VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Multifaceted C-X-C Chemokine Receptor 4 (CXCR4) Inhibition Interferes with Anti—Vascular Endothelial Growth Factor Therapy—Induced Glioma Dissemination

Jean-Pierre Gagner,^{*†} Yasmeen Sarfraz,^{*†} Valerio Ortenzi,^{*†} Fawaz M. Alotaibi,^{*†} Luis A. Chiriboga,[†] Awab T. Tayyib,^{*†} Garry J. Douglas,[‡] Eric Chevalier,[‡] Barbara Romagnoli,[‡] Gérald Tuffin,[‡] Michel Schmitt,[‡] Guillaume Lemerrier,[‡] Klaus Dembowski,[‡] and David Zagzag^{*†§¶||}

From the Microvascular and Molecular Neuro-Oncology Laboratory,^{*} the Division of Neuropathology,[§] Department of Pathology,[†] and the Department of Neurosurgery,[¶] New York University Langone Medical Center, New York, New York; Polyphor Ltd.,[‡] Allschwil, Switzerland; and the New York University Langone Laura and Isaac Perlmutter Cancer Center,^{||} New York, New York

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Address correspondence to
David Zagzag, M.D., Ph.D.,
Department of Pathology, NYU
Langone Medical Center, 550
First Ave, New York,
NY 10016. E-mail: david.zagzag@nyumc.org.

Resistance to antiangiogenic therapy in glioblastoma (GBM) patients may involve hypoxia-induced expression of C-X-C motif chemokine receptor 4 (CXCR4) on invading tumor cells, macrophage/microglial cells (MGCs), and glioma stem cells (GSCs). We determined whether antagonizing CXCR4 with POL5551 disrupts anti—vascular endothelial growth factor (VEGF) therapy—induced glioma growth and dissemination. Mice bearing orthotopic CT-2A or GL261 gliomas received POL5551 and/or anti—VEGF antibody B20-4.1.1. Brain tissue was analyzed for tumor volume, invasiveness, hypoxia, vascular density, proliferation, apoptosis, GSCs, and MGCs. Glioma cells were evaluated for CXCR4 expression and polymorphism and POL5551's effects on CXCR4 ligand binding, cell viability, and migration. No CXCR4 mutations were identified. POL5551 inhibited CXCR4 binding to its ligand, stromal cell—derived factor-1 α , and reduced hypoxia- and stromal cell—derived factor-1 α —mediated migration dose—dependently but minimally affected cell viability. In vivo, B20-4.1.1 increased hypoxic foci and invasiveness, as seen in GBM patients receiving anti-VEGF therapy. Combination of POL5551 and B20-4.1.1 reduced both glioma invasiveness by 16% to 39% and vascular density compared to B20-4.1.1 alone in both glioma models. Reduced populations of GSCs and MGCs were also seen in CT-2A tumors. POL5551 concentrations, evaluated by mass spectrometry, were higher in tumors than in neighboring brain tissues, likely accounting for the results. Inhibition of CXCR4-regulated tumoral, stem cell, and immune mechanisms by adjunctive CXCR4 antagonists may help overcome antiangiogenic therapy resistance, benefiting GBM patients. (*Am J Pathol* 2017, 187: 2080–2094; <http://dx.doi.org/10.1016/j.ajpath.2017.04.020>)

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J.-P.G. and Y.S. contributed equally to this work.

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Current address of Y.S., Department of Radiation Oncology, Weill Cornell Medical College, New York, NY; of V.O., Division of Pathology, University Hospital of Pisa and University Hospital of Cagliari, Pisa, Italy; of F.M.A., Division of Neurosurgery, The Ottawa Hospital, University of Ottawa, Ontario, ON, Canada.

Despite the promising initial radiographic and progression-free survival responses of patients with glioblastoma (GBM) to antiangiogenic therapy, most patients who receive the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab (BEV; Avastin; Genentech, Inc., South San Francisco, CA) fail to demonstrate an overall survival benefit and develop disseminated relapse^{1–3} associated with vascular remodeling and metabolic adaptation to tumor hypoxia.^{4–6} Unlike cancers that develop resistance to therapy from genetic mutation of drug targets, gliomas either possess intrinsic resistance or acquire secondary resistance to BEV by bypassing antiangiogenic therapy (while VEGF signaling remains inhibited) through several mechanisms,^{1,2} leading to further tumor growth and dissemination. These mechanisms include upregulation of alternate proangiogenic factors by tumor or stromal cells [eg, stromal cell–derived factor (SDF)-1 α (CXCL12) and C-X-C motif chemokine receptor 4 (CXCR4)],⁷ accumulation of tumor-initiating glioma stem cells (GSCs),⁸ and infiltration of glioma-associated myeloid cells that promote tumor growth, migration, and immunosuppression.^{8–10} Combining angiogenesis inhibitors with drugs targeting these parallel mechanisms of resistance may provide more durable antiangiogenic efficacy and improve patient outcome.^{1,2,11,12} Recent clinical trials with BEV in combination with other therapies have renewed interest in targeting angiogenesis in GBMs. For the first time, an overall survival benefit was observed with BEV combined with the alkylating agent lomustine in patients with recurrent GBM^{13,14} or with temozolomide and radiotherapy in patients with proneural GBM in the AVAglio trial.¹⁵ Improved survival was also demonstrated in a subset of newly diagnosed GBM patients who showed increased tumor blood perfusion after VEGF receptor kinase inhibitor cediranib and chemoradiation.¹⁶

The SDF-1 α receptor CXCR4, which is overexpressed in numerous human cancers,¹⁷ including glioma,^{18,19} has been shown to promote tumor growth, invasion, angiogenesis, metastasis, relapse, and therapeutic resistance.^{20,21} In GBMs, BEV may promote invasion by hypoxic glioma cells into the brain, at least in part, through mechanisms that involve hypoxia-induced expression of CXCR4 on invading tumor cells and migration of these cells toward central nervous system neurons and blood vessels, which are known to express SDF-1 α .^{22,23} This effect may be mediated by enhanced phosphorylation of proline-rich tyrosine kinase 2,²⁴ a signaling effector of CXCR4-regulated glioma chemotaxis.¹⁸ Similar mechanisms have been implicated in the migration of CXCR4-expressing GSCs^{8,25–27} and the tropism of glioma-associated CXCR4-expressing myeloid cells in regions with heightened glioma invasion.^{8–10} Inhibition of SDF-1 α /CXCR4 signaling has been found to reduce brain tumor growth and/or recurrence,^{28,29} the viability and number of GSCs,^{28,30} and the recruitment of glioma-associated macrophage/microglial cells (MGCs).^{9,10}

