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Review

Engineering and screening the N-terminus of chemokines for drug discovery

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ABSTRACT

Chemokines are small chemoattractive proteins involved in many important physiological and pathological processes such as leukocyte mobilisation, inflammation, cancer and HIV-1 infection. The N-terminus of chemokines was shown to be crucial for interaction and activation with their cognate receptors. Therefore, multiple strategies including elongation, truncation, mutagenesis or chemical modifications of chemokine N-terminus were developed to identify analogues with modified selectivity displaying antagonist or enhanced agonist activities. Library approaches allowed fast screening of a large number of such chemokine variants and led to the identification of promising therapeutic candidates.

Additional studies were able to reduce the chemokine to the size of a peptide while retaining its receptor affinity and selectivity. In analogy to full length chemokines, peptides derived from the chemokine N-terminal sequence were improved by mutagenesis, elongation and truncation approaches to develop potential therapeutic molecules used in various clinical trials.

Altogether these studies demonstrated the pharmacophore potential of the chemokine N-terminus and its vast modulation properties to develop analogues with great therapeutic value for a large set of pathologies.

Contents

1. Introduction

Since the discovery of Interleukin 8 (IL-8/CXCL8) [1,2] and the monocyte chemoattractant protein (MCP-1/CCL2) [3] in the late 1980s, the human chemokine family kept on increasing constantly with fifty chemokines and twenty receptors identified to this date [4]. Chemokines are a family of small cytokines produced by

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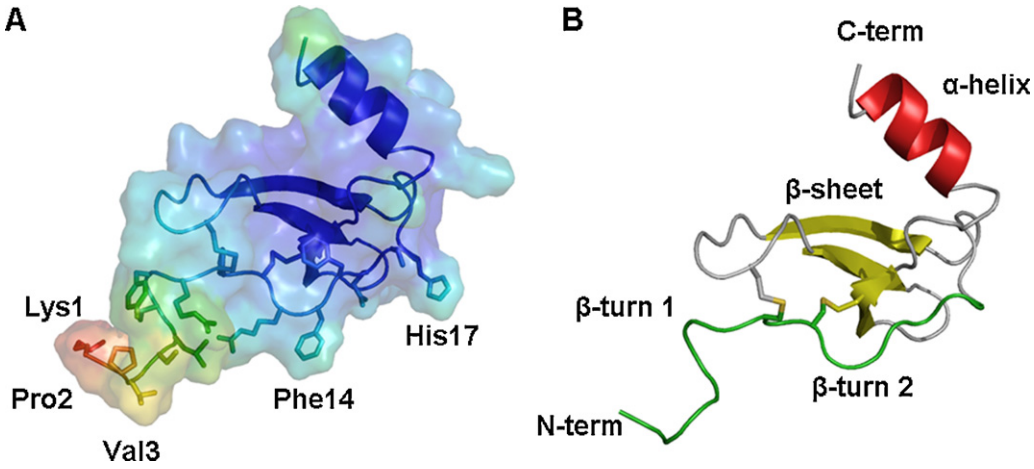

Fig. 1. Representation of the chemokine folding and flexibility of the N-terminus. (A) Mapping of the flexibility of the chemokine CXCL12a (PDB 1SDF). The flexibility is evaluated using the b factor (distance displacement) including thermal displacement, static disorder and dynamic disorder. Deep blue specifies minimal flexibility whereas red indicates maximal flexibility. Residues of the flexible N-terminus (1–17) are shown as stick model. (B) Folding and secondary structures of the chemokine CXCL12a. The flexible N-terminus (1–17) is coloured in green and displays a β-turn structure. The β-sheet and the C-terminal α-helix are coloured in yellow and red, respectively.

various cells and their main function is the induction of direct cellular movement. Chemokines are small-sized proteins (70–130 amino acids) with conserved cysteine residues and an organised three-dimensional shape. This structure is characterised by a flexible N-terminal region attached to a structured compact core (Fig. 1) [5].

According to the spacing between the first two cysteine residues, chemokines were classified into four different subfamilies (CC, CXC, CX3C and C) [6].

Chemokines are produced and released by cells in response to infections and agents causing physical injury [7,8]. These inducible chemokines attract leukocytes, monocytes, neutrophils and other effector cells to the sites of infection or damage and are critical at establishing efficient innate and adaptive immune responses, wound healing and organ repair [9]. In contrast, some other chemokines are involved in controlling migration of cells during physiological processes such as tissue homeostasis, embryonic development, neo-vascularisation and angiogenesis and do not require specific stimulation for production and release [4].

Biological activities of chemokines are mediated through interaction with G protein coupled transmembrane receptors (GPCR) also called seven transmembrane receptors (7TM) [10]. Upon chemokine (agonist) binding, the structure of the GPCR undergoes conformational changes inducing activation of G-coupled proteins that trigger a set of downstream signalling and cellular responses. The magnitude and duration of this activation is closely regulated by various mechanisms including receptor desensitisation, internalisation and endocytic trafficking [11–13]. In general, activated GPCRs are rapidly desensitised at the cell surface and become refractory to further agonist stimulation by phosphorylation and arrestin binding [12]. GPCRs are then internalised by clathrin-mediated endocytosis, resulting in the reduction of cell surface receptor concentration. In this review, the term down-modulation will be used to refer to this reduction in GPCR at the cell surface. Depending on the nature of the ligand and the length of the treatment, GPCRs are either sequestered from the cell surface to intracellular compartments, dephosphorylated and recycled back to the cell surface in a resensitised form or degraded into lysosomes, a process important for signal termination [11–13].

Chemokines and their cognate receptors are not exclusive and redundancy was observed resulting in an intricate complex network with a high level of regulation and crosstalk. Ligand–receptor binding was proposed to follow a two-site mechanism. A first interaction between the ligand core residues and the N-terminal receptor domain ensures the binding of the chemokine to its receptor (site 1). The second interaction involves the ligand N-terminal residues and the receptor's transmembrane domain and extracellular loops (site 2) triggering a conformational change of the receptor resulting in signalling through the G proteins pathway [14] (Fig. 2). The first interaction supports the "addressing" of the

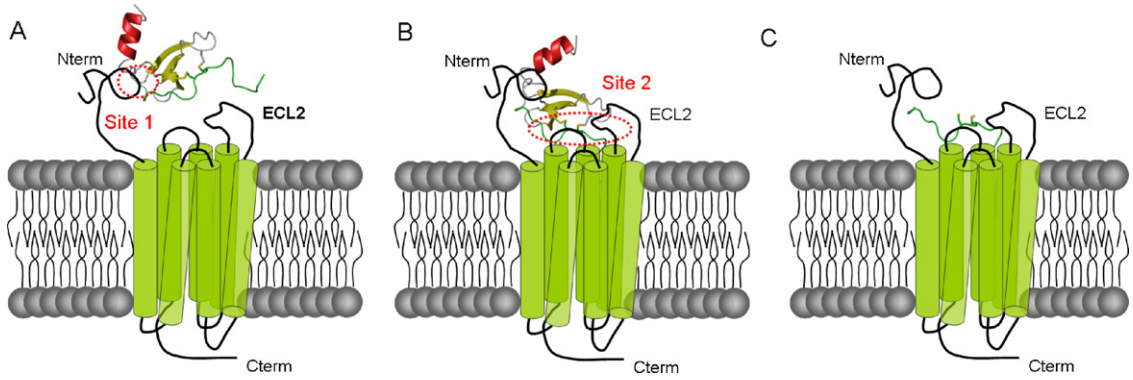

Fig. 2. Model of the interaction between chemokines and their receptors. (A and B) Putative two-site mechanism of the interaction between the chemokine CXCL12a and the receptor CXCR4. (A) First step: interactions between the core of the chemokine and the N-terminal domain of the receptor (addressing of the chemokine). (B) Interactions between the flexible N-terminus (dark green) of the chemokine, the extracellular loops and the transmembrane domain of the receptor (induction of the message) [14–16]. (C) Chemokine size reduction. Interaction of a peptide derived from the N-terminus of chemokines (dark green) with the receptor surface.

chemokine to the receptor (specificity) while the second interaction site is related to the induction of the "message" (signalling).

Although chemotaxis is the major role of chemokines, these proteins are also involved in cell adhesion, degranulation and play a critical role in angiogenesis and pathological processes such as tumor cell growth [9,17], chronic and acute inflammation [18], autoimmune diseases and HIV-1 infection [19,20]. In this regard, chemokines and their receptors represent valuable targets for drug discovery and various strategies were developed to specifically inhibit their interactions. One approach was inspired by the identification of naturally truncated or extended chemokine variants exhibiting distinct biological properties when compared to full length chemokines [21–23]. These analogues are generated by natural polymorphism [24] or by post-translational modifications such as proteolytic degradation or glycosylation, mainly at the N-terminal part of the chemokine [25]. These truncated, mutated or glycosylated chemokine analogues displayed variable binding affinities or antagonist properties. Biological activities of these naturally modified chemokines were extensively reviewed by Mortier et al. [26]. This review will focus on the different approaches developed to engineer the N-terminus of full length chemokines and to reduce their size to a peptide level maintaining sufficient receptor affinity and activity.

2. Engineering the N-terminus of full length chemokines

2.1. N-terminal truncations and mutations

The first studies on N-terminally mutated or truncated chemokines were performed on the CXC chemokine CXCL8 (IL-8), a major chemoattractant for neutrophils implicated in a number of inflammatory diseases. These studies revealed the importance of the N-terminal domain including the clustered disulfide bridge and the N-terminal loop region (residues 10–17) for biological activity and receptor selectivity [27–29]. A particular motif constituted of the residues Glu^4–Leu^5–Arg^6 (ELR motif) was critical for receptor binding and neutrophil-stimulating activity. Indeed, the truncated analogue $CXCL8_{(5-72)}$ displayed a decreased biological activity and further amino acid deletion resulted in completely inactive analogues ($CXCL8_{(6-72)}$ and $CXCL8_{(7-72)}$). The maximal biological activity was obtained with $CXCL8_{(4-72)}$, a truncated analogue comprising the ELR motif [28]. These data were further confirmed when the first 15 residues of the chemokine (except Cys^9 and Cys^{11}) were replaced by Ala. Mutations of residues Glu^4–Leu^5–Arg^6 abolished calcium release and diminished the receptor binding by a factor of 30 to 100. In addition, replacement of Glu^4 and Arg^6 by residues with the same physico-chemical properties did not restore the binding to the level of wild type CXCL8 [27]. This mutagenesis analysis also highlighted the role of Lys^3 and Ile^{10} for receptor binding and neutrophil stimulation. Mutation of Lys^3–Glu^4–Arg^6 to Ala clearly reduced binding to the receptor and abolished cytosolic calcium release in neutrophils. Mutation of the charged residues in other regions than the N-terminus did not affect receptor binding affinity [27]. Interestingly, the truncated analogue $CXCL8_{(6-72)}$ devoid of agonist activity displayed antagonist properties and inhibited binding of wild type CXCL8 to the receptor, CXCL8 induced exocytosis, chemotaxis and respiratory burst. The same switch from agonist to antagonist was observed when mutating Glu^4 and Leu^5 of the ELR motif in the truncated analogue $CXCL8_{(4-72)}$ [30].

