Future Drug Discovery

CXCR4 as a novel target in immunology: moving away from typical antagonists

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CXCR4 has been a target of interest in drug discovery for numerous years. However, so far, most if not all studies focused on finding antagonists of CXCR4 function. Recent studies demonstrate that targeting a minor allosteric pocket of CXCR4 induces an immunomodulating effect in immune cells expressing CXCR4, connected to the TLR pathway. Compounds binding in this minor pocket seem to be functionally selective with inverse agonistic properties in selected GPCR signaling pathways (G_i activation), but additional signaling pathways are likely to be involved in the immunomodulating effects. In depth research into these CXCR4-targeted immunomodulators could lead to novel treatment options for (auto)-immune diseases.

G protein coupled receptors (GPCRs) represent the largest family of transmembrane proteins translating extracellular stimuli into intracellular action, therefore being involved in the regulation of many physiological functions. Among the different sub-families of GPCRs, the group of chemokine receptors became a target of special interest in drug discovery research due to their close connection to cancer and immunology. The 50 human chemokines (the natural ligands of the chemokine receptors) are divided into C, CC, CXC and CX3C classes based on the number and spacing of conserved cysteine residues in their N-terminal region [1,2]. The receptors were then named after their first discovered ligand. Apart from the classification based on structure, chemokines are also classified into three groups: inflammatory chemokines expressed during inflammation, homeostatic chemokines constitutively expressed or chemokines with dual functions [3].

Among the members of the chemokine receptor family, one of the most interesting receptors currently under investigation as a therapeutic target is the CXCR4 also known as cluster of differentiation 184 (CD184) or fusin. The endogenous chemokine ligand of CXCR4 is CXC motif ligand 12 (CXCL12), also known as stromal cell-derived factor-1 alpha (SDF-1α). The pleiotropic role for CXCR4 is well illustrated by the complexity of the mechanisms underlying its biological function including receptor cross talk, receptor and ligand isoforms and non canonical ligands, such as the macrophage migration inhibitory factor, HIV protein gp-120, β-defensin and extracellular ubiquitin. While the CXCL12-CXCR4 signaling events have been extensively studied, non canonical pathways remain to be fully characterized [4,5].

Under physiological conditions CXCR4 is found throughout the body and expressed mostly on hematopoietic, progenitor, metastatic and immune cells [6,7]. It is best known for its function in the mobilization of cell migration including the connected vascularization, angiogenesis, neurogenesis, mobilization of immune cells and homing of immune cells in the bone marrow [8]. The CXCL12/CXCR4 axis plays a determinant role in different types of cancer [9–11]. CXCR4 also acts as a co-receptor for CXCR4-tropic HIV entry to CD4$^+$ T cells [12].
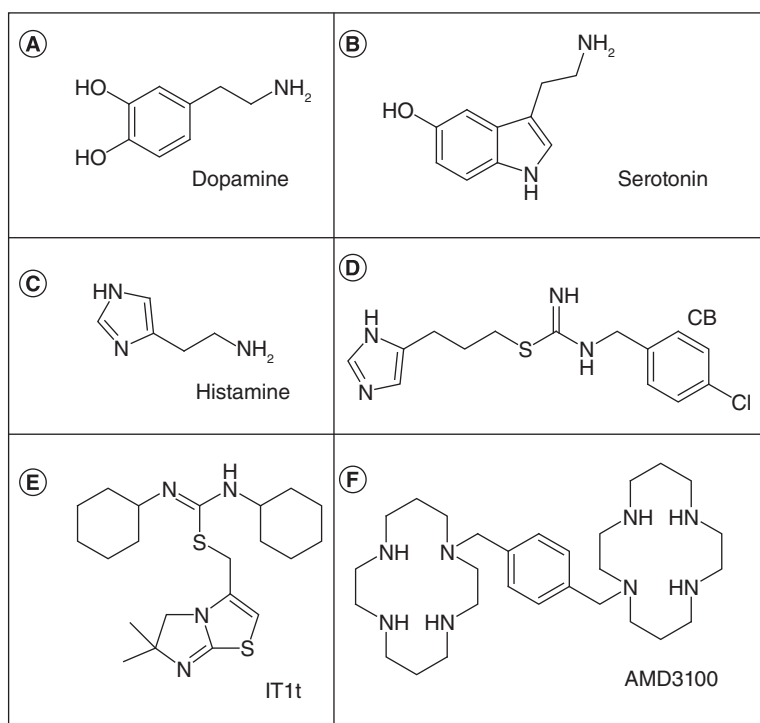
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Figure 1.   **Selected small molecule CXCR4 ligands.** Structure of small molecules activating a CXCR4 dependent anti-inflammatory effect **(A–E)** through binding into the minor pocket of CXCR4. Minor pocket ligands: **(A)** dopamine, **(B)** serotonin, **(C)** histamine, **(D)** clobenpropit (CB) and **(E)** IT1t. **(F)** Structure of small molecule antagonist AMD3100.

