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A synthetic bivalent ligand of CXCR4 inhibits HIV infection

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ABSTRACT

G-protein-coupled receptors (GPCRs) are cell membrane protein receptors that transduce signals across the cell membrane and are important targets for therapeutic interventions. As members of the GPCR superfamily, chemokine receptors such as CXCR4 play critical roles in normal physiology as well as the pathology of many human diseases including cancer, inflammation, autoimmune diseases, and human immunodeficiency virus (HIV) infection. Here we report the discovery and study of a novel peptide ligand of CXCR4 using D-amino acids and bivalent ligand approach. This peptide, DV1-K-(DV3), shows very high affinity for CXCR4 with an IC_{50} of 4 nM in anti-CXCR4 monoclonal antibody (mAb) 12G5 competitive assay, which is more potent than full length natural ligand SDF-1α, even though the peptide is less than half of the number of residues of SDF-1α. This peptide can block the calcium influx stimulated by SDF-1α and inhibit cancer cell migration *in vitro* via CXCR4, thus functioning as a CXCR4 antagonist. Furthermore, DV1-K-(DV3) peptide displayed anti-HIV activity by inhibiting HIV-1 infection mediated by CXCR4. With its high receptor affinity and stability from D-amino acids, this peptide may be a new probe of CXCR4 functions in physiology and pathology and promising lead for therapeutic development.

1. Introduction

Chemokine receptors are seven transmembrane (7TM) helical proteins which belong to the superfamily of G-protein-coupled receptors (GPCRs). Chemokine receptor CXCR4 is expressed on many types of cells, such as leukocytes, hematopoietic stem cells (HSCs), endothelial cells, and tumor cells. In recent years, there has been intensive biological and chemical research of CXCR4, highlighted and exemplified by the recent determination of the three-dimensional crystal structures of CXCR4 in complex with small molecule and peptide ligands [1], to study the mechanisms of chemokine receptor–ligand interactions and functions [2–4]. CXCR4 and its natural ligand stromal cell-derived-factor-1 (SDF-1α) are involved in number of human diseases, such as cancer, inflammation, auto-immune diseases, and acquired immunodeficiency syndrome (AIDS) [5,6]. The development of new ligands targeted to CXCR4 has become a promising therapeutic approach for cancer, AIDS, and other diseases [7,8].

Like many GPCRs, CXCR4 can form dimers or even oligomers in the active state [4,9]. However, the role of CXCR4 dimerization in normal physiology and disease still awaits further investigation. The use of a linker to conjugate synthetically two identical or different ligand molecules to form a bivalent, dimeric ligand molecule as a probe of the function of the receptor dimer is becoming an important chemical biology strategy [10,11]. Previously, we reported the CXCR4 antagonist peptides DV1 and DV3 [12], both of which are D-amino acid peptides derived from the N-terminal sequences of viral macrophage protein-II (vMIP-II) and display moderate affinity to CXCR4 with IC_{50} of 236 nM and 440 nM, respectively, in an anti-CXCR4 mAb 12G5 competitive assay. We have applied the bivalent ligand approach to synthesize a high affinity peptide DV1-K-(DV3) by chemically linking DV1 and DV3 through the side chain of an added Lys residue. DV1-K-(DV3) competes with the CXCR4 binding of mAb 12G5 with an IC_{50} value of ~4 nM, which is 59 and 110 fold more potent than monomeric DV1 and DV3, respectively. It is noteworthy that the CXCR4 receptor affinity of DV1-K-(DV3) as measured by mAb 12G5 competitive binding assay is even higher than the natural ligand of CXCR4, SDF-1α, whereas DV1-K-(DV3) is much smaller in size (less than half of the residues in SDF-1α) and more amenable for synthesis and further chemical modifications.

2. Materials and methods

2.1. Peptide synthesis

All the peptides were prepared by manual solid-phase synthesis using a TentaGel S RAM (0.24 mmol/g) resin as the solid support

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and 9-fluorenylmethoxy carbonyl (Fmoc) chemistry. A 5-fold excess of Nα-Fmoc-amino acid, diisopropyl-carbodiimide (DIC), hydroxybenzotriazole (HOBt) was used in every coupling reaction step. Removal of the N-terminal Fmoc group was accomplished by 20% piperidine in dimethylformamide (DMF) for two cycles (5 min and 15 min). Dde (N-[1-(4, 4-dimethyl-2, 6-dioxocyclohex-1-ylidene)ethyl]) group was deprotected with 2% hydrazine in DMF for 2 min, and the step was repeated three times. The bivalent peptide was synthesized starting from a DV3/DV1 peptide with a C-terminal Lys. The second DV3 moiety was synthesized from C-terminal to N-terminal by coupling it through the ε-amine group of the Lys side chain. The cleavage of a peptide from the resin was carried out with cleavage cocktail comprised of water (5%, vol/vol), thiophenol (5%, vol/vol), and trifluoroacetic acid (TFA) (90%, vol/vol) for 2 h at room temperature with gentle stirring. The peptides were precipitated by adding ice-cold diethyl ether and washed repeatedly in cold diethyl ether. The crude peptides were dissolved in 20% acetonitrile in deionized water before being lyophilized. Then the crude peptides were dissolved in water/acetonitrile and purified using semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) [13]. The fractions containing the peptides were pooled together and lyophilized. The purity of the final products was assessed by analytical reverse-phase HPLC [13], and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). All peptides were at least 95% pure.

