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Structural Characterization and in Vivo Evaluation of β -Hairpin Peptidomimetics as Specific CXCR4 Imaging Agents

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Abstract

The CXCR4 chemokine receptor is integral to several biological functions and plays a pivotal role in the pathophysiology of many diseases. As such, CXCR4 is an enticing target for the development of imaging and therapeutic agents. Here we report the evaluation of the POL3026 peptidomimetic template for the development of imaging agents that target CXCR4. Structural and conformational analyses of POL3026 and two of its conjugates, DOTA (POL-D) and PEG₁₂-DOTA (POL-PD), by circular dichroism, two-dimensional NMR spectroscopy and molecular dynamics calculations are reported. *In silico* observations were experimentally verified with *in vitro* affinity assays and rationalized using crystal structure-based molecular modeling studies. [¹¹¹In]-labeled DOTA conjugates were assessed *in vivo* for target specificity in CXCR4 expressing subcutaneous U87 tumors (U87-stb-CXCR4) through single photon emission computed tomography (SPECT/CT) imaging and biodistribution studies. *In silico* and *in vitro* studies show that POL3026 and its conjugates demonstrate similar interactions with different micelles that mimic cellular membrane and that the ϵ -NH₂ of lysine⁷ is critical to maintain high affinity to CXCR4. Modification of this group with DOTA or PEG₁₂-DOTA led to the decrease of IC₅₀ value from 0.087 nM for POL3026 to 0.47 nM and 1.42 nM for POL-D and POL-PD, respectively. In spite of the decreased affinity toward CXCR4, [¹¹¹In]POL-D and [¹¹¹In]POL-PD demonstrated high and significant uptake in U87-stb-CXCR4 tumors compared to the control U87

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Author Contributions

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tumors at 90 min and 24 h post injection. Uptake in U87-stb-CXCR4 tumors could be blocked by unlabeled POL3026, indicating specificity of the agents in vivo. These results suggest POL3026 as a promising template to develop new imaging agents that target CXCR4.

Keywords

molecular imaging; CXCR4; SPECT/CT; chemokine; chemokine receptor

INTRODUCTION

The G-protein coupled chemokine receptor 4 (CXCR4), along with its ligand CXCL12 (also known as stromal derived factor1, SDF1), play critical roles in the directional migration of cells during development and in normal physiology.¹ The CXCR4-CXCL12 axis is important for homing of immune cells in several pathological states, including asthma, lupus, and arthritis.¹ CXCR4 is also a coreceptor for the entry of HIV into T-cells. In cancer, CXCR4 is overexpressed in more than 23 tumor types and contributes to tumor growth, angiogenesis, and drug resistance.² CXCR4 expression in tumors often correlates with a propensity for metastasis and poor prognosis.² CXCL12 is present at all known cancer metastatic sites.³ It has been suggested that cancer cells “hijack” the CXCR4-CXCL12 axis to establish distant organ metastases.³ The important roles played by the CXCR4-CXCL12 axis in cancer and other diseases make it an important therapeutic target. A number of CXCR4-targeted low-molecular-weight (LMW) agents, peptides, and antibodies are currently in clinical trials.⁴

Expression of CXCR4 in several tissues and its importance in normal biological functions suggests the development of diagnostic agents for therapeutic guidance and monitoring. Radio-iodinated anti-CXCR4 antibodies have shown specific accumulation in tumors.⁵ Cyclam-based LMW agents labeled with ⁶⁴Cu have been used to evaluate graded levels of CXCR4 expression in breast cancer xenografts and experimental lung metastasis models.⁶ Similarly, CXCL12 and polyphemusin II-derived peptide analogs labeled with a variety of radionuclides have been reported.^{1,7-9} Of the reported agents, the CXCR4 pentapeptide radiolabeled with ⁶⁸Ga and LMW agents, such as AMD3465 and AMD3100, radiolabeled with ⁶⁴Cu demonstrated superior imaging characteristics.¹⁰⁻¹⁷ Most of the reported peptide-based imaging agents, while having shown a measure of specific uptake in CXCR4-positive tumors, demonstrated low target-to-nontarget ratios due to metabolic instability and significant nonspecific binding to red blood cells.^{8,7,14} Some limitations observed with peptides in general have been circumvented through the development of peptidomimetics that allow for high affinity and improved stability with tunable pharmacokinetics.^{18,19}

While a variety of peptidomimetic strategies exist, the β -hairpin protein epitope mimetics (PEM) technology was successfully used to create CXCR4 binding peptidomimetics.¹⁸ PEM technology uses the ^DPro-^LPro dipeptide template with a cysteine disulfide bridge to stabilize the β -hairpin structure. With that general structure in place, important residues from a peptide of interest, TC14011 in the case of CXCR4,⁹ are incorporated into the β -hairpin mimetic to generate high-affinity, stable analogs. Several peptidomimetics based on

TC14011 have been synthesized using that technology, including the monocyclic POL1638 and the more stable and potent bicyclic peptide analogs, POL2438 and POL3026.¹⁸ Of those, POL3026 demonstrated superior inhibitory activity with an IC₅₀ of 1.2 nM for displacement of SDF-1 from CXCR4 receptors.¹⁸ Although shown to possess high selectivity for CXCR4 relative to other chemokine receptors, the biophysical characteristics accounting for the selectivity of CXCR4-POL3026 have not been described, which is important for further optimization of these agents. Also, the prolonged in vivo stability of POL3026 observed in human plasma ($t_{1/2} > 300$ min) makes it a suitable template on which to build imaging agents that target tissues expressing CXCR4.

The aim of the present study was to explore POL3026 as the CXCR4 imaging agent and gain insight into the modes of receptor–ligand interactions. We evaluated POL3026 and two of its DOTA conjugates, the latter in preparation for radiolabeling, for binding to CXCR4. We used circular dichroism (CD), nuclear magnetic resonance (NMR) spectroscopy, molecular dynamic simulations, molecular modeling, and in vitro CXCL12 displacement assays to characterize those interactions. To validate the in silico and in vitro observations, ¹¹¹In radiolabeled POL3026 conjugates were investigated in U87 glioblastoma tumor models stably expressing CXCR4 by SPECT/CT imaging and biodistribution studies. Our data show that the ε-amino group of Lys⁷ proved critical for retaining high affinity to the receptor. Furthermore, in vivo studies demonstrated that Lys⁷-based POL3026 conjugates accumulate specifically within CXCR4-positive tumors.

MATERIALS AND METHODS

Materials

POL3026 and CVX15 peptides were synthesized by CPC Scientific (Sunnyvale, CA) with >95% purity. All chemicals were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise specified. Fmoc-*N*-PEG₁₂-NHS-ester, DOTA-NHS-ester, and ¹¹¹InCl₃ ($t_{1/2}$, 2.81 days) were purchased from Quanta Biodesign Ltd., Macrocyclics and Nordion, respectively. All reagents and solvents were used as received without further purification.