In the past years, numerous small molecules have been studied and developed to target CXCR4 [13]. These efforts resulted in one US FDA approved small molecule antagonist: AMD3100. AMD3100 is a bicyclam molecule that reversibly antagonizes CXCR4 [14]. It was initially developed as an anti-HIV agent [15] but was eventually approved for short-term treatment to mobilize hematopoietic stem cells in patients suffering from multiple myeloma and lymphoma. This might seem contradictory at first glance however the reason for this is that CXCL12 is highly expressed in the bone marrow keeping a reservoir of CXCR4 expressing hematopoietic stem cells at sight for rapid deployment. Consequently, blocking of the CXCL12/CXCR4 interaction by AMD3100 (Figure 1F) causes the mobilization of cells from this reservoir [16,17].

Other molecules targeting CXCR4 that have been or currently are in clinical trials include small molecules like mavorixafor (X4P-001) or TG-0054 (burixafor) and (cyclic) peptides like LY2510924, POL6326 (balixafortide) or BL-8040 (BKT140). All these compounds are published to be antagonists of CXCR4 and developed for application in oncology, HIV or WHIM syndrome [18]. Unfortunately, many potential CXCR4 blockers failed in clinical trials because of low efficacy, high toxicity or poor pharmacokinetic properties [19].

CXCR4 has been a target of interest in autoimmunity and inflammation, based on its involvement in leukocyte chemotaxis toward sites of inflammation [20–22], but also in the inappropriate retention of activated innate inflammatory cells at inflammation sites [23].

An unexpected and equally exciting new role of CXCR4 has recently been described in the suppression of the production of type-I interferons with potential application in a set of auto-immune diseases including interferonopathies such as systemic lupus erythematosus (SLE), or rheumatoid arthritis, or also in the context of controlling viral infections and their spread [24,25]. This signaling pathway is initiated by the minor pocket compounds acting as inverse agonists or potentially by activating further selective pathways which have yet to be studied in detail.

In this review, we will discuss CXCR4 as a new target in immunology, moving away from blocking the activation of CXCR4 with antagonists. Instead, we will focus on a new mode of action for CXCR4-targeted ligands, discussing the binding and signaling of IT1t [26] a compound first described in 2008 and the best characterized compound demonstrating an immunomodulating function at CXCR4. Based on the data generated around IT1t, novel small molecules could be designed to target the minor pocket of the receptor, either acting as inverse agonists or resulting in the activation of a non canonical CXCR4-pathway ultimately leading to immunomodulating effects.

CXCR4 as target for inflammation

CXCR4 plays a key role in inflammation due to its abovementioned function in mobilizing cell migration. CXCL12, the endogenous ligand of CXCR4 and an agonist, classically classified as a homeostatic chemokine, causes chemotaxis of immune cells to sites of inflammation [27–29].

