n = 3$) or saline ($n = 5$) commenced on day 8 when the tumor became palpable and continued until the end of study. B and C, primary MFP tumor growth as measured by bioluminescence imaging, representative image showing the primary tumors on day 21. D and E, distant metastasis to the chest as measured by bioluminescence imaging, representative image showing the distant metastases on day 41 ($P < 0.05$). F, human CK-18 staining of MDA-MB-231 tumor cells in lung tissue. G, quantification of CK18-positive cells in lung tissue; $**$, $P < 0.001$; H, ex vivo BLI of bone metastases ($P < 0.05$).
anthracycline and taxane chemotherapies (50–52). Eribulin decreased MDA-MB-231 viability in vivo (Supplementary Fig. S5A–S5C) and prolonged mice survival in a dose-dependent manner (Supplementary Fig. S5D). However, the higher and most effective doses were associated with greater chemotherapy-induced weight loss (Supplementary Fig. S5E). Aiming at identifying a combination regimen that would reduce toxicity and reveal synergistic effects with CXCR4 inhibition, we selected a submaximal effective dose of eribulin (0.1 mg/kg) that yielded 50% primary breast tumor reduction when used as single agent (Supplementary Fig. S5F). After orthotopic MFP tumors were established, neoadjuvant treatment was initiated on day 10, and mastectomy was performed when all tumors reached 1 cm³, so that metastasis-free survival after surgery could be fairly evaluated for all treatment groups. Because of the antitumor effect of eribulin on primary growth, chemotherapy-treated mice required additional time (roughly 5 extra days) to reach primary tumor mass equivalent to the vehicle group. All doses of eribulin were administered before mastectomy, whereas POL5551 administration continued after mastectomy. As expected, eribulin decreased the primary tumor growth rate, whereas POL5551 had no direct or additive effects on primary MDA-MB-231 MFP tumors (Fig. 5A–C). In contrast, combination POL5551 plus eribulin treatment significantly decreased distant metastases to the chest and bone as measured by BLI (Fig. 5D and E). Finally, POL5551, in combination with eribulin, prolonged survival (58 days) compared with eribulin alone (51 days) or vehicle controls (45 days; Fig. 5F). Breast cancer cell interactions with the stromal environment can contribute to resistance to cytotoxic chemotherapy (11). We hypothesized that antagonizing CXCR4 with POL5551 for short...
periods of time around each dose of chemotherapy—a "framing dose" strategy—could disrupt CXCR4/SDF-1–mediated tumor–stromal cell interactions and potentially enhance chemotherapy efficacy. To test this hypothesis, metastases were established by intracardiac inoculation in NSG mice, and on day 10 mice were treated as following: A, vehicle (n = 4): saline (s.c. and i.v.); B, eribulin alone (n = 5): eribulin (0.2 mg/kg, i.v.) alone and saline (s.c.); C, combination treatment (n = 5): POL5551 (20 mg/kg, s.c.) was administered 4 hours before eribulin and 4 and 18 hours after eribulin (0.2 mg/kg, i.v.) chemotherapy. B and C, BLI of tumor burden in liver and bone (\( P < 0.05; \nonumber P < 0.001 \)). D, G, representative X-Ray images of tumor-associated osteolytic lesion in leg bone. E, quantification of osteolytic lesion area. F, representative image showing extent of metastatic burden in liver and leg bone on day 17 (\( P < 0.05); \nonumber **, P < 0.01 \)). G, mouse weight lost between days 9 and 17. Statistical analyses are as compared with vehicle treatment (ns, \( P > 0.05); **, \nonumber P < 0.01 \)).

Figure 6. Reduction of bone tumor burden: POL5551 in combination with eribulin is superior to eribulin alone. A, schema of bone metastasis model utilizing "framing dose" strategy: MDA-MB-231 bone metastases were established by intracardiac inoculation in NSG mice, and on day 10 mice were treated as following: A, vehicle (n = 4): saline (s.c. and i.v.); B, eribulin alone (n = 5): eribulin (0.2 mg/kg, i.v.) alone and saline (s.c.); C, combination treatment (n = 5): POL5551 (20 mg/kg, s.c.) was administered 4 hours before eribulin and 4 and 18 hours after eribulin (0.2 mg/kg, i.v.) chemotherapy. B and C, BLI of tumor burden in liver and bone (\( P < 0.05; \nonumber P < 0.001 \)). D, representative X-Ray images of tumor-associated osteolytic lesion in leg bone. E, quantification of osteolytic lesion area. F, representative image showing extent of metastatic burden in liver and leg bone on day 17 (\( P < 0.05); \nonumber **, P < 0.01 \)). G, mouse weight lost between days 9 and 17. Statistical analyses are as compared with vehicle treatment (ns, \( P > 0.05); **, \nonumber P < 0.01 \)).

