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Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1 infection

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ABSTRACT

The chemokine receptor CXCR4 interacts with a single endogenous chemokine, CXCL12, and regulates a wide variety of physiological and pathological processes including inflammation and metastasis development. CXCR4 also binds the HIV-1 envelope glycoprotein, gp120, resulting in viral entry into host cells. Therefore, CXCR4 and its ligands represent valuable drug targets. In this study, we investigated the inhibitory properties of synthetic peptides derived from CXCR4 extracellular loops (ECL1-X4, ECL2-X4 and ECL3-X4) towards HIV-1 infection and CXCL12-mediated receptor activation. Among these peptides, ECL1-X4 displayed anti-HIV-1 activity against X4, R5/X4 and R5 viruses (IC_{50} = 24 to 76 μM) in cell viability assay without impairing physiological CXCR4–CXCL12 signalling. In contrast, ECL2-X4 only inhibited X4 and R5/X4 strains, interfering with HIV-entry into cells. At the same time, ECL2-X4 strongly and specifically interacted with CXCL12, blocking its binding to CXCR4 and its second receptor, CXCR7 (IC_{50} = 20 and 100 μM). Further analysis using mutated and truncated peptides showed that ECL2 of CXCR4 forms multiple contacts with the gp120 protein and the N-terminus of CXCL12. Chemokine neutralisation was mainly driven by four aspartates and the C-terminal residues of ECL2-X4. These results demonstrate that ECL2 represents an important structural determinant in CXCR4 activation. We identified the putative site for the binding of CXCL12 N-terminus and provided new structural elements to explain the recognition of gp120 and dimeric CXCR4 ligands.

1. Introduction

The chemokine receptor CXCR4 is a class A G-protein-coupled receptor (GPCR) expressed at the surface of a large variety of cells including T lymphocytes, monocytes, neutrophils, dendritic and endothelial cells [1–3]. The interaction of CXCR4 with its unique endogenous ligand, the chemokine CXCL12, also named SDF1α, plays a crucial role in various processes such as hematopoietic stem cell [4,5] and leukocyte trafficking [6,7], vascular and neuronal development as well as inflammation and immune-modulation [5,7,8]. In addition to its physiological role, CXCR4 is involved in several pathologies including inflammatory diseases, WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelokathexis) syndrome, cancer and HIV-1 infection [9–13]. CXCR4 and another chemokine receptor, CCR5, act as co-receptors for the entry of HIV-1 into host cells by interacting with the viral envelope protein gp120 after its engagement with CD4 [11,14–21]. Viruses enter via CCR5 or CXCR4 (termed "R5 viruses" and "X4 viruses"), or use both co-receptors (termed "R5/X4" or dual tropic viruses). Viruses using CCR5 are believed to be preferentially transmitted as they infect effector memory CD4+ T-cells as well as macrophages and dendritic cells, which are abundant underneath the epithelial layer where infection occurs. Viruses utilising CXCR4, preferentially infecting naïve CD4 T-cells, generally appear later during infection and are associated with a decline of the immune response and with the onset of AIDS [22–27].

CXCR4 is also expressed on a large number of cancer cells and its interaction with CXCL12 has been demonstrated to favour tumour cell survival, proliferation and mobility leading to metastasis development [13,28–30]. Besides CXCL12, CXCR4 also interacts with the broad-spectrum human herpes virus 8-encoded chemokine vCCL2, which acts as an antagonist [31]. In 2005, CXCR7 was identified as the second CXCL12-binding chemokine receptor [32,33]. Similar to CXCR4, CXCR7 promotes cancer metastasis and its over-expression is often associated with more aggressive tumour phenotypes and bad prognosis [34–36]. Importantly, the biology and regulation of the activity of CXCR4, CXCR7 and their common ligand CXCL12 were suggested to be interdependent [37].

