Preclinical Development of a Novel Class of CXCR4 Antagonist Impairing Solid Tumors Growth and Metastases

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Abstract

The CXCR4/CXCL12 axis plays a role in cancer metastases, stem cell mobilization and chemosensitization. Proof of concept for efficient CXCR4 inhibition has been demonstrated in stem cell mobilization prior to autologous transplantation in hematological malignancies. Nevertheless, CXCR4 inhibitors suitable for prolonged use as required for anticancer therapy are not available. To develop new CXCR4 antagonists a rational, ligand-based approach was taken, distinct from the more commonly used development strategy. A three amino acid motif (Ar-Ar-X) in CXCL12, also found in the reverse orientation (X-Ar-Ar) in the vMIP-II inhibitory chemokine formed the core of nineteen cyclic peptides evaluated for inhibition of CXCR4-dependent migration, binding, P-ERK1/2-induction and calcium efflux. Peptides R, S and I were chosen for evaluation in in vivo models of lung metastases (B16-CXCR4 and KTM2 murine osteosarcoma cells) and growth of a renal cells xenograft. Peptides R, S, and T significantly reduced the association of the 12G5-CXCR4 antibody to the receptor and inhibited CXCL12-induced calcium efflux. The four peptides efficiently inhibited CXCL12-dependent migration at concentrations as low as 10 nM and delayed CXCL12-mediated wound healing in PES43 human melanoma cells. Intrapertoneal treatment with peptides R, I or S drastically reduced the number of B16-CXCR4-derived lung metastases in C57/BL mice. KTM2 osteosarcoma lung metastases were also reduced in Balb/C mice following CXCR4 inhibition. All three peptides significantly inhibited subcutaneous growth of SN12C-EGFP renal cancer cells. A novel class of CXCR4 inhibitory peptides was discovered. Three peptides, R, I and S inhibited lung metastases and primary tumor growth and will be evaluated as anticancer agents.

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Introduction

Chemokines are a large family of 8 to 12 kDa peptides that serve as chemoattractants for cellular activation, differentiation and trafficking. To date, about 50 chemokines have been identified in humans, and these have been grouped into four families - CXC, CC, CX3C, and XC - based on the arrangement of cysteine residues involved in the formation of disulfide bonds [1–3]. The biological activities of chemokines are exerted via seven transmembrane domain G-protein coupled chemokine receptors having long disordered N and C-terminal regions and three transmembrane domain G-protein coupled chemokine receptors having long disordered N and C-terminal regions and three transmembrane domain G-protein coupled chemokine receptors having long disordered N and C-terminal regions and three transmembrane domain. The CXCR1/CXCL10 (stromal cell-derived factor-1α) binds to the CXCR4 and CXCR7 receptors, initiating divergent signaling pathways that result in chemotaxis, cell survival and/or proliferation, increased intracellular calcium and transcription of genes critical for cell inflammation and cancer metastases [4,5]. CXCR4 receptor activation is mediated by coupling to an intracellular heterotrimeric G-protein associated with the inner surface of the plasma membrane [4,5]. Although it was initially thought that CXCR4 only transduces through an intracellular heterotrimeric G-protein subunit Gzι [4], recent evidence suggests CXCR4 involves Gzq, Gzο, and Gzς and thus activates different downstream pathways. A newly discovered receptor, CXCR7, binds CXCL12 with higher affinity than CXCR4 [6,7] and regulates CXCR4 function [8]. While CXCR4 activity is primarily G-protein mediated, the transduction pathway originating from the CXCR7 receptor seems to involve the β-arrestin pathway and is G-protein independent [9,10].

The CXCL12/CXCR4 axis function in adults is integral to lymphocyte trafficking and to the retention and homing of hematopoietic stem cells in the bone marrow microenvironment [11,12]. In cancer, CXCR4 expression was first correlated with the metastatic capability of breast and melanoma cancer cells [5];
then a direct correlation between receptor upregulation and tumor progression, neovascularization, invasion and metastasis was demonstrated [13–20]. CXCL12 is constitutively expressed in lung, liver, skeletal muscle, brain, kidney, heart, skin and bone marrow and is induced in tissue damage such as myocardial infarction, limb ischemia, toxic liver damage, excessive bleeding, total body irradiation, and chemotherapy [17–20]. It has also been implicated in the recruitment of bone marrow derived cells (BMDCs) into tumors [20,21].