Modifications of the POL3026

POL3026-DOTA (POL-D)—Five milligrams of the peptide (1.87 μmol) was dissolved in 0.5 mL of DMF and mixed with 2.1 mg of DOTA-NHS-ester (2.79 μmol in 0.5 mL of DMF). The pH of the reaction mixture was adjusted to 7 using diisopropylethylamine (DIPEA). Product was separated on a reversed phase-high performance liquid chromatography (RP-HPLC) system (Varian ProStar) with an Agilent Technology 1260 Infinity photodiode array detector using a semipreparative C-18 Luna column (5 mm, 10 × 250 mm Phenomenex) and a gradient elution starting with 98% H₂O (0.1% TFA) and 2% MeOH (0.1% TFA) reaching 90% of MeOH in 65 min at a flow rate of 2 mL/min. The desired POL-D at ~41.25 min of elution time was collected and evaporated. The resulting residue was dissolved in deionized water and lyophilized, yielding 3.85 mg (1.3 μmol) of the product as a white powder (yield: 61.3%), which was used for further experiments. To confirm modification, the resulting conjugate was analyzed by mass spectrometry. Theoretical chemical formula, C₁₁₁H₁₆₆N₃₆O₂₇S₂; exact mass, 2499.22; molecular weight,

2500.86; observed m/z 2498.47, $(M + 1)^{+1}$; 1250.06, $(M + 2)^{+2/2}$; 833.76, $(M + 3)^{+3/3}$ (Figure S1, panels A–C, of the Supporting Information).

POL3026-PEG₁₂-DOTA (POL-PD)—Twenty-five milligrams of POL3026 (9.31 μ mol) was dissolved in 0.5 mL DMF and mixed with 13.1 mg of Fmoc-N-PEG₁₂-NHS (14 μ mol in 0.5 mL DMF). The pH of the reaction mixture was adjusted to 7 using DIPEA. Resulting conjugate was precipitated in ice-cold ether, followed by deprotection of the primary amine using 20% piperidine/DMF, purification by RP-HPLC, and lyophilization, yielding 32 mg (9.76 μ mol) of product (97.5%). Five milligrams of the lyophilized POL-PEG₁₂-NH₂ conjugate (1.52 μ mol) was subsequently reacted with 1.74 mg of DOTA-NHS-ester (2.3 μ mol) and purified by RP-HPLC. The fraction related to POL-PD was collected at 43.78 min and evaporated. The obtained residue was dissolved in deionized H₂O and lyophilized yielding 4.5 mg (1.27 μ mol) of the desired product in the form of a white powder (yield: 74.3%), which was used in the subsequent studies. Theoretical chemical formula, C₁₃₈H₂₁₉N₃₇O₄₀S₂; exact mass, 3098.57; molecular weight, 3100.57; observed m/z 1549.65, $(M + 2)^{+2/2}$; 1033.66, $(M + 3)^{+3/3}$; and 775.53, $(M + 4)^{+4/4}$ (Figure S2, panels A and B, of the Supporting Information).

Circular Dichroism Measurements

CD spectra of POL, POL-D, and POL-PD in phosphate buffer at pH 7.4 and in the buffered micellar solutions of DPC, SDS, and mixed DPC:SDS micelles at molar ratios 9:1 and 5:1 were acquired using a Jasco J-815 spectropolarimeter. All measurements were done using 0.15 mg/mL peptide solutions and a 2 cm/min scan speed at 298 K. Experiments were performed in triplicate to increase signal-to-noise ratios and carried out over the 190–260 nm range. Final spectra were corrected by background subtraction and analyzed as mean molar ellipticity Θ (degree $\times$ cm² $\times$ dmol^{−1}) versus wavelength λ (nm). The content of the secondary structure was calculated from the spectra using a SELCON 3 method.²⁰

NMR Measurements

Sample was prepared by mixing 35 mg of DPC-*d*₃₈ and 5 mg of POL in 0.5 mL of partially deuterated phosphate buffer (H₂O:D₂O 9:1, v/v, pH 7.4) and sonicated for several minutes to increase homogeneity of the peptide–micelle complex. A 0.32 mL of the resulting solution was transferred to the Shigemi tube and the NMR spectra were recorded on a Varian Unity Plus 500 MHz spectrometer (¹H resonance frequency 499.83 MHz). Initially NMR spectra of POL were recorded at 298 K; however, the quality of resulting NMR spectra was not satisfactory, and the complete assignment of signals was not feasible. Elevated temperature to 310 K resulted in a decrease of the line-broadening and small increase of the chemical shift dispersion. Further improvement of spectral quality was observed upon changing pH of the sample to 5.5 with dilute acetic acid solution, which

ASSOCIATED CONTENT

Supporting Information

Schematic of POL3026 modification with DOTA, low-resolution mass spectrum of POL, and ESI-MS analysis; schematic representation of the synthetic pathway leading POL-PD formation; NMR analysis; molecular modeling studies; purification of radiotracers; inhibition of CXCL12-Red binding to CXCR4; structural evaluation of POL, POL-D, and POL-PD. This material is available free of charge via the Internet at <http://pubs.acs.org>.

provided high-quality NMR spectra of POL in DPC micellar solution (Figure S3, panels A, B, and C, of the Supporting Information). TOCSY spectra were recorded with mixing times of 15 and 60 ms using modified MLEV-17 spin-lock sequence.²¹ 2D $^1\text{H} - ^1\text{H}$ NOESY data sets were acquired with 100, 150, and 200 ms mixing times for exclusion of spin-diffusion effects.²² Hetero-nuclear 2D $^1\text{H} - ^{13}\text{C}$ HSQC experiment was performed on a natural abundance ^{13}C isotope.^{23–25} Double-quantum filtered correlation spectroscopy (DQF-COSY) was used to determine the $^3J_{\text{HNH}\alpha}$ coupling constants.²⁶ The DQF-COSY spectrum was processed to enhance the resolution to 1.2 Hz per point in F2. All chemical shifts were referenced with respect to external 2,2-dimethyl-2-silapentanesulfonic acid (DSS) using $\Xi = 0.251449530$ ratio for indirectly referenced ^{13}C resonance.²⁷ All recorded NMR data were processed by VNMR 6.1B (Varian Inc., Palo Alto, CA) and analyzed with Sparky.²⁸