CXCR4 was shown to adopt a typical GPCR fold consisting of a seven-transmembrane helix bundle. However, the location and shape

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of its ligand-binding pocket differs from that of other protein-binding GPCRs and is situated closer to the receptor surface [38] suggesting a greater implication of the N-terminus and the three extracellular loops (ECL1, ECL2 and ECL3) in ligand binding and receptor activation [39–41].

The tridimensional structure of chemokines consists of (1) an elongated and flexible N-terminus, (2) a cysteine motif, (3) a loop of approximately ten residues, often referred to as the N-loop, (4) a single-turn of 3_{10} helix, (5) three antiparallel β-strands and (6) a C-terminal α-helix. These secondary structures are connected by turns known as the 30s, 40s, and 50s loops, which reflects the numbering of residues in the mature protein [42,43]. The chemokine structure is further stabilised by two disulphide bridges connecting the cysteine residues of the N-terminus with those located on the 30s and 50s loops [42].

On the basis of the large amount of information available for CXCL12 and CXCR4, a general two-step mechanism was proposed to describe the interaction of chemokines with their cognate receptors [44,45]. The initial step of this model corresponds to the anchoring of the chemokine to the receptor's N-terminus (Chemokine Recognition Site 1, CRS1) and is followed by the binding of the flexible N-terminus of the chemokine to a second site (CRS2) located in the vicinity of the transmembrane segments (TMs) and the extracellular loops of the receptor. In line with this model, studies using sulfated peptides derived from the N-terminal domain of CXCR4 demonstrated that peptide corresponding to CRS1 binds the surface of CXCL12 in an extended conformation close to the chemokine N-loop [46,47]. Furthermore, these studies highlighted the importance of sulfotyrosines present on CRS1 and sulfotyrosine-binding pockets present on the chemokine. Binding of the chemokine N-terminus to CRS2 was suggested to induce conformational changes in the receptor and in its subsequent activation. In agreement with this model, short peptides derived from the flexible N-terminus of CXCL12 were shown to be sufficient to specifically bind CXCR4, and displayed agonist activity [48–51]. Further analyses conducted with affinity purified CXCR4 identified several amino acids located on the CXCL12 β-sheet and 50s loop as additional receptor interacting residues [52]. Although all these results corroborate the two-step binding model, the exact stoichiometry of the CXCR4–CXCL12 interaction as well as the receptor determinants forming the CRS2 remain to be clarified [38,46].

The critical role of CXCR4 in cancer biology and HIV-1 infection makes this receptor and its ligands valuable targets for drug development. To date, several small CXCR4 antagonists including AMD3100, T140 or CTCE-9908 have been described [53–57]. Although these molecules are very potent in blocking HIV-1 infection and metastasis development, they are often associated with important side effects and/or inverse action on other chemokine receptors [53,58,59]. Therefore, other inhibition strategies need to be explored. Over the last few years, ligand neutralisation by small molecules, peptides and antibody fragments has emerged as an interesting alternative to the classical receptor inhibition [60–67]. However, peptidic derivatives of receptor extracellular loops have never been reported as potential chemokine neutralisers. In the context of CXCR4 and CXCR7, targeting their common chemokine, CXCL12, would allow the simultaneous interference with its binding to both receptors [68]. On the other hand, the development of molecules neutralising the HIV-1 envelope protein gp120 rather than the receptor would confer T-cell protection against viral infection without impeding the physiological functions of CXCR4.

In the present study, we investigated the neutralising properties of individual peptides corresponding to the first, second and third extracellular loops (ECL1, ECL2, ECL3) of CXCR4 towards CXCL12 binding and HIV-1 infection. Analyses with mutated and truncated peptides provided new insights on the molecular basis of receptor–ligand recognition opening new perspectives for the development of CXCR4 ligand neutralisers.