The crucial ELR motif is also present in rabbit and sheep CXCL8 and is shared by other neutrophil-activating proteins such as the neutrophil activating peptide-2 (NAP-2/CXCL7) but not by non-neutrophil agonists [27]. Ala ($Glu^4 > Ala$ and $Arg^6 > Ala$) and conserved ($Glu^4 > Asp$, and $Arg^6 > Lys$) mutations in the ELR motif of CXCL7 abolished the neutrophil activation properties of the mutants as observed for CXCL8 [31]. Interestingly, grafting the ELR motif to a chemokine (platelet factor 4/CXCL4) lacking this motif and the ability to activate neutrophils turned the hybrid chemokine (CXCL4-ELR) into a potent neutrophil activator. Mutations of the ELR motif in the hybrid chemokine markedly reduced its neutrophil activating properties [31,32]. However, introduction of the ELR motif into the N-terminus of the interferon-inducible protein 10 (IP10/CXCL10) or CCL2 did not convert these chemokines to neutrophil activators, suggesting the requirement of additional binding sites to trigger biological activity. Indeed, replacement of Tyr^{28} and Arg^{30} in CCL2 by Leu and Val, the corresponding amino acids in CXCL8, conferred neutrophil chemotaxis activity to the CC chemokine [33].

In analogy with CXCL8, truncation of Glu^6–Leu^7 in the growth regulated oncogene-alpha chemokine (GROα/CXCL1) resulted in a chemokine analogue ($CXCL1_{(8-73)}$) with antagonist properties [34]. While $CXCL8_{(6-72)}$ blocked CXCR1 and CXCR2, both $CXCL1_{(8-73)}$ and the hybrid chemokine ELR-CXCL4 specifically inhibited CXCR2 functions. These data were further confirmed *in vivo* in two acute inflammation mouse models. Pre-treatment of mice with $CXCL1_{(8-73)}$ or ELR-CXCL4 inhibited neutrophil recruitment in subcutaneous tissue and in the peritoneal cavity [35].

Further efforts were conducted to engineer CXCL8 analogues with antagonist properties. Bovine CXCL8 truncated of three N-terminal residues and displaying 2 point mutations ($Lys^{11} > Arg$ and $Gly^{13} > Pro$) acted as a potent antagonist. Efforts were undertaken to humanise this potent antagonist. A chimera composed of the N-terminus of bovine CXCL8 and the core of human CXCL8 displayed the same potency. Further humanisation by mutating the N-terminal residues different from the human CXCL8 using site directed mutagenesis ($Thr^3 > Lys$, $His^{13} > Tyr$, $Thr^{15} > Lys$, $Glu^{35} > Ala$ and $Ser^{37} > Thr$) did not improve the potency [36].

The importance of the N-terminal domain preceding the first cysteine in receptor recognition and activation was also confirmed for other chemokines including CXCL11 (I-TAC), CCL26 (Eotaxin-3), regulated on activation, normal T expressed and secreted (RANTES/CCL5), CCL2 (MCP-1), CCL7 (MCP-3) as well as macrophage inflammatory protein (MIP-1β/CCL4, MIP-3α/CCL20 and MIP-4/CCL18) and fractalkine (CX3CL1) [37–45].

Replacement of Glp (pyroglutamate), the first residue of CCL2, by other non-cyclic amino acids or individual Ala mutations of the ten first N-terminal residues had weak effect on the biological activity of the chemokine [39]. In contrast, extension of CCL2 by an Ala or Met as well as deletion of the five N-terminal amino acids ($CCL2_{(2-76)}$ to $CCL2_{(6-76)}$) significantly reduced its chemotaxis activity and its binding affinity to monocytes and basophils [37,46]. Surprisingly, $CCL2_{(2-76)}$ was a potent chemoattractant of eosinophils that do not express CCR2 and are not sensitive to full length chemokine. This effect is most likely due to the interaction of the truncated analogue with CCR3, a receptor not accessible to full length CCL2. Further truncation of the chemokine resulted in analogues ($CCL2_{(7-76)}$ to $CCL2_{(11-76)}$) devoid of biological function but maintaining high affinity binding to monocytic cells [37,46]. $CCL2_{(9-76)}$ and $CCL2_{(10-76)}$ acted as antagonist presumably by blocking CCR2 and prevented chemotaxis induced by CCL2, CCL8, and CCL7 but not by CCL5, CCL3 and CCL4.

Among all engineered mutants, $CCL2_{(9-76)}$, Met-$CCL2_{(9-76)}$ and $CCL2_{(1+9\ to\ 76)}$ were potent CCL2 inhibitors and their anti-inflammatory potential was investigated in various *in vivo* models of acute and chronic inflammation [39,47–53]. In a murine model of arthritis, prophylactic injection of $CCL2_{(9-76)}$ prevented the onset of disease while injection of full length CCL2 had detrimental effects [47]. Moreover, delivery of the antagonist when arthritis was already well established resulted in a significant reduction of symptoms, an effect considerably enhanced by simultaneous

administration of $CXCL1_{(8–73)}$, a polymorphonuclear monocyte antagonist [48]. The addition of a Met to this promising CCL2 analogue (Met-$CCL2_{(9–76)}$) resulted in a chemokine variant delaying the initiation and the progression of lupus nephritis in a murine model of systemic lupus erythematosus [49]. In parallel to these studies, a third CCL2 mutant engineered by truncation of amino acids two to eight and known as $CCL2_{(1+9\ to\ 76)}$, was extensively studied for its ability to improve clinical conditions associated with arteriosclerosis and other vascular diseases in mice and rats by inhibiting monocyte recruitment into the animal arterial wall [52,53]. This mutant was also used in anti-CCL2 gene therapy based on transfection of the $CCL2_{(1+9\ to\ 76)}$ mutant gene into skeletal muscles. Intramuscular transduction of this mutant in animal models inhibited arteriosclerosis formation and progression [54], restenosis [54,55], neointimal formation [56,57] and brain ischemia [58]. Gene therapy using the same chemokine analogue was also successfully used in various animal disease models to delay initiation and progression of pulmonary hypertension [59], Alzheimer-related neuroinflammation [60], cerebral aneurysm progression [61], autoimmune encephalomyelitis [62] or myocarditis [63], tumor angiogenesis [64], nephropathy [65–67] as well as renal [68] or hepatitis fibrosis [69].

Recently, Shahrara et al. [70] investigated the anti-inflammatory potential of a novel endogenous CCL2 inhibitor in a rat model of adjuvant-induced arthritis. Mutation of Pro^8 to Ala resulted in a monomeric variant of the chemokine (P8A-CCL2) displaying full activity on a human monocytic cell line (THP-1 cells) *in vitro* while unable to induce murine monocyte chemotaxis *in vivo*. Therapeutic treatment with the P8A-CCL2 analogue decreased the severity of the disease by reducing macrophage accumulation and cytokine expression in the joints of the animals. This analogue acted most likely by displacing endogenous CCL2 from the endothelial surface reducing monocyte recruitment. P8A-CCL2 also had a beneficial impact in murine models of airway inflammation and autoimmune encephalomyelitis [71].

Other N-terminally truncated chemokines including the secondary lymphoid tissue chemokine CCL21 (SLC), CCL19 (MIP-3β) and CXCL11 (I-TAC) proved to inhibit T cell migration. Sasaki et al. [72] used a N-terminally truncated CCL21 to block homing of donor CCR7-expressing T cells in a murine model of chronic graft-versus-host disease (GVHD). Truncation of the first four residues of CCL21 resulted in an antagonist analogue inhibiting CCL21-induced chemotaxis and calcium influx in CCR7-expressing cells. In addition, the CCL21 antagonist blocked homing of donor CCR7-positive T cells into secondary lymphoid organs, resulting in a reduced number of activated host B cells. All together, these results suggested that the CCL21 antagonist had beneficial effects on the prevention of chronic GVHD.

N-terminal truncations and mutations of the chemokine CCL19, also implicated in T cell and dendritic cell migration through interaction with CCR7, were extensively studied. Ala mutations of Asn^3, Asp^4 and Asp^7 resulted in a reduced receptor binding and activation compared to wild type CCL19 revealing the importance of these residues for high affinity receptor binding. Truncation of the first two N-terminal residues reduced the receptor activation potency. A gradual decrease of the activation properties was observed by further deleting three to six residues and the corresponding truncated analogues behaved as partial agonists. The analogue $CCL19_{(8–77)}$ (truncation of seven residues) showed no detectable receptor activation and inhibited CCL19 induced chemotaxis in CCR7 positive cells. The residues Glu^6 and Asp^7 appeared to be major contributors to the agonist activity [73]. Pilkington et al. [74] further investigated the role of the truncated $CCL19_{(8–83)}$ analogue in a murine model of allogenic immune response. Although mice treated with the antagonist showed no significant reduction of cell recruitment into lymph nodes, the generation of cytotoxic T cells towards allogenic dendritic cells was inhibited.

The stromal cell-derived factor-1 (SDF-1α/CXCL12a), a CXC chemokine lacking the ELR motif, is a potent chemoattractant for B- and T-cells. Mutagenesis and sequential truncation analysis of CXCL12a demonstrated the critical role of the first two N-terminal residues (Lys^1 and Pro^2) for CXCR4 activation [14]. Truncation of Lys^1 resulted in an analogue with reduced activity whereas further deletion of the chemokine led to completely inactive derivatives ($CXCL12a_{(3–67)}$–$CXCL12a_{(9–67)}$). These data were further confirmed by single mutation analysis. Among all engineered analogues, the four derivatives $CXCL12a_{(2–67)}$, $CXCL12a_{(3–67)}$, K1R-CXCL12a ($Lys^1 > Arg$) and P2G-CXCL12a ($Pro^2 > Gly$) displayed a considerable binding affinity for the receptor without inducing significant receptor signalling. In combination with $CXCL11_{(4–79)}$, a CXCR3 antagonist, P2G-CXCL12a ($Pro^2 > Gly$), inhibited experimental autoimmune encephalomyelitis in a mouse model of multiple sclerosis and reduced migration and gathering of $CD4^+$ T cells in the central nervous system [75].

In analogy with P2G-CXCL12a, a similar CXCL12b mutant was designed ($Pro^2 > Gly$). While this mutant induced CXCR4 receptor internalisation and blocked efficiently wild type CXCL12b-induced chemotaxis, no cell migration, calcium influx or Akt phosphorylation was observed. This mutant improved angiogenesis and muscle regeneration in an ischemic mouse model without inducing inflammatory or apoptotic effects in heart, liver and kidneys. In comparison to AMD3100, a small molecule CXCR4 antagonist, the modified chemokine was less toxic [76].

N-terminal and N-loop (residues 12–20) mutagenesis of CCL5 revealed the residues involved specifically in CCR1, CCR3 and CCR5 binding. In contrast to neutrophil agonists, receptor binding was not associated with a particular motif. Mutation of Arg^{17}, $Phe^{12} + Try^{14}$ or $Pro^2 + Phe^{12} + Ile^{15}$ resulted in the loss of CCR1, CCR3 and CCR5 binding, respectively. Different but overlapping epitopes are involved in CCR1, CCR3 and CCR5 binding [77]. These data suggested that mutagenesis in or near the N-terminus affect receptor selectivity and could be useful for the design of a selective CCR5 agonist/antagonist. However, considering the complexity of the chemokine network involved in inflammatory pathways, antagonists inhibiting multiple chemokine receptors may be a promising strategy to tackle inflammatory diseases. Gong et al. [38] engineered various analogues by truncation of CCL5, CCL2 and CCL7. $CCL5_{(9–68)}$, $CCL7_{(10–76)}$ and $CCL2_{(9–76)}$ were selected for their antagonist properties and further characterised in a cross reactivity study. While $CCL7_{(10–76)}$ and $CCL2_{(9–76)}$ retained the same binding selectivity as observed with the parent chemokines, $CCL5_{(9–68)}$ interacted with CCR2, a receptor not accessible to wild type CCL5. Among all engineered analogues, $CCL5_{(9–68)}$ was the most potent antagonist of wild type CCL5. When administered alone or simultaneously with a low dose of cyclosporine A to rats, $CCL5_{(9–68)}$ improved clinical conditions associated with heart graft transplantation and prolonged tissue survival [78,79].