Discussion

Our work shows that high CXCR4 expression in primary breast cancer tissue defined a subgroup of patients that, despite the lack of bone marrow DTCs and other favorable characteristics, had high and early incidence of metastasis and poor survival. It has been proposed that CXCR4 antagonism could directly affect tumor cell survival at two stages of metastasis. Early in progression, CXCR4 inhibitors may inhibit tumor cell homing from the primary tumor to metastatic sites expressing high SDF-1. After tumor cells have disseminated to secondary sites and are protected...
from chemotherapy, CXCR4 antagonists may mobilize tumor cells into the peripheral blood, as occurs with HSCs. Once in circulation, tumor cells are no longer protected by interactions with stromal cells in the HSC niche and could become more susceptible to therapeutic agents, including chemotherapy, resulting in reduced metastases and tumor burden. We hypothesized that inhibition of CXCR4 could decrease metastasis. We found that the CXCR4 antagonist POL5551 blocked breast cancer cell migration and resistance to anoikis in vitro, and reduced breast cancer metastasis despite having little effect on primary tumor growth in vivo. When administered in combination with eribulin chemotherapy, POL5551 decreased metastases and improved survival compared with chemotherapy alone. We hypothesized that POL5551 could “mobilize” tumor cells from their stromal and vascular niches, and heighten sensitivity to chemotherapy. We found that only 3 doses of POL5551 given just before and just after eribulin further decreased metastatic tumor growth compared with chemotherapy alone. Taken together, our findings suggest that POL5551 is capable of disrupting interactions between cancer cells and their environment that contribute to their metastatic potential and resistance to chemotherapy.

It has been previously reported that POL5551 is a potent mobilization agent that can mobilize hematopoietic stem and progenitor cells from bone marrow niche with greater efficiency than AMD3100 (plerixafor; ref. 26). In confirmation of this, the POL5551 dose we used in vivo rapidly mobilized leukocytes into the circulation (Supplementary Fig. S6). During metastatic dissemination, CXCR4-expressing cancer cells are thought to use the HSC-specific CXCR7 to preferentially home to SDF-1–rich sites, such as bone, lymph nodes, and lungs. In a mouse metastasis model, Shiozawa and colleagues (8) demonstrated that prostate cancer cells can directly compete with HSC to occupy the HSC niche in the bone marrow through the CXCR4–SDF-1 pathway, and that administration of the CXCR4 antagonist AMD3100 mobilized DTCs into the peripheral blood. Stromal environments in the bone or lung have been shown to be protective and contribute to chemoresistance to cytoxic therapies (11); mobilization of tumor cells from the protective microenvironment may, therefore, increase their susceptibility to cytoxic chemotherapy.

Two main chemokine receptors capable of SDF-1 binding have been found to play some role in breast cancer: CXCR4 and CXCR7. Although CXCR7 may play a role in transendothelial migration (53), Hernandez and colleagues (54) demonstrated that CXCR7 overexpression enhanced tumor growth and vascularization, but reduced invasiveness and metastases. Specifically designed to selectively target CXCR7, POL5551 showed no effect on SDF-1 binding to overexpressed CXCR7 by a Guassia luciferase complementation assay in breast cancer cells. Moreover, in agreement with previously published datasets (55), we did not detect CXCR7 expression in MDA-MB-231 cells. We therefore consider it unlikely that CXCR7 might play a major role in the current set of experiments.

Targeting a physiologic characteristic of the cell of origin, together with a protumor gain-of-function, a number of clinical trials have used CXCR4 antagonists in hematologic malignancies, such as multiple myeloma, acute myelogenous leukemia, and lymphoma [14]. In solid tumors, CXCR4 overexpression has been associated with poorer prognosis, and functional studies have shown that CXCR4 plays a critical role in the metastatic dissemination of solid tumor cells (56, 57). In accordance with this hypothesis, we found that, even in patients with no bone marrow micrometastases (DTC negative), high CXCR4 expression in the primary tumor was associated with earlier metastases and poorer prognosis.

In the host, the CXCR4 gene plays an important role in a number of cell types and physiologic processes, such as HSCs and leukocytes trafficking. In our previous work, we have shown that targeted gene disruption of host hematopoietic CXCR4 resulted in enhanced osteoclastogenesis and consequent tumor growth in bone, although no differences were found in lung and subcutaneous tumor burden (58). By pharmacologically targeting the CXCR4 receptor with an antagonist, especially with an intermittent regimen, CXCR4 expression by normal tissues would not be constantly disrupted, thus potentially having less sustained effects on leukocytosis. Indeed, mice did not show overt hematopoietic toxicity as measured by serial blood counts following a framing dose of POL5551 in combination with eribulin (Supplementary Fig. S7). It should be noted that not all HSCs and/or DTCs are mobilized after a single dose of a CXCR4 antagonist, so repeated dosing may have higher efficacy. In this study, we found that POL5551 did not induce bone loss and in fact resulted in decreased tumor-associated bone loss. This is likely due to decreased tumor burden in the bones, which is consistent with the reduction of metastatic tumor burden in extra-skeletal sites (lungs and kidneys), and with the observed effects on the migration and survival of isolated tumor cells. We also anticipate that the pharmacologic dosing of POL5551 would not affect osteoclasts to the same extent as complete genetic disruption.