Molecular Dynamics Calculations

MD simulations were carried out using AMBER 11.²⁹ The starting coordinates of the DPC micelle–water system were taken from simulations carried out by Tieleman et al.³⁰ The prebuilt hydrated DPC micelle consisted of 65 DPC monomers and 6305 water molecules. The DPC topology was adapted to AMBER. The structures of nonstandard residues (2-Nal, PEG12, and DOTA) were modeled using bond lengths, valence, and torsion angles of appropriate residues within the AMBER library or taken from the CSD database.³¹ The point charges were optimized by fitting them to the ab initio molecular electrostatic potential (6–31G* basis set, GAMESS'04, ab initio molecular electronic structure program)³² for three different conformations, followed by consecutive averaging of the charges over all conformations, as recommended by the RESP protocol.³³ Parameters of nonstandard citrulline were adopted from literature.³⁴ To build the peptide–micelle complexes, POL, POL-D, and POL-PD were placed into the simulation DPC-water box with their center of mass coinciding with that of the DPC micelle. The starting configuration of each peptide was picked from preliminary calculations with a simulated annealing (SA) algorithm, including time-averaged distance restraints (TAV) and dihedral angle restraints derived from NMR data of POL. Due to spherical symmetry of the DPC micelle, the orientation of the peptide was unimportant. The side chains of Lys and Arg were protonated and POL had a total charge of +5. Total positive charge for POL-D and POL-PD was +4. Chloride ions were added to neutralize the entire resulting systems. The entire systems were subjected to energy minimization (steepest descent method). To eliminate the initial adverse interactions between peptide and the micelle core and to prevent penetration of water during equilibration, for the first 20000 steps of minimization, peptide and water were kept under weak harmonic constraints with force constants of 10 and 5 kcal/mol Å, respectively. In the next 20000 steps of minimization, all constraints were removed. Afterward, each system was subjected to NTP dynamics for 24 ns at 310 K using a nonbonded cutoff of 9 Å and time step of 2 fs (Figure S3D of the Supporting Information). The time-averaged distance restraints (TAV) and dihedral angle restraints derived from NMR spectroscopy of POL were included during the entire NTP MD simulations. The interproton distances were restrained with the force constants $f = 50 \text{ kcal}/(\text{mol} \times \text{\AA}^2)$, and the dihedral angles with $f = 5 \text{ kcal}/(\text{mol} \times \text{rad}^2)$. The improper dihedral angles centered at the C_α atoms were restrained with $f = 50 \text{ kcal}/(\text{mol} \times \text{rad}^2)$. The geometry of peptide groups was kept fixed according to NMR data (all trans) [$f = 50 \text{ kcal}/(\text{mol} \times \text{rad}^2)$]. The coordinates were collected at each 2000th

step. The 20 structures obtained in the last steps of MD simulations were considered in further analysis. The figures were prepared by MOLMOL³⁵ and PyMOL (the PyMOL Molecular Graphics System, version 1.4.1, Schrödinger, LLC).

Molecular Modeling of Peptides Binding to CXCR4

All molecular graphics and modeling experiments were performed using Discovery Studio 4 (Accelrys, Inc. San Diego, CA). The X-ray structure of CXCR4 cocrystallized with the cyclic peptide CVX15 (PDB: 3OE0) was downloaded from the protein data bank (RCSB, <http://www.rcsb.org/pdb/home/home.do>). The cocrystallized ligand, with water molecules removed, was used as a template to sketch POL and POL-D using the Sketch Molecules module in Discovery Studio.

In Situ Ligand Minimization—Each ligand was minimized while binding to its target protein using the in situ ligand minimization module with the following parameters: CHARMM as an input force field, minimization algorithm as a smart minimizer, maximum minimization steps equal to 1000, minimization with RMS gradient equal to 0.001 Å, and minimization energy change set equal to zero. After the minimization protocol was executed, the in-situ-minimized ligands were stripped of their nonpolar hydrogens to simplify the overall view. The protein (CXCR4) was depicted in the form of a light gray line ribbon. The bound ligands (CVX15, POL, and POL-D) are depicted as a stick with atoms color coded according to element: carbon (gray), nitrogen (blue), and oxygen (red) (Figure S4 of the Supporting Information).

Radiolabeling

For radiolabeling, approximately 10 µg of POL-D (3.39 pmol) or POL-PD (2.81 pmol) peptide conjugates in 100 µL of 0.1 M of sodium acetate were mixed with ~37 MBq (~1 mCi) of ¹¹¹InCl₃ in 0.02 M HCl and incubated at 65 °C for 30 min. Products were purified on a C-18 (Luna, 5 µm, 10 × 250 mm; Phenomenex) semipreparative column using a Varian ProStar system equipped with a radioactive single-channel radiation detector (model 105S; Bioscan) and a Varian ProStar UV absorbance detector set to 275 nm. Gradient elution starting with 98% H₂O (0.1% TFA) and 2% MeOH (0.1% TFA) reaching 90% MeOH over 65 min at flow rate of 2 mL/min was applied. Radiolabeled peptide conjugates [¹¹¹In]POL-D and [¹¹¹In]POL-PD were collected at ~38.42 and ~44.59 min, respectively, evaporated, diluted with buffered saline, sterile filtered, and used for in vitro and in vivo evaluation (Figure S5, panels A and B, of the Supporting Information).

Cell Lines

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. The human glioblastoma cell line U87 and CHO-K1 cell lines were purchased from American Type Culture Collection. A U87 cell line stably transfected with human CD4 and CXCR4 (U87-stb-CXCR4) was obtained from the NIH AIDS Research Reference Reagent Program.³⁶ The CHO-K1 cell line stably expressing SNAP-CXCR4 was generated in our laboratory. All the cell lines were cultured and maintained as described previously.³⁷

Competitive Binding Assays

Competitive binding assays using fluorescent CXCL12 were performed as described previously.³⁷ IC_{50} and K_i values were calculated by fitting the data to a sigmoidal dose response curve and the Cheng-Prusoff equation (with derived $K_D = 19$ for CXCL12-Red and concentration of 15 nM), respectively (Figure S6 of the Supporting Information).

In Vitro Receptor Binding Assay of Radiolabeled Analogs

U87 and U87-stb-CXCR4 cells (1×10^6) in 1 mL PBS buffer containing 2 mM of EDTA and 1% of FBS were incubated with $\sim 1 \mu\text{Ci}$ of [^{111}In]POL-D or [^{111}In]POL-PD at 37 °C for 60 min. Following incubation, cells were washed three times with PBS buffer, and the cell pellets were counted in a gamma spectrometer.

Animal Models

All experimental procedures using animals were conducted according to protocols approved by the Johns Hopkins Animal Care and Use Committee. Female NOD/ SCID mice, 6 to 8 weeks old were purchased from The Johns Hopkins Immune Compromised Animal Core. Mice were implanted subcutaneously (s.c.) with U87 and U87-stb-CXCR4 cells (4×10^6 cells/100 μL) in opposite flanks. Animals were used for ex vivo biodistribution and SPECT/CT imaging experiments when the tumor size reached approximately 200– 400 mm^3 .

