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Antibodies targeting chemokine receptors CXCR4 and ACKR3

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d) List of nonstandard abbreviations

mAb, monoclonal antibodies

GPCR, G-protein coupled receptor

CXCR4, C-X-C chemokine receptor type 4

CXCR7, C-X-C chemokine receptor type 7

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ACKR3, atypical chemokine receptor 3

CXCL12, C-X-C motif chemokine 12

WHIM, warts, hypogammaglobulinemia, infections and myelokathexis syndrome

FDA, Food and Drug Administration

Fv, variable fragments; Fab, fragment antigen-binding

scFv, single-chain variable fragment

Fc, fragment crystallizable

CDR, complementarity determining region

ADCC, antibody-dependent cell mediated cytotoxicity

CDC, complement-dependent cytotoxicity

VHH, variable heavy-chain region of a heavy-chain only antibody

Nb, nanobodies

Nb-Fc, nanobody fused with fragment crystallizable

IgNAR, immunoglobulin new antigen receptor

VNAR, variable fragment of IgNAR

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Abstract

Dysregulation of the chemokine system is implicated in a number of autoimmune and inflammatory diseases, as well as cancer. Modulation of chemokine receptor function is a very promising approach for therapeutic intervention. Despite an interest from academic groups and pharmaceutical companies, there are currently few approved medicines targeting chemokine receptors. Monoclonal antibodies (mAb) and antibody-based molecules have been successfully applied in the clinical therapy of cancer and represent a potential new class of therapeutics targeting chemokine receptors, belonging to the class of G protein-coupled receptors (GPCRs). Besides conventional mAb, single-domain antibodies and antibody scaffolds are also gaining attention as promising therapeutics. In this review we provide an extensive overview of mAbs, single-domain antibodies and other antibody fragments targeting the C-X-C chemokine receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3), formerly referred to as CXCR7. We discuss their unique properties and advantages over small molecule compounds, and also refer to the molecules in preclinical and clinical development. We focus on single-domain antibodies and scaffolds and their utilisation in GPCR research. Additionally, structural analysis of antibody binding to CXCR4 is discussed.

Significance statement

Modulating the function of GPCRs and, particularly, chemokine receptors draws a high interest. A comprehensive review is provided for monoclonal antibodies, antibody fragments and variants directed at CXCR4 and ACKR3. Their advantageous functional properties, versatile applications as research tools and use in the clinic are discussed.

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Introduction

CXCR4 and ACKR3: role in physiology and diseases

Chemokines and chemokine receptors are key for immune system homeostasis, controlling the activation, differentiation, migration, and survival of leukocytes and other hematopoietic cells (Scholten et al., 2012). Due to their role in leukocyte migration, the chemokine receptor system is an important mediator of inflammation. Prolonged or deregulated expression of chemokines or chemokine receptors promotes an abnormal infiltration of leukocytes into inflamed tissue. This may result in inflammation, autoimmune diseases, tumour growth, survival and metastasis (Scholten et al., 2012). Most chemokine receptors, belonging to the superfamily of GPCRs, activate G-protein dependent signalling pathways upon agonist binding. There are some chemokine receptors, however, that signal via G protein-independent systems, and are therefore referred to as atypical chemokine receptors (Nibbs and Graham, 2013). CXCR4 activates the G_i family of heterotrimeric G proteins, while ACKR3, previously known as C-X-C chemokine receptor type 7 (CXCR7), signals in a G protein-independent and β-arrestin-dependent manner. ACKR3 downregulates CXCR4 signalling by either scavenging its ligand C-X-C motif chemokine 12 (CXCL12) or by forming heterodimers with CXCR4 (Rajagopal et al., 2010). There may also be crosstalk between CXCR4 and ACKR3 upon ligand binding that is mediated by intracellular signalling effectors (Zabel et al., 2009).