As result of its pleiotropic role in tumor development, the CXCR4-CXCL12 pathway is considered an important potential cancer therapeutic target. Plerixafor (previously known as AMD3100) is a CXCR4 antagonist that has provided proof of concept for inhibition of the pathway. Mobilization with G-CSF plus Plerixafor reduces the incidence of failure to collect the minimum number of CD34 stem cells necessary for autologous stem cell transplantation. Consequently, Plerixafor in combination with G-CSF has FDA approval for hematopoietic stem cell mobilization in patients with non-Hodgkin lymphoma and multiple myeloma [22]. Plerixafor, a metal-chelating bicyclam, has been reported to cause cardiotoxicity and other adverse events, leading to the consensus opinion that it is not a suitable agent for long-term use as an anticancer agent [23–24].

To develop new CXCR4 antagonists suitable for anticancer therapy, a ligand-based approach was taken. Like other members of the chemokine family, CXCL12 has a short N-terminal region, an ~10-amino acid loop that follows the CC/CXC motif (N-loop), a large well-folded core characterized by a three-strand antiparallel β-sheet, a C-terminal α-helix and N- and C-terminal distal regions [25,26]. Searching for short structural motifs in the ligand receptor-binding region, a three-residue segment was identified in CXCL12 that was similar to, in reverse order, a peculiar inhibitory chemokine secreted by herpes virus 8 (HHV8) known as vMIP-II. The motif Ar1-Ar2-R, where Ar is an aromatic residue [27–31], constitutes the core of a cyclic peptide library that was tested in in vitro and in vivo for its ability to inhibit CXCR4 function [32].

Materials and Methods

Synthesis/Design Methods

The 19-membered library, consisting of different cyclic peptides, was synthesized on solid phase by using Fmoc chemistry standard protocols. All compounds were obtained in good yield and with high purity grade (>95%) after RP-HPLC purification. They were fully characterized for their identity by mass spectrometry. Starting structures were modeled from the experimental CXCL12 backbone conformation of the R-Ar2-Ar1 motif by mutating the preceding and following residues into cysteine residess, while the sequence sense was alternatively derived from vMIP-II or CXCL12. The designed peptides were energy minimized and then subjected to molecular dynamics in solution.

Cell Culture

PES 43 human melanoma cells were grown at 37°C in 5% CO₂ in IMDM with 10% fetal bovine serum (FBS). CCRF-CEM human T-Leukemia cells were grown in RPMI-1640 with 10% FBS and 2 mM glutamine. B16-CXCR4 murine melanoma cell line were transfected with pYF1-fusin plasmid containing CXCR4 gene (kindly provided by Dr Aloj, NCI “Pascale”, Naples, Italy) SN12C human renal cancer cells were transfected with pEGFP-1 (BD biosciences Clontech). The transfected cells were grown at 37°C in 5% CO₂ in IMDM with 10% FBS and 2 mM glutamine 50 μg/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL G418. B16 and SN12C cells were transfected with CXCR4 according to FuGEN 6 protocol (Roche Applied Science, Indianapolis, IN). All the tested cell lines were morphologically identified monthly.

Migration Assay and Wound Closure

Migration was assayed in 24-well Transwell chambers (Corning Inc., Corning, NY) using inserts with an 8-μm pore membrane. Membranes were precoated with collagen (human collagen type I/III) and fibrinectin (20 μg/mL each). PES43 cells were placed in the upper chamber (2.5 × 10⁵ cells/well) in IMDM containing 1% BSA (migration media) in the presence of AMD3100 or peptides; 100 ng/mL CXCL12 was added to the lower chamber. After 16 h incubation, cells on the upper surface of the filter were removed using a cotton wool swab; migration of cells in migration media alone was compared with migration in media containing CXCL12. The cells were counted in ten different fields (original magnification ×40). The migration index was defined as the ratio between migrating cells in the experimental group and migrated cells in the control group. For the wound closure assay, tested cells were allowed to reach confluence in six-well plates containing serum-depleted growth medium and scratched with pipette tips to make wounds. The wound closure was observed microscopically 6 hours post-wounding (OKO Time Lapse).