2. Materials and methods

2.1. Peptides, proteins and cells

Peptides corresponding to the extracellular loops of CXCR4 (ECL1-X4, ECL2-X4 and ECL3-X4) were designed based on the receptor topology predicted prior to its X-ray structure resolution [39] (Table 1). All peptides including scrambled control peptides ECL1-$X4_{scrbl}$ (FNYSGAKFVNDLWA) and ECL2-$X4_{scrbl}$ (DVQDPRVLDWRNDVYSFYAFQFVCINE) were purchased from JPT and contained an amide group at the C-terminus to avoid additional negative charges. Peptide ECL2-X4 was also purchased biotinylated at its N-terminus and the biotin moiety was separated from the peptide by a Ttds linker ([N1-(9-Fluorenylmethoxycarbonyl)]-1,13-diamino-4,7,10-trioxatridecan-succinamic acid). The CXCL12 N-terminal peptide comprised the first 17 residues of the chemokine (KPVSLSYRCPCRFFESH). Control peptide (SPAPERRGYSGYDVPDY) (Ctrl) corresponded to a HCDR3 sequence binding to an antibody directed against human influenza haemagglutinin [69]. Chemokines CXCL12 (SDF1α), vCCL2 (vMIP-II), CCL5 (RANTES), CCL3 (MIP-Iα) and CCL4 (MIP-Iβ) were purchased from Peprotech. Alexa Fluor 647-labelled CXCL12 was purchased from Almac. MT-4, Cf2Th-CXCR4, CEM.NK^R, CEM.NK^R-CCR5 and U87.CD4.CXCR4 cell lines were obtained through the NIH AIDS programe from Dr. D. Richman, Dr. J. Sodroski and Dr. A. Trkola [70–72]. Cells stably expressing CXCR7 were obtained by transfecting U87.CD4 cells with pBABE-CXCR7 vector.

2.2. HIV-1 infection inhibition assay

Inhibition of HIV infection of MT-4 cells and peptide cytotoxicity were measured as previously described [73,74]. MT-4 cells ($6 \cdot 10^4$ cells/well) were incubated with or without X4 (III_B), R5/X4 (89.6) or R5 (Ba-L) viruses (100 $TCID_{50}$) for five days in the presence of three-fold dilutions of ECL-X4 and control peptides starting at a concentration of 100 μM. Viral entry inhibition and peptide cytoxicity were evaluated by monitoring the absorbance at 540 nm (Abs) corresponding to MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) reduction by mitochondrial enzymes using a Multiskan Ascent spectrophotometer (Thermo-fisher). Protection (%) was calculated using the following equation: $(Abs_{cells + virus + peptide} - Abs_{cells + virus}) / (Abs_{cells} - Abs_{cells + virus}) \times 100$.

Luciferase-tagged recombinant viruses harbouring the NL4.3 Env and VSV-g pseudovirions were produced as previously described [75–77]. U87.CD4.CXCR4 cells (10,000 cells/well) were infected in 96-well plates with Env-recombinant viruses (200 pg p24, quantified by Perkin Elmer kit) for 48 h at 37 °C. Medium was replaced and cells were cultured for another 48 h, after which luciferase activity was assayed using the Promega Luciferase kit (Promega) and read on a Polarstar Omega microplate reader (BMG labtech). All infections were performed in triplicate. Peptide cytotoxicity was determined in uninfected cells using the MTT method as described above.

2.3. Binding of fluorescently labelled CXCL12 to CXCR4

Alexa Fluor 647-labelled CXCL12 (100 ng/ml) was incubated for 30 min at room temperature with CXCR4 ECL-X4 peptides (50 μM) prior addition to Cf2Th-CXCR4 cells. After 90 min incubation at 4 °C, cells

Table 1
Sequence and length of peptides derived from CXCR4 extracellular loops.

Name	Length	Position	Sequence
ECL1-X4	14	97–110	DAV**ANWYF**GNFLCK
ECL2-X4	27	176–202	**NVSEADDRYICDRFYPN**DLWVVVFQFQ
ECL3-X4	21	262–282	DSFILL**EIIKQG**CEFENTVHK

Residues in bold are solvent exposed in the CXCR4 X-ray structure.