CXCR4 and ACKR3 have been widely studied in relation to their role in a large number of diseases. CXCR4 was the first HIV coreceptor discovered and has since then become an interesting target for the treatment of AIDS (De Clercq, 2003). X4 HIV strains bind to CD4 and CXCR4 through the envelope glycoprotein gp120 to infect the target cell (Murphy and Heusinkveld, 2018). Mutations truncating the C-terminus of CXCR4 also cause the rare immunodeficiency syndrome known as WHIM (warts, hypogammaglobulinemia, infections and myelokathexis). The CXCR4-CXCL12 axis is also implicated in chronic inflammatory

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diseases such as asthma and rheumatoid arthritis (Tamamura et al., 2004). Multiple cancer cells show elevated expression of CXCR4, including breast, ovarian, and prostate cancer cells, melanomas, gliomas, neuroblastomas, osteosarcomas, leukaemia, T- and B cell lymphomas, colorectal, pancreatic, and uterine cancers, amongst others (Peled et al., 2012). ACKR3 expression is also increased in a number of cancer types including leukaemia, breast, pancreatic, colon, and lung (D'Alterio et al., 2016; Guo et al., 2016; Melo et al., 2014; Miao et al., 2007). Both receptors are involved in different stages of tumorigenesis including growth, survival, and metastasis, according to in vitro and in vivo experimental data (Burns et al., 2006; Peled et al., 2012; Scholten et al., 2012; Teixido et al., 2018). Thus, a specific blockage of CXCR4 and/or ACKR3 function has a great potential for clinical therapy of cancer and other disorders. Antibodies and their fragments can be ideal candidates for such clinical interventions and serve as important tools in fundamental research.

Monoclonal antibodies and their first approvals for targeting GPCRs

In 1985, the United States Food and Drug Administration (FDA) approved the first monoclonal antibody directed against CD3, muromonab (Orthoclone OKT3), for the treatment of kidney transplant rejection (Smith, 1996). Since then, 76 mAbs have received marketing approval by the European Medicines Agency and FDA, with around 30 mAbs approved for oncological indications (http://www.ema.europa.eu, www.fda.gov, as of April 2019). mAbs and antibody-based therapeutics is a relatively young but fast-growing field with approximately 30 candidates in late-stage clinical trials for cancer therapy and with many more in earlier phases of development, indicating a further increase of approvals in the upcoming years (Kaplon and Reichert, 2018).

Antibodies are normally produced by B-cells of the immune system to regulate immune response and specifically bind and neutralise foreign pathogens and microbes. Specific target

recognition is mediated by variable fragments (Fv) located on the ends of two fragment antigen-binding (Fab) arms composed of heavy and light chains (Figure 1 and 2). Fabs are connected to the fragment crystallisable region (Fc), and the resulting molecule features a Y-shaped heterotetrameric structure with an approximate molecular weight of 150 kDa. In 1975, Köhler and Milstein introduced the first technique for isolating murine mAbs from hybridomas, triggering development of antibodies for academic research, clinical diagnostics, and, eventually, therapeutics, representing today an industry with billion-euro revenues (Kohler and Milstein, 1975). Afterwards several methods were introduced to generate fully human mAbs to overcome immunogenicity issues associated with rodent or humanised antibodies. Examples include *in vitro* antibody display technologies or transgenic animals modified to have the human antibody repertoire (Boder and Wittrup, 1997; de Haard et al., 1999; Hanes et al., 2000; Lonberg et al., 1994). Currently, multiple different platforms are in use by biotech companies and research groups to select antibodies and antibody fragments with desired specificity and affinity against targets of interest, including GPCRs.

Historically, GPCRs are targeted with small molecule drugs. Some of them face issues including fast receptor dissociation, poor oral bioavailability and other pharmacokinetic aspects (Pease and Horuk, 2012). Currently, there are only two antibodies against GPCRs approved for clinical therapy. One is mogamulizumab (Poteligeo) targeting CCR4, which received the first marketing approval in 2012 in Japan for the treatment of CCR4-positive adult T-cell leukaemia-lymphoma (Beck and Reichert, 2012). In May 2018, the FDA approved another GPCR-targeting mAb erenumab (Aimovig), a fully-human antagonistic antibody against the calcitonin gene-related peptide (CGRP) receptor for prevention of migraine (Dolgin, 2018).Erenumab is a highly potent antibody and, in comparison to small-molecule compounds targeting CGRP receptor e.g. telcagepant (MK-0974), offers greater specificity with no measurable activity on other receptors in the family reducing possible off-target effects (Shi et al., 2016; Walker et al.,