We sought to disrupt the growth and dissemination of GBM by targeting CXCR4-regulated mechanisms using a

novel, more potent antagonist. POL5551³¹ belongs to a class of novel and selective CXCR4 antagonists developed by Polyphor, Ltd (Allschwil, Switzerland) using the proprietary protein epitope mimetic (PEM) technology³² and has shown to be markedly more potent (approximately 200-fold) than the bicyclam AMD3100 (plerixafor; Genzyme Corp., Cambridge, MA) in functional assays.³¹ Using two orthotopic immunocompetent murine glioma models previously shown to emulate the human GBM phenotype (eg, finger-like protrusions and single-cell infiltration), CT-2A³³ and GL261,^{23,34} we sought to determine whether POL5551 could disrupt glioma tumor growth and/or invasion triggered by antiangiogenic therapy with anti-murine VEGF neutralizing monoclonal antibody B20-4.1.1 (Genentech, Inc.).³⁵ To evaluate the mechanisms of action of POL5551 in GBMs, its effects on tumor hypoxia, vascular density, proliferation, and apoptosis were examined. Because GBMs have been reported to contain GSCs that express CXCR4^{26,27,30} and are infiltrated by tumor-associated myeloid cells that express CXCR4,^{9,36} we also assessed the tumor content of GSCs and glioma-associated MGCs. Our studies are supported by analyses of glioma *CXCR4* gene polymorphism and POL5551 pharmacokinetics as well as *in vitro* studies of POL5551 binding to CXCR4 and its effects on glioma cell viability and migration.

In the present study, using mouse glioma models in which administration of anti-VEGF antibody B20-4.1.1 recapitulated the invasive phenotype seen in human GBM during BEV therapy, we demonstrated, for the first time, that when combined with B20-4.1.1, the CXCR4 antagonist POL5551 reduced glioma invasion and the number of tumor-associated MGCs, known to promote glioma growth and dissemination. In addition, no *CXCR4* mutations were identified in glioma cells that could alter their function or response to treatment.

Materials and Methods

Cell Culture and Pharmaceuticals

Murine CT-2A (a gift from Dr. Thomas Seyfried, Boston College, MA) and GL261 (purchased from the Division of Cancer Treatment and Diagnosis, National Cancer Institute, Frederick, MD) glioma cells were cultured to 80% confluence in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 2 mmol/L glutamine, and 1% penicillin/streptomycin (CT-2A) or 0.25% gentamicin (GL261) at 37°C in a humidified 5% CO₂ incubator. These cell lines were authenticated using mouse short tandem repeat DNA profiling and tested by PCR to be free of *Mycoplasma* contamination (DDC Medical, Fairfield, OH). After trypsinization, cells were resuspended in RPMI 1640 medium (Cellgro) without serum for animal experiments or DMEM with 1% FBS for *in vitro* assays. POL5551

acetate was supplied by Polyphor, Ltd, and B20-4.1.1 antibody by Genentech, Inc.; both drugs were dissolved in phosphate-buffered saline (PBS) before use. Recombinant mouse SDF-1 α and AMD3100 were purchased from R&D Systems Inc. (Minneapolis, MN) and Sigma-Aldrich (St. Louis, MO), respectively.

Animal Experiments

In accordance with the NIH's *Guide for the Care and Use of Laboratory Animals*³⁷ and a protocol approved by the New York University Institutional Animal Care and Use Committee, glioma cells were implanted into the brains of mice, as previously described.²³ Briefly, 10- to 12-week-old female C57BL/6 mice (20 g) (Taconic Biosciences, Germantown, NY) were housed under pathogen-free conditions. After general anesthesia with 90 mg/kg ketamine i.p. and 10 mg/kg xylazine chloride hydrate i.p., mice were secured in a stereotactic frame, and a burr hole was drilled into the skull 0.1 mm posterior to the bregma and 2.3 mm lateral to the midline. CT-2A or GL261 cells (1×10^5) in 2 μ L of RPMI 1640 medium were injected to a depth of 2.35 mm from the cortical surface into the right caudate/putamen. On day 14 after implantation, mice were randomized into four treatment groups ($n = 9$ to 10 per group): i) control, ii) POL5551 (5 mg/kg s.c. twice daily), iii) B20-4.1.1 antibody (5 mg/kg i.p. twice weekly), and iv) combined POL5551 and B20-4.1.1. Mice were examined daily, and only one moribund mouse was excluded. On day 26, 60 mg/kg pimonidazole (Hypoxyprobe, Burlington, MA) was injected i.p. 1 hour before sacrifice to detect foci of tumor hypoxia. Mice were anesthetized and perfused intracardially with PBS, followed by 4% paraformaldehyde; the brains were harvested, placed in cold 4% paraformaldehyde overnight, sliced into 2-mm coronal sections, and embedded in paraffin. Tissue sections (4 μ m thick) were stained with hematoxylin and eosin or immunostained, digitized, and analyzed by image processing or visual scoring, as described below.

Immunohistochemistry

Immunohistochemistry was performed on a Discovery XT platform (Ventana Medical Systems, Tucson, AZ) using Ventana's reagents and detection kits, unless otherwise noted. Sections were immunostained to measure hypoxia (pimonidazole), vascular density (CD105/endoglin), cellular proliferation [phosphohistone H3 (pHH3)],³⁸ apoptosis (cleaved caspase 3), macrophage/microglial cells [ionized calcium binding adaptor molecule 1 (Iba1)],^{39,40} and glioma stem cells (nestin)^{41,42} using primary antibodies diluted in Dulbecco's PBS: mouse monoclonal anti-pimonidazole (1:50 dilution, HP2-100; Hypoxyprobe), goat anti-mouse CD105 (1:200 dilution, AF1320; R&D Systems, Minneapolis, MN), rabbit anti-mouse pHH3 (1:400 dilution, 369A-15; Cell Marque, Rocklin, CA), rabbit anti-mouse cleaved caspase 3 (1:100 dilution, number 9661; Cell

Signaling Technology, Danvers, MA), rabbit anti-mouse Iba1 (1:500 dilution, number 019-19741; Wako Chemicals USA, Richmond, VA), and rabbit anti-mouse nestin tail (1:2000 dilution, PRB-336C; BioLegend, San Diego, CA) antibodies. After deparaffinization and rehydration, antigen retrieval was performed for pHH3 (10 minutes), caspase 3 (10 minutes), pimonidazole (20 minutes), and CD105 (20 minutes) by 1200-W microwave heating in 10 mmol/L sodium citrate, pH 6.0. Iba1 and nestin were processed without epitope retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 4 minutes for all samples. Primary antibodies against pimonidazole, CD105, pHH3, and caspase 3 were incubated overnight, and those against Iba1 and nestin were incubated for 3 and 6 hours, respectively, at room temperature. Pimonidazole was detected with rabbit anti-fluorescein isothiocyanate-horseradish peroxidase antibody (1:150) and incubated for 30 minutes, CD105 with biotinylated horse anti-goat antibody (1:100) for 30 minutes, and pHH3 and caspase 3 with biotinylated goat anti-rabbit antibody (1:200) for 30 minutes (all from Vector Laboratories, Burlingame, CA), followed by application of streptavidin-horseradish peroxidase conjugate. Iba1 and nestin were detected with rabbit anti-horseradish peroxidase antibody (Ventana Medical Systems) and incubated for 8 minutes. Immune complexes were visualized with 3,3'-diaminobenzidine and enhanced with copper sulfate. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with Permunt mounting medium. Appropriate positive and negative tissue controls were included with the study sections.