CXCR4 Binding

CXCR4 binding was evaluated as previously described [33]. Briefly 5 × 10⁵ CCRF-CEM cells were pre-incubated with 10 μM peptides or AMD3100 in binding buffer (FBS 1 x plus 0.2% BSA and 0.1% NaNO₃) for 30 minutes at 37°C, 5% CO₂ and then labeled for 30 minutes with anti-CXCR4 PE-antibody (FAB170P,
the presence of peptides or AMD3100 bound was assessed indirectly by flow cytometry using the PE-labelled anti-CXCR4 antibody (clone 12G5). Calcium Mobilization Assay

Calcium Mobilization Assay

For the calcium efflux studies a 2 mg/mL stock solution of Fluor-3 acetoxymethylester (Invitrogen, Carlsbad, CA) in anhydrous DMSO was used. A 20% w/v stock solution of the detergent pluronic acid F-127 (Invitrogen, Carlsbad, CA) was also prepared in DMSO. CCRF-CEM cells were resuspended in cell loading buffer (PBS, 1% FCS, 1 mM MgCl₂, and 1 mM CaCl₂) at 5 x 10⁶ cells/mL. Fluor-3AM and Pluronic acid were added to each sample to increase Fluor-3AM solubility and improve dye loading into the cells. Baseline calcium efflux was established, then chemokines were added as indicated and chemokine-induced calcium efflux was measured.

Immunoblotting

Cells were homogenized in lysis buffer (40 mM Hepes pH 7.5, 120 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM EDTA, 1% Triton X-100) containing protease (Complete Tablets- EDTA-free, Roche) and phosphatase inhibitors (20 mM α-glycerol-3-phosphate, 2,5 mM Na-pyrophosphate). The following primary antibodies were used: anti-p-ERK (sc7383, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-ERK2 (sc 154G, Santa Cruz Biotechnology CA, USA). P-Erk induction was plotted as ratio P-Erk in the presence of CXCL12 (100 ng/ml)/P-Erk in serum free.

In Vivo Assays

All mice were obtained from Harlan (Bar Harbor, ME, USA) and their care was in accord with institutional guidelines. Twenty-five 6-8-week-old female C57BL/6 mice were inoculated into the tail vein with 5 x 10⁶ B16-CXCR4 cells pre-treated for 30 minutes with peptide S (10 μM), peptide R (10 μM), peptide I (10 μM), or AMD3100 (10 μM). Six hours later intraperitoneal (IP) treatment started with AMD3100 (1.25 mg/kg) or each peptide (2 mg/kg) in sterile PBS once a day for 10 days. A week later the mice were sacrificed. The experiment was repeated three times. The lung metastases assay was also conducted with K7M2 murine osteosarcoma cells. Twenty-five 6-8-week-old female Balb/c mice were injected via tail vein with 2.5 x 10⁵ K7M2 cells pre-treated for 30 minutes with AMD3100 (10 μM), peptide S (10 μM), peptide R (10 μM), peptide I (10 μM) and animals were treated IP daily for 15 days with 2.5 mg/kg for AMD3100 group and 10 mg/kg for each peptide groups. A week later the mice were sacrificed. Xenograft tumor growth was evaluated using pEGFP-SN12C cells in exponential growth phase subcutaneously injected into the right flank of twenty-five 6-8-week-old female CD1 nude mice. 2 x 10⁵ SN12C-pEGFP were injected in the right flank of each mouse and treated with AMD3100 (1.25 mg/kg) and peptides (2 mg/kg) for 10 days ip. A week later mice were euthanized and tumor volume evaluated through Leica MacroFluo fluorescence stereomicroscope (Leica microsystem, Houston, TX). The experiment was repeated three times. The Istituto Nazionale per lo Studio e la Cur a dei Tumori, Fondazione Giovanni Pascale Independent Ethical Committee approved the study (GEI/600/12).