Ex Vivo Biodistribution

NOD/SCID mice harboring U87 and U87-stb-CXCR4 xenografts were injected intravenously with 20 μCi of [^{111}In]POL-D and [^{111}In]POL-PD in 200 μL of saline. At 1.5 and 24 h post injection of radiotracers, animals were sacrificed, blood, tumors, and selected tissues were harvested and weighed, and the radioactivity was measured in an automated gamma spectrometer. To demonstrate the in vivo specificity of [^{111}In]POL-D, mice were subcutaneously injected with blocking doses of 0.27, 0.85, and 2.33 mg/kg (corresponding to ~ 5 , 15, and 45 μg of total dose, respectively) of POL at 0.5 h prior to the injection of radiotracer. Mice were sacrificed 1.5 h after injection of [^{111}In]POL-D. For each experiment, aliquots of the injected dose were counted as reference standards for the calculation of percent of injected dose per gram of tissue (% ID/g) values. Three to four animals were used per group.

SPECT/CT Imaging and Analysis

Whole-body SPECT/ CT images were acquired on an X-SPECT small animal SPECT/CT system (Gamma Medica Ideas, Northridge, CA) as described previously.⁵ After an intravenous injection of $\sim 350 \mu\text{Ci}$ of radiolabeled peptides, mice were induced with 3% and maintained under 1.5% isoflurane (v/v), and images were acquired between 1–2 h and 24–25 h post injection. The tomographic data were acquired in 64 projections over 360° at 30s per projection using medium energy pinhole collimators. CT was acquired in 512 projections to allow anatomic coregistration. Data were reconstructed using the ordered subsets-expectation maximization algorithm, and 3D volume-rendered images were generated using Amira 5.3.0 software (Visage Imaging Inc.).

Data Analysis

Statistical analysis was performed using an unpaired two tailed *t* test using a Prism 5 Software (GraphPad). *P*-values <0.05 were considered to be significant, and the comparative reference was cell line or tumor with low CXCR4 expression.

RESULTS AND DISCUSSION

Modifications of POL3026

POL3026 (Figure 1A) has a single reactive primary amine on Lys⁷ that could be used for site-selective modification to enable radiolabeling. Modification of lysine side chain with chelating agent or fluorescent dyes was shown to impact the binding affinity of various polyphemusin II-derived peptides to CXCR4, even though this part of the peptide was shown to be positioned away from binding cavity of the receptor.^{8,38,39} To investigate the effect of lysine ϵ -NH₂ group modification on POL conformation, interaction with cellular membranes, affinity to CXCR4, biodistribution, and pharmacokinetics, the DOTA chelator was either directly conjugated to the lysine side chain (POL-D) or via polyethylene glycol PEG₁₂ (POL-PD) (Figures 1S, panels A–C, and 2S, panels A and B, of the Supporting Information). PEG spacers were previously shown to affect the metabolic stability and pharmacokinetics of peptides.^{40–42} While PEG spacers >3000 Da decrease affinity of the peptides to the receptors,⁴¹ very short PEG spacers (<250 Da) do not significantly affect the pharmacokinetics of the peptides.⁴² Therefore, an intermediate 600 Da PEG₁₂ spacer was selected to evaluate the influence of PEGylation on the pharmacokinetics of POL without the loss of affinity to the receptor.

Structural Evaluation and Interactions with Lipid Bilayers

Circular Dichroism Studies—To evaluate if conjugation of DOTA or PEG₁₂-DOTA to POL affects its conformation and interactions with cellular membrane mimicking micelles, a series of circular dichroism spectra were recorded (Figure 1, panels B, C, and D). Binding of the peptide to a membrane is controlled by electrostatic and hydrophobic interactions, which drive respective peptide residues toward different parts of the membrane such as hydrophobic core, hydrophilic surface, or aqueous phase. Thus, the conformational studies of POL, POL-D, and POL-PD were carried out in different micellar environments, dodecylphosphocholine (DPC), sodium dodecyl sulfate (SDS), and mixed DPC:SDS micelles. DPC provides a zwitterionic surface on the micelle that adequately imitates the eukaryotic cell membrane. However, to mimic the electrostatic properties of the membrane with a slight prevalence of negative charge, mixed DPC and SDS micelles were used. In turn, pure SDS micelle usually mimics a negatively charged bacterial cell membrane.^{43–46} Both detergents are compatible with and routinely used for CD and NMR studies. The 200 nm region of POL CD spectra consisted of a negative band near 215 nm and a positive band at ca. 199 nm typical for β -sheet structure (Figure 1B). The observed changes in the CD spectra of POL in the presence of detergents indicate alteration of the peptide conformation resulting from interactions with the micelles. Comparison of the CD spectra of POL recorded in pure DPC and mixed DPC:SDS micelles showed that an addition of negatively charged SDS only slightly influences the intensity of the major minimum at 215 nm. This indicates that the conformation of POL and its interactions with DPC and the mixed

DPC:SDS micelles remain very similar, which suggests stabilization and positioning of POL in DPC and DPC:SDS mixed micelles occurs mainly through hydrophobic interactions. On the contrary, in the presence of pure anionic SDS micelle, the major negative band was blue-shifted, which may be explained by the increased role of local electrostatic interactions that occur between the positively charged residues of POL and the negatively charged polar head groups of the SDS micelle. Due to those electrostatic interactions, POL prefers a location at the SDS micelle–water interface. Observed results are well-supported by the previously described two-step GPCR ligand transportation model, where the peptide initially interacts with the cellular membrane, leading to a change of its conformation and orientation.^{47,48} Subsequently, the peptide undergoes a two-dimensional diffusion on the membrane surface to the receptor surface membrane, where it is recognized and bound. A quantitative analysis of secondary structure performed with the CDPRO program²⁰ revealed that POL showed a minimum of ~33% β -sheet content in the SDS micelle. In the remaining solutions, including detergent-free phosphate buffer, buffered solution of the DPC and mixed DPC:SDS micelles, the β -sheet content varies from 37 to 46%, where it was highest upon binding of the peptide to DPC micelles (46%). A comparable spectral profile was observed for POL-D and POL-PD (Figure 1, panels C and D), indicating that POL conjugates retain similar secondary structure and interactions with micelles.