Image Analysis

All histological sections were digitized (resolution, 0.5 μ m/pixel) using the SCN400 Slide Scanner (Leica Microsystems, Buffalo Grove, IL) and analyzed in a double-blinded manner by at least two independent observers (Y.S., V.O., F.M.A., and/or A.T.T.). Using ImageJ software version 1.47 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>), tumor volume was calculated from tissue sections near the site of tumor cell implantation using the following equation: Tumor Volume = (Length $\times$ Width²)/2, for a prolate ellipsoid, where length and width are the length and width of the tumor, respectively. As the irregularity of the tumor border increases with its invasiveness, the ratio between the perimeter and surface area increases as well. Tumor invasiveness (INV), a unitless value, was calculated by comparing the ratio of the perimeter (P) and surface area (S) of tumors using the following equation: $INV = P / \sqrt{4\pi S}$.⁴³ A noninvasive, spherical tumor would have $INV = 1.0$, whereas a tumor with a highly irregular border made of finger projections and islands of invasive cells would have a high INV value.⁴³ Using ImageJ, the entire tumor perimeter and surface area, including all clusters of tumor cells ($>20 \mu\text{m}^2$), were delineated, measured, and summed for each tumor.

To determine the mitotic rate, pHH3-stained sections were analyzed for positive staining and morphological features of mitotic figures³⁸ after scale setting (100 pixels/ μm), color channel split, and threshold optimization using ImageJ. To determine tumor hypoxia, vascular density, and apoptosis, sections immunostained for pimonidazole, CD105, or caspase 3 were enumerated for the number and area of hypoxic foci, number of positive vessels, and number of apoptotic cells, respectively.²² Finally, to quantify MGCs and GSCs, sections immunostained for Iba1^{39,40} and nestin^{41,42} were enumerated by scoring the percentage of positively stained cells in tumors on a 1 to 4 scale (1 if <1%, 2 if 1% to 10%, 3 if 11% to 50%, and 4 if 51% to 100%). The Iba1 staining pattern of tumors was also described qualitatively as strongly central, central, central and peripheral, peripheral, or strongly peripheral. Discrepancies in scoring between observers were resolved by reaching consensus.

Pharmacokinetics of POL5551

Mice bearing CT-2A or GL261 tumors were randomized into four treatment groups ($n = 4$ to 6 per group) in separate experiments on day 14 after implantation and administered B20-4.1.1 (5 mg/kg i.p. twice weekly) and/or POL5551 [either as a single dose (5 mg/kg s.c. at 0.5, 2, 6, and 24 hours before collection) or repeatedly (5 mg/kg s.c. twice daily for 12 days, with the last doses at 0.5 and 2 hours before collection on day 26 after implantation)]. Blood samples were collected in lithium heparin Microtainer tubes with plasma separators (BD, Franklin Lakes, NJ) and spun at $800 \times g$ at 4°C , and the plasma was stored at -80°C . Brains were harvested and dissected into tumor tissue, brain adjacent to tumor, and contralateral brain tissue. Each piece was weighed, snap frozen in liquid nitrogen, and stored at -80°C . Samples (50 μL) were spiked with a PEM internal standard (P0006582) and extracted with acetonitrile plus 2% formic acid, and the supernatant was evaporated and reconstituted in water/acetonitrile (95:5, v/v) plus 0.2% formic acid. The tissue concentration of POL5551 was determined by reverse phase liquid chromatography (Acquity UPLC BEH C18 sorbent column, 1.7- μm particles, 2.1×100 mm; Waters, Milford, MA), coupled to a mass spectrometer with a positive ion mode electrospray interface (4000 QTRAP; AB Sciex, Framingham, MA), and expressed in ng/mL of plasma or ng/g of tissue.

CXCR4 Gene Sequencing Analysis

After isolation of genomic DNA from CT-2A and GL261 glioma cells using the QIAmp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA), Sanger sequencing of the PCR-amplified products of the *CXCR4* gene locus was performed and analyzed by Genewiz LLC (South Plainfield, NJ). Briefly, the two exons of the *CXCR4* gene of each glioma cell line were individually amplified by PCR using four pairs of primers:

Table 1 List of PCR Primers and Sequencing Primers Used for Sanger Sequencing of the Mouse *CXCR4* Gene

Exon	Primer name	Primer sequence
PCR primers		
Exon 1	CXCR4-Ex1-F	5'- <u>TGGCGGAGGTTCCAACATTG</u> -3'
	CXCR4-Ex1-R	5'- <u>CTCCCCCTATAGCCATGTTTC</u> -3'
Exon 2	CXCR4-Ex2-1-F	5'- <u>TTGGAGTTTCCTGGGAAAGG</u> -3'
	CXCR4-Ex2-1-R	5'-TCAGGTATAGTCAGGAGGAG-3'
	CXCR4-Ex2-2-F	5'-ACTGTCAACCTCTACAGCAG-3'
	CXCR4-Ex2-2-R	5'-TTTGGAAAGGATCTTGAGGC-3'
	CXCR4-Ex2-3-F	5'-CAAGTGGATCTCCATCACAG-3'
	CXCR4-Ex2-3-R	5'- <u>CTGCCTAGACACTCATTAC</u> -3'
Sequencing primers		
Exon 1	CXCR4-Ex1-F	5'- <u>TGGCGGAGGTTCCAACATTG</u> -3'
	CXCR4-Ex1-SeqR	5'- <u>TTCCCGCTCCTCCTTGTGAC</u> -3'
Exon 2	CXCR4-Ex2-1-F	5'- <u>TTGGAGTTTCCTGGGAAAGG</u> -3'
	CXCR4-Ex2-1-R	5'-TCAGGTATAGTCAGGAGGAG-3'
	CXCR4-Ex2-2-F	5'-ACTGTCAACCTCTACAGCAG-3'
	CXCR4-Ex2-2-R	5'-TTTGGAAAGGATCTTGAGGC-3'
	CXCR4-Ex2-3-F	5'-CAAGTGGATCTCCATCACAG-3'
	CXCR4-Ex2-3-SeqR	5'- <u>GTCTTTGCATAAGTGTTAGC</u> -3'

Nucleotides which are underlined indicate noncoding regions.

one pair for the first exon and 350-bp upstream region and three pairs for the second exon (Table 1). Each amplicon was purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) and subsequently sequenced in the forward and reverse directions using the BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The sequences obtained were compared with the reference sequences for *CXCR4* mRNA and *CXCR4* gene locus of C57BL/6J mice (<https://www.ncbi.nlm.nih.gov/nucleotide>; GenBank accession numbers NM_009911.3 and NC_000067.6, respectively) using SnapGene software version 2.3.2 (GSL Biotech LLC, Chicago, IL). The threshold for single-nucleotide polymorphism detection was set to 10% of the major peak. All sequencing traces were of quality value >41 (probability of error, <0.010%).