Results

Rational Design of a Novel Class of Cyclic Peptides that Inhibit CXCR4

To develop new CXCR4 antagonists suitable for anticancer therapy, a ligand-based approach was employed. Previous NMR studies on the vMIP-II N-terminal tail showed that a structured motif encompassing tryptophan 5 to proline 8 (W5-P8) preserved its conformation in the intact protein, making this motif a promising candidate scaffold to design short CXCR4-ligand peptides. A three-residue motif, Trp-His-Arg (Ar1-Ar2-R), was identified in the vMIP-II N-terminus [25–31] and after comparison with the CXCL12 structure, a similar motif was identified in CXCL12, but in the N-loop and with the sequence reversed, Arg-Phe-Arg (R-Ar1-Ar2) [30]. Consequently, the two motifs were used as templates to design cyclic peptides with the structure G-Ar1-Ar2-R-C and C-R-Ar1-Ar2-G, with the cysteines at each end in a disulfide-bridge to stabilize the structure and provide...
protection from proteases. In addition, peptides were either 
elongated at their C-termini or, after sequence-reversal, at their 
N-termini, so as to mimic another possibly conserved basic residue 
motif. The peptide series shown in Table 1 was synthesized to 
undergo biochemical and biological characterization (Italian 
Patent n\textsuperscript{u} MI2010A000093; International Patent n\textsuperscript{u} WO2011/ 
092575 A1) \[32\]. Peptides were CXCL12-mimetic (R, S and T, 
shown in bold) and v-MIP-II mimetic (I and all others). 

Representative structures for Peptides R, I, O and D are showed 
in \textbf{Figure S1}.

**Peptides R, I, S and T Impair the Binding of 12G5 to 
CXCR4**

An initial biological characterization regarded possible peptide 
toxicity. Peptides R, I and S were evaluated for cytotoxicity on 
several human cancer cell lines and showed no toxicity \[Figure 
S2\]. The anti-CXCR4 cyclic peptides were first evaluated for their 
ability to inhibit the association of the 12G5 anti-CXCR4 
antibody with the CXCR4 receptor \[22\]. CCRF-CEM cells were 
incubated with the anti-CXCR4 cyclic peptides (10 \mu \text{M}) or with 
the CXCR4 inhibitor, AMD3100 (10 \mu \text{M}) for 30 minutes. 

**Figure 1A** shows the effect of the peptides R, I, S, and T 
(10 \mu \text{M}) on the binding of 12G5 to CXCR4. Peptides R, S, and T, 
seven-residue CXCL12-mimetics with elongation at the N-
terminus, reduced the association of the 12G5-CXCR4 antibody 
to the receptor to 21, 17 and 22\% of control, respectively, a level 
of inhibition comparable to that obtained with AMD3100; the 
five-residue peptide I, a v-MIP-II-mimetic, inhibited the association 
of the 12G5-anti-CXCR4 antibody to 45\% of control. Since 
CXCL12 also binds CXCR7, its association with the CXCR7 
receptor in the CXCR7 expressing cell line, MCF7, was evaluated 
in the presence of peptides R, I, S and T. The unrelated CXCR3-
expressing cell line, COLO205, was also evaluated. \textbf{Figures S3A-} 
\textbf{C} show that peptide S inhibited 12G5-CXCR4 binding to 
CXCR4 comparably to AMD3100 while the binding to CXCR3 
or CXCR7, which was inhibited by the specific ligand, CXCL11, 
was unaffected by peptides R, I and S.

**Peptides R, I, S and T Inhibit Calcium Release**

As seen with other G-protein coupled receptors (GPCR), 
CXCR4 activation results in recruitment of G\textsubscript{q} protein that in 
turn leads to PLC\textsubscript{q} activation and Ca\textsuperscript{2+} influx. \[4\]. The effect of 
peptides R, I, S, and T (10 \mu \text{M}) on CXCL12-induced calcium 
release was evaluated using CCRF-CEM cells. The cells were 
icubated in the presence of peptide and then loaded with Fluo-
3AM in the presence of F-127 Pluronic Acid. As shown in 
**Figure 1B**, the peptides clearly inhibited CXCL12 induced 
calcium efflux: R (51.25\% of control), S (49.75\%), and T (33.65\%).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{**Figure 2. Peptides R, I, S, T Inhibit CXCL12 dependent cell migration, wound healing and p-ERK induction.** Experiments were conducted using PES43 cells. (A) Migration was assayed in 24-well Transwell chambers using inserts with 8-\mu \text{m} pore membrane. PES43 cells were placed in the upper chamber (2.5 \times 10^5 cells/well) in IMDM containing 1% BSA (migration media) in the presence of AMD3100 (10 \text{M}) or peptides at several concentrations (10 nM, 100 nM, 1 \mu \text{M}, 10 \mu \text{M}; 100 ng/mL CXCL12 was added to the lower chamber. Migrated cells on the lower surface were fixed, stained with H&E and counted microscopically. The results are expressed as the migration index relative to migration in presence of BSA alone. (B) Delay in wound healing after 6 hours in presence of peptides R, I, S and T compared with CXCL12. Images were acquired with OKO Time Lapse. (C) Effect of peptides R, I, S, and T on CXCL12 p-ERK induction at 5 minutes. PES43 cells were serum-starved and incubated with CXCL12 (100 ng/ml) alone or in presence of AMD3100/peptides. Each peptide was tested in at least three different experiments. Double tailed T-Test was used for statistical analyses. Differences were considered significant at $P < 0.05$ compared to control.
\textbf{doi:10.1371/journal.pone.0074548.g002}}
\end{figure}
Minimal effect on calcium efflux was observed with Peptide I. A representative experiment with peptide T is shown in Figure S3D.