The discovery of CCR5 as a co-receptor for HIV entry and the potent antiviral activity of its physiological ligand, CCL5, resulted in an extensive research for novel CCL5 analogues as potent anti-HIV drugs. The inherent pro-inflammatory properties of wild type CCL5 were a major drawback to its development as a therapeutic compound. Pre-treatment of primary monocytes, macrophages and T lymphocytes with β-chemokines resulted in an increased viral replication [80,81]. In this regard, chemokine analogues devoid of agonist activity were absolutely required. Arenzana-Seisdedos et al. [82] investigated the anti-HIV effects of the previously described $CCL5_{(9–68)}$ antagonist. Although $CCL5_{(9–68)}$ exhibited a lower affinity for CCR5 than the full length chemokine, this truncated analogue was still able to inhibit infection of activated PBMC by the R5-tropic HIV strain YU2. These data were

further supported by Ylisastigui et al. [83] who compared the antiviral effects of full length CCL5 and $CCL5_{(8-68)}$, another CCR5 antagonist, on peripheral blood mononuclear cells (PBMC) and primary macrophages. $CCL5_{(8-68)}$ protected T lymphocytes and macrophages from HIV-1 infection although its antiviral potency was 10-fold less compared to full length chemokine.

Similar to the design of truncated analogues, single mutations were engineered in the N-terminal domain of full length chemokines to gain better insights in their binding modes and selectivity as well as to develop novel therapeutic chemokine variants. Polo et al. [84] generated five recombinant CCL5 analogues based on wild type CCL5 sequences with an additional N-terminal Leu and mutations at the following positions in the N-terminus: $Ser^1 > Cys + Ser^5 > Cys$ (C1.C5 CCL5), $Ser^1 > Cys$, $Tyr^3 > Ala$ and $Asp^6 > Arg$. Only the C1.C5 CCL5 mutant displayed a 3–5-fold improved antiviral activity compared to wild type CCL5 in antiviral assays using primary cells (PBMC and *in vitro* derived-macrophages) and primary HIV isolates. While this mutant had slower kinetics for the down-modulation of CCR5 compared to wild type CCL5, the binding affinity for the receptor (K_D = 0.93 nM) was higher than that of wild type CCL5 (K_D = 6.22 nM). Calcium mobilisation and chemotaxis properties were dramatically reduced for the C1.C5 CCL5. However, the CCL5 analogue triggered signalling through CCR1 although at significantly lower concentration than the antiviral doses [84]. This mutant was used in a proof of concept study to develop microbicides using *Lactobacillus jensenii* as a delivery vehicle to prevent HIV transmission. Microbicides in the field of HIV are topically applied agents preventing the transmission of HIV. These agents can be formulated as gels, creams, films, or suppositories for vaginal or rectal applications. The bacterial strain *L. jensenii* belongs to the human commensal microbiota and populates the gastrointestinal tract and vagina. Its ability to colonize and express exogenous recombinant proteins makes this strain very attractive as vehicle to deliver drugs at the vaginal mucosal sites, one of the main entry sites of HIV. Additionally, lactobacilli expressing anti-HIV proteins present lower manufacturing costs providing the possibility for large scale treatment and global prevention of HIV transmission. The engineered C1.C5 CCL5 *Lactobacillus* inhibited HIV infection but with higher IC_{50} as its synthetic counterpart [85,86].

In summary, extensive studies on CXCL8 demonstrated the importance of the N-terminal domain of chemokines for receptor binding and activation. Mutation and truncation studies on a large variety of chemokines not only confirmed the importance of the N-terminal domain but also resulted in the discovery of antagonists of which some had beneficial effects in experimental animal disease models. Minor substitutions were sufficient to switch from agonist to antagonist properties. Furthermore, single mutations also affected the selectivity of chemokines providing a unique opportunity to fine-tune therapeutic effects on specific subsets of cells. Although the neutrophil activating proteins shared a common motif (ELR motif) crucial for binding and activation, such motifs were absent in the majority of the chemokines. It became clear that a general rule to switch from agonist to antagonist could not be established. Chemokines need to be studied individually to determine the residues implicated in receptor binding/activation and to identify variants of clinical importance.

2.2. N-terminal elongation and mutations

The serendipitous discovery of Met-CCL5, a new CCL5 analogue further emphasised the impact of the N-terminal modification on chemokine bioactivity [87]. N-terminal extension of CCL5 with a Met transformed the chemokine into a potent antagonist binding CCR5 and CCR1 with nanomolar affinities [87,88]. *In vitro*, Met-CCL5 abolished calcium mobilisation as well as monocyte chemotaxis and eosinophil effector functions induced by the natural ligands of CCR5 and CCR1 [87,89,90].

Therefore, during the last fifteen years several studies investigated the impact of this analogue on various animal models of Th1 and Th2 mediated inflammation such as adjuvant induced arthritis [91,92], colitis [93], allograft rejection [94–97], glomerulonephritis [98] and airway inflammation [90,99,100]. In addition, the antagonist properties of Met-CCL5 were also studied for oncology applications such as tumor growth inhibition [101]. Considering the number of *in vivo* experiments using Met-CCL5, an individual description in this paragraph is not feasible and the outcomes of these studies is summarized were Table 1.

Although Met-CCL5 was used to improve clinical conditions associated with inflammatory processes in numerous investigations, for some particular diseases or animal models, its therapeutic potential remained controversial. Moreover, several studies reported detrimental effects of this analogue. This may be explained by the complexity and the multiple functions of the chemokine network. Although chemokines are a main mediator in inflammatory processes causing tissue damage, these proteins are also critical for triggering an efficient immune response and regulating inflammation [119]. Moreover, although Met-RANTES was initially considered as a potent CCR5 antagonist, further *in vitro* studies suggested that this antagonist exhibited residual agonist activities. Indeed, in transfected cells over-expressing CCR5, Met-RANTES induced receptor phosphorylation, internalisation and weak but detectable calcium mobilisation [132–134]. However, it still remains controversial whether these properties are triggered by the applied N-terminal modification or by the proteolytic degradation of the analogue, resulting in products displaying agonist properties [133]. These data suggest therefore a precautious use of chemokine receptor antagonists and a need for further investigation of their long-term effects.

In the field of HIV, Met-CCL5 showed weaker HIV inhibitory properties than wild type CCL5 in CCR5 transfected and primary cells (PBMC and primary macrophages) [135]. Polo et al. [84] engineered a second extended analogue of CCL5 similar to Met-CCL5 by addition of a hydrophobic Leu at the N-terminus. Both analogues displayed striking differences regarding their anti-HIV activity. In contrast to Met-CCL5, Leu-CCL5 was a potent HIV inhibitor and 1.6 to 2.4-fold more effective than full length chemokine in blocking HIV infection in primary cells (PBMC and differentiated macrophages). This effect was correlated with the strong affinity of Leu-CCL5 for CCR5 (K_D = 0.38 nM), associated with a dramatic loss of signalling function as monitored by a reduced calcium mobilisation via CCR3 and CCR5. Despite its strong antagonist activity (IC_{50} = 60.2 nM), Leu-CCL5 still showed residual CCR1 signalling and chemotaxis activity on primary monocytes. However, these effects were only observed at doses significantly higher than required for HIV inhibition.

Development of potent HIV inhibitors prompted further research on CXCL12b, the natural ligand of CXCR4, another major co-receptor of HIV entry. In contrast to Met-CCL5, extension of CXCL12b by Met resulted in a potent agonist with enhanced functional activity (monitored by calcium mobilisation) and a 4-fold reduced affinity for its receptor (K_D = 12.4 nM). Modified CXCL12b was 100 to 1000-fold more potent in suppressing viral replication in cell lines (U87 cells) and primary T cells compared to wild type chemokine [136]. The analogue had no HIV inhibitory properties on R5-tropic viruses. This strong antiviral activity was explained by a prolonged down-modulation of CXCR4 expression upon incubation with Met-CXCL12b. Combining this analogue with a chemically modified CCL5 analogue (AOP-CCL5) resulted in inhibition of dual infections with R5 and X4 viruses by 95 to 99% whereas single drugs reduced the HIV infection by only 32 to 61% [137].

Table 1
Therapeutic potential of Met-CCL5 in various inflammatory *in vivo* models.

Pathologies	Model	Species	Therapeutic properties	Reference
Autoimmune diseases	Serum-induced glomerulonephritis	Mice	Reduction in crescent formation, renal leukocyte influx and proteinuria but failed to inhibit collagen deposition.	[98,102]
	Horse apoferritin-induced glomerulonephritis	Mice	Development of pro-inflammatory macrophages resulting in a more severe disease.	[103]
	Anti-Thy-1.1 induced glomerulonephritis	Rats	Abolishment of bone marrow endothelial cell recruitment in injured tissue.	[104]
	Collagen or adjuvant-induced arthritis	Mice	Reduction in inflammation, swelling and bone erosion of the articulation by interfering with neutrophils and macrophages influx into the animal joints.	[91,92]
	Proteoglycan-induced arthritis	Mice	Enhancement of arthritis progression.	[105,106]
	Experimental autoimmune encephalomyelitis (EAE)	Mice	Limited efficacy on chronic-relapsing EAE.	[107]
	Experimental autoimmune gastritis (EAG)	Mice	No influence on the induction of EAG	[108]
	Experimental autoimmune uveitis (EAU)	Rats	Variable therapeutic effects depending on antigen used to trigger the disease. Amelioration of oral tolerance.	[109]
Allergy	OVA-induced skin allergy	Mice	Reduction of lymphocyte and eosinophil infiltration as well as eotaxin and CCL5 expression into the lungs.	[110]
	OVA-induced airway inflammation	Mice	Inhibition of leukocyte recruitment into the lungs by 59% but failed to affect bronchial hyperreactivity.	[99,100]
	Contact skin allergy	Mice	Decrease by 68% eosinophil influx into the skin.	[111]
Allograft rejection	Renal transplant	Rats	Diminution in lymphocyte and macrophage recruitment limiting vascular and tubular damages and therefore reducing the pace of acute allograft rejection.	[95,112]
	Small bowel transplant	Rats	Decrease of endothelial cell–leukocyte interactions and inhibition of perfusion failure.	[96,113]
	Heart transplant	Mice	Amelioration of fibrous airway obliteration.	[114]
	Tracheal transplant	Mice	Reduction in leukocyte recruitment, inflammatory chemokine expression and immune response against donor antigens.	[97]
Inflammatory bowel diseases	TNBS-induced colitis	Rats	Decrease of monocytes, mast cells and neutrophils recruitment into the colon and diminution in tissue injury and inflammation.	[115]
	Colitis	Rats	Amelioration of the ileal fluid accumulation and myeloperoxidase activity induced by toxin A.	[93]
	Toxin A-induced enteritis	Rats	Inhibition of colonic damages and bacterial translocation	[116]
Airway inflammation	Bacterial-induced inflammation	Rats	Abolished dendritic cell influx into airway when the inflammation was induced by heat-killed bacteria but not by live virus or soluble antigen.	[90]
	Pneumonia virus induced infection	Mice	Significant reduction in morbidity and mortality when co-administered with ribavirin.	[117]
	Respiratory syncytial virus (RSV) infection	Mice	Recruitment of antiviral T cells into the bronchial epithelium was abolished resulting in an increase in RSV replication and delayed viral clearance.	[118]
Tumor Growth	Breast cancer model	Mice	Reduction in tumor growth and macrophage influx.	[101]
HSV infection	Herpes simplex virus-2	Mice	Decrease in NK cell influx into the peritoneum but impaired the antiviral response (higher viral load into the liver of infected mice).	[119]
	Herpes simplex virus-1	Mice	Reduction in leukocyte influx and interaction with meningeal endothelial cells, viral load into the brain was not affected.	[120]
Atherosclerosis	Diet-induced atherosclerosis	Mice	Decrease of atherosclerosis plaque formation by reducing leukocyte infiltration as well as CCR5 and CCR2 expression.	[121]
	Angiotensin II-induced atherosclerosis	Rats	Strong inhibition of mononuclear adhesion with the arteriolar and venular endothelium	[122]
Pancreatitis	Caerulein-induced pancreatitis		Reduction in lung damages.	[123]
Liver inflammatory diseases	ConA-induced hepatitis	Mice	Decrease of CCR-1$^+$ NK cells recruitment into the liver and amelioration of hepatitis injury.	[124]
	Liver fibrosis		Decrease in macrophages and T cells migration into the liver and reduction in liver injury and fibrosis regression.	[125]
Adhesion formation	Postoperative intraperitoneal adhesion formation	Rats	Reduction in adhesion formation after visceral injury.	[126]