Western Blot Analysis

The expression of CXCR4 protein in glioma cells was demonstrated by Western blotting, as previously described.^{22,23} Briefly, CT-2A and GL261 cells (5×10^5 /10-cm plates) were incubated under normoxic (21% O_2) and hypoxic [1% O_2 in an incubator chamber (Billups-Rothenberg, Del Mar, CA)] conditions for up to 24 hours and lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with complete protease inhibitor (Roche Diagnostics, Indianapolis, IN) and 2 mmol/L Na orthovanadate (Sigma-Aldrich). Equal amounts of protein [40 to 60 μg , measured by micro bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL)] in the presence of 5% β -mercaptoethanol were resolved on 8% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting at 4°C overnight. Western

blotting was performed using rabbit anti-murine CXCR4 (1:250 dilution, NB100-56437; Novus Biologicals, Littleton, CO), rabbit anti-murine hypoxia-inducible factor (HIF)-1 α (1:1000 dilution, A300-286A; Bethyl Laboratories, Montgomery, TX), and rabbit anti-murine β -actin (1:5000 dilution, A2066; Sigma-Aldrich) antibodies. After incubation with horseradish peroxidase—conjugated enhanced chemiluminescence anti-rabbit IgG (1:5000 dilution; GE Health Care, Chalfont, UK), chemiluminescence was detected with SuperSignal West Pico (CXCR4 and β -actin) or Femto (HIF-1 α) Substrate Kits (Thermo Fisher Scientific), and densitometry of the protein bands was performed using ImageJ software.

CXCR4 Ligand Binding Assay

A CXCR4 competitive binding assay (Tag-lite; Cisbio Bioassays, Codolet, France) based on time-resolved fluorescence resonance energy transfer as a readout for binding activity⁴⁴ was performed according to the manufacturer's instructions. Briefly, labeled CXCR4-expressing HEK293 cells (C1TT1CXCR4) were plated in 384-well microplates. A fixed concentration (15 nmol/L) of red-labeled fluorescent SDF-1 α ligand (L0012RED) was added to increasing concentrations of the CXCR4 antagonists POL5551 (5×10^{-11} to 5×10^{-7} mol/L) or AMD3100 (5×10^{-10} to 5×10^{-6} mol/L) diluted in Tag-lite buffer. After incubation for 3 hours at 4°C, plates were read at the donor (620 nm) and acceptor (665 nm) emission wavelengths after excitation at 340 nm using a Victor 2V microplate reader (Perkin Elmer, Waltham, MA). The responses were normalized using controls without antagonist or with excess (500 nmol/L) of unlabeled SDF-1 α (100% and 0% binding, respectively). The half-maximal inhibitory concentration (IC₅₀) for each compound tested was calculated using GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA).

Intracellular Calcium Flux Assay

Murine CXCR4-transfected 300-19 murine pre-B cells were labeled with Calcium 4 reagent (Molecular Devices, Sunnyvale, CA) in Hanks' balanced salt solution (without phenol red) containing 20 mmol/L HEPES and 0.1% bovine serum albumin at 37°C for 40 minutes, dispensed (8×10^4 /well) in black-wall 384-well microplates, centrifuged, and placed in a FLIPR 384 fluorometric imaging plate reader (Molecular Devices). After reading a 20-second baseline, the cells were stimulated with a fixed concentration (10 nmol/L) of SDF-1 α and treated with increasing concentrations of POL5551 or AMD3100 in Hanks' balanced salt solution containing 20 mmol/L HEPES and 0.1% bovine serum albumin at room temperature. Calcium flux signals were recorded for 200 seconds before and after addition of SDF-1 α or CXCR4 antagonists. The maximum and minimum signals were determined from control wells without CXCR4 antagonists or SDF-1 α , respectively. The resulting

percentage of inhibition of SDF-1 α —induced calcium flux was used to calculate the IC₅₀ values for POL5551 or AMD3100 using GraphPad Prism.

Cytotoxicity Assay

The cytotoxicity of POL5551 was determined by MTT assay. Briefly, CT-2A, GL261, and U87 glioma cells (2×10^4 /well) were cultured in DMEM with 10% FBS in 96-well plates to reach 40% confluence at 24 hours and incubated in quadruplicate with various concentrations of POL5551 (1 nmol/L to 1 μ mol/L) for an additional 16 hours. After removing the culture medium, MTT (0.5 mg/mL; Sigma-Aldrich) in PBS was added and incubated at 37°C for 2 hours. After solubilization of the formazan product with 10% SDS in PBS and incubation at 37°C for 2 hours in the dark, the absorbance was recorded at 540 nm (test) and 690 nm (reference) using a SpectraMax M5 microplate reader (Molecular Devices), and the cellular viability was calculated as a percentage of controls.

Cell Migration Assay

The effect of POL5551 on glioma cell migration was evaluated under normoxic or hypoxic conditions using Boyden chamber assays, as previously described.^{22,23} Briefly, glioma cells cultured to 80% confluence were detached using Accutase (Innovative Cell Technologies, San Diego, CA) to minimize CXCR4 proteolysis,⁴⁵ and cells (5×10^4 /well) in 400 μ L DMEM with 1% FBS were seeded in triplicate in BioCoat polyethylene terephthalate filter inserts with 8- μ m porosity (354578; Corning, Lowell, MA) placed in 24-well plates containing 600 μ L DMEM with 1% FBS per well for 2 hours to allow cells to adhere. After replacing the upper chamber medium with DMEM containing 1% FBS and various concentrations of POL5551 (0 to 500 nmol/L), cells were allowed to migrate for 16 hours in the absence or presence of murine SDF-1 α [0 or 50 nmol/L (equivalent to 400 ng/mL) in the lower chamber] under normoxic or hypoxic conditions at 37°C. After incubation, the inserts were washed twice with PBS, fixed with 100% methanol, and stained with 1% toluidine blue in 1% borax, and the number of migrated cells on the entire lower surface was counted using an Eclipse inverted microscope (Nikon Instruments, Melville, NY). To assess whether cellular proliferation contributed to the observed changes, cells were allowed to migrate in the absence or presence of the ribonucleotide reductase inhibitor hydroxyurea (10 mmol/L; Sigma-Aldrich) for 16 hours,⁴⁶ and the numbers of migrated cells were compared.

Statistical Analysis

Continuous measures were expressed as means $\pm$ SEM and tested for normality using D'Agostino-Pearson K2 and Shapiro-Wilk tests. Because of the observed skewness of the tumor volume and invasiveness data, these data were logarithmically transformed before statistical analysis.