CXCR4 has been a target of interest in (auto)immune disorders such as multiple sclerosis or rheumatoid arthritis, and lung inflammation/fibrosis, based on its involvement in leukocyte chemotaxis toward sites of inflammation [20–22] and the prevention thereof by CXCR4 antagonists like AMD3100 [30–32]. However, the CXCL12/CXCR4 axis is also mediator of inappropriate retention of activated innate inflammatory cells at inflammation sites, a key progression factor of chronic inflammatory diseases [23]. This demonstrates how the activation but also the blocking of CXCR4 could be beneficial in a disease dependent on a patient's clinical condition.

Apart from this already known important role in inflammation, CXCR4 has recently been shown to have an additional regulatory function by directly suppressing the activation of TLR signaling pathways and consequently having a direct influence on type-I interferon levels [33]. Many auto immune diseases are directly connected to these elevated interferon levels making CXCR4 a promising target [34]. Moreover, CXCR4 is overexpressed on circulating B cells in patients with active SLE [35,36] and pathologic T cell–B cell interactions are a hallmark of SLE. In this context, T follicular helper cells and some subsets of T peripheral helper cells are expanded in SLE patients.

A set of small molecules including endogenous monoamines (histamine, dopamine, serotonin) and the histamine synthetic analogue clobenpropit (CB) [24,37] as well as the CXCR4 specific isothiourea secondary amine IT1t [25] downmodulate inflammation in different settings (see Figure 1A–E for compound structures). IT1t was, until now, most often reported as an antagonist of CXCR4 resulting in potent and dose-dependent inhibition of the CXCL12/CXCR4 interaction [38].

All compounds reverse the effect of Resiquimod (R848), a TLR7 and 8 agonist (Figure 2), through downmodulation of the production of inflammation markers (IFNα) *ex vivo* in innate immune cells such as plasmacytoid dendritic cells, and *in vitro* in monocytic THP-1 cells. In addition, they have also been shown to counteract inflammation from a more physiological cause by reducing the production of type I interferons in peripheral blood mononuclear cells and isolated plasmacytoid dendritic cells after incubation with influenza orCXCR4-tropic HIV-1. These results translate into *in vivo* settings with the compounds displaying pharmacodynamic responses in a lupus mouse model with lowered IFNα, TNFα, IL-1β, IL-17 and anti-ds-DNA antibodies, a relevant efficacy readout for SLE. Furthermore, *ex vivo* peripheral blood mononuclear cells from lupus patients show reduced levels of IFNα, TNFα, IL-6, TRAIL and STAT1/3 phosphorylation after pre-incubation with IT1t [25]. Recently, the immunomodulatory effect of CB was confirmed on whole blood from COVID-19 patients as TNFα, IL-1β, IL-6 and IL-10 expression levels were also inhibited [37]. These studies using patient samples demonstrate the direct relevance of the compounds in a disease setting and their potential as therapeutic application.

Importantly, the blocking effect of IT1t, CB and histamine on the secretion of interferons as a response to incubation with influenza and HIV is reverted when (immune) cells are treated with siRNA targeting CXCR4 and also when cells are pre-incubated with the CXCR4 antagonist AMD3100. In contrast, the modulation of histamine receptors with different blockers or by siRNA had no impact on the functionality of CB or histamine [24,25]. This clearly demonstrates the CXCR4 dependency of the compounds to induce the immunomodulating effects. Moreover, neither CXCL12 nor AMD3100 show the same immunomodulating activity demonstrating that the minor pocket ligands activate signaling pathways distinct from a classical agonist or antagonist.

Structurally, all these compounds contain an amino group and a ring structure. IT1t binds into the minor pocket of CXCR4 [39], the conserved amino moiety is in the case of IT1t a key interactor of the minor pocket discussed more in detail in the following.