Peptides R, I, S, and T Inhibit CXCL12-dependent Cell Migration, Wound Healing and P-ERK Induction

Functional CXCR4 transduces signals that activate chemotaxis and phosphorylation of ERK through multiple and complex pathways, such as Gqi-AC, β-arrestin-RAF and Goq-Pi3K [4]. To further characterize the effect of peptides R, I, S, and T on CXCR4 function, CXCL12-dependent p-Erk induction and migration were analyzed in PES43, a human melanoma cell line previously demonstrated to exhibit high levels of a functional CXCR4 [34]. Figure 2A shows that peptides R, I, S, T inhibited migration at concentrations as low as 10 nM. Also, given that CXCL12 primarily is a lymphocyte chemoattractant, effects of the peptides R, S and I were evaluated on CCRF-CEM human T- Leukemia cells migration. As shown in Figure S4 Peptide R inhibited CCRF-CEM human T-Leukemia cells migration in a dose-dependent manner. Figure 2B demonstrates that the peptides R, I, S and T delayed CXCL12-mediated wound healing in PES43 human melanoma cells. Figure 2C depicts the inhibition of CXCL12 induced p-ERK by peptides R, I, S, T. Like AMD3100, peptides R, I, and S (but not peptide T), reduced CXCL12-mediated p-ERK induction after 5 minutes. A representative experiment with peptide R is shown in Figure S5. In the absence of CXCL12, the peptides had a negligible effect on P-ERK induction. The in vitro evaluation of the CXCR4 inhibitory efficacy resulted in the selection of four peptides R, S, T and I that were consistently impairing CXCR4 function although performing differently in the tested assays (Peptide R revealed the best efficacy in inhibiting CXCL12 dependent migration, P-Erk induction and wound healing; Peptide S efficiently competed with the anti CXCR4 antibody binding and Peptide I showed the best efficacy in inhibiting Calcium efflux ). This is not surprising since the 12G5-anti CXCR4 antibody binding versus inhibition in migration, calcium efflux or P-Erk induction- CXCL12 measured really different properties that involves different sites of interaction.

Peptides R, I and S Inhibited Murine Lung Metastases and Human Primary Tumor Growth

To evaluate the potential of the peptides for clinical development, the efficacy of peptides R, I and S was evaluated in in vivo lung metastasis models [35,36]. While these models are often used
to evaluate inhibition of metastases, we used the assays to detect the ability of the peptides to inhibit lung colonization after the injection of tumor cells. Peptide T could not be evaluated in vivo because of poor solubility. 5
6 10^5 B16 melanoma cells transduced with CXCR4 were pre-treated with peptides R, I or S (10 \( \mu \)M) or AMD3100 (10 \( \mu \)M) and inoculated into the tail vein of C57/BL6 mice. Mice were then further treated intraperitoneally for 15 days with 2.5 mg/kg AMD3100 or 10 mg/kg peptide R, peptide I or peptide S. (B) Graphical representation of the number of lung metastases in treated mice. Double tailed T-Test was used for statistical analyses. The experiments were repeated three times.