Figure 1 Tumor volume, invasiveness, and hypoxic foci in glioma-bearing mice treated with CXCR4 antagonist POL5551 (POL) and/or anti-murine VEGF antibody B20-4.1.1 (B20). Beginning on day 14 after tumor implantation, mice bearing CT-2A or GL261 glioma were administered POL5551, B20-4.1.1, and/or vehicles. On day 26, the brains were harvested and tissue sections were stained with hematoxylin and eosin to measure the tumor volume (A and B) and invasiveness (C and D) or immunostained to count the number of hypoxic foci (E and F). Tumor invasiveness (INV) was calculated using the equation, $INV = P / \sqrt{4\pi S}$, where P is the perimeter and S is the surface area of the tumor; a noninvasive spherical tumor would have $INV = 1.0$, whereas INV is >1.0 for a tumor with increasingly irregular/invasive borders. The results were analyzed by analysis of variance. Data are expressed as means $\pm$ SEM. $n = 9$ to 10 per group (A–F). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Statistical differences between the means of groups were evaluated by one- or two-way analysis of variance, followed by the Tukey post hoc test. For the Iba1 and nestin score rank analysis, these categorical data were expressed as mean ranks $\pm$ 95% confidence intervals, and the differences between the mean ranks of groups were evaluated by Kruskal-Wallis test, followed by Dunn post hoc test. All tests were two sided, and $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 6.

Results

Tumor Volume and Invasiveness in Glioma-Bearing Mice Treated with POL5551 Alone and in Combination with B20-4.1.1

In CT-2A glioma-bearing mice, treatment with anti-VEGF antibody B20-4.1.1 alone significantly reduced the tumor volume compared with controls (by 57%, $P < 0.01$) or POL5551 alone (by 62%, $P < 0.01$). The combination of POL5551 with B20-4.1.1 did not decrease the tumor volume further (ie, by 60%, $P < 0.01$) (Figure 1A). No significant effect between treatment with B20-4.1.1 alone or in combination with POL5551 was apparent on GL261 tumor

volume either (Figure 1B). As seen in the latter, anti-VEGF agents have been found to not always produce a significant alteration of tumor size.⁴⁷

In both CT-2A and GL261 glioma, treatment with B20-4.1.1 alone increased tumor invasiveness compared to controls [twofold ($P < 0.001$) and 1.4-fold ($P < 0.05$), respectively] (Figure 1, C and D), also consistent with observations in GBM patients treated with bevacizumab.^{1–3} When POL5551 was combined with B20-4.1.1, tumor invasiveness was reduced compared with B20-4.1.1 alone in GL261 glioma (by 39%, $P < 0.01$) (Figure 1D and Supplemental Figure S1, A–D). A lesser reduction (16.4%) of CT-2A glioma invasiveness was measured compared with B20-4.1.1 alone (Figure 1C and Figure 2, A–D). The presence of single glioma cell infiltration into brain parenchyma was not assessed in the present study, but has been demonstrated to be present at least in a GL261 glioma model using green fluorescent protein–labeled GL261 cells.³⁴

Tumor Hypoxia, Vascular Density, Cell Proliferation, and Apoptosis in Glioma-Bearing Mice Treated with POL5551 Alone and in Combination with B20-4.1.1

Administration of B20-4.1.1 alone induced more tumor hypoxic foci compared to controls in CT-2A glioma

Figure 2 Representative immunohistopathologic images of tumor invasion, hypoxic foci, vascular density, proliferation, and apoptosis in CT-2A glioma tumors. Tissue sections from CT-2A glioma-bearing mice treated with vehicles (**first column**), POL5551 alone (**second column**), POL5551 and B20-4.1.1 (**third column**), or B20-4.1.1 alone (**fourth column**) were stained with hematoxylin and eosin to show tumor invasive borders (**A–D**) or immunostained to demonstrate the presence of hypoxic foci (**E–H**), vascular density (**I–L**), cellular proliferation (**M–P**), and apoptosis (**Q–T**). Original magnification: $\times 10$ (**A–D** and **I–L**); $\times 2$ (**E–H**); $\times 20$ (**M–T**).

(4.1-fold, $P < 0.05$) and to a lesser extent in GL261 glioma (1.9-fold) (**Figure 1**, E and F). However, B20-4.1.1 alone only slightly reduced (by 16% to 28%) the vascular density, measured as CD105⁺ endothelial cell density in both models compared to controls (**Figure 3**, A and B). These data are consistent with those previously reported for GL261 tumor after a more frequent dosing of B20-4.1.1 (5 mg/kg i.p. three times/week).⁴⁸ Although POL5551 combined with B20-4.1.1 did not significantly affect the number of hypoxic foci induced by B20-4.1.1 in both tumors (**Figure 1**, E and F), the combination of POL5551 with B20-4.1.1 led to approximately a 49% reduction ($P > 0.05$) of the vascular density in both models compared to controls (**Figure 3**, A and B). Representative images of hypoxic foci

and vascular density of glioma-bearing mice treated with POL5551 and/or B20-4.1.1 are shown for CT-2A (**Figure 2**, E–L) and GL261 gliomas (**Supplemental Figure S1**, E–L). Considering tumor proliferation and apoptosis, B20-4.1.1 alone also inhibited tumor proliferation significantly (67%, $P < 0.05$) in GL261 glioma (**Figure 3D**) compared to controls. POL5551 alone similarly attenuated tumor proliferation by 56% ($P > 0.05$) in GL261 glioma (**Figure 3D**), a result consistent with the known stimulatory effect of SDF-1 α on glioma cell proliferation⁴⁹; the combination treatment yielded only a small additional benefit (ie, a 67% reduction, $P > 0.05$). However, combined treatment significantly increased apoptosis in GL261 glioma compared to controls (2.5-fold, $P < 0.05$) (**Figure 3F**), a

Figure 3 Tumor vascular density, cell proliferation, and apoptosis in glioma-bearing mice treated with POL5551 (POL), B20-4.1.1 (B20), and/or vehicles. Brain tissues from the same glioma-bearing mice were immunostained to measure vascular density (CD105; **A** and **B**), tumor cell proliferation (pHH3; **C** and **D**), and apoptosis (caspase 3; **E** and **F**), and positively stained cells were enumerated per section. The results were analyzed by analysis of variance. Data are expressed as means $\pm$ SEM. * $P < 0.05$.

finding in agreement with the known stimulation of glioma cell survival by SDF-1 α .⁴⁹ Representative images of tumor proliferation and apoptosis of glioma-bearing mice treated with POL5551 and/or B20-4.1.1 are shown for CT-2A (Figure 2, M–T) and GL261 gliomas (Supplemental Figure S1, M–T). Because CT-2A tumors did not show statistically significant changes in proliferation and apoptosis (Figure 3, C and E) that could account for reductions in tumor volumes after B20-4.1.1 or combined therapy (Figure 1A), we determined whether the tumor content of other major cell types changed.