Table 1 (*Continued*)

Pathologies	Model	Species	Therapeutic properties	Reference
Cardiomyophathy	*T. cruzi* infection	Mice	Decrease in parasite load and fibronectin expression in the cardiac tissue of infected animals. Beneficial effect on heart injury and amelioration of the survival rate. No modulation in inflammatory cell recruitment into the central nervous system.	[127–129]
	T. cruzi infection	Rats	Increase in cardiac inflammation, fibrosis and parasitism.	[130]
Periodontitis	Periodontopathogen infection	Mice	Attenuation in alveolar bone loss and in development of inflammatory reaction in response to pathogen infection.	[131]

Further optimisation of CCL5 was performed by chemical modifications of the N-terminus. Based on the success of Met-CCL5, the first residue of CCL5 (Ser) was replaced by a chemical isostere, the *n*-pentyl chain resulting in AOP-CCL5 (aminooxy-pentane-CCL5) [135]. AOP-CCL5 was initially considered as a potent CCR5 antagonist with respect to its ability to inhibit CCL5- and CCL4-induced calcium signalling and chemotaxis in primary monocytes [135,138]. In an experimental glomerulonephritis murine model, AOP-CCL5 abolished glomerular infiltration of monocytes by 60% and slowed down disease progression [139]. However, although AOP-CCL5 did not induce calcium mobilisation in primary monocytes, this effect was observed when cells evolved to the CCR5-expressing macrophage phenotype and in CCR5 transfected cells [89]. Moreover, AOP-CCL5 was more potent than wild type chemokine in triggering receptor phosphorylation and desensitisation [88,140]. AOP-CCL5 also exhibited an improved ability to down-modulate CCR5 and prevent its recyclisation at the cell surface [138], a property explaining its strong anti-HIV properties. Indeed, compared to Met-CCL5, AOP-CCL5 exhibited an 8-fold increase in inhibiting viral infection in primary cells (PBMC and macrophage) [135]. In this regard, AOP-CCL5 was considered as a CCR5 superagonist (Fig. 3) [141].

In analogy with AOP-CCL5, Townson et al. [144] designed a CCL3 derivative as antiviral inhibitor. AOP-CCL3L1 (or LD78β), a non-allelic isoform of the CCL3 chemokine, displayed an enhanced affinity for CCR1 and CCR5 receptors and induced calcium signalling and chemotaxis at levels comparable to wild type chemokine. However, this analogue induced a stronger CCR5 internalisation and was thus significantly more potent than CCL3L1 and AOP-CCL5 at inhibiting CCR5-mediated HIV entry [144,145].

Supported by the successful strategy of the addition of a hydrophobic residue to CCL5 and CCL3, a second CCL5 analogue displaying an *n*-nonanoyl chain instead of Ser (NNY-CCL5) was designed. Continuous administration of AOP-CCL5 or NNY-CCL5 in a murine HIV-1 model resulted in a decrease in viral load and a reduced loss of $CD4^+$ T cells. Upon treatment interruption (seven days post-infection), mice treated with AOP-CCL5 developed viremia whereas 60% of the animals treated with NNY-CCL5 were protected against infection [146]. The improved antiviral activity of NNY-CCL5 was most probably due to its higher receptor affinity and its enhanced ability to prevent recycling of internalised CCR5 [147]. These promising results were further confirmed by a preclinical study. Toossi et al. [148] assessed the antiviral potential of AOP- and NNY-CCL5 in preventing spread of HIV-1 in patients co-infected with tuberculosis. While incubation of PBMC and pleural fluid mononuclear cells isolated from co-infected patients enhanced HIV-replication, simultaneous incubation with CCL5 analogues blocked HIV-1 replication although the effects were patient dependent.

Based on studies described above, Hartley et al. [149] used a medicinal chemistry approach to improve the N-terminal pharmacophore domain of CCL5. Protein analogues were designed by using NNY-CCL5 as scaffold to develop a third semi-synthetic CCL5 analogue called PSC-CCL5. This new derivative was similar to native CCL5 except that the first three amino acids were replaced by a nonanoyl, a thioproline and a cyclohexylglycine, respectively. In a human T-cell line, AOP-, NNY- and PSC-CCL5 induced long term internalisation of CCR5 and prevented HIV-1 infection. However, PSC-CCL5 was 50 times more potent than AOP-CCL5 [150]. In the context of the future development of CCL5 analogues as microbicide, Kawamura et al. [151] evaluated their abilities to block HIV-1 infection in immature human Langerhans cells. A short incubation (20 min) with each CCL5 analogue was sufficient to prevent infection in a dose-dependent manner. Here, PSC-CCL5 exhibited the most potent antiviral activity. Finally, Lederman et al. [152], demonstrated that a topical prophylactic administration of high doses of PSC-CCL5 ($\geq$100 μM) fully protected macaques from vaginal challenge with a R5-tropic virus without exhibiting detectable toxicity.

Inspired by NNY-CCL5, two other chemokines, CCL11 (Eotaxin-1) and CCL14 (Hemofiltrate CC chemokine 1/HCC-1) were transformed by truncation of one or nine amino acids, respectively and addition of a *n*-nonanoyl group [153,154]. Modification of CCL11 resulted in an agonist (NNY-$CCL11_{(2-74)}$) with reduced receptor selectivity (CCR3 and CCR5) compared to wild type (CCR3) [154]. NNY-$CCL14_{(10-74)}$ is a potent agonist of CCR1, CCR3 and CCR5 receptors and induced chemotaxis and activation of human eosinophils and PBMC at nanomolar concentrations [153,155]. In addition, NNY-$CCL14_{(10-74)}$ induced receptor internalisation and sequestration preventing further interaction with their natural ligands. When tested for its ability to improve clinical conditions related to allergic airway inflammation, NNY-$CCL14_{(10-74)}$ reduced inflammatory cell influx into the lungs and improved airway hyper-responsiveness without displaying side effects related to its agonist properties [153,155].

The discovery of Met-CCL5 as a potent antagonist triggered the engineering of a new generation of promising CCL5 analogues characterised by addition of various hydrophobic chemical groups at the chemokine N-terminus (AOP, PSC, NNY). The particularity of these derivatives comes from their potency to induce a prolonged receptor sequestration (Fig. 3). Although the early steps of this inhibitory mechanism were relatively well defined, details by which these analogues interfere with the recycling pathway have long been controversial. A recent study suggested that these derivatives induced a longer occupancy of the receptor during the desensitisation process [143]. However, the exact reasons of this phenomenon remain to be elucidated. Chemical modifications were also successfully applied to other chemokines to develop analogues with anti-inflammatory and anti-HIV properties. To date, the chemical modified chemokine PSC-CCL5 is in phase I clinical trial as an anti-HIV candidate. However, several drawbacks like leukocyte recruitment, stimulation of HIV-1 replication [156], high potency variability [157] and protease susceptibility [133,158] remain limiting factors that need to be addressed before considering human applications.

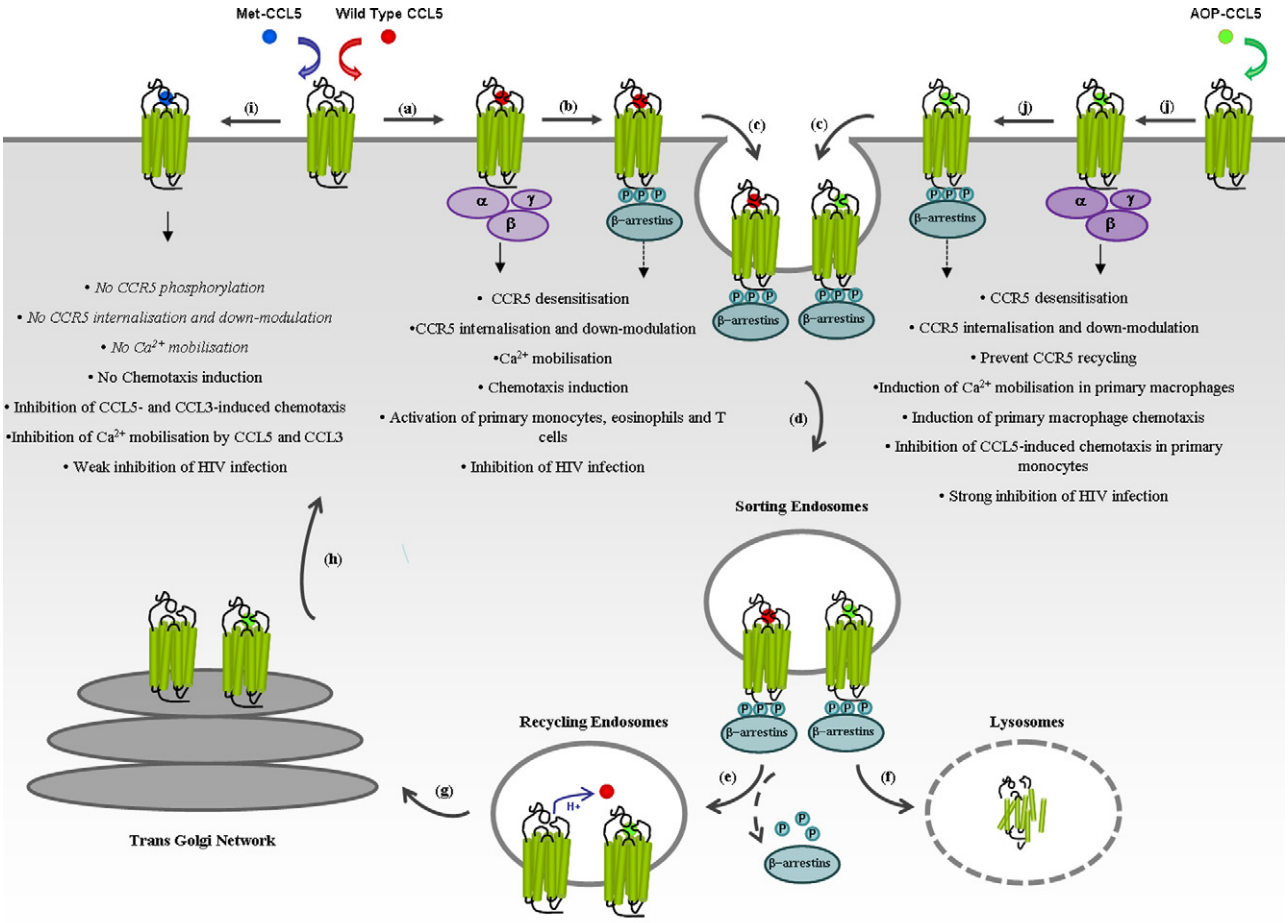

Fig. 3. CCR5 activation and modulation pathways by wild type and modified CCL5 chemokines. (a) Binding of wild type agonist CCL5 chemokine activates G proteins and triggers downstream signalling leading to various cellular responses. (b) Activated CCR5 receptor is then phosphorylated and binds β-arrestins blocking activation of the G proteins. Recruitment of β-arrestins may initiate signalling distinct from G proteins [142]. (c) Phosphorylated CCR5 is internalised via clathrin-mediated endocytosis and (d) traffics through the sorting endosomes towards (e) the recycling pathway or (f) lysosomal degradation. (g) Dephosphorylated CCR5 follows a retrograde trafficking via the Trans Golgi network [143]. (h) Exportation of the recycled CCR5 receptor to the cell surface. (i) Binding of antagonist modified CCL5 (Met-CCL5) prevents further interaction with natural ligands without triggering cellular responses. (j) The binding of "superagonist" modified CCL5 chemokine (AOP-CCL5) induce signalling, cellular responses and receptor internalisation with long term sequestration in the Trans Golgi network. *Italic represents controversial data regarding Met-CCL5 induced internalisation and signalling* [132–134].
Source: Adapted from [11].