2015). CGRP has an extensive binding epitope. Therefore, a broad coverage of extracellular sites with multiple interactions on both subunits of CGRP-receptor complex by erenumab can offer superior specificity and potency in competing with CGRP over small-molecule analogues (Hollenstein et al., 2014; Shi et al., 2016). Application of small-molecule CGRP-receptor antagonists in clinical trials was associated with liver toxicity and discontinued (Moore and Salvatore, 2012). Antibodies, erenumab included, are mostly eliminated via proteolytic degradation in a liver- and renal-independent manner what greatly reduces the risk of hepatotoxicity and drug-drug interactions (Silberstein et al., 2015; Wu and Dall'Acqua, 2005). Additionally, overall prolonged blood half-life of antibodies allows less frequent administration of erenumab which has advantages for prophylactic treatment of migraine and better therapy adherence (Raffaelli and Reuter, 2018).

The limited number of currently available therapeutic antibodies targeting GPCRs may in part be explained by technical difficulties during the antibody development phase. Those difficulties have been reflected in several reports demonstrating poor specificity or reactivity of a few GPCR-directed commercial antibodies, including some against ACKR3 (Berahovich et al., 2010; Bodei et al., 2009). This might in part be explained by usage of short synthetic peptide fragments as antigens for immunization and/or material for antibody selection. Such peptides lack important posttranslational modifications and, overall, hardly represent unique conformational features of the target GPCR. As a result, a final antibody can possibly bind multiple antigens sharing similar linear epitopes or have a poor affinity. Caution is needed for experimental application and data interpretation using such antibodies (Kirkpatrick, 2009). In addition, several validation techniques, including disappearance of staining in the target knock-out animals and reduction of staining upon knock-down approaches (Michel et al., 2009), or evaluation of specific functionality on a panel of closely related receptors (Griffiths et al., 2016) are necessary to ensure the antibodies are specifically targeting the receptor of interest.

Using GPCRs in physiologically relevant conformations during antibody generation phase appears to be crucial for development of highly potent and specific therapeutic candidates, minimizing potential off-target effects. Purified forms of GPCRs are only available for limited number of receptors. Additionally, it also requires stabilisation of a purified receptor conformation with detergents, which can mask extracellular epitopes needed for antibody binding (Hutchings et al., 2010). The transmembrane topology and absence of purified protein material necessitate usage of membranes, virus-like particles or cells presenting a target receptor for immunisation and/or phage display selection (Baribaud et al., 2001; Silence et al., 2014; Tamura and Chiba, 2009; Van Hout et al., 2018). As an alternative to a live cell immunisation, which often results in a broad off-target immune response, DNA immunisation represents another approach for transmembrane receptors (Bobkov et al., 2018a; van der Woning et al., 2016). An additional limiting factor is generally the low surface expression levels of GPCRs.

In this review we will focus on therapeutic monoclonal antibodies, nanobodies and other functional fragments developed against CXCR4 and ACKR3. Information regarding other GPCR-targeting antibodies can be found in a recent review from Marshall and co-authors (Hutchings et al., 2017).

Targeting CXCR4 and ACKR3 with antibodies

Given the key role of the CXCR4 and ACKR3 receptors in a variety of diseases, they have received increasing attention from academia and pharmaceutical industry to develop drugs that specifically target them. Despite the considerable effort, there is only one small molecule compound against CXCR4 that is approved by the FDA, AMD3100 (plerixafor) (Brave et al., 2010). As molecules for therapeutic intervention, antibodies can offer several benefits in comparison to small-molecule compounds (Table 1). Longer blood half-life, up to several

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weeks for immunoglobulin G (IgG), can offer less-frequent patient dosing regimens in treatment of certain conditions e.g. in preventive therapy (Chames et al., 2009). Depending on the IgG subclass, mAbs can possess Fc domain-mediated effector functions that result in the elimination of target-expressing cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Vidarsson et al., 2014). In the context of targeting chemokine receptors, proof of concept for this approach was demonstrated by mogamulizumab, a glyco-engineered mAb with enhanced ADCC activity that efficiently eliminates CCR4-overexpressing tumour cells (Ito et al., 2009). Multiple other Fc engineering approaches have been described for fine-tuning the effector functions and blood half-life (Saxena and Wu, 2016). Additionally, antibodies can be made as bispecific molecules to specifically recognise two different epitopes or as antibody-drug conjugates for the targeted delivery of cytotoxic agents, enabling additional therapeutic modalities (Beck et al., 2017; Godar et al., 2018).