Tumor Macrophage/Microglial and Stem Cell Fractions in Glioma-Bearing Mice Treated with POL5551 Alone and Combined with B20-4.1.1

Infiltrating most human glioma tumors, macrophage/microglial cells comprise up to 30% of the tumor mass.¹⁰ In the CT-2A glioma model, combined treatment reduced the number of Iba1-positive macrophage/microglial cells by 54% ($P < 0.05$) compared to POL5551 alone (Figure 4A and Figure 5, B and C); this correlated with similar changes (57% reduction, $P < 0.01$) in tumor volume (Figure 1A). In mice treated with B20-4.1.1 alone or in combination with POL5551, Iba1-positive cells were noted predominantly in areas with heightened tumor invasion (Figure 5, C and D)

compared to controls, which corroborates the pattern of Iba1 immunopositivity seen in U87 glioma treated with the soluble VEGF decoy receptor drug aflibercept.³⁹ Considering tumor-initiating GSCs that also comprise a significant portion of the tumor,^{42,50} the number of nestin-positive cells was reduced by 69% ($P < 0.001$) by POL5551 alone compared to B20-4.1.1 alone in the CT-2A model (Figure 4C), a finding that validates the subjective reduction of nestin-positive cells seen in U87 glioma treated with POL5551.⁵¹ In GL261 glioma treated with B20-4.1.1 and/or POL5551, the preferential distribution of Iba1 immunopositivity at the tumor border or center was also seen, but lesser effects on Iba1 and nestin were observed in that model (Figure 4, B and D). Representative images of tumor MGCs and tumor-initiating GSCs of glioma-bearing mice treated with POL5551 and/or B20-4.1.1 are shown for CT-2A (Figure 5) and GL261 glioma (Supplemental Figure S2). Treatment-induced loss of microglia/macrophage and/or stem cell tumor content appears to reflect more closely the changes in tumor volumes than tumor proliferation and apoptosis.

CXCR4 Gene Mutation Analysis in Glioma Cells

Because CT-2A and GL261 tumors did not always show similar responses to POL5551 and B20-4.1.1, we

Figure 4 Tumor macrophage/microglial cells (MGCs) and glioma stem cells (GSCs) in glioma-bearing mice treated with POL5551 (POL), B20-4.1.1 (B20), and/or vehicles. Brain tissues from the same glioma-bearing animals were immunostained to measure tumor MGCs (Iba1; **A** and **B**) and GSCs (nestin; **C** and **D**). Positively stained cells per section were scored on a 1 to 4 scale (1 if <1%, 2 if 1% to 10%, 3 if 11% to 50%, and 4 if 51% to 100%). The results were analyzed by Kruskal-Wallis test. Data are expressed as mean score ranks $\pm$ 95% confidence intervals. * P < 0.05, *** P < 0.001.

determined whether some of these differences resulted from *CXCR4* mutations. A common *CXCR4* polymorphism (*rs2228014* C/C genotype) has recently been found to predict progression-free survival in metastatic colorectal cancer patients treated with first-line bevacizumab-based chemotherapy.⁵² The nucleotide sequence of the *CXCR4*

gene and 350-bp upstream regulatory region from CT-2A and GL261 glioma genomic DNA was analyzed by Sanger sequencing of PCR-amplified products, but no *CXCR4* mutations were identified in glioma cells that could alter their function or response to the treatments (Figure 6 and Table 1).

Figure 5 Representative immunohistopathologic images of macrophage/microglial cells (MGCs) and glioma stem cells (GSCs) in CT-2A glioma-bearing mice treated with vehicles (first column), POL5551 alone (second column), POL5551 and B20-4.1.1 (third column), or B20-4.1.1 alone (fourth column) were immunostained to demonstrate the presence of Iba1-positive MGCs [shown at two magnifications, low (**A–D**) and high (**E–H**), the latter with two subpanels separated by a black line showing the tumor center (left sides) and tumor periphery (right sides)] and nestin-positive GSCs (**I–L**). Original magnification: $\times 3.5$ (**A–D**); $\times 20$ (**E–L**).

Figure 6 Schematic diagram of the mouse *CXCR4* gene locus on chromosome 1 showing the location of the two exons, coding regions, 3'- and 5'-untranslated regions (3'UTR and 5'UTR, respectively), and 5' regulatory region. Also indicated is the alignment of the Sanger sequencing reads for CT-2A and GL261 genomic DNA with mouse genome GRCm38/mm10. Each pair of primers for exon 2 overlapped by 100 bp to enhance the alignment of DNA sequences.

Pharmacokinetics of POL5551 in Glioma-Bearing Mice Treated with POL5551 Alone and Combined with B20-4.1.1

In naïve mice, administration of POL5551 (5 mg/kg s.c.) resulted in a maximal plasma concentration at 1 hour after administration of 5800 ng/mL (2.96 μ mol/L) and a half-life of 0.6 hours. A model-predicted exposure profile is shown in [Supplemental Figure S3](#). We next measured the concentration of POL5551 in the plasma and brain tissues (tumor tissue, brain adjacent to tumor, and contralateral brain) of glioma-bearing mice treated or not with B20-4.1.1. In a first experiment, CT-2A glioma-bearing mice received a single dose of POL5551 at various times before sacrifice ([Figure 7](#)). At the 30-minute time point, the concentration of POL5551 was higher in tumor tissues (286 ± 38 ng/g) than in brain adjacent to tumor (48 ± 9 ng/g) or contralateral brain (53 ± 23 ng/g), consistent with the concentrations of POL5551 detected in U87 glioma.⁵¹ Administration of B20-4.1.1 reduced the concentration of POL5551 in tumors by 4.8-fold ($P < 0.01$) at 30 minutes, despite comparable plasma levels ([Figure 7](#)), suggesting that this anti-VEGF agent decreased the delivery of POL5551 to tumors. In an experiment in which CT-2A glioma-bearing mice were administered POL5551 repeatedly for 2 weeks, the tumor concentration of POL5551 (205 ± 34 ng/g) at 30 minutes after the final dose was similar to that measured after a single dose of POL5551 ($P > 0.05$) ([Supplemental Figure S4](#)). However, the concentration of POL5551 (134 ± 26 ng/g) achieved in tumors treated with B20-4.1.1 was 2.2-fold higher than that (60 ± 15 ng/g) after a single dose of POL5551 at 30 minutes, suggesting a cumulative effect of repeated POL5551 in the setting of VEGF inhibition.

Expression of CXCR4, and the Effect of POL5551 on CXCR4 Ligand Binding, Glioma Cell Viability, and Migration *in Vitro*

In a time-resolved fluorescence resonance energy transfer CXCR4 ligand binding assay, POL5551 completely

displaced labeled SDF-1 α with an IC_{50} of 2.68 nmol/L ([Figure 8A](#)). In the same assay, the IC_{50} of AMD3100 was >100 -fold higher (388 nmol/L). In addition, in Calcium 4 reagent-labeled CXCR4-transfected murine 300-19 pre-B cells, POL5551 completely inhibited the stimulatory effect of SDF-1 α with an IC_{50} of 0.31 nmol/L compared to 63.6 nmol/L for AMD3100 ([Supplemental Figure S5](#)). These results with murine CXCR4-transfected cells are likely to be applicable to both CT-2A and GL261 cells given the wild-type *CXCR4* genotype in these murine glioma cell lines ([Figure 6](#)). Similar potency (IC_{50}) values of 1.7 and 495 nmol/L have been reported in a comparison of POL5551- and AMD3100-mediated inhibition of cellular Ca^{2+} flux in hematopoietic stem/progenitor cells, respectively.³¹ The high binding affinity of POL5551 to CXCR4 suggests that the concentrations of POL5551 (30 to 150 nmol/L) measured in tumor and normal tissues ([Figure 7](#) and [Supplemental Figure S4](#)) were likely sufficient to account for its biological effects *in vivo*.