NMR Studies—In light of results obtained based on the CD studies, solution NMR studies were performed only for POL, and spectra were recorded in DPC micelles (Figure 2A and Figure S3 of the Supporting Information). To obtain high-quality NMR spectra allowing for assignment of all signal-related POL in DPC micellar solution (Figure 2A), spectra were recorded at 310 K and pH 5.5. The assignment of ^1H resonances were performed on the basis of 2D homonuclear TOCSY spectra acquired with 15 and 60 ms mixing times (Table 1). Sequence-specific assignment procedure was applied in a standard way based on $^1\text{H}_\alpha - ^1\text{H}_\text{N}$, $^1\text{H}_\alpha - ^1\text{H}_\delta$ (X-Pro), and $^1\text{H}_\text{N} - ^1\text{H}_\text{N}$ regions on 2D $^1\text{H} - ^1\text{H}$ NOESY spectrum. Analysis of NOE and the $^3J_{\text{HNNH}\alpha}$ coupling constants (Figure 2B) clearly indicated existence of a β -sheet structure, which is consistent with results obtained based on CD spectroscopy. The $^3J_{\text{HNNH}\alpha}$ coupling constant values varied between 10.0 and 12.1 Hz, which is typical for the extended conformation typical for the extended conformation with typical for the extended conformation ϕ dihedral angles in the range of -140° to -100° .⁵⁰ All intrastrand interproton distances, other than sequential, were too long to be observed in the NOESY spectra. On the other hand, long-range connectivity such as $d_{\alpha\text{N}}$, $d_{\beta\text{N}}$, d_{NN} , or $d_{\alpha\alpha}$ between residues in neighboring strands of the β -sheet were sufficiently short to be observed in the NOESY spectra of POL. The long-range $^1\text{H} - ^1\text{H}$ interactions comprised in the central part of the peptide and the continuous run of sequential d_{NN} contacts, which are not typical for β -structure, in the range of Arg¹-Tyr⁵, suggested presence of β -sheet from residues 4 to 13 with suggested presence suggested presence β -turn at position 8,9 (numbers correspond to two central proline residues of β -turn). In addition, the presence of $d_{\alpha\text{N}}$ (16,2) NOE cross peaks indicates a β -turn in the head-to-tail fragment Gly¹⁵-D-Pro¹⁶-Arg¹-Arg².

Molecular Dynamic Simulations—To determine the 3D structure of POL, molecular dynamic simulations explicitly utilizing DPC micelle model were performed using a set of 216 (133 intraresidue, 51 sequential, 6 medium-range, and 26 long-range) time-averaged

distance constraints (Table 2) derived from NOESY spectrum (150 ms) and 7 ϕ dihedral angles constraints obtained on the ϕ dihedral angles constraints obtained on the basis of the $^3J_{\text{HNH}\alpha}$ coupling constants. Initially POL was placed along the micelle diameter. As presented in Figure 3A, during molecular dynamics simulations POL diffused from the core of the DPC micelle to the water–DPC interface to adopt more energetically favorable conformation. The conformation and position of the peptide was stabilized in about 16 ns of MD simulations and remains nearly constant to the end of the simulations (Figure S3D of the Supporting Information). The 3D structure of POL evaluated after 24 ns of MD simulations appears as a β -hairpin motif (Figure 3B), involving two antiparallel β -strands extending in the fragments Cys⁴-Lys⁷ and Tyr¹⁰-Cys¹³ and connected with β II'-turn in position 8,9. A head-to-tail cyclization resulted in a second loop, linking both β -strands with β II'-turn in the fragment Gly¹⁵-D-Pro¹⁶-Arg¹-Arg². Previous analysis of another polyphemus II analog T140, showed that Arg², Nal³, Tyr⁵, and Arg¹⁴ are crucial for CXCR4 binding. Conformational analysis of T140 predicted that the side chains of Nal³, Tyr⁵, and Arg¹⁴ lie on the same plane of the β -sheet and form protrusion opposed to the side chain of Arg².⁵¹ In the case of POL, the side chains of all these residues extend to the same side of the backbone. However, due to clear backbone chain bending in the head-to-tail part of the peptidomimetic, the Arg² side is located below the mean plane of the β -sheet (Figure 3B). Similar mutual arrangement of the side chains essential for receptor binding is observed in the crystal structure of monocyclic antagonist CVX15 bound to the CXCR4 chemokine receptor (Figure 3C), which further corroborates with molecular modeling studies described in the later sections.³⁹

The interactions of POL with DPC micelle were further analyzed with radial distribution function (RDF) evaluated with PTRAJ program within AMBER 11 (Figure S7 of the Supporting Information). A detailed analysis showed that Nal³, Gln⁶, D-Pro⁸, and Pro⁹ residues are deeply immersed into the hydrophobic micelle core. Residues located just opposite to Nal³ and Gln⁶ in the neighboring β -strand (i.e., Arg¹⁴ and Arg¹¹, respectively) are placed in the interface of the DPC micelle and, consequently, are more accessible to the water. The corner residues of the β II'-turn in the head-to-tail part of the peptide (D-Pro¹⁶ and Arg¹) are more exposed to the aqueous environment than the D-Pro⁸-Pro⁹ template at the opposite pole of the molecule. These results suggest that the peptide is tilted to the micelle surface. This is also consistent with the electrostatic potential map of POL (Figure 3D) because the D-Pro⁸-Pro⁹ dipeptide template is a hydrophobic pole of the peptide and consequently anchored into the micelle hydro-phobic core. Kalinina and co-workers proposed that the first step of the ligand–receptor binding process is characterized by long-range electrostatic interactions between a receptor and its ligand, governing the formation of a nonspecific encounter complex.⁵² In the second step, the binding partners reorient themselves to increase the complementarity of the electrostatic surfaces. Taking into account that the binding pocket of CXCR4 and the area near its entrance are negatively charged,⁵² and POL has a positive potential on the surface, we postulate the reorientation of the peptide, when it undergoes two-dimensional diffusion from the membrane surface into the receptor. Therefore, in the micelle-bound state, POL is immersed into the micelle core with D-Pro⁸-Pro⁹ dipeptide template, whereas upon binding to the CXCR4 receptor, this pole of the peptide would be exposed to the extracellular milieu, which is consistent with the

CVX15-CXCR4 crystal structure and our molecular modeling of the peptidomimetics binding to CXCR4.

Previous studies of PEG interactions with different types of micelles showed that it exhibits a preference for interaction with anionic surfactants such as SDS, whereas interactions with cationic or zwitterionic micelles are rather limited.⁵³ The Gadolinium–DOTA complex (Gd(DOTA)), which is generally used as a paramagnetic probe in NMR studies, preferably localizes at the water–micelle interface and enables the detection of the solvent-accessible fragments of the peptide.⁵⁴ Taking this into consideration and the fact that the lysine side chain of POL in our conformational studies is exposed to aqueous phase, one can reasonably assume that modification of the Lys⁷ side chain with hydrophilic DOTA or PEG₁₂-DOTA will not significantly influence the interactions with the DPC micelle. Supporting this assumption, our molecular dynamic analysis of POL-D and POL-PD indicate that conjugation of Lys⁷ side chain with DOTA or PEG₁₂-DOTA only slightly affects their interactions with the DPC micelle, which could also be attributed as a direct consequence of removing the positive lysine charge (Figure 3, panels E and F, and Figure S7 of the Supporting Information). In both conjugates, the uncharged lysine hydrocarbon chain is immersed into the micelle interior. In POL, it is strongly water exposed, most likely due to repulsive electrostatic interactions with positively charged choline groups of DPC. Some differences were also observed for the D-Pro⁸-Pro⁹ pole of the conjugated peptidomimetics, which in the case of POL is deeply buried in the DPC micelle core (vide supra). In the case of POL conjugates, D-Pro⁸ remains still deeply immersed into the hydrophobic micelle core, but Pro⁹ is located closer to the micelle surface. The 3D structures of POL and both conjugates obtained after 24 ns of MD simulations show high similarity (Figure 3, panels A and G), including the arrangement of all side chains, requiring a two-dimensional diffusion in a similar manner for receptor binding. However, removal of the positive charge from lysine side chain in the case of POL-D and POL-PD may also affect interaction with CXCR4 compared to the unmodified peptidomimetic, since entrance to the receptor is negatively charged.