In contrast to mogamulizumab, ulocuplumab (BMS-936564/MDX1338) is a fully human IgG4 lacking ADCC and CDC effector functions. It is currently the most advanced anti-CXCR4 therapeutic antibody in clinical trials, actively studied for the treatment of Waldenström's macroglobulinemia and acute myeloid leukaemia (Table 2). Ulocuplumab binds to the second extracellular loop (ECL2) of CXCR4, prevents CXCL12 binding and inhibits CXCL12-mediated signalling and cell migration. The anti-cancer activity of this mAb is solely mediated by blocking CXCR4 function and inducing apoptosis (Kashyap et al., 2016; Kuhne et al., 2013). LY2624587 is another anti-CXCR4 IgG4 antibody with similar mode of action, which has also reached the clinical trial phase (Table 2) (Peng et al., 2016b). The combination of CXCR4 inhibition, induction of apoptosis and ADCC and CDC effector functions is employed in an IgG1 from Pfizer, PF-06747143, and shows a strong effect in multiple hematologic tumour models (Table 2) (Kashyap et al., 2017; Liu et al., 2017; Zhang et al., 2017). Utilisation of

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different antibody formats and Fc engineering strategies provides an attractive flexibility in pursuing specific therapeutic needs.

Small molecule drugs often target orthosteric binding pockets, which are highly conserved among the family of GPCRs, especially for the receptors sharing common ligands. Antibodies can overcome this off-target issue and provide better specificity by binding extracellular loops and the N-terminus, the regions of GPCRs representing the greatest diversity (Venkatakrishnan et al., 2013). Also, the overall druggability of chemokine receptors appears to be challenging for small molecule competitive antagonists due to the extensive chemokine binding interface (Kufareva et al., 2017). In this context it is worth noting that for the anti-CXCR4 antibody MEDI3185 the paratope and binding epitope have been described in detail. The interaction of the antibody is likely to be into a β-strand/β-strand fashion between CDR3H and ECL2, explaining the inhibitory activity of the antibody through a steric hindrance with CXCL12 (Peng et al., 2016a). This binding mode appears to be different from those of small molecules and peptides and might be beneficial for inhibition of chemokine receptors.

Autoantibodies against CXCR4 have been reported in patients with systemic sclerosis (Weigold et al., 2018). While most autoantibodies directed at GPCRs were shown to have agonistic properties (Cabral-Marques et al., 2018; Wallukat and Schimke, 2014), the functionality and binding epitopes of the reported CXCR4 autoantibodies have not been described (Recke et al., 2018). In contrast, antagonistic properties would be advantageous in therapeutic antibodies to block receptor function involved in disease progression. So far only antagonistic therapeutic antibodies have been developed to CXCR4 (Table 2). Binding epitopes of some of these antibodies and nanobodies, and their functional activities will be further discussed in the paper. To our knowledge currently there are no reported therapeutic mAbs against ACKR3 in preclinical or clinical development. One group reported an application of ^{89}Zr-11G8, a radiolabelled murine IgG1, for positron emission tomography (PET) studies in xenograft mice

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but without any therapeutic effects described (Behnam Azad et al., 2016). This contrasts with a relatively broad panel of mAbs targeting CXCR4, summarized in Table 2. Although it is beyond the scope of this review, it is worth mentioning that most of the generated ACKR3-targeting small molecule compounds and peptides to date function as agonists (Wang et al., 2018). Functional inhibition of ACKR3 by nanobodies and an antibody-based fusion construct have been reported and will be discussed further. Overall, antibodies represent a promising new class of therapeutics with improved specificity and potency inhibiting chemokine receptors such as CXCR4 and ACKR3.

Single-domain antibodies as therapeutics and tools in GPCR research

Although small molecules are mostly used to target GPCRs, their use has been accompanied with issues relating to selectivity, specificity and potency (Hutchings et al., 2017; Sriram and Insel, 2018). Antibodies and antibody fragments are a good alternative to resolve some of these issues. A variety of different antibody formats was engineered in the past with each format having distinct advantages and disadvantages (Chames et al., 2009; Fernandes, 2018). Some of the more commonly used formats can be found in Figure 1.