We used hypoxic conditions *in vitro* to simulate the hypoxic environment associated with intracerebral glioma *in vivo*. As we previously reported in human glioma cells,^{22,23} HIF-1 α -regulated CXCR4 protein was constitutively expressed at low levels under normoxia and induced threefold after 18 to 24 hours of exposure to hypoxia in CT-2A cells, and twofold after 16 hours of exposure to hypoxia in GL261 cells ([Figure 8B](#)). The presence of dual bands for HIF-1 α and CXCR4, as we have previously reported,²² may reflect the detection of constitutive and inducible forms of HIF-1 α (115 and 120 kDa), according to Bethyl Laboratories, and the formation of glycosylated isoforms of CXCR4 (42 and 49 kDa),⁵³ respectively. The effects of POL5551 do not appear to be because of direct cytotoxicity as incubation with various concentrations of POL5551 (1 nmol/L to 1 μ mol/L) had little effect on the viability of CT-2A, GL261, and U87 glioma cells ([Figure 8C](#)), in agreement with data obtained in adherent cultures of breast cancer cells treated with POL5551.⁵⁴ In Boyden chamber assays, increasing concentrations of

Figure 7 Plasma and brain tissue concentrations of POL5551 (POL) after a single dose of POL5551 or in combination with repeated B20-4.1.1 (B20) administration in CT-2A glioma-bearing mice. Beginning on day 14 after implantation, mice were administered B20-4.1.1 or vehicles for 12 days. On day 26 after a single dose of POL5551 (5 mg/kg s.c.), 0.5, 2, 6, or 24 hours before sacrifice, plasma was collected and the brains were dissected into tumor tissue (A), brain adjacent to tumor (B), and contralateral brain (C) to determine the concentration of POL5551 in plasma (ng/mL) and brain tissue (ng/g). The results were analyzed by analysis of variance. Data are expressed as means $\pm$ SEM. $n = 4$ per group (A–C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

POL5551 led to a gradual decrease in the rate of migration of glioma cells, requiring only 10 to 50 nmol/L for GL261 cells compared to 100 to 500 nmol/L for CT-2A cells (Figure 8, D–F), consistent with the inhibitory effect of POL5551 on the migration of breast cancer cells.⁵⁴ The apparently greater sensitivity of GL261 cell migration to POL5551 is consistent with the larger inhibitory effect of POL5551 on the invasiveness of GL261 glioma *in vivo*

compared to CT-2A glioma (Figure 1, C and D). The migration of CT-2A (Figure 8, D and F) and GL261 cells (Figure 8E) increased approximately twofold under 24-hour hypoxic conditions ($P < 0.001$) and to a lesser extent in response to SDF-1 α . The higher concentration of POL5551 needed to inhibit CT-2A cell migration after hypoxic exposure (500 nmol/L) compared to normoxia (100 nmol/L) is consistent with a concurrent induction of CXCR4 on

Figure 8 Expression of CXCR4, and effect of POL5551 on CXCR4 ligand binding, cell viability, and migration in glioma cells *in vitro*. **A:** Using the Tag-lite CXCR4 ligand binding assay, CXCR4-expressing HEK293 cells were incubated with increasing concentrations of POL5551 or AMD3100 in the presence of a fixed concentration of fluorescent SDF-1 α ; the normalized responses were plotted, and the half-maximal inhibitory concentration was calculated. **B:** CT-2A and GL261 cells were both incubated under normoxic (N) and hypoxic (H; 1% O₂) conditions for up to 24 hours and for 16 hours, respectively, and the protein expression of CXCR4, HIF-1 α , and β -actin was determined by Western blot analysis. **C:** CT-2A, GL261, and U87 glioma cells were incubated in quadruplicate with POL5551 (1 nmol/L to 1 μ mol/L) for 16 hours, and cell viability was assessed by MTT assay. **D and E:** In a Boyden chamber assay, CT-2A and GL261 cells were incubated in triplicate with POL5551 (0 to 500 nmol/L) under normoxic and hypoxic conditions and allowed to migrate in the absence or presence of an SDF-1 α gradient (50 nmol/L) for 16 hours, after which the number of migrated cells was counted. **F:** To assess whether cellular proliferation contributed to the observed changes, cells were allowed to migrate in the presence of 10 mmol/L hydroxyurea for 16 hours. The results were analyzed by analysis of variance. Data are expressed as means $\pm$ SEM (A and C–F). *** $P < 0.001$, **** $P < 0.0001$.

glioma cell surface by hypoxia (Figure 8B). Overall, POL5551 reduced hypoxia- and SDF-1 α -mediated migration dose-dependently *in vitro*, consistent with the inhibitory effect of POL5551 on the invasiveness of glioma cells *in vivo*. The migratory rate of CT-2A glioma cells treated with POL5551 was unchanged in the presence of the cytostatic drug hydroxyurea (Figure 8F), indicating that the effect of POL5551 on migration did not result from concurrent inhibition of cell proliferation.

Discussion

Preclinical studies suggest that combining antiangiogenic therapy with inhibitors targeting the tumor invasive phenotype could be a promising approach to overcoming cancer resistance to antiangiogenic therapy.^{11,12} Because enhanced tumor hypoxia during bevacizumab resistance is associated with increased SDF-1 α receptor CXCR4 expression on invading tumor, tumor-initiating GSCs, and tumor-associated MGCs, we chose to target CXCR4 using a highly potent PEM antagonist, POL5551. In novel immunocompetent mouse glioma models of antiangiogenic therapy, POL5551 achieved higher concentrations in intracranial glioma tumors than normal brain tissues, and reduced glioma invasion by inhibiting hypoxia-induced glioma migration, and the populations of GSCs and MGCs known to promote glioma proliferation and invasion, compared to controls. Our study differs from a previous study⁵¹ by the use of syngeneic invasive CT-2A and GL261 glioma^{23,33,41} in immunocompetent mouse models (as opposed to minimally invasive U87 cells in nude mice). In addition, we have used B20-4.1.1, an anti-murine VEGF antibody comparable to BEV (both produced by Genentech Inc.), and demonstrated, for the first time, that a low dose (5 mg/kg) of POL5551 diminished B20-4.1.1-induced glioma invasion and glioma-associated MGCs, which are known to promote glioma invasion³⁹ and participate in glioma-elicited immunosuppression.^{55,56}