Among breast cancers, the TNBC subtype has the most aggressive phenotype with a poorer prognosis [59–61]. Unlike ER– or HER2–positive breast cancers, which benefit from targeted therapies, the treatment of TNBC patients largely relies on surgery and systemic chemotherapy. The microtubule inhibitor eribulin has shown significant survival benefit in patients with metastatic breast cancer refractory to anthracyclines and taxanes (51, 62). As with most antiproliferative drugs, in addition to the killing effect of chemotherapeutic agents, eribulin is also antiangiogenic and antiproliferative [63]. The CXCR4 antagonist POL5551 significantly improved survival, but to the expense of greater treatment-associated weight loss due to chemotoxicity. In addition, optimal eribulin dosing reduced tumor burden to a point that was below the level in which our in vivo bioluminescence assays were able to consistently and confidently measure tumor burden, precluding our ability to detect any synergistic effects of POL5551. For these reasons, suboptimal and less toxic dosing of eribulin (0.1–0.2 mg/kg) was used for this preclinical study. In agreement with previous research that has shown CXCR4 inhibition to sensitize tumor cells to chemotherapy (13, 63), we found that combining POL5551 with low-dose eribulin (0.1–0.2 mg/kg) decreased metastatic tumor burden compared with mice administered eribulin alone. Together with other studies in published literature, our preclinical data suggest that combining CXCR4 antagonism and cytoxic chemotherapy could decrease metastasis by mobilizing tumor cells or otherwise disrupting their interactions with chemoprotective stromal environments. In the present study, POL5551 administered just before and after eribulin was sufficient to decrease tumor burden.

Few clinical trials have so far targeted CXCR4 in solid tumors, despite encouraging preclinical studies and associations between expression and prognosis. A phase I study using a peptide CXCR4 antagonist, LY2510924, in patients with advanced cancer was
recently completed in the United States [64]. However, no dedicated breast cancer trial has so far been designed to evaluate CXCR4 antagonism. This study has provided important preclinical rationale and proof-of-principle data to examine the combination of CXCR4 antagonism and eribulin chemotherapy in metastatic breast cancer patients. To translate these findings from bench to bedside, a multicenter phase I clinical trial (*"Dose escalation of POL6326 in combination with eribulin in patients with metastatic breast cancer," ClinicalTrials.gov identifier: NCT01837095, sponsored by Polphor), has been initiated. This study will evaluate POL6326, a close analogue of POL5551, which differs at only a single amino acid, has nearly identical bioactivity profiles, but has slightly more favorable pharmacokinetic properties than POL5551. Here, POL6326 will be evaluated in combination with eribulin in a framing dose sequence similar to what we used in the preclinical studies presented in Fig. 6. This is the first clinical trial in breast cancer patients with a CXCR4 antagonist in combination with chemotherapy. In addition, POL6326 is currently in clinical studies for HSC mobilization. Although the overall magnitude of survival extension in this preclinical study was modest (as compared with vehicle, median survival was increased 12% with eribulin alone and 22% with eribulin + POL555), it is important to note that a suboptimal dose of eribulin was used for this study. This was necessary to permit a level of tumor growth sufficient for detection by in vivo bioluminescent assays. Thus, when optimal doses of eribulin are used, as in the ongoing phase I clinical trial above, additional benefit may be seen. Our preclinical data suggest that CXCR4–SDF-1 interactions play a critical role during breast cancer metastasis, and that CXCR4 antagonism administered in sequence with chemotherapy can disrupt tumor cell spread. Future studies to evaluate the impact of CXCR4 inhibition on tumor cell dormancy, tumor microenvironment, and immune modulation will be important to better define which patients to treat and how to combine CXCR4 antagonist with ongoing cancer therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Xiang, M.A. Hurchla, X. Su, G.J. Douglas, T. Pluard, E. Chevalier, K. Dembowsky, K.N. Weilbaecher

Development of methodology: J. Xiang, M.A. Hurchla, X. Su, K.E. Luker, G.D. Luker, K.N. Weilbaecher

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Xiang, M.A. Hurchla, F. Fontana, S.R. Amend, A.K. Esser, K.E. Luker, R.L. Aft, K.N. Weilbaecher

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Xiang, M.A. Hurchla, F. Fontana, S.R. Amend, G.J. Douglas, M. Bauer, J. Zimmermann, K. Dembowsky, K.N. Weilbaecher

Writing, review, and/or revision of the manuscript: J. Xiang, M.A. Hurchla, F. Fontana, S.R. Amend, A.K. Esser, G.J. Douglas, T. Pluard, F.O. Ademuyiwa, B. Romagnoli, G. Tufﬁn, E. Chevalier, G.D. Luker, M. Bauer, J. Zimmermann, K. Dembowsky, K.N. Weilbaecher

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Xiang, X. Su, J. Zimmermann, K.N. Weilbaecher

Study supervision: J. Xiang, K.N. Weilbaecher

Other (review and discussion on concept, conduct, and data analysis): M. Bauer

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Molecular Cancer Therapeutics

CXCR4 Protein Epitope Mimetic Antagonist POL5551 Disrupts Metastasis and Enhances Chemotherapy Effect in Triple-Negative Breast Cancer

Jingyu Xiang, Michelle A. Hurchla, Francesca Fontana, et al.

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