Apart from the previously discussed compounds there are several compounds targeting CXCR4 which could be of interest. AMD070/AMD11070 has been reported to bind into the minor pocket of CXCR4 based on docking and mutational studies [40,41] and might be an interesting molecule to evaluate further. TG-0054 developed and screened for cell mobilization displayed an immune suppressive function in minipigs [42], however the authors of the paper proposed that the mobilization of stem cells is the cause of this effect. At last, EPI-X4, a natural fragment of albumin and EPI-X4 derived peptides have been shown to inhibit inflammatory cell infiltration in a mouse model of allergic hypereosinophilia [43] and reduce skin inflammation in a mouse model of atopic dermatitis [44]. Moreover, these peptides share key interactions at CXCR4 with IT1t [45].
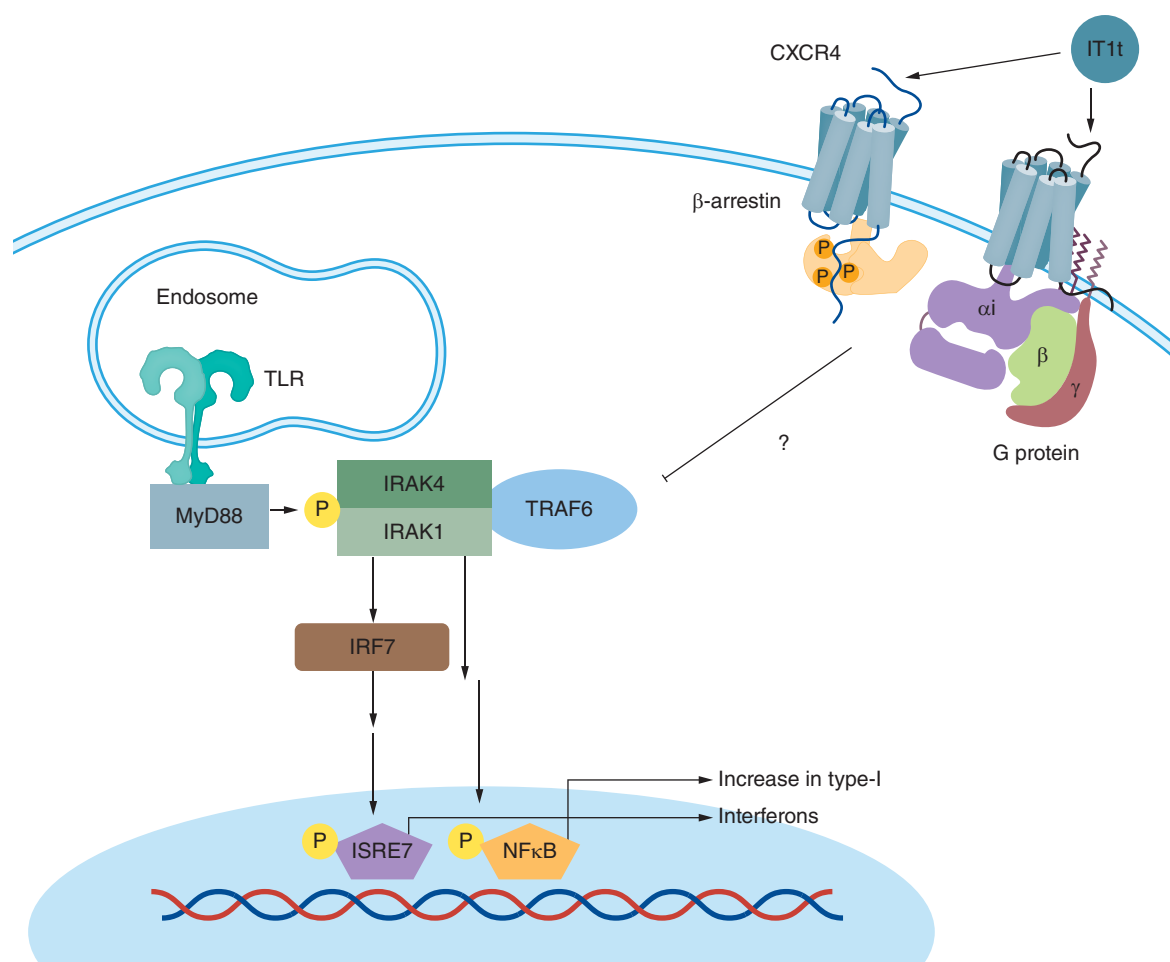
Figure 2.   CXCR4 blocks TLR activation. Activation of TLRs and their down-steam signaling ultimately results in elevated type-I interferons. This is initiated by pathogens but can also be chronically elevated in auto-immune disease. Novel CXCR4 minor pocket ligands cause inhibition of TLR downstream signaling and subsequent reduction of interferon type-I levels, the exact mode of action remains unclear.