In malignant glioma, chronic exposure of tumor tissues to low oxygen tension produces necrotic areas surrounded by hypoxic pseudopalisading tumor cells that trigger HIF-1 α -regulated cellular processes, such as tumor invasion and angiogenesis.^{4,22,23} Among these HIF-1 α -regulated events, we have demonstrated that hypoxia-induced expression of CXCR4 in pseudopalisading glioma cells promotes their migration at the invasive edges toward SDF-1 α -secreting neurons and blood vessels.^{22,23} Consistent with our reports, distant glioma recurrence (>4 cm from the margin of the original tumor) after total resection was found to be closely associated with high levels of SDF-1 α and CXCR4 expression in glioma tissues.²¹ Similarly, hypoxia-induced migration of neural stem cells to the glioma site is mediated by HIF-1 α -dependent upregulation of SDF-1 α /CXCR4, VEGF/VEGF receptor 2, hepatocyte growth factor/c-MET, and urokinase-type plasminogen activator and its

receptor.²⁵ Hypoxia also favors microglia migration via enhanced CXCR4 expression.^{9,10} Inhibition of SDF-1 α /CXCR4 signaling has been found to reduce brain tumor growth or recurrence,^{28,29} the viability and number of GSCs,^{28,30} and the recruitment of glioma-associated MGCs.^{9,10} In our study, potent CXCR4 inhibition with POL5551 disrupted the interactions between cancer cells and their environment by reducing the invasiveness of glioma cells through a direct effect on glioma cell migration rather than an antiproliferative effect.

In GBMs, the uptake of drugs from the systemic circulation may be facilitated by the increased permeability of the blood-brain barrier associated with the pathologically structured microvessels and expression of VEGF and other angiogenic factors.^{4,5} The capacity of POL5551 (approximately 2 kDa) to cross this disrupted barrier and reach a high concentration in glioma tissue is evidenced by the lower tumor concentration of POL5551 in B20-4.1.1-treated mice, possibly because of transient vascular normalization¹⁶ during antiangiogenic therapy. Although B20-4.1.1 only slightly reduced the vascular density in our glioma models, the lower number of tumor hypoxic foci may reflect other effects that we did not examine, such as blood vessel lumen size, blood flow, hemorrhage, and/or thrombosis.

Recent advances in the classification of tumors of the central nervous system have demonstrated that different genetic subtypes of morphologically identical tumors have different natural histories and may differ significantly in their responses to treatment.^{57,58} Likewise, the histomorphologically similar high-grade astrocytoma CT-2A and GL261 tumors may be of different genetic subtypes that differ significantly in their responses to treatment. Both CT-2A and GL261 models were derived from malignant astrocytoma induced by intracranial implantation of methylcholanthrene (20- and 3-methylcholanthrene, respectively) in C57BL/6 mice. The characterization of the underlying oncogenic mechanisms of CT-2A and GL261 tumors is limited. For example, both tumor types have *p53* mutations and are phosphatase and tensin (*PTEN*) homolog deficient,^{59,60} features that are seen in subsets of human GBM. In an attempt to further characterize the genetic landscape of these glioma cell lines, we evaluated whether *CXCR4* mutations may be present, but found no evidence of mutations in the *CXCR4* gene and its 5' regulatory region in both glioma cell lines. In addition, the differences in the *CXCR4* expression level and *CXCR4*-mediated migratory responses that we observed in CT-2A and GL261 tumors support the notion that the *CXCR4* signal transduction pathways and therapeutic outcomes to *CXCR4* inhibition may be distinct in these two cell lines despite being histomorphologically similar glioma tumors, as previously reported for primary patient-derived GBM cells.⁶¹ That CT-2A and GL261 gliomas did not always show similar responses to POL5551 and B20-4.1.1 may be best accounted for by being of different genetic subtypes that differ in their responses to treatment.

Infiltrating most glioma tumors, macrophages/microglia comprise up to 30% of the tumor mass and have been associated with poor clinical prognosis in patients with high-grade glioma.^{8–10} Microglia, which commonly express Iba1 together with CD11b, are the main effector cells of the innate immune system in the central nervous system and perform a number of critical functions. Microglia promote glioma invasiveness not just by degrading the extracellular matrix through proteinase activity but also by stimulating cell mobility through the release of factors that activate the promigratory proline-rich tyrosine kinase 2 signaling pathway in glioma cells.⁶² Upon administration of anti-VEGF therapy with aflibercept, a dramatic accumulation of Iba1-positive microglia/macrophages was noted at the tumor/normal brain interface in regions with heightened tumoral invasion, suggesting the presence of a myeloid population implicated in the escape of malignant glioma from anti-VEGF therapy.³⁹ In addition to corroborating this finding using B20-4.1.1 antibody, we report that combined treatment with POL5551 significantly reduced the number of Iba1-positive cells in CT-2A glioma by half compared to control. This finding raises the possibility that CXCR4 PEM antagonists may interfere with the microglial mechanism of escape of malignant glioma from anti-VEGF therapy and have application in glioma immunotherapy analogous to that reported for AMD3100 in multikinase inhibitor (sorafenib)—treated hepatocellular carcinoma.⁶³

GBMs contain a subpopulation of cells referred to herein as GSCs that possess self-renewal and tumor-initiating capacity and express neural stem cell markers, such as CD133 and nestin.^{25–27} CXCR4 was found to be highly expressed in a subpopulation of GSCs expandable by hypoxia, and treatment with AMD3100 inhibited the highly invasive growth of GSC engraftment and cell migration.²⁶ Targeting of a similar population of GSCs by POL5551 may account for the significant reduction of nestin-positive cells observed in the CT-2A glioma model.

POL5551, combined with existing therapies, has previously been found to improve survival in immunodeficient mouse models.^{51,54} In U87 glioma-bearing mice, combined treatment with POL5551 (up to 80 mg/kg by osmotic pump) prolonged mouse survival compared to treatment with the anti-VEGF antibody mcr84 alone (35 versus 18 days).⁵¹ In an MDA-MB-231 breast cancer model, resection of the primary tumor followed by combined treatment with POL5551 also increased the survival compared with single-agent eribulin, a chemotherapeutic microtubule inhibitor (58 versus 51 days).⁵⁴ CXCR4 antagonists are now being evaluated in clinical trials of cancer therapy. In patients with recurrent high-grade glioma, a clinical trial (NCT01339039) with combined treatment of AMD3100 and BEV is ongoing and recruiting patients. In patients with metastatic breast cancer, a clinical trial (NCT01837095) with combined treatment of the PEM CXCR4 antagonist POL6326 (a close analog of POL5551 that differs by a single amino acid) and eribulin was recently initiated.⁵⁴

In conclusion, our study demonstrates that disruption of several CXCR4-regulated mechanisms with POL5551 can reduce intracranial glioma invasion through inhibition of hypoxia-induced glioma cell migration, as well as reduce the populations of tumor-initiating GSCs and tumor-associated MGCs known to promote glioma proliferation and invasion. Our results suggest that CXCR4-regulated mechanisms contribute to antiangiogenic therapy resistance. Adjunctive therapy with PEM CXCR4 antagonists may also help overcome antiangiogenic therapy resistance in patients with GBM and possibly in patients with other CXCR4-expressing cancers.

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Supplemental Data

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