One interesting type of antibody fragments that is used to target GPCRs are nanobodies. These fragments are derived from heavy-chain only antibodies, which are uniquely found in animals from the camelid family. Nanobodies are roughly ten times smaller than conventional mAbs and have a more convex binding surface, which makes it easier to target cryptic epitopes that conventional mAbs cannot reach (De Genst et al., 2006; Muyldermans, 2013). GPCR-targeting nanobodies are more easily generated, even though conventional mAbs have been developed against GPCRs. To date, multiple publications show the potential of GPCR-targeting nanobodies both as research tools and therapeutics.

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Antibody-fragments as tools in crystallization studies

In the past few years, GPCR crystal structures have proven to be essential tools in drug development. However, obtaining crystal structures is a challenging tasks due to multiple conformations of receptors (Manglik and Kobilka, 2014). To overcome this, nanobodies, and other antibody fragments, are used to stabilize distinct GPCR conformations to enable the crystallization of these receptors by reducing the conformational heterogeneity. To obtain these conformation-stabilizing nanobodies, also referred to as confobodies, llamas or alpacas are immunized with purified receptors that are in complex with agonists or antagonist to stabilize the active or inactive conformation. Subsequent selections with the ligand-receptor complex are performed to obtain conformational stabilizers, which have been successfully used to crystallize GPCRs. By this means active and/or inactive conformations of different receptors have been obtained. Targets so far include the β2 adrenergic receptor (β2AR), M2 muscarinic acetylcholine receptor, μ-opioid receptor, κ-opioid receptor and the viral chemokine receptor US28 (Burg et al., 2015; Godar et al., 2018; Huang et al., 2015; Kruse et al., 2013; Rasmussen et al., 2011; Rasmussen et al., 2007; Ring et al., 2013). The use of (high affinity) stabilizing nanobodies even makes it possible to crystallize GPCRs in complex with low-affinity ligands, which was previously only possible with high affinity ligands (Ring et al., 2013). Next to crystallization, these nanobodies have also been used in drug discovery programmes to obtain agonists for different receptors by stabilizing the active conformation (Chevillard et al., 2018; Pardon et al., 2018).

GPCR-targeting intrabodies

Small molecules modulate GPCRs by binding to the extracellular but also the intracellular side of the receptor. In a similar manner, antibodies and their fragments can also target intracellular epitopes. However, their therapeutic value is limited due to their inability to cross the plasma

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membrane. Despite this, intracellular expression of these mAbs or fragments, which are referred to as intrabodies, overcomes this problem making them interesting research tools for investigating GPCR signalling.

Modulation of GPCR signalling and receptor expression has been achieved by expressing β2AR-specific intrabodies (Staus et al., 2014). Besides modulating GPCR signalling, intrabodies can also be used as biosensors. For example, nanobodies used for the crystallization of GPCRs, were fused to GFP and used for the visualization of spatiotemporal receptor trafficking and activation. For various GPCRs, intrabodies have provided greater insight into trafficking and signalling, in particular from endosomes, of these receptors (Irannejad et al., 2013; Stoeber et al., 2018).

Next to receptor-specific intrabodies, more generic intrabodies can target GPCR-interaction partners. For example, intrabodies targeting β-arrestin can act as generic GPCR endocytosis inhibitors without affecting β-arrestin mediated-signalling (Ghosh et al., 2017). Another study generated an intrabody that targeted Gβγ and competed with the binding Gβγ-interaction partners while having no effect on Gαq or Gαs mediated-signalling (Gulati et al., 2018). In addition, a generic Nb-biosensor was generated that bound the active form of Gαs and was used to detect the activation of GPCRs in endosomes (Irannejad et al., 2013; Ismail et al., 2016).