Receptor Binding Affinity

To validate the MD simulations, the CXCR4 receptor-binding affinities of POL and its derivatives were determined by a competitive binding assay using fluorescently labeled CXCL12-Red as a ligand and CHO-K1-CXCR4-SNAP-Lumi4-Tb cells based on the fluorescence resonance energy transfer technology (Table 3, Figure 4A and Figure S6 of the Supporting Information).³⁷ POL is a close analog of CVX15, which was previously used for determination of the CXCR4-ligand crystal structure and exhibits similar conformation to 3D structure of POL obtained based on our simulations.³⁹ The difference between these two peptides is replacement of 1-Nal with 2-Nal and a head-to-tail cyclization to obtain POL. Therefore, we also used CVX15 for in vitro CXCR4 affinity comparison studies. All analytes demonstrated a concentration-dependent inhibitory effect on the CXCL12-Red binding to CXCR4. The lowest IC₅₀ value was observed for CVX15 (0.02 nM), while the bicyclic POL demonstrated a 4.35-fold lower affinity (0.087 nM) compared to the parent analog. Further decrease in binding affinity of POL to CXCR4 by 5.4 (0.47 nM)- and 16.3 (1.42 nM)-fold was observed upon modification of Lys⁷ with DOTA or PEG₁₂-DOTA,

respectively. These results indicate that removal of the positive charge from the lysine side chain perturbs the interaction of the peptidomimetic with the targeted receptor and suggests that changes observed in our MD simulation studies are more likely due to loss of the positive charge on lysine.

Molecular Modeling Studies

To understand the decrease in binding affinity from CVX15, POL, and POL-D conjugate to CXCR4, a series of in situ ligand minimization experiments were conducted using Discovery Studio. This algorithm minimizes the agent built within the binding domain of a given protein structure. The results of three individual minimization experiments, in which CVX15, POL, or POL-D were subjected to the minimization procedure using a reported CVX15-CXCR4 X-ray crystal structure,³⁹ (3OE0) in Figure 4B (and Figure S4 of the Supporting Information) shows superimposed CVX15, POL, and POL-D bound to the receptor. Molecular modeling showed that N-terminus of CVX15 forms a salt bridge with the carboxylate side chain of Asp187 in the extracellular loop II (ECL-II) of CXCR4 and also engages in a π -cation interaction with the Trp⁹⁴ in helix II of CXCR4. Cyclization of CVX15 to form POL cancels the π -cation interaction with Trp⁹⁴ and replaces the Asp¹⁸⁷ salt bridge with a weaker H bond between the Arg¹ side chain and Asp¹⁸⁷. These perturbations in the binding interaction may have contributed to a decreased binding affinity as manifested by the increase of IC₅₀ from 0.02 nM for CVX15 to 0.08 nM for POL. Furthermore, the protonated ϵ -amino group of Lys⁷ of POL maintains a salt bridge with Asp¹⁹³ of CXCR4 (helix V). Conjugation of POL with DOTA replaces the salt bridge with a weaker H bond contributing to further decrease in binding affinity as indicated by an increase in the IC₅₀ value (0.47 nM) for POL-D. The increase of entropy associated with the long PEG linker could be a contributing factor to the further loss of binding affinity observed for POL-PD.

Radiolabeling and in Vitro Cell Binding Assays

To evaluate the distribution, specificity, and clearance of the synthesized peptidomimetic analogs, POL-D and POL-PD were labeled with ¹¹¹In(III). Radiolabeling resulted in 70–75% yields with > 95% radiochemical purity (Figure S5, panels A and B, of the Supporting Information). Specific activities were 4.9 MBq/ μ g (132 μ Ci/ μ g) and 4.8 MBq/ μ g (131 μ Ci/ μ g) for [¹¹¹In]POL-D and [¹¹¹In]POL-PD, respectively. Incubation of the resulting [¹¹¹In]POL-D and [¹¹¹In]POL-PD with U87-stb-CXCR4 and U87 tumor cells showed significantly higher uptake of radioactivity by U87-stb-CXCR4 cells (Figure 4C). The U87-stb-CXCR4/U87 ratios were 3.98, and 4.93 for [¹¹¹In]POL-D and [¹¹¹In]POL-PD, respectively. In spite of its lower affinity to the CXCR4 receptor, improved in vitro specificity observed with [¹¹¹In]POL-PD could be due to its increased hydrophilicity and the resulting washout of non-specifically bound peptide.

Biodistribution

To evaluate the in vivo CXCR4 binding properties of [¹¹¹In]POL-D and [¹¹¹In]POL-PD, biodistribution studies were performed in NOD/SCID mice bearing U87 and U87-stb-CXCR4 brain tumor xenografts. At 90 min post injection, both tracers showed specific and similar uptake in the U87-stb-CXCR4 tumors compared to the U87 tumors (Figure 5A). The U87-stb-CXCR4-to-U87 ratio for [¹¹¹In]POL-D was 2.26. In addition to the tumor, uptake

of [^{111}In]POL-D was also present in the liver, spleen, lungs, and kidneys, similar to the results observed with other polyphemusin II-derived peptides.^{7,12,8} The blood pool, $5.54 \pm 0.56\%$ ID/g, is in agreement with the high plasma half-life reported for the POL.¹⁸ This high retention of radioactivity resulted in tumor-to-blood ratios of 1.80 and 0.79 for U87-stb-CXCR4 and U87 tumors, respectively. Nonspecific tissues, such as the muscle, accumulated $0.54 \pm 0.02\%$ ID/g of radioactivity leading to tumor-to-muscle ratios of 18.33 and 8.1 for U87-stb-CXCR4 and U87 tumors, respectively.

For [^{111}In]POL-PD, the % ID/g in the U87-stb-CXCR4 and U87 tumors was 12.73 ± 0.68 and 2.62 ± 0.22 , respectively, resulting in an increased U87CXCR4/U87 tumor ratio of 4.86. Radioactivity uptake in lung and spleen were nearly 10% and 15% lower than those observed with the POL-D. The muscle and blood % ID/g values were 0.29 ± 0.05 and 2.66 ± 0.35 , respectively. Similar lower accumulation of radioactivity was observed in a majority of other tissues except for the liver, suggesting that PEGylation of the peptide results in improved clearance. Overall, [^{111}In]POL-PD radiotracer demonstrated a similar distribution profile as that of the [^{111}In]POL-D but with faster clearance, resulting in high target-to-nontarget, tumor-to-blood, and tumor-to-muscle ratios.