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Figure 4. Peptides R, I and S inhibit murine osteosarcoma lung metastases. (A) Twenty-five 6-8-week-old female Balb/c mice were injected via tail vein with 2.5 \( \times \) 10^5 K7M2 cells pre-treated for 30 minutes with AMD3100 (10 \( \mu \)M), peptide R (10 \( \mu \)M), or peptide I (10 \( \mu \)M) or peptide S (10 \( \mu \)M). The animals were then further treated intraperitoneally for 15 days with 2.5 mg/kg AMD3100 or 10 mg/kg peptide R, peptide I or peptide S. (B) Graphical representation of the number of lung metastases in treated mice. Double tailed T-Test was used for statistical analyses. The experiments were repeated three times.

CXCR4 Antagonists Impaired Solid Tumor Metastases

Discussion

In this manuscript we describe a new class of ligand based, cyclic peptide inhibitors of CXCR4. Three novel cyclic peptides are shown to impair CXCR4 function in vivo and in vitro. Nineteen peptides based on an Ar1-Ar2-R motif, identified in vMIP-II and found to be present in the opposite orientation as R-Ar1-Ar2 in the N-loop of CXCL12, were initially assessed. The nineteen peptides were based on the identified motif, in both sequence sense, plus elongation at their C- or -N-termini or modification of aromatic residues. The entire peptide library was then screened in vitro for CXCR4 interfering activity. Based on the rational design, while seeking for CXCR4 antagonistic peptides, we were not expecting all of them to work. Thus peptides showing concomitant antagonistic activity in the four in vitro assays (competition with anti CXCR4 antibody binding, ligand dependent migration, calcium efflux and P-Erk induction) were further pursued.
To identify the best CXCR4 inhibitors suitable for anticancer therapy peptides R, I, S and T were selected for in vivo evaluation. Solubility limitations prevented the evaluation of peptide T in vivo, but peptides, R, I and S, reduced lung metastases in mice injected with B16-CXCR4 mouse melanoma cells and K7M2 mouse osteosarcoma cells. In addition, peptides R, I and S inhibited primary tumor growth in a xenograft model of human renal cancer cells, SN12C.

CXCR4 antagonism was defined on the basis of competition with anti-12G5-CXCR4 antibody, as well as inhibition of migration, calcium flux and P-Erk induction. While the peptides were designed to block CXCR4 and they clearly inhibit its function, we recognize that the competition assays we used do not directly measure peptide binding to CXCR4.

According to the initial design, three of these peptides are CXCL12-mimetic (R, S and T) and one is vMIP-II mimetic (I); interestingly three of the four contained the RA-NH2 extension deemed crucial for binding. Theoretical complexes obtained for peptides R, I and S showed their binding sites lay entirely in the intrahelical site, similar to AMD3100 and CVX15, a cyclic peptide inhibitor of CXCR4. Although AMD3100 and peptides R, I and S share a common core of ligand-receptor interactions, they span different subsites possibly explaining differences in activities.

The CXCR4-CXCL12 axis has become more complex since the discovery of CXCR7, a deorphanized receptor that binds CXCL12. CXCL12 binds CXCR7 with an affinity even higher than CXCR4. Recent evidence shows that TC14012, a peptido-mimetic inverse agonist of CXCR4, is also a CXCR7 agonist and that two structurally unrelated CXCR4 antagonists, TC14012 and AMD3100 also perform as agonists on CXCR7 [38]. Thus, CXCR4 and CXCR7 while transducing on the beta-arrestin pathway differently share similarities in their binding sites for synthetic ligands, suggesting CXCR4 inhibitors may also be active on CXCR7. Preliminary results demonstrate that peptides R, I and S do not affect CXCR7 and CXCR3 binding (See Figure S2), and none of our experiments suggest agonistic effects.

The development of CXCR4 inhibitors is of increasing interest with AMD3100 (Plerixafor) having been approved by the FDA for stem cell mobilization [21,39]. We evaluated peptides R, I and S for their ability to mobilize hematopoietic precursors in a DBA/2 mouse model in a CXCR4 dependent manner. Preliminary evidence shows that peptides R, I, and S induce as potent stem cell and neutrophil mobilization as does AMD3100. A single treatment resulted in a rapid and dose-related 2 to 5-fold increase in