CXCR4 binding pockets

There are multiple reviews and publications describing the binding positions of chemokines and small molecules at CXCR4 based on the crystal structures of the receptor fused to the T4 lysozyme including the structure with IT1t (structure with cyclic peptide CVX15 and IT1t: [39]; structure with viral chemokine vMIP-II: [46]). Moreover, the signaling transmission through the transmembrane helices was described in a mutational study [47]. For further reading Xu *et al.*, 2013 [48], Wescott *et al.*, 2016 [47] or Adlere *et al.*, 2019 [13] can be recommended.

While CXCR4 maintains the overall structure of a GPCR with its seven transmembrane helices, it displays some structural differences in comparison to other GPCRs which impact its extracellular structure and thereby the binding interface of CXCR4. The extracellular interface of CXCR4 consists of the N-terminus, extracellular loop 1 (ECL1) linking helices II and III, ECL2 linking helices IV and V and ECL3 linking helices VI and VII, with two disulphide bonds critical for ligand binding. Major differences in the extracellular space in comparison with other GPCRs include a rotation of helix II redefining the binding pocket of CXCR4 in comparison with predictions made by homology models. Helix V and VII reach further into the extracellular space, helix I, IV and VI are shifted and there is an additional disulphide bond in the extracellular space connecting the N-terminal region and helix VII of CXCR4. The ECL2 and N-terminal regions of CXCR4 are constrained through two disulphide bonds that shape the entrance to the ligand-binding pocket [39].

Comparison of the predicted binding positions of CXCL12, AMD3100 (docking and mutational studies) and IT1t (crystal structure) at CXCR4 show clear differences (Table 1).

Table 1. Key interaction residues between CXCR4 and CXCL12, IT1t and AMD3100.

	Residue (CXCR4)	CXCL12	IT1t	AMD3100
N-terminus	E2	[48]		
	E14	[48,49]		
	E15	[48,49]		
	D20	[48,50]		
	Y21	[50]		
	E26	[48]		
H I	E32	[48]		
	Y45			[41]
H II	F87	[48]		
	W94	[47]	[39]	[41]
	D97	[47,49]	[39]	
ECL1	W102		[39]	
H III	V112		[39]	
	Y116		[39]	[41]
H IV	D171			[51,52]
	A175			[52]
ECL2	R183		[39]	
	I185		[39]	
	C186	[48]	[39]	
	D187	[47–49]	[39]	[52]
	R188	[48]		
	F189	[47]		[41]
	Y190			[41]
H VI	Y255			[52]
	D262	[47,48]		[41,51,52]
	E268	[50]		
ECL3	K271	[48]		
H VII	E277	[48]		
	V280	[48]		
	H281	[47]		
	E288	[48]	[39]	[41,51,52]
	F292	[53]		

The binding mechanism of CXCL12 to CXCR4 as for chemokine receptors in general is described by a sequential two-step model [54,55]. CXCL12 occupies the entire pocket of CXCR4 causing a conformational change of the receptor N-terminal and extracellular domain, through H-bonds, salt bridges, polar and hydrophobic interactions. Key residues for interaction with CXCL12 are primarily located in the N-terminus, in helix I, II, VI and VII as well as in ECL2 and 3 [48].