In vivo imaging tools

mAbs and antibody derivatives can also be used as in vivo imaging tools. Most commonly used labelling strategies involve the incorporation of radiolabels or fluorescent groups into mAbs (Freise and Wu, 2015). mAbs targeting CXCR4 or ACKR3 were used as in vivo imaging tools. In this case, antibodies were radiolabelled with ^{89}Zr and could detect GPCR expression in xenograft tumors in mice using positron emission tomography (Azad et al., 2016; Behnam Azad et al., 2016). To our knowledge, no GPCR-targeting nanobodies have been used so far for in

vivo imaging so far. However, numerous studies show that nanobodies in combination with different tracers can be used as imaging tools. This could be an interesting approach for in vivo imaging of GPCRs (Iezzi et al., 2018; Massa et al., 2016).

Nanobodies as therapeutics

Conventional antibodies are well established as therapeutics, while nanobodies are emerging as potential therapeutics. As described earlier, nanobodies are a good alternative to conventional mAbs for difficult targets including membrane bound receptors and enzymes. Nanobodies are also relatively low-cost and high yielding in terms of manufacturing processes. They can also be administered via multiple routes (injection, nebulisation, oral and ocular administration) which makes them therapeutically interesting (Frenzel et al., 2013; Sheridan, 2017). To date, one nanobody, Caplacizumab, has obtained positive phase III results and has been launched in Europe in October 2018 (Kaplon and Reichert, 2018). It targets the von Willebrand factor as a treatment against acquired Thrombotic Thrombocytopenic Purpura.

Multiple studies describe nanobodies that modulate GPCRs, which show the potential of GPCR-targeting nanobodies as therapeutics. Other GPCRs targets, besides CXCR4 and ACKR3, include CXCR2, ChemR23, mGlu2 receptor and US28 (Bradley et al., 2015; Heukers et al., 2018; Peyrassol et al., 2016; Scholler et al., 2017).

Single-domain antibodies and scaffolds targeting CXCR4

Two nanobody candidates against CXCR4, 238D2 and 238D4 (Table 2), are able to inhibit CXCL12 binding to CXCR4 with high affinity (Jahnichen et al., 2010). Despite binding two different epitopes of the extracellular part of CXCR4, the two nanobodies demonstrated full inhibition of CXCR4-mediated Gi signalling via CXCL12 in terms of both inositol phosphate accumulation and cAMP production inhibition. These nanobodies were also able to inhibit

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CXCL12-induced Jurkat cell migration. Linking the two nanobodies resulted in increased affinity and potency. The increased potency of such biparatopic nanobodies may be a result of simultaneous binding to two CXCR4 molecules in close proximity or within a dimer or higher order oligomer. The biparatopic nanobodies, in contrast to their monovalent counterparts, exhibited inverse-agonistic properties in inositol phosphate accumulation when tested with the CXCR4 constitutively active mutant N3.35A. Both monovalent and bivalent nanobodies could effectively inhibit CXCR4 mediated HIV entry in cells (Jahnichen et al., 2010). Just as the marketed CXCR4 antagonist AMD3100 (plerixafor) (Cashen, 2009), the bivalent nanobody potently triggered the mobilisation of both white blood cells and CD34+ stem cells in cynomolgus monkeys (Jahnichen et al., 2010).

Similarly, another nanobody called 10A10 (Table 2) was generated (de Wit et al., 2017), targeting wild type CXCR4 as well as WHIM variants (called CXCR4-R334X and CXCR4-S338X) with a truncated C-terminal tail. This nanobody was able to fully displace CXCL12 on all CXCR4 constructs, and similar to the nanobodies 238D2 and 238D4, showed improved affinity when generated as a bivalent construct (10A10-10A10). Both monovalent and bivalent formats of 10A10 were able to inhibit CXCL12-mediated CXCR4 cAMP production, ERK1/2 phosphorylation and inositol phosphate accumulation. The bivalent 10A10 could also inhibit CXCR4 calcium mobilisation in K652 myeloid leukemic cells. As for WHIM syndrome linked to human papillomavirus (HPV)-associated malignancies (Beaussant Cohen et al., 2012), 10A10-10A10 was tested in a HPV related assay and partially inhibited HPV-driven migration of human keratinocytes, which is mediated by the CXCL12-CXCR4 axis.