2.3. Chimeric and modified chemokines

As mentioned above the network of chemokine–receptor interactions is extremely complex and the molecular basis for this complexity and redundancy as well as the determination of the selectivity is not well understood. Engineering of chimeric chemokines called "fusokines" represented an interesting approach to modulate their selectivity and activity [159]. This strategy was mainly described for the fusion of the flexible N-terminus and the structured core domain of two different chemokines. These chimeras allowed to interchange or decouple the "message" delivered by the N-terminus from the "address" encoded in the core domain and were therefore used as a tool to investigate the molecular basis of chemokine selectivity [45,160–163]. The fusokine constituted of the first eight residues of the N-terminal domain of CCL3 and the core domain of CCL5 remained a potent antiviral inhibitor of R5-tropic HIV-1 strains and induced chemotaxis of monocytes. This chimera also displayed reduced CCR1 affinity and no interaction with CCR3, thereby differing from CCL5 [164]. In similar experiments, Davis et al. [165] used the low selectivity of the core of vCCL2 (vCCL2) as template to identify the determinants of CX3CL1 essential for binding to CX3CR1. The authors demonstrated the importance of the N-terminus and in particular the three residues located between the two cysteines for CX3CR1 binding and selectivity.

In parallel to these fundamental purposes, fusokines were also developed to generate therapeutic molecules with antagonist or superagonist properties or modified selectivity. Gaertner et al. [161] improved the selectivity of the PSC-CCL5 analogue by fusing increasing parts of its N-terminal pharmacophore region to the core of CCL4, a more selective CCR5 ligand. Indeed, the obtained fusokine (Ch8-CCL4) had an antiviral potency comparable to PSC-CCL5 but displayed the receptor binding profile of CCL4. However, as previously reported for the parental CCL4 chemokine, this analogue presented an additional undesired binding to CCR1 [166–169]. In 2005, Dong et al. [163] replaced the eight N-terminal amino acids of CXCL12a by the first 10 D-amino acids of vCCL2. This chemokine analogue (RCP222) interacted with CXCR4 to the same extent as CXCL12a but was devoid of agonist activity.

More recently, Wang et al. [170] engineered a fusokine by substituting the N-terminal loop of CXCL10 with the N-terminus of CXCL11. The chimera exhibited the respective antitumor effects of both parental chemokines. In addition, a remarkable synergistic antitumor activity not reachable by the native chemokines alone or

in combination was observed. Furthermore, it is important to note that the new epitope created at the junction of the chimera was not immunogenic during the treatment and no significant toxicity was observed.

To further extend the possibility of modification, Kumar et al. [171] developed the concept of *de novo* synthetically and modularly modified chemokines (SMM-chemokines). These SMM-chemokines represent a versatile tool to create new chemokines by fusion, deletion or incorporation of non-natural amino acids. SMM-chemokines were used to study chemokine/receptor functions and to design novel ligands with modified activity, enhanced receptor selectivity and reduced toxicity [163,171]. The proof of concept of this approach was provided by transformation of the non-selective vCCL2 chemokine into more selective analogues. Modification of the first ten residues of vCCL2 with non-natural D-amino acids resulted in four vCCL2 analogues (RCP168, RCP169, RCP188, RCP189) with enhanced selectivity for CXCR4 and/or CCR5. In particular, the RCP168 derivative displayed potent antiviral properties equivalent to drugs commonly used in the clinical treatment of AIDS and was devoid of CXCR4 signalling or internalisation. Furthermore, this analogue did not interfere with CXCL12a binding which is a prerequisite to maintain the physiological functions of the receptor [172].

In summary, fusokines and SMM-chemokines represent two useful tools to study the chemokine–receptor network and to develop lead protein therapeutics. Development of synthetic chemokines in particular, opened a new area in the engineering of chemokines allowing the introduction of an almost unlimited range of unnatural amino acids and chemical modifications at any given site(s) of the chemokine [171].

2.4. Library approach

The chemokine analogues earlier described were engineered by introducing single mutations in a single molecule to study the importance of the different positions in the N-terminal domain of the chemokine. Peptide scanning and mutagenesis studies delivered valuable data on the pharmacophore properties of the N-terminus of chemokines. However, this approach is not efficient for the development of new therapeutic molecules. High throughput technologies in which large numbers of chemokine variants can be engineered and analyzed are required.

The phage display technology is a high throughput approach by which millions of variants can be explored in a rapid manner. The proof of concept to use this technology in the chemokine field was provided by Hartley et al. [173] who identified novel chemokine variants as candidate anti-HIV therapeutics.

The phage display technology is based on the introduction of foreign degenerated DNA in the pIII or pVIII gene of filamentous phage [174]. The insertion of foreign degenerated DNA results in the expression of a fusion protein constituted of the protein/peptide variants encoded by the foreign DNA and the phage pIII/pVIII protein. These fusion proteins are displayed at the phage surface. The collection of phage each displaying different protein variants constitutes a phage library. Variants specific to a given target can then be isolated based on affinity selection strategies (biopanning).

Hartley et al. [173] engineered a phage library in which the C-terminus of CCL5 analogues was fused to the N-terminus of the pIII protein of filamentous phage. Previous studies demonstrated that N-terminal extension of CCL5 with a hydrophobic residue improved the anti-HIV activity of the chemokine (see Section 2.2). The CCL5 phage library was engineered by adding a supplementary residue at position 0 and introducing fully or partially randomised positions in the first nine amino acids of the N-terminus of CCL5. The motif of the N-terminal domain of the CCL5 variants phage library was X_0–Ser_1–$\#_2$–X_3–Ser_4–Ser_5–X_6–$\#_7$–$\#_8$–$\#_9$– with X corresponding to fully randomised positions comprising all twenty amino acids and # corresponding to Ala, Pro, Ser or Thr. The phage library was screened on the chemokine receptor CCR5 expressed on living cells. In contrast to a classical biopanning strategy in which phage binding to the target are recovered, this particular strategy aimed to enrich for phage internalised by the cell. This strategy potentially recovers phage sequestering the CCR5 receptor. After three selection rounds, a consensus N-terminal motif Leu–Ser–Pro–*X*–Ser–Ser–Gln–Ser–Ser–Ala with *X* corresponding to Leu, Met or Val was identified. Two representative clones displaying the LSPVSSQSSA- and FSPLSSQSSA-CCL5 sequences and known as P1 and P2, respectively, were further characterised. These CCL5 analogues inhibited fusion of the HIV envelope in the nanomolar range, as observed for AOP-CCL5. Interestingly, the HIV inhibitory activity of these CCL5 analogues on laboratory adapted viruses and primary isolates was improved with a factor 5–10 compared to AOP-CCL5. Both analogues displayed enhanced affinities for CCR5 suggesting a more efficient blocking of the binding of the HIV envelope to the receptor. While P1 displayed no CCR5 down-modulation, P2 induced CCR5 down-modulation comparable to AOP-CCL5. Both CCL5 analogues acted as agonists in a calcium flux assay but had no activity on CCR1 and CCR3 receptors and thus displayed a higher selectivity than wild type CCL5 binding CCR1, CCR3 and CCR5.

This study provided the proof of concept for the phage library approach to engineer chemokines with improved therapeutic properties. The technology provides a fast access to improved CCL5 analogues with increased receptor affinity, selectivity and enhanced anti-HIV activity [141,173]. Characterisation of a recombinant (*E. coli* produced) P2-CCL5 highlighted the monomeric properties of this variant compared to the dimeric character of wild type CCL5. Further more, P2-CCL5 displayed a lowered affinity to heparin compared to its wild type counterpart [175].

The P1 and P2 CCL5 analogues were further optimised using the same phage library approach and selection strategy on living cells [176]. The objective of the study was to develop a strong CCL5 analogue with a comparable HIV entry inhibitory activity to the potent PSC-CCL5 (see Section 2.2) but devoid of agonist activity. This analogue could then be successfully produced in a cost-effective manner as a microbicide. Three cycles of optimisation were applied using the P1 and P2 sequences as starting point for the first optimisation (X_0–X_1–Pro_2–X_3–X_4–X_5–Gln_6–$\#_7$–Thr_8–Pro_9) and the sequences obtained during the first optimisation for a second and third round of optimisation ((Gln_0–Gly_1–Pro_2–Pro_3–Leu_4–Met_5–X_6–X_7–X_8–X_9) and (Gln_0–Gly_1–Pro_2–O_3–Σ_4–X_5–X_6–X_7–X_8–X_9, with *O* is Gly, Leu, Pro and Σ is Gly, Leu, Met). In total, 45 sequences were isolated of which three were further characterised based on their (i) no signalling and no sequestration, (ii) no signalling but sequestration and (iii) signalling and sequestration properties. All three analogues were potent anti-HIV molecules comparable to PSC-CCL5. The two analogues 5P12- and 6P4-CCL5 (QGPPLMATQS-CCL5 and QGPPGDIVLA-CCL5) displaying no signalling/no sequestration and signalling/sequestration properties, respectively were also fully protective in a macaque HIV vaginal challenge model when topically applied [177]. Clinical safety trials will now be launched with the 5P12 CCL5 analogue.

The phage library approach was also applied for the engineering of the CX3CL1 chemokine [178]. This chemokine exists in two forms with distinct functions i.e. adhesion of leucocytes for the soluble form and strong chemo-attractant for the membrane bound structure. As many other chemokines, CX3CL1 is involved in inflammatory conditions and the chemokine represents a valuable target to develop anti-inflammatory drugs. The objective of the study was to develop CX3CL1 analogues with antagonist activity

on the human CX3CR1 receptor. The first six residues of the human CX3CL1 were fully or partially randomised according the following consensus X_0–Z_1–X_2–X_3–Σ_4–δ_5–Ω_6 with X being any amino acid; Z being Leu, Pro, Gln, or Arg; Σ being Val, Ala, Asp, or Gly; δ being Leu, Met, or Val; and Ω being Ser, Pro, Thr, or Ala. An additional amino acid was added at the N-terminus as for the CCL5 library [173]. Selections with the phage library were performed on living cells. In contrast to the previous study, phage were rescued by addition of an excess of soluble CX3CL1 (competitive elution). After four selection rounds a consensus sequence was assigned: Ile–Leu–Asp–*X*–Gly–Leu/Val–Ala/Ser, where *X* is any amino acid and the most frequently isolated sequence corresponded to Ile–Leu–Asp–Asn–Gly–Val–Ser. This phage interacted specifically with CX3CR1 expressing cells and with an anti-CX3CL1 antibody. The most frequently isolated clone was produced by chemical synthesis or as chimeric fusion protein with an immunoglobulin and further characterised. These analogues had a 12 times lower receptor affinity than wild type CX3CL1 (1.9 nM versus 0.16 nM) but were receptor-specific. Both analogues were devoid of calcium and chemotaxis responses and did not induce receptor internalisation. These proteins displayed antagonist properties towards CX3CL1-induced calcium release (34 and 72 nM, respectively) and chemotaxis (IC_{50} = 2.7 nM and 6.1 nM, respectively). The analogues also antagonised the CX3CL1/CX3CR1 mediated cell adhesion but with higher IC_{50} values indicating that the binding and adhesion mechanisms are not the same. Finally, the analogues acted as antagonists *in vivo* in a peritonitis mouse model.