Figure 5. Peptides R, I, and S inhibit the growth of primary human SN12C-pEGFP tumors. 6 to 8-week-old female CD1 nude mice were injected subcutaneously with 2 × 10^6 pEGFP-SN12C cells pre-treated with 10 μM AMD3100 or 10 μM of each peptide. Beginning the following day mice were further treated intraperitoneally for 10 days with 1.25 mg/kg AMD3100 or 2 mg/kg of peptides R, I or S. A week later mice were euthanized and tumor volume evaluated directly from the flank of mice using a Leica MacroFluo fluorescence stereomicroscope (Leica microsystem, Houston, TX) (A). The experiments were repeated three times. B. SN12C-pEGFP cells migration toward CXCL12 in the presence of AMD3100 or Peptide R, S and I. doi:10.1371/journal.pone.0074548.g005
neutrophils and hematopoietic stem and progenitor cells in peripheral blood. Interestingly the mobilization effect was more durable in mice treated with peptides R, I, and S compared to peripheral blood. Interestingly the mobilization effect was more neutrophils and hematopoietic stem and progenitor cells in the studies presented here both binding site. Peptide CXCR4 antagonist activity has been shown for several classes of CXCR4 inhibitors. Comparative studies between peptidic inhibitor LY2410924 [NCT 01439568] have evaluated the efficacy of the CXCR4 peptide inhibitor LY2410924 (Eli Lilly and Company) in combination with sunitinib and carboplatin/etoposide respectively. While these clinical trials are evaluating these agents in patients with advanced cancer, the ideal setting to demonstrate the efficacy of CXCR4 inhibitors will be as metastasis-preventing agents in the adjuvant and neoadjuvant setting with tumors such as colorectal cancer [47] and in the prevention of first recurrence after radiotherapy in glioblastoma [8,18,40].

In summary we describe a new family of peptides that were rationally designed and not derived from the naturally occurring CXCR4 inhibitor polyphephinus-II, used as a template to design several classes of CXCR4 inhibitors. Comparative studies between the new family of peptides and Plerixafor suggest a common binding site. Plerixafor CXCR4 antagonist activity has been shown in the studies presented here both in vitro and in vivo and a first in human, Phase I trial is planned with peptide R in patients with advanced tumors.

Supporting Information

Figure S1 Representative structures for Peptides R, I, O and D are showed. (TIF)

Figure S2 Peptides R, I and S were not toxic on human cancer cell lines (SN12C, RXF393, A498 and PES43). (TIF)

Figure S3 Peptide S specifically inhibits 12G5-CXCR4 binding to CXCR4. A. CCRF-CEM cells were preincubated for 30 minutes with Peptide S (10 \( \mu \)M) or AMD3100 (10 \( \mu \)M) and then incubated with 12G5 anti CXCR4 antibody. B. CEM cells were preincubated for 30 minutes with CXCL11 (100 nM), Peptide S (10 \( \mu \)M) or AMD3100(10 \( \mu \)M) and then incubated with anti CXCR7 antibody; C. CCRF-CEM cells were preincubated for 30 minutes with CXCL11 (100 nM), Peptide S (10 \( \mu \)M) or AMD3100(10 \( \mu \)M) and then incubated with anti CXCR3 antibody; D. Baseline calcium efflux was established, then chemokines were added as indicated and chemokine induced calcium efflux was measured. CCRF-CEM cells were preincubated for 30 minutes with CXCL12 (100 nM), Peptide T (10 \( \mu \)M) or AMD3100 (10 \( \mu \)M) or iomycin as positive control. Fluo-3AM and Phuronic acid were added to each sample to increase Fluo-3AM solubility and improve dye loading into the cells. (TIF)

Figure S4 Peptide R inhibited CCRF-CEM human T-Leukemia cells migration in a dose-dependent manner. (TIF)

Figure S5 Peptide R inhibits the CXCL12 induced p-ERK. PES43 cell lines were serum starved for 16 hours. Then the cells were preincubated for 30 minutes with Peptide R (10 \( \mu \)M) or AMD3100 (10 \( \mu \)M) and then treated with CXCL12 (100 nM). (TIF)

Author Contributions

Conceived and designed the experiments: SS PA LP AC GC CA. Analyzed the data: LP RMV CD MN CI SS PA. Contributed reagents/materials/analysis tools: CA AB AL. Wrote the paper: LP SS GC. Synthesis peptides: LM SDL. In vivo experiment: AL.

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