The modelled binding interface between CXCL12 and CXCR4 shows a clear overlap with the binding positions of the small molecule IT1t [48]. In comparison to other crystalized class A GPCRs, the binding cavity for IT1t is larger, more open and located closer to the extracellular surface [56]. The more open pocket of chemokine receptors is partially covered by the ECL2. One reason explaining this structure could be that chemokines, the natural ligands of chemokine receptors, are larger than the natural ligands of other receptors. Additionally, the binding pockets of chemokine receptors present several negatively charged amino acidic residues involved in ligand binding. Whereas the globular cores of the chemokines interact with the extracellular surface of the receptors, including the top of the TM helices, the EC loops and the receptor N-terminus [1].

IT1t binds the minor pocket of CXCR4 located in the core of the TM domain (Figure 3), defined by side chains from helices I, II, III and VII, without making any contacts with helices IV, V and VI. The nitrogens of the symmetrical isothiourea group are both protonated with a net positive resonance charge, one of them forming a salt bridge with the Asp97 side chain. Both cyclohexane rings fit into small sub pockets, making hydrophobic contacts
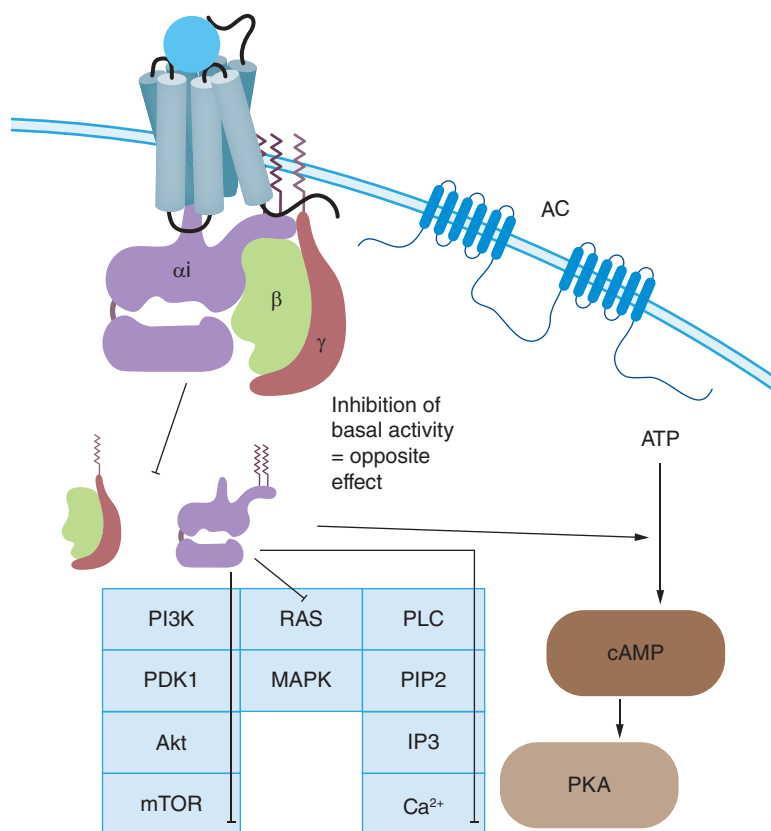
Figure 3.   Comparison of binding sites at CXCR4. Key interaction partners of IT1t are highlighted in red [39], key residues interacting with AMD3100 are highlighted in blue [41,57] and shared residues in purple. **(A)** top view of the receptor and **(B)** snake plot of CXCR4. CXCR4 plots adapted from GPCRdb [58].

with CXCR4. Connected by a short flexible linker, the imidazo-thiazole ring system is the only part of the ligand that contacts helix VII, by making a salt bridge between the protonated imidazo-thiazole and Glu288 [39].