The relatively high percentage of injected dose observed in the blood at 90 min post injection reflects the high plasma stability and circulation times observed with POL.¹⁸ The four positive charges associated with the POL conjugates and lipophilicity may have contributed to the increased lung, liver, and kidney uptake. Several of the previously investigated CXCR4-binding radiolabeled polyphemusin II derivatives demonstrated nonspecific binding to the red blood cells.^{7,12,8} In these studies, use of low doses of cold peptide was shown to increase the tumor uptake, reduce the red blood cell bound radioactivity, and accumulation in nontarget tissues.⁷ To further evaluate the in vivo specificity of [^{111}In]POL-D and investigate if similar improvements can be achieved, we performed blocking studies with increasing concentrations of the cold POL peptide (Figure 5B). In mice pretreated with 5 μg of blocking dose, a significant reduction in % ID/g was observed in blood, lungs, spleen, control U87 tumors, and several other peripheral tissues. No reduction in % ID/g was observed in the U87-stb-CXCR4 tumor or kidneys, but a significant increase in uptake, from 31 to 63% ID/g, was observed in the liver. Further increase of the blocking dose to 15 or 45 $\mu\text{g}/\text{mouse}$ resulted in considerably lower accumulation of radioactivity in all organs and the U87-stb-CXCR4 tumor. An increase in % ID/g was also observed in kidneys presumably due to faster renal clearance. These blocking studies suggest that high uptake observed with [^{111}In]POL-D in several tissues was partly due to nonspecific binding. Also, blocking observed with low doses of the unlabeled peptide without impairing the tumor uptake suggests that the POL peptidomimetic is highly protein bound and further supports the previous protein binding studies reported with POL¹⁸ and differs from the distribution profile observed with other reported CXCR4 binding peptides.

In view of the high tumor uptake observed and the suitable plasma/metabolic stability reported for POL and the relatively long half-life of ^{111}In radioisotope, we investigated the biodistribution profile of both [^{111}In]POL-D and the [^{111}In]-POL-PD at 24 h post injection of the tracers (Figure 5C). Biodistribution results for [^{111}In]POL-D showed retention of radioactivity mainly in the U87-stb-CXCR4 tumor, liver, spleen, and kidneys. Tumor uptake

remained similar to that observed at 90 min, while a significant decrease in % ID/g values was observed in the blood and lungs compared to the distribution at 90 min. Interestingly, [^{111}In]POL-PD demonstrated nearly 50% lower % ID/g in all the tissues and tumors. While PEGylation could have resulted in faster clearance of [^{111}In]POL-PD, the loss of affinity to the receptor associated with the conjugation of the linker may have contributed to the low retention observed in the U87-stb-CXCR4 tumor.

SPECT-CT Imaging

The whole body SPECT/CT images of NOD/SCID mice bearing U87 and U87-stb-CXCR4 tumors acquired between 1–2 h and 24–25 h post injection of [^{111}In]POL-D and [^{111}In]POL-PD clearly delineated the U87-stb-CXCR4 tumors with obvious and specific accumulation of radioactivity (Figure 6). Highest uptake was also seen in the liver and kidneys with both imaging agents. At 1 h post injection, [^{111}In]POL-PD demonstrated an improved contrast compared to the [^{111}In]POL-D and seemed to clear faster from U87-stb-CXCR4 by 24 h, which is in good agreement with the biodistribution data. The biodistribution and imaging studies of the POL conjugates illustrate the interplay between the affinity and hydrophilicity in target-specific binding, retention, and in vivo distribution.

Because of the wide variety of radionuclides and tumor models used in the previous studies, a fair comparison of the performance of POL peptidomimetics with other published work is difficult. Nevertheless, the observed tumor uptake with [^{111}In]POL-D and [^{111}In]POL-PD suggest that other short half-life radionuclides, including $^{99\text{m}}\text{Tc}$, ^{64}Cu , ^{18}F , and ^{68}Ga , could be incorporated once an analog with suitable pharmacokinetics is identified. In addition, our studies suggest that POL3026 analogs with an alkylated lysine⁷ secondary amine may possess improved affinity to the receptor. Furthermore, the fact that PEGylated POL retains binding affinity to CXCR4 at the nanomole level provides a multitude of opportunities to use this peptidomimetic for synthesis of targeted nanoparticles for specific drug delivery to tumors.

CONCLUSIONS

Structural evaluation of POL and its conjugates showed that modification of the lysine side chain with DOTA or PEG₁₂-DOTA does not significantly alter the conformation of the peptidomimetics or their interactions with micelles. However, there was loss of affinity to CXCR4 upon conjugation, which is more pronounced for PEG₁₂-DOTA in comparison to DOTA. In vivo evaluation of ^{111}In radiolabeled conjugates showed high and specific CXCR4 accumulation and retention in the U87-stb-CXCR4 tumors even at 24 h post injection despite decreased affinity observed upon modification of POL. PEGylation resulted in improved imaging contrast and faster clearance. Our observations suggest POL peptidomimetics offer a suitable template for the development of pharmacokinetically optimized imaging agents.

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ABBREVIATIONS

POL	POL3026 β -hairpin protein epitope mimetic
POL-D	POL3026 conjugated to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
POL-PD	POL3026 conjugated to polyethylene glycol 12, terminated with 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid
RP-HPLC	reversed phase-high performance liquid chromatography
RT	retention time

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Figure 1.

CD spectroscopy of POL, POL-D, and POL-PD. (A) Schematic depiction of POL3026 and the derivatives. The CD spectra of (B) POL, (C) POL-D, and (D) POL-PD at pH 7.4 in detergent-free phosphate buffer (black) and the following micellar solutions DPC (red), SDS (green), DPC:SDS 9:1 (blue), and DPC:SDS 5:1 (cyan).

Figure 2.

NMR structural analysis of POL. (A) The fingerprint region of the ${}^1\text{H}$ - ${}^1\text{H}$ TOCSY spectrum recorded for POL in DPC micellar solution at 310 K and pH 5.5. (B) Graphical representation of NOE effects and the vicinal coupling constants, ${}^3J_{\text{HNH}\alpha}$ [Hz], observed for POL in DPC micellar solution at 310 K and pH 5.5. The thickness of the lines corresponds to NOE intensities. Disulfide bridge and head-to-tail linkage are shown by a solid line. Asterisk in 2B corresponds to $d_{\alpha\delta}(i,i+1)$ connectivity.

Figure 3.