All together, these data demonstrate the potency of the phage display technology to engineer chemokines as therapeutic proteins. Potent antagonists were obtained in a very fast process and more interestingly, these analogues were also functional *in vivo* in experimental animal models. Despite the great success of this technology, few papers are available using this approach to discover new therapeutic chemokine variants. To the best of our knowledge this technology was only applied on two chemokines, CCL5 and CX3CL1. Screening on living cells is not straightforward and care needs to be taken not to select for irrelevant receptors present at the cell surface. Strategies to represent GPCR in a more suitable and stable fashion without altering to a great extent their structure would probably enhance the use of phage libraries and render the quest for GPCR drugs easier.

3. Chemokine size reduction

In parallel to the engineering of full length chemokines, several studies were conducted to evaluate the possibility to reduce the size of the chemokine to a peptide level while maintaining parental activity and selectivity. Indeed, with their small size peptides are easier to produce and modulate and were proposed to hold great potential for the design of small chemical compounds compared to macromolecular proteins [179–181].

Although size reduction was reported for several chemokines including CXCL8, this approach was mainly applied to the chemokines CCL5, CXCL12a and vCCL2 binding the receptors CCR5 and CXCR4 implicated in HIV entry [5,28,182,183]. These studies provided the proof of concept that peptides derived from the N-terminus of chemokines can be developed as short agonists or antagonists of their cognate receptors [179–181,184–186] (Table 2).

Table 2
Chemokine N-terminus derived peptides and analogues.

Chemokine	Sequence	Form	Effect	Affinity	Reference
CCL5	Residues 1–68	Complete	Agonist	K_D = 6.2 nM, IC_{50} = 0.78–12.18 nM	[84]
$CCL5_{(1-10)}$	SPYSSDTTPC	Monomer	ND	ND	[187,188]
CXCL12a	Residues 1–67	Complete	Agonist	EC_{50} = 3.6 nM, IC_{50} = 79 nM	[14]
$CXCL12a_{1-8)}$	KPVSLSYR	Monomer	Agonist	EC_{50} = 37.5 μM	[184]
$CXCL12_{a(1-9)}$	KPVSLSYRC	Monomer	Agonist	EC_{50} = 5.2 μM	[184]
$CXCL12a_{(1-13)}$	KPVSLSYRCPCRF	Monomer	Agonist	K_i = 12.4 μM, IC_{50} = 44 μM IC_{50} = 22 μM	[179,181] [179]
$CXCL12a_{(1-17)}$	KPVSLSYRCPCRFFESH	Monomer	Agonist	EC_{50} = 2.2 μM, K_i = 850 nM	[184]
C9W–$CXCL12a_{(1-13)}$	KPVSLSYRWPCRF	Monomer	ND	IC_{50} = 10.5 μM	[179]
L5H–$CXCL12a_{(1-13)}$	KPVSHSYRCPCRF	Monomer	–	K_i = 4.8 μM, IC_{50} = 12.5 μM IC_{50} = 4 μM	[179,181] [179]
$CXCL12a_{(3-14)}$	VSLSYRCPCRFF	Monomer	ND	IC_{50} = 20 μM	[179]
$CXCL12a_{(5-16)}$	LSYRCPCRFFES	Monomer	ND	IC_{50} = 18 μM	[179]
$CXCL12a_{(5-14)}$	LSYRCPCRFF	Monomer	ND	IC_{50} = 10.2 μM	[179]
RSVM–$CXCL12a_{(1-17)}$	RSVMLSYRCPCRFFESH	Monomer	Agonist	EC_{50} > 100 μM	[189]
ASLW–$CXCL12a_{(1-17)}$	ASLWLSYRCPCRFFESH	Monomer	Agonist	EC_{50} > 60 μM	[189]
C9W, F13–14F-$CXCL12a_{(5-14)}$	LSYRWPCR*FF*	Monomer	Antagonist	K_i = 3.7 μM, IC_{50} = 1 μM	[181]
C9W, F13–14F-$CXCL12a_{(5-14)}$	$(LSYRWPCR\mathit{FF})_2$	Dimer	Antagonist	K_i = 290 nM, IC_{50} = 130 nM	[181]
$CXCL12a_{(1-9)}$	$(KPVSLSYRC-)_2$	Dimer	Antagonist	EC_{50} = 500 nM, K_i = 730 nM	[184]
P2G-$CXCL12a_{(1-9)}$	$(KGVSLSYRC-)_2$	Dimer	Antagonist	K_i = 2.5 μM	[184]
P2G-$CXCL12a_{(1-8)}$ (CTCE-9908)	$KNH_2(-KGVSLSYR)_2$	Dimer	Antagonist	ND	[190]
$CXCL12a_{(12-17)}$ retro	HSFFRCPCRFFESH	Retropeptide	Agonist	ND	[191]
$CXCL12a_{(5\ to\ 14+56\ to\ 67)}$	LSYRCPCRFF-GGGG-LKWIQEYLEKALN	Extented	Agonist	ND	[192]
Cyclo($K^{24}E^{28}$)-$CXCL12a_{(1\ to\ 14+56\ to\ 67)}$	KPVSLSYRCPCRFF-GGGG-LKWIQEYLEKALN	Extented	Agonist	IC_{50} = 254 nM, EC_{50} = 106 nM	[193]
Cyclo($E^{24}K^{20}$)-$CXCL12a_{(1\ to\ 14+56\ to\ 67)}$ (CTCE-0021)	KPVSLSYRCPCRFF-GGGG-LKWIQEYLEKALN	Extented	Agonist	IC_{50} = 225 nM, EC_{50} = 147 nM	[193]
Cyclo($K^{24}E^{28}$)C9A, C11F-$CXCL12a_{(1\ to\ 14+56\ to\ 67)}$ (CTCE-0214)	KPVSLSYRAPFRFF-GGGG-LKWIQEYLEKALN	Extented	Agonist	IC_{50} = 226 nM, EC_{50} = 1.35 μM	[194]
vCCL2	Residues 1–71	Complete	Antagonist	IC_{50} = 14.8 nM	[186]
$vCCL2_{(1-21)}$	LGASWHRPDKCCLGYQKRPLP	Monomer	Antagonist	IC_{50} = 190 nM	[186]
$vCCL2_{(1-10)}$	LGASWHRPDK	Monomer	Antagonist	IC_{50} = 5.2 μM	[195]
$vCCL2_{(6-18)}$	RPDKCCLGYQKR	Monomer	Antagonist	IC_{50} > 100 μM	[186]
$vCCL2_{(1-11)}$	LGASWHRPDKC–	Dimer	Antagonist	ND	[195]
$_{D}$-$vCCL2_{(1-21)}$	*LGASWHRPDKCCLGYQKRPLP*	Monomer	Antagonist	IC_{50} = 13 nM	[196]

Italic, D-amino acids; ND, not determined; (–) residue involved in the dimerisation.

3.1. Linear peptides derived from the N-terminus of CCL5, CXCL12a and vCCL2

Different studies were performed to determine the smallest CCL5 fragments retaining agonist or/and antiviral properties. Wells et al. [197] were the first to analyze the chemotaxis effect and signal induction of synthetic overlapping peptides (10-mers) spanning the complete CCL5 sequence. Only the 10-mer peptides $CCL5_{(1-10)}$, $CCL5_{(3-12)}$ and $CCL5_{(5-14)}$ induced chemotaxis with EC_{50} values ranging from 2 to 8 nM. In contrast to the full length chemokine, $CCL5_{(1-10)}$, displayed no antiviral activity against the R5-tropic HIV-1_{JR-CSF} strain. However, upon N-terminal acetylation and C-terminal amidation a significant antiviral activity was recorded with this peptide [180,185,187]. Further analysis with truncated peptides revealed that only $CCL5_{(1-10)}$ and $CCL5_{(5-14)}$ exhibited significant antiviral activities while shorter peptides $CCL5_{(6-14)}$, $CCL5_{(7-14)}$ and $CCL5_{(8-14)}$ were not effective [185]. $CCL5_{(5-14)}$ was approximately 2 times less potent than the $CCL5_{(1-10)}$ peptide. In another study, Ramnarine et al. [188] observed antiviral properties with peptides $CCL5_{(1-14)}$ and $CCL5_{(3-14)}$ but not with peptide $CCL5_{(2-14)}$. These data suggested that the biological activity of these peptides was dependent on the presence of a hydrophilic residue. More particularly, Ser or Tyr residues at the N-terminus were proposed to create hydrogen bounds with the receptor. This hypothesis could also explain the antiviral activity recorded for the peptide $CCL5_{(5-14)}$ also presenting a N-terminal Ser [180]. Finally, the smallest peptide displaying antiretroviral activity corresponded to the 5-mer peptide $CCL5_{(6-10)}$ [187].

Attempts to reduce the size of the chemokine CXCL12a while retaining activity were proven very successful [179,181,184] (Table 2). In contrast to the majority of the chemokines, CXCL12a was considered for many years as an exclusive CXCR4 chemokine and thus represented a very interesting candidate for the development of CXCR4 specific and selective antagonists [179]. Recently, CXCR7 was identified as another receptor for this chemokine [198]. Analysis with overlapping peptides covering the complete sequence of CXCL12a demonstrated that the 13-mer peptide corresponding to the N-terminus of the chemokine ($CXCL12a_{(1-13)}$) displayed significant CXCR4 specific anti-HIV and signalling activities (IC_{50} = 22 μM) (Table 2). Systematic substitution of each position in this peptide by the other 19 amino acids revealed two motifs, Lys^5–Ser^6–Tyr^7 and Pro^{10}–Cys^{11}–Arg^{12} required for anti-HIV activity [179]. Interestingly, replacement of Cys^9 with an aromatic residue (C9W-$CXCL12a_{(1-13)}$) resulted in an enhanced antiviral activity (IC_{50} = 10.5 μM). For almost all positions with the exception of Tyr^7, substitution by charged residues such as Arg or His resulted in a significant increase of the antiviral activity by 20–40%. Particularly, mutant L5H-$CXCL12a_{(1-13)}$ displayed an IC_{50} of 12.5 μM for HIV inhibition and failed to induce signalling even in presence of the two first residues (Lys^1 and Pro^2) suggesting that Lys^1 and Pro^2 are only a part of a larger motif required for efficient signalling [179].