The antagonist AMD3100 (Figure 1F) is composed of two cyclam moieties connected by a conformationally constraining heteroaromatic phenylenebis(-methylene) linker. In contrast to IT1t it has been postulated that AMD3100 binds into the major binding pocket with one cyclam ring interacting with Asp171 in Helix IV, whereas the other ring is sandwiched between the carboxylic acid groups of Asp262 and Glu288 from Helix VI and VII, respectively [59]. Another model proposed for AMD3100 binding mode consists of the positively charged bicyclam rings associated with Asp262 and Glu288 and the phenylenebis(methylene) linker associated with Phe189 and Tyr190 [41].

Signaling of minor pocket compounds

Since neither CXCL12 nor AMD3100 demonstrate a similar immunomodulating effect as IT1t or the monoamines, it is apparent that the signaling of minor pocket ligands must be distinct from the canonical agonist like CXCL12 or a classical antagonist like AMD3100. Within the group of compounds binding into the minor pocket of CXCR4, IT1t is best characterized from a signaling point of view. However, IT1t is also partially binding into the major pocket of CXCR4 leaving it up for debate which portion of the signaling cascade is related to the anti-inflammatory function. Connections between the TLR pathway and GPCRs have previously been published for neuropeptides [60] and similar mechanisms could be happening in the case of CXCR4.

CXCR4 signaling, including CXCL12-mediated canonical signaling, has been explored in detail and is subject of excellent reviews [8,61]. In brief, CXCL12 promotes dimer formation of CXCR4, and primarily activates $G_{i/o}$ pathways but also dependent on cell environment G_q or G_{13} signaling. CXCL12 has also been shown to recruit β-arrestin-2 and to a minor extent β-arrestin-1 after C-terminal phosphorylation of the receptor through G-protein coupled receptor kinases. This recruitment is followed by internalization of the receptor through clathrin-coated pits.
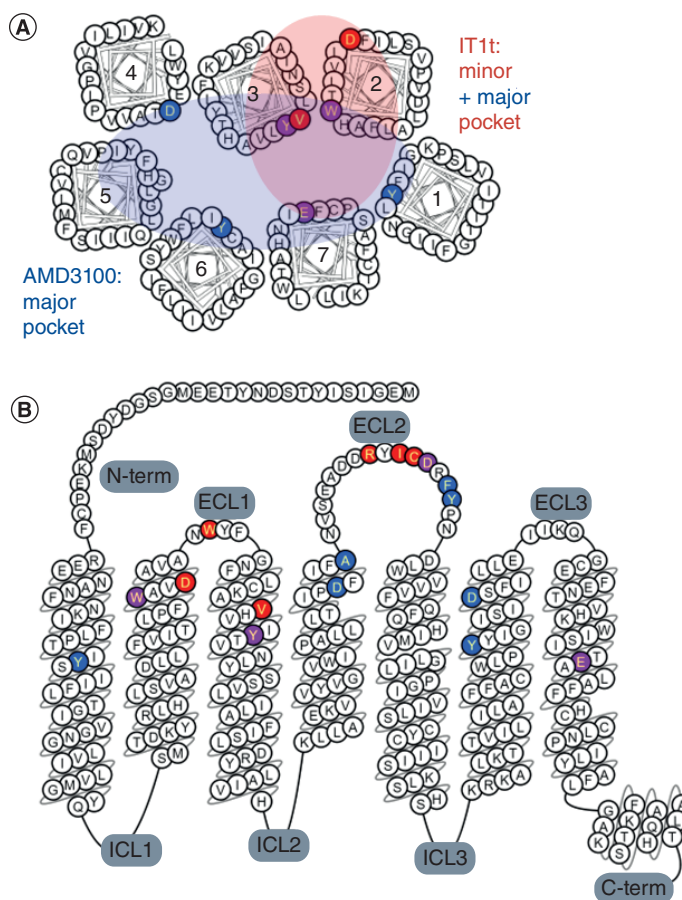
Figure 4. Hypothetical signalling of IT1t at CXCR4. IT1t has been published to inhibit G_{i2} signaling. Hypothetical signaling promoted further downstream at CXCR4 by IT1t and potentially other minor pocket ligands include the cAMP, PI3K/Akt and MAPK pathway; several of these pathways have been shown to regulate inflammation.