2.2. CXCR4 competitive binding assay

HEK293 cells were grown in RPMI1640 medium with 10% (v/v) FBS, 100 IU penicillin, 0.1 mg/mL streptomycin and 2 mM L-glutamine. The presence of 400 μg/mL geneticin was used as a selection agent to make the HEK293 cells stably express CXCR4. Before performing the assay, HEK293 cells were collected and washed twice with FACS buffer (0.5% BSA, 0.05% sodium azide in PBS) by centrifugation. The cells were then seeded in v-shaped 96-well plates at 5×10^5 cells/well, and co-incubated with various concentrated peptides and primary antibody (1:3000, mouse anti-human CD184 antibody, BD Biosciences, USA) for 40 min on ice. After incubation, cells were washed twice with assay buffer by centrifugation, and then co-incubated with secondary antibody (1:250, anti-mouse IgG-FITC antibody, Sigma, USA) for 30 min on ice. After incubation, the cells were washed twice with assay buffer by centrifugation and 50 μL FACS buffer was added per well finally. The fluorescence ($485_{EX}/528_{EM}$) was recorded using a Synergy 2 microplate reader (BioTek, USA). The mean values of fluorescence were expressed as a percentage of the control group values. CXCR4 competitive binding assays were performed in duplicate and the results were presented as means ± SEMs. Experimental data were generated from at least three independent experiments. Binding curves were fitted using a sigmoidal dose–response model, and the IC_{50} values were calculated using GraphPad Prism 4.

2.3. CXCR4 chemotaxis assay

SUP-T1 cells were grown in RPMI1640 medium with 10% (v/v) FBS, 100 IU penicillin, 0.1 mg/mL streptomycin and 2 mM L-glutamine. Before performing the chemotaxis assay, SUP-T1 cells were collected and washed twice with assay buffer (RPMI1640 medium with 0.5% BSA) by centrifugation. A sample containing 1×10^6 cells were first co-incubated with variant concentrated of peptides for 2 h, and then were seeded in the upper chambers of HTS transwell 96-well plates with 5 μm pore size (Corning, USA). The upper chambers were placed into the lower chambers, which contained 200 μL assay buffer and 1 nM SDF-1α as chemoattractant. Background groups were run by adding only assay buffer in the lower chambers. The transwell plate was placed in a 37 °C cell culture incubator in a 5% CO_2 for 3 h while the cells were allowed to migrate. After incubation, the upper chambers were removed and SUP-T1 cells that had migrated to the lower chambers were quantified by Cell Titer-Blue reagent (Promega, USA).

2.4. Calcium mobilization assay

SUP-T1 cells were collected by centrifugation and washed twice with assay buffer (HBSS buffer with 20 mM HEPES) by centrifugation. Probenecid sodium (5 μM, Sigma, USA) and Fura-2 AM dye (2 μM, Molecular Probes, USA) were added to the cells and incubated in 37 °C cell culture incubator in a 5% CO_2 for 30 min with gently vortex every 5 min. After incubation, the cells were washed twice with assay buffer and resuspended at cell density of 1×10^6/mL. The assays were performed by pre-incubating the cells with CXCR4 antagonist peptides at variant concentrations for 2–3 min, followed by stimulation with 50 nM SDF-1α. The fluorescence ($340_{EX}/510_{EM}$) was measured by a fluorescence spectrometer measurement program.

2.5. HIV drug screening assay

Antiviral activity was evaluated by inhibition of the replication of HIV as measured by the production of p24 antigen in culture supernatants (Perkin Elmer's Alliance HIV-1 P24 ELISA kit, catalog #NEK050001KT). Non-adherent CEM cells were infected in suspension with the NL4-3 strain of HIV-1 at an MOI of 0.001 for 2 h at 37 °C. The cells were washed and centrifuged three times (300*g*, 20 °C, 6 min) with Dulbecco's PBS and then diluted with plus 2 μg/ml of Polybrene (Sigma, catalog N028) to a cell density of 500,000 cells/mL, and dispensed into 96 well plates (Falcon round bottom clear plate, catalog 353077) at 100 μL/well. Each drug was diluted to a 2× concentration and 100 μL dispensed into each respective well. The infected cells were incubated at 37 °C for 7 days. 50 μL of supernatant were collected and stored at −20 °C. The supernatants were assayed for p24 at a 1/100 dilution with PBS. The percent compound inhibition represented the percent p24 value relative to infected cells not exposed to drug.

2.6. Drug cytotoxicity assay

The LIVE DEAD assay (Invitrogen/Molecular Devices, Catalog L3224) was used to assess cytotoxicity from drug exposure through perturbation of fluorescent dyes. The conditions mimicked the HIV drug screen (minus the virus) and the cell viability was determined after 7 days of exposure to the two highest concentrations of drug tested in the drug inhibition assay, 10 μM and 100 μM. The protocol followed manufacturers' specifications and the dyes Calcein (ex/em – 485/530 nm) and Ethidum homodimer-1 (ex/em – 530/645 nm) were applied to the test cells for 1.5 h then the values read on a Molecular Devices Spectra Max 96 well reader. Subsequently, the values were normalized relative to internal controls cells without drug exposure as a percent of the control.

3. Results and discussion

3.1. Dramatic enhancement of CXCR4 binding affinity of DV1-K-(DV3)

Bivalent ligand DV1-K-(DV3) contains two CXCR4 monomer ligands, DV1 and DV3 linked chemically via the side chain of a Lys residue. DV1 is an all D-amino acid peptide derived from the N-terminus (residues 1–21) of vMIP-II and displays moderate binding affinity with CXCR4 and anti-HIV entry activity [12]. The mutation