Further size reduction resulted in peptides $CXCL12a_{(3-14)}$, $CXCL12a_{(5-16)}$ and $CXCL12a_{(5-14)}$ with even higher antiviral activities displaying IC_{50} of 20, 18 and 10 μM, respectively [179]. In particular, the two Phe residues located at the C-terminus of peptides $CXCL12a_{(3-14)}$ and $CXCL12a_{(5-16)}$ were proposed to be important for receptor binding. Indeed, Phe^{14} is solvent exposed and could be involved in the binding of the receptor [14] (Fig. 1). Peptides shorter than nine residues displayed reduced affinity [184]. The peptide corresponding to the first nine residues ($CXCL12a_{(1-9)}$) was 7-fold more potent than the 8-mer $CXCL12a_{(1-8)}$ peptide (Table 2). Elongation of the 9-mer peptide $CXCL12a_{(1-9)}$ with the RFFESH motif resulted in a peptide $CXCL12a_{(1-17)}$ with a 150-fold increased potency when compared to $CXCL12a_{(1-9)}$ and only 100 times less potent than the full length CXCL12a. As observed for the complete chemokine, elimination or mutation of Lys^1 and Pro^2 turned the peptide into an antagonist [14,179,181,184]. Most of the peptides derived from the CXCL12a N-terminus induced calcium signalling representing a limiting factor for their therapeutic use.

Viral CCL2 encoded by human herpes virus 8 (HHV-8) was also explored as a source of short CXCR4 inhibitors [186,199]. In contrast to CXCL12a, this chemokine is a very broad spectrum chemokine and binds to a large variety of CC and CXC receptors including CCR5 and CXCR4 [200,201]. This CC and CXC binding property and its natural antagonist activity towards these receptors is unique among chemokines and makes vCCL2 an attractive template to develop dual tropic anti-HIV drugs targeting CCR5 and CXCR4 [186,201]. Zhou et al. [186] analyzed the antagonist potential of the N-terminus of vCCL2 on the receptors CCR5 and CXCR4. Surprisingly, peptides corresponding to the first twenty one residues of vCCL2 ($vCCL2_{(1-21)}$) displayed strong binding to CXCR4 (IC_{50} = 190 nM) but not to CCR5 which markedly differed from the behaviour of the parental chemokine. The peptide $vCCL2_{(1-21)}$ was 70 times less potent than full length chemokine CXCL12 whereas $CXCL12_{(1-17)}$ was 1000 times less potent than the parental chemokine [186,199] (Table 2). The peptide $vCCL2_{(1-21)}$ showed antiviral protection in the micromolar range against X4-tropic and dual tropic viruses but not against R5-tropic viruses consistent with its specific binding to CXCR4 [186,201]. The surprising discrepancy in CXCR4 and CCR5 binding of $vCCL2_{(1-21)}$ peptides and full length chemokine is not elucidated yet. These data suggest distinctive sites on vCCL2 for CXCR4 and CCR5 binding; the N-terminus being implicated in CXCR4 binding while other undefined domains would be implicated in CCR5 binding [186]. The recently resolved structure of CXCR4 revealed a ligand binding site different from other GPCRs located more closely to the extracellular surface [202]. This larger contribution of the extracellular parts in ligand binding could partially explain the improved binding of vCCL2 peptides to CXCR4.

Truncation analysis of peptides $vCCL2_{(1-10)}$ and $vCCL2_{(6-18)}$ demonstrated the importance of the first five residues of the peptide for CXCR4 binding which markedly differed from results recorded with the peptide $CXCL12a_{(5-14)}$ deleted from the first five residues [186]. The binding of peptide $vCCL2_{(1-21)}$ was improved by introducing non-natural D-amino acids [196]. These analogues revealed the importance of the residues Leu^1, Arg^7 and Lys^{10} residues for CXCR4 binding [199]. In particular, as observed for CXCL12, substitution of the two Cys residues had important effects on CXCR4 binding. Mutants C11A-vCCL2 (Cys^{11} > Ala), C11F-vCCL2 (Cys^{11} > Phe) and C11AC12G-vCCL2 (Cys^{11} > Ala/Cys^{12} > Gly) were 8, 14 and 17 times more potent than the non-mutated $vCCL2_{(1-21)}$ peptide.

Despite their differences in sequence and signalling behaviour, peptides derived from the N-terminus of CXCL12a and vCCL2 displayed common features. These peptides exhibited low overall positive charges (+2.5 and +3.5) compared with the parental chemokines (+8 and +9) and other highly positive CXCR4 binding molecules [203–205]. This observation suggests that electrostatic interactions are not the sole interaction mode of these peptides with the receptors [199]. Additionally, NMR and circular dichroism analyses revealed a conserved β-turn structure in the N-terminus of CXCL12a and vCCL2 derived peptides as well as in their parental chemokines [206]. The importance of the β-turn in receptor binding is further emphasised by the presence of this structure in CXCR4 antagonists derived from polyphemusin such as T22, T134 and T140 [205,207]. These data together with the resolved three-dimensional conformation of CXCR4 provide new key elements for the optimisation and the design of peptidomimetics based on β turn motifs.

Importantly, binding of vCCL2 peptides induced receptor internalisation without activating the intracellular signalling pathways representing a valuable property for therapeutic development as antiviral lead compounds. However, like for CXCL12a derived peptides, the antiviral activities recorded with vCCL2 peptides were proposed to be mainly associated with receptor surface occupancy, rather than related to receptor internalisation. Peptides inducing receptor internalisation and a sustained sequestration in the intracellular compartment devoid of receptor signalling might provide better therapeutic candidates [208]. Another therapeutic application of the N-terminal CXCL12a and vCCL2 peptides is their use as a vehicle to deliver molecules to CXCR4 expressing cells [209]. The CXCR4 receptor is upregulated in tumor cells and provides a marker to target these cells and deliver therapeutic molecules into their cytoplasm [210]. Egorova et al. [209] demonstrated enhanced DNA delivery to CXCR4 cells by linking DNA to peptides CXCL12a$_{(1-17)}$, CXCL12a$_{(1-8)}$ and vCCL2$_{(1-10)}$. The best results were observed with longer CXCL12a peptides displaying higher affinity for the receptor as well as agonist properties and most probably increased receptor endocytosis.

3.2. Dimeric peptides derived from the N-terminus of chemokines

Although linear peptides derived from CXCL12a proved their usefulness, their lack of high affinity represents a limitation for further therapeutic development. In parallel to the study on linear CXCL12a peptides, Loetscher et al. [184] demonstrated a 40-fold increased potency of a dimeric peptide composed of the residues one to nine when compared to the monomeric format. Moreover, Pro^2 > Gly substitution turned the dimeric peptide into an antagonist (Table 2). Similarly, Heveker et al. [181] dimerised an optimised CXCL12a$_{(5-14)}$ peptide containing a Cys^9 > Trp mutation and additional replacements of the two last L-Phe by D-Phe. Again, dimerisation resulted in a 10-fold increase in binding and antiviral activity compared to the linear format. Until the resolution of the three-dimensional structure of CXCR4 the underlying mechanisms of the observed enhanced potency of dimerised peptides was not understood. The CXCR4 structure revealed the formation of a consistent homodimer with an interface including helices V and VI [202]. The improved affinity of the dimeric peptides might be thus related to an increased avidity as proposed by Heveker et al. (Fig. 4) [181].

Similar results were reported with dimeric peptides derived from the N-terminus of vCCL2 (vCCL2$_{(1-11)}$) [195] (Table 2). vCCL2 peptides in mono- and dimeric format were more potent than CXCL12a derived peptides [195]. To date, no data on the size and chemical nature of the linker for optimal dimerisation were reported.

Until today, the most promising dimeric peptide based on the chemokine N-terminus is the CTCE-9908 analogue (British Canadian Biosciences Corp.). This short CXCR4 antagonist corresponds to the peptide P2G-CXCL12a$_{(1-8)}$ (Pro^2 > Gly) dimerised via a Lys residue (Table 2). This analogue was reported to inhibit metastasis in osteosarcoma, melanoma, prostate and breast cancer murine models [211–215] and displays synergic effects when combined with other anti-cancer molecules [211]. In addition, safety of this analogue was described in a single dose Phase I trial in healthy adults [216]. In July 2005, CTCE-9908 was granted orphan-drug by the FDA and this derivative is currently in a phase I/II clinical trial for the treatment of solid tumours.

In summary, these results demonstrate that peptides derived from the chemokine N-terminus represent an alternative source of promising chemokine receptor modulators holding great potential for the development of small agonists or antagonists. Mutagenesis and dimerisation of peptides provided tools to enhance the affinity and modulate their biological activity (agonist versus antagonist).

3.3. D-Peptides and retropeptides

To obtain protease resistant peptides, a chemical analogue approach based on the use of D-amino acids and non-natural amino acids was applied to the CXCL12a and vCCL2 peptides. Against all expectations, D-amino acid substitutions at all positions in the linear peptide vCCL2$_{(1-21)}$ improved binding and activity of the peptide by at least a factor 10. Replacement with L- and D-amino acids demonstrated a positive correlation between the higher binding capacity and mutations introduced in the first ten amino acids of the N-terminus [196]. This remarkable stereochemical flexibility of the peptide-receptor interface opens interesting opportunities for further chemical improvement (solubility, stability) and the development of small molecules (peptidomimetics).

On the other hand, a retro-peptide approach was explored for CXCL12a. Palladino et al. [191] coupled head to head two CXCR4 binding motifs derived from CXCL12a (12-RFFESH-17) to obtain the following retro-peptide, HSEFFRCPCRFFESH. This non-natural arrangement did not result in an improvement of the affinity. However, surprisingly this peptide displayed agonist activity even though residues Lys^1 and Pro^2 were absent. This confirms that Lys^1 and Pro^2 are not the sole residues implicated in signalling [184].

3.4. N-terminus peptide extension

The previous studies clearly demonstrated the potential of chemokine derived peptides as improved agonists or antagonists. However, up to date no study reported on the optimisation of their biological activity by addition of amino acids as described for full length chemokines. Addition of N- or C-terminal amino acids may enhance their affinity by occupying additional sites on the receptor. On the other hand, addition of hydrophobic moieties or other chemical tails may serve as an anchorage into the cell membrane and consequently enhance the concentration of the peptide nearby the target.

Addition of special moieties could stabilise the structure of the peptide and enhance its affinity for the receptor. Nowadays, the only extension described corresponds to the attachment of the C-terminal part of CXCL12a, CXCL12a$_{(56-67)}$ to its N-terminal peptide CXCL12a$_{(5-14)}$ via a glycine linker, a modification described for the first time in 1999 by Luo et al. [192,193]. Although the C-terminus of CXCL12a had no activity and did not bind the receptor, linkage to CXCL12a$_{(5-14)}$ dramatically increased chemotaxis and calcium

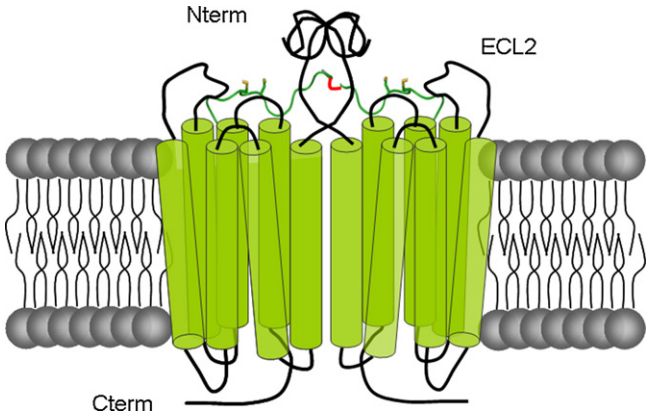

Fig. 4. Model of the interaction of dimeric N-terminal derived peptides with dimerised receptor. Peptides (dark green) dimerised via a disulfide bridge between the Cys at their C-terminus (red) interact with the transmembrane domain and the extracellular loops of the two receptor monomers.

Table 3
Phage MIMOKINE libraries derived from the N-terminus of CXCL12a.