CXCR4 forms dynamic homodimers and -oligomers that increase in number with an increase in expression level. While CXCL12 promotes dimerization and AMD3100 has no effect, IT1t has been shown to prevent the formation of these dynamic CXCR4-CXCR4 homodimers or oligomers [62,63]. It is unclear if these changes in dimerization status have direct influence on signaling. On the one hand, it was reported that mutants uncapable of dimerization ($Trp^{195}Ala$-CXCR4; $Leu^{194}Ala$, $Trp^{195}Ala$, $Leu^{267}Ala$, $Glu^{268}Ala$-CXCR4) are still capable of signaling through CXCL12 with comparable potencies despite CXCL12 promoting dimerization [63]. On the other hand, the influence in dimerization status correlated well with the influence on CXCR4 basal activity; compounds that prevented dimerization also inhibited basal activity of G_{i2} [62] with one exception, a nanobody preventing dimer formation due to its size, that has no influence on basal activity. The inhibition of basal G_i activity by IT1t has been shown by many groups [62,64–66] and would cause all following classical CXCR4 signaling to be reversed (Figure 4). This would hypothetically lead to an increase in cAMP, and a reduction of Ca^{2+}, PI3K/AKT and MAPK activation. These signaling pathways include messengers that have been shown to play an influence in inflammation and represent targets for drug development in themselves, for example, the increase of cAMP levels has been shown to be connected to a reduction in pro-inflammatory mediators and an increase in anti-inflammatory factors [67,68]. The reduction of the PI3K/AKT pathway activity as well as the MAPK pathway have been shown to reduce the severity of inflammation in various *in vitro* and *in vivo* mouse disease models [69,70].

Lastly, also G protein independent signaling has been investigated. There has been, to our knowledge, no reports on GRK phosphorylation or β-arrestin recruitment itself. However, it has been shown that IT1t can completely block the CXCL12-mediated internalization of CXCR4 and has no effect on its own [38,71,72]. Notably, in a membrane proteome of Jurkat cells treated with IT1t, GNB2L1 is downregulated in comparison to the buffer control, a receptor involved in increasing protein kinase C (PKC) phosphorylation [71]. This might suggest that IT1t regulates PKC phosphorylation below basal levels.

Future perspective

The exploration of this non canonical pathway of CXCR4 directly interacting with the downstream signaling of activated TLRs has the potential to bring a new set of molecules to the market with application in (auto)immune disease. Today there are already companies exploring this potential. These novel compounds would especially be helpful for people not responding well to current therapies as it represents an entirely new mechanism.

Executive summary

Conclusion

- CXCR4 has been a target of drug discovery and development for multiple diseases, including HIV infection and cancer metastasis. Drug discovery efforts have therefore been focused on finding antagonists to block CXCR4 function. However, recent data have shown that molecules binding into the minor pocket of CXCR4 activate the receptor selectively ultimately reducing the production of type I interferons. This immunomodulating property could be of great interest in inflammatory diseases especially when taking into account the high expression levels of CXCR4 on immune cells and its upregulation on immune cells in lupus and arthritis correlating with disease progression [35,36,57,73].
- IT1t, the most understood example, seems to have dual function, behaving like an antagonist similar to AMD3100 in some assays, probably due to the partly shared binding pocket and the associated binding competition with CXCL12. The distinct signaling of IT1t, different from AMD3100 and CXCL12, shows inverse agonist activity in G_i activation assays.
- However, the exact mode of action and downstream signaling of IT1t leading to the immunomodulating effects is not yet fully elucidated. Novel compounds, exclusively binding the minor pocket, and devoid of CXCR4 antagonism could represent potent and safe immunomodulating treatment option for autoimmune diseases and should be explored in more detail.

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