Molecular dynamic simulations of POL, POL-D, and POL-PD. (A) Diffusion of POL from the hydrophobic core of the DPC micelle to the interface over molecular dynamic time. (B) Stereoview of 20 conformers of POL obtained in the last steps of 24 ns molecular dynamics simulations with time-averaged distance constraints and ϕ dihedral angle constraints as well as POL structure obtained after 24 ns of MD simulations. The side chains of Arg², 2-Nal³, Tyr⁵, and Arg¹⁴ that crucial for binding to CXCR4 are marked in red. (C) Stereoview of a comparison of the 3D structure of POL obtained in this study (cyan) with the 3D crystal

structure of CVX15 (orange) extracted from the CXCR4-CVX15 complex taken from PDB database (3OE0). (D) Surface electrostatic potential of POL. Electrostatic potential is presented as blue for positive and white for neutral potentials. Figure was prepared with MOLMOL. Position of (E) POL-D and (F) POL-PD in the DPC micelle after 24 ns of MD simulations. (G) Stereoview of a comparison of the 3D structures of POL (blue), POL-D (green), and POL-PD (firebrick) obtained after 24 ns of MD simulations.

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Figure 4.

POL peptidomimetics affinity. (A) Representative normalized curves of in vitro inhibition of CXCL12-red binding to CXCR4, illustrating decrease of IC_{50} upon transition from CVX15 to POL and conjugation of DOTA or PEG₁₂-DOTA. (B) Superimposed binding modes of CVX15 (red), POL (blue), and POL-D (green) to CXCR4 as predicted by an in situ ligand minimization protocol (Discovery Studio 3.1 client). The in situ experiments used 3OE0 X-ray coordinates of CXCR4. The protein is presented as a light gray line ribbon. (C) In vitro binding of [¹¹¹In]POL-D and POL-PD to U87 and U87-stb-CXCR4 cells; ***, $P < 0.001$.

Figure 5.

Specificity of POL-D and POL-PD binding to CXCR4. Ex vivo biodistribution of [¹¹¹In]POL-D and [¹¹¹In]POL-PD in NOD/SCID mice bearing U87 and U87-stb-CXCR4 glioblastoma xenografts brain tumor ($n = 3$), presented as percent of injected dose per gram tissue (% ID/g) at 90 min post injection (A). (B) Blocking experiments, in which mice were injected with different doses (5, 15, and 45 μ g) of POL3026 30 min prior to the administration of [¹¹¹In]POL-D. (C) Ex vivo biodistribution 24 h after administration of [¹¹¹In]POL-D and [¹¹¹In]POL-PD. **, $P < 0.01$; ***, $P < 0.001$.

Figure 6. Imaging CXCR4 expression with POL peptidomimetics: Representative whole body SPECT/CT images of NOD/SCID mice bearing U87 and U87-stb-CXCR4 glioblastoma xenografts on left (unfilled arrow) and right (solid arrow) flanks, respectively, recorded (A) 2 h and (B) 24 h after tail vein injection of ~350 μ Ci of [^{111}In]POL-D (left panel) and [^{111}In]POL-PD (right panel). All images were scaled to the same maximum threshold value. K, kidney; L, liver; B, bladder. Images clearly indicate that CXCR4 expression can be detected using both radiotracers due the high and specific accumulation in U87-stb-CXCR4 tumors.

¹H Chemical Shifts of POL3026 in DPC Micelle at 37°C and pH = 5.5. Chemical Shifts are Measured ±0.02 ppm at 500 MHz Frequency^a

Table 1

residue	proton chemical shifts (ppm)				
	HN	Hα	Hβ	Hγ	others
Arg ¹	8.49	4.06	1.73,1.95	1.52	Hδ 3.13, Hε 7.19
Arg ²	7.94	4.21	1.61	1.18,1.39	Hδ 2.65, 2.73, Hε 7.23
Nal ³	8.34	4.69	3.22, 3.33		H ₁ 7.61, H ₃ 7.36, H ₄ 7.45, H ₅ 7.75, H ₆ 7.42, H ₇ 7.37, H ₈ 7.74
Cys ⁴	8.60	5.70	2.55, 3.00		
Tyr ⁵	8.93	4.83	2.92		H _{2,6} 6.89, H _{3,5} 6.60
Gln ⁶	8.55	n	1.93, 2.00	2.28	ε-NH ₂ 6.53, 7.40
Lys ⁷	8.89	4.83	1.55,1.91	1.37, 1.73	Hδ 1.73, 1.77, Hε 2.98
DPro ⁸	–	4.69	1.89, 2.24	2.00, 2.13	Hδ 3.53, 3.82
Pro ⁹	–	4.33	1.60, 2.02	1.23, 1.78	Hδ 3.61, 3.79
Tyr ¹⁰	7.62	4.63	2.90, 3.05		H _{2,6} 7.14, H _{3,5} 6.82
Arg ¹¹	8.33	4.70	1.57, 1.64	1.34	Hδ 3.05, Hε 7.13
Cit ¹²	8.50	4.51	1.27, 1.50	1.35	Hδ 3.05, Hε 6.28
Cys ¹³	8.57	5.36	2.92		
Arg ¹⁴	8.89	n	2.10	1.84	Hδ 3.22, 3.30, Hε 7.66
Gly ¹⁵	8.73	3.84, 4.43			
DPro ¹⁶	–	4.38	1.95, 2.27	2.17	Hδ 3.63

^a n: not determined. These protons were probably bleached due to water suppression.

Table 2

Statistic of Distance Constraints Used for High-Resolution 3D Structure Calculations and Quality Ensemble of the 20 Conformations of POL3026 Peptide Obtained in the Last Steps of MD Simulations

Total Number of NOE Restraints ^a	216
intraresidual ($ i - j = 0$)	133
sequential ($ i - j = 1$)	51
medium-range ($ i - j \leq 5$)	6
long-range ($ i - j > 5$)	26
Rmsd to the Mean Structure for Residues 4...13 (Å)	
backbone atoms	0.133
all heavy atoms	0.286
Hydrogen Bonds	
HN ² -CO ¹⁵	
HN ⁵ -CO ¹²	
HN ⁷ -CO ¹⁰	
HN ¹⁰ -CO ⁷	
HN ¹² -CO ⁵	
HN ¹⁴ -CO ³	
HN ¹⁵ -CO ³	

^aThe range of interproton distances was evaluated based on the residue number in the sequence, therefore the distance constraint, such as for example dN_α(1,16), was considered as a long-range one.

Half Maximum Inhibitory Concentrations (IC₅₀) and Inhibition Constants Obtained Based on Inhibition of CXCL12-Red Binding to CXCR4^a

Table 3

no.	peptide	IC ₅₀ (nM)	IC ₅₀ 95% CI	K _i (nM)	K _i 95% CI
1	CVX15	0.02	0.008–0.031	0.009	0.005–0.017
2	POL	0.087	0.035–0.125	0.0489	0.019–0.071
3	POL-D	0.47	0.28–0.96	0.26	0.12–0.53
8	POL-PD	1.42	0.71–1.81	0.817	0.395–1.01

^a CI: confidence intervals.