Name	Randomization	*X*	*Z*	Theoretical complexity	Observed complexity	Inserts (%)
Mimo12.1	MGVSLSYRXPXRFFESH	2	0	1 024	3.5×10^7	94.4
Mimo12.2	MGZZZZZ(R/K)XPX(R/K)ZZ(E/D)ZH	2	8	1.3×10^{11}	6.5×10^7	96.3
Mimo12.3	MGZXZXZ(R/K)X(P/G)X(R/K)ZZZZZ	4	8	3.6×10^{15}	2.0×10^8	95.6
Mimo12.4	MXVXLXYRXPXRXFESH	6	0	1×10^9	2.3×10^8	93.3
Mimo12.5	MPXSLXYRXPXRFXESH	5	0	3.36×10^7	1.0×10^8	95.5

Randomisation, complexity and percentage of inserts of the CXCL12a phage displayed libraries. *X* represents any of the 20 amino acids and *Z* represents subsets of amino acids displaying similar physico-chemical properties.

signalling activities of the peptide. The authors hypothesised that the dramatic increase in affinity was related to the local higher concentration of the peptide via binding of the C-terminus to glycosaminoglycans [192]. Further optimisation by lactam cyclization in the C-terminal extension either between Lys^{20} and Glu^{24} (CTCE-0021) (IC_{50} = 225 nM) or between Glu^{24} and Lys^{28} (IC_{50} = 254 nM) resulted in peptides displaying a 114-fold increased receptor affinity when compared to the linear form (IC_{50} = 29 μM) [193]. Compared to the parental CXCL12a chemokine, CTCE-0021 displayed a 5 times reduced binding affinity and was 2000 times less potent in inducing cell migration (IC_{50} = 226 nM and EC_{50} = 1.35 μM) [194]. However, *in vivo* administration of CTCE-0021 to mice induced a long term elevation of the blood polymorphonuclear neutrophils in a dose dependent manner within minutes after injection [194]. Based on these data, a second analogue (CTCE-0214) containing two additional mutations ($Cys^9 > A$ and $Cys^{11} > F$) was engineered. CTCE-0214 exhibited higher plasma stability and increased circulating hematopoietic cells after *in vivo* administration to mice [217]. In synergy with supplementary cytokines including thrombopoietin, this analogue also improved *ex vivo* survival and expansion of hematopoietic progenitor cells as well as their engraftment in non-obese diabetic/severe combined immunodeficient mice [214]. Therefore, the CTCE-0214 derivative is a promising drug to mobilise stem cells and early precursors from the reservoir to the circulation representing a valuable property for blood cell transplantation purposes. Currently, CTCE-0214 is in a Phase I study for haematological support (British Canadian Biosciences Corp.).

Additional CXCL12a N-terminus derived peptides are currently in development and in preclinical studies for vascular diseases (CTCE-0324), wound healing (CTCE-0422) and stroke prevention (CTCE-0501) (British Canadian Biosciences Corp.).

In summary, the introduction of non-natural amino acids or addition of C-terminal extensions provides a strategy to efficiently improve the properties of the peptides in order to envisage their use in clinical trials.

3.5. N-terminus library screening

Site directed mutagenesis demonstrated that while certain positions were critical for binding or signalling other positions were more permissive to mutations and allowed the identification of higher affinity binders [179]. This apparent plasticity provides the possibility to further optimise these peptides in order to modulate and fin-tune their affinity, biological activity and selectivity.

Sachpatzidis et al. [189] explored the potential of a library based approach to identify CXCL12a N-terminally derived peptides with enhanced agonist properties. By screening a cDNA library encoding 160 000 N-terminal $CXCL12a_{(1-17)}$ peptides displaying random amino acids at position one to four in a yeast functional assay, the authors identified two new CXCR4 agonists (1-RSVM-4 and 1-ASLW-4). Peptide RSVM behaved as a partial agonist in chemotaxis assays while peptide ASLW had a superagonist effect. To the best of our knowledge the study of Sachpatzidis and co-authors, represents the first and only description of biological high throughput screening of peptides derived from the N-terminus of a chemokine. Although successful, their screening approach was restricted to the discovery of allosteric agonists.

Based on the success of the phage technology approach on full length chemokines and the proof of concept on the biological activity of peptides derived from the N-terminus of the chemokines, we proposed a strategy to optimise chemokine derived peptides. We focussed on the N-terminus of CXCL12a and engineered phage libraries displaying peptide variants with different degrees of randomisation to identify short antagonist peptides. These peptidic antagonists *mim*ic the binding of the chem*okine* to the receptor and are called MIMOKINES.

Five different phage libraries based on the first seventeen amino acids of the N-terminus of CXCL12a were engineered (Table 3).

For all libraries, Cys^9 and Cys^{11} implicated in the formation of two disulfide bridges with the core of the chemokine, were fully randomised (20 amino acids) to create new binding interactions. Libraries Mimo 12.1, 12.2, 12.3 were designed to display increasing complexities by introducing soft randomisations (replacement by residues with equivalent physico-chemical properties) (*Z*) and increasing number of fully randomised positions (*X*). In contrast, libraries Mimo 12.4 and 12.5 displayed, respectively, five and six fully randomised positions. In all libraries, the N-terminal Lys^1 responsible for the agonist properties of the chemokine CXCL12a was replaced by a Met (ATG codon) [179]. These libraries were screened on a peptide mimicking the extracellular surface of CXCR4. Using competitive elution with full length CXCL12a, we identified at least three variants with a 2–3-fold increase in their binding ability compared to the wild type $CXCL12a_{(1-17)}$.

The applicability to isolate either agonist or antagonist peptides from the MIMOKINE libraries may represent an original and useful tool for the identification of new CXCR4 therapeutic peptides.

4. Discussion and future challenges

Chemokines and their cognate receptors are involved in a broad range of pathologies such as inflammation, cancer and HIV-1 infection and represent therefore a valuable target for drug discovery. During the last two decades, the pharmaceutical industry has mainly focused on chemical compound library screening to develop drugs interfering with the chemokine–receptor interactions.

Simultaneously, studies were conducted to identify chemokine analogues with modified activity and selectivity by engineering chemokines and in particular their N-terminal domain. The studies on CXCL8 were innovative and triggered further research on the importance of the N-terminal domain of the chemokines in receptor interaction and activation. N-terminal mutations, truncations, elongations with natural and non-natural amino acids or with chemical moieties and chimeric chemokines (fusokines) resulted in an incomparable source of information on chemokine/

receptor interactions. More importantly, these studies demonstrated the pharmacophore potential of the N-terminal domain and provided tools to modulate the biological activity and selectivity of chemokines. These approaches resulted in promising therapeutic analogues derived from among others CCL2, CCL3, CXCL12a and CCL5 [35,47,48,70,71,76,78,137,144]. These analogues acted as antagonists or superagonists and had beneficial effects in experimental animal models of a large number of pathologies including inflammation, autoimmune diseases, allergies and infectious diseases.

To our opinion, three important milestones affected the engineering of the N-terminus of chemokines: (I) the feasibility to produce fully synthetic chemokines, (II) the library approach for chemokine analogue discovery and (III) the size reduction of chemokines to a peptide level.

The first production of a fully synthetic chemokine (CCL5) was reported by Wilken et al. and further exploited by Hartley et al. [149,218]. This technical milestone opened new perspectives to drug discovery and provided chemokines in a milligram to gram scale, often required for a complete investigation of their activity [218]. In addition, chemical tails or non-natural amino acids can also be introduced into the synthetic chemokine in a reliable and controllable manner with a high degree of purity [171,218]. However, major drawbacks including the absence of some posttranslational modifications such as glycosylation [25,26], expensive costs and technical requirements limit its use for high throughput screening approaches.

A second milestone was the introduction of the library approach for chemokine analogue discovery by Hartley et al. [173]. This technology allows fast and efficient screening of a large collection of engineered chemokine variants (library). A CCL5 analogue based on natural amino acids (5P12-CCL5) and as potent as its chemically modified counterpart (PSC-CCL5) was identified by this approach. Consequently, safety clinical trials with 5P12-CCL5 will be launched in the near future. In contrast to the chemically modified chemokines, the manufacturing costs for natural analogues have decreased and thus, they have become more attractive for therapeutic development as novel anti-HIV microbicides. This aspect is particularly important in developing countries representing a very high HIV incidence.

Despite the large number of chemokines studied, a general strategy to improve the therapeutic properties of these proteins cannot be deduced. Only a small subset of chemokines represented a conserved motif in the N-terminus implicated in receptor binding and activation (the ELR motif of neutrophil agonists). Modifications resulting in antagonist analogues cannot be translated to all chemokines as demonstrated for Met-CCL5 and Met-CXCL12[87,135–137].

The complexity of the chemokine network renders the rationalization of interaction models difficult and chemokine–receptor interactions need to be addressed individually. Distinct but overlapping epitopes were identified in CCL5 as responsible for the interaction with CCR1, CCR3 and CCR5 [77]. However, this complexity can also be used to fine-tune and target therapeutic effects on a specific subset of cells.

Furthermore, beneficial effects of chemokine analogues on a particular receptor might be detrimental for other receptor interactions. This applies particularly to CCL5 and CXCL12a analogues developed to inhibit HIV infection while preserving the physiological roles of these chemokines. Several chemokine derivatives such as AOP-CCL5 induced mobilisation of leukocytes and stimulated viral replication through T cell activation [156]. The analogue Met-CCL5 induced pro-inflammatory events and hampered immune response in *in vivo* models [103–105,119].

A major drawback to the use of full length chemokine analogues is their potential residual agonist activity as illustrated in studies with Met-CCL5 [88,132–134,140]. As observed with their natural counterparts, some chemokine analogues were subject to N-terminal proteolysis or degradation consequently changing their activity, affinity and selectivity [133].

A third important discovery is related to the size reduction of chemokines to a peptide level. Different studies demonstrated that peptides derived from the N-terminus of chemokines retained the parental biological activity and affinity albeit at a reduced level [179,181,184,186,199]. CCL5, CXCL12 and vCCL2 derived peptides inhibited HIV infection and a peptide as small as five amino acids inhibited viral infection [188]. As observed for full length chemokines, truncation, mutation and introduction of non-natural amino acids provided peptides with antagonist properties, modulated receptor selectivity and improved affinity [181,184,196]. To further enhance the affinity of these peptides, dimerisation strategies were proven to be very successful. To date, the best candidates displayed affinities in the nanomolar range [181,196]. Other strategies such as amino acid extension, linker nature and size optimisation for dimeric peptides as well as addition of chemical moieties in combination with high throughput technologies could lead to more potent peptidic antagonists. The N-terminal derived peptides described in this review exhibited antagonist properties acting mainly in a competitive manner by site receptor occupancy. The potential of these analogues may also be improved by exploring their abilities to induce receptor internalisation and sequestration.

As previously described for full length chemokines, screening of N-terminal derived peptide variants was also performed by library approach and resulted in the identification of agonists. Studies with newly engineered phage libraries displaying degenerated N-terminal peptide variants and providing the possibility to identify antagonists with receptor internalising and sequestering properties are on their way.

In comparison to complete chemokines, peptides can easily be manufactured and highly modified to change their activity or to improve their resistance to serum modification and activation [190,205]. Clinical trials launched with the CXCL12 derived peptides CTCE-9908 and CTCE-0214 for anti-metastasis and haematological support purposes demonstrate the therapeutic value of N-terminal peptides [216]. Finally, we believe that the resolution of the three-dimensional structure of the first chemokine receptor (CXCR4) will provide new possibilities to further improve the development of chemokine and peptide variants [199].

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