Using an alternative approach, we have created bivalent nanobodies against CXCR4 by fusing them with an Fc domain from human IgG1. Initially developed monovalent nanobodies VUN400-402 (Table 2), which bind distinct but overlapping epitopes, demonstrated divergent activity in inhibition of HIV entry and CXCR4-related functions (Van Hout et al., 2018).

VUN402 showed selective activity towards inhibiting a broad range of HIV strains with only poor blockage of CXCL12 binding, chemotaxis and CXCL12-induced cell morphology change. This shows the potential of nanobodies as selective HIV-blocking agents. In a follow-up study, these three nanobodies were formatted as bivalent nanobody-Fc (Nb-Fc) constructs (VUN400-Fc, VUN401-Fc and VUN402-Fc). They demonstrated significantly higher affinities to CXCR4, and also increased potencies towards inhibiting CXCL12 binding, signalling, cell morphology change and HIV entry. Additionally, Fc-mediated induction of ADCC and CDC was demonstrated with a human leukemia cell line CCRF-CEM (Bobkov et al., 2018b). Nb-Fc fusion constructs could potentially show reduced blood clearance, in comparison to nanobodies, with their increased size and FcRn-mediated recycling via Fc domain. This approach entails a new class of molecules targeting GPCRs with the combined favourable properties of nanobodies and the functional utility of conventional antibodies.

Immunoglobulin new antigen receptor (IgNAR) is another class of single-domain antibody found in the immune system of sharks (Fig.1). Their antigen-binding domains are represented by small variable fragments (VNAR), which have similar binding properties as nanobodies and were selected for specific diagnostic and therapeutic purposes against different targets, such as AMA-1, Ebolavirus nucleoprotein, TNFα and VEGF (Camacho-Villegas et al., 2013; Camacho-Villegas et al., 2018; Goodchild et al., 2011; Nuttall et al., 2004). The structural similarity between VNAR and I-set family of human immunoglobulin domains, for example neural cell adhesion molecule 1 (NCAM), has been reported previously (Streltsov et al., 2004). The usage of NCAMs as scaffolds by incorporating binding loops, mimicking complementarity determining regions (CDR), allows construction of fully human analogues of VNAR, designated as ‘i-bodies’ (Griffiths et al., 2016). Griffiths et al. also reported a selection of several CXCR4 antagonistic i-bodies which bind deep in the ligand binding pocket of the receptor via the elongated CDR3 loop and showed inhibition of cell migration and HIV

infection but not mobilisation of hematopoietic stem cells. Of interest, the i-body AD-114 (Table 2) demonstrated to have an anti-fibrotic effect and diminished the level of lung injury in an *in vivo* murine model of pulmonary fibrosis (Griffiths et al., 2018). This anti-CXCR4 i-body was proposed as a potential candidate for the treatment of idiopathic pulmonary fibrosis (IPF). Another scaffold engineering approach involves grafting a modified CXCR4-targeting peptide CVX15 into a bovine antibody with ultralong CDRH3 (Liu et al., 2014). The resulting antibody scaffolds, bAb-AC1 and bAb-AC4 (Table 2), with the peptide grafted into CDRH3 or CDRH2, respectively, showed specific binding to CXCR4 expressing cells with nanomolar affinities, inhibition of CXCL12-mediated signalling and cell migration. This approach illustrates an application of antibodies as scaffolds for peptides targeting GPCRs, broadening their therapeutic prospects.

Nanobodies and other fragments targeting ACKR3

Three nanobodies against ACKR3, NB1-3, exhibit CXCL12 displacement properties. Whilst NB2 and NB3 fully inhibit CXCL12 binding to ACKR3, NB1 only shows a partial inhibition despite having a good affinity (Maussang et al., 2013). Consistently, NB2 and NB3 could completely inhibit CXCL12 induced β-arrestin2 recruitment with respectively submicromolar and nanomolar potencies, whereas NB1 was unable to inhibit recruitment even at high concentrations. Comparable to the CXCR4 nanobodies, the ACKR3-targeting nanobodies were also formatted as bivalent NB2-NB2 and biparatopic NB1-NB3 constructs. For in vivo purposes, both constructs were genetically linked to a nanobody targeting albumin for increased half-life. As expected, these new constructs demonstrated increased affinity and potency toward ACKR3. In the head and neck cancer cell line 22A, which highly expresses ACKR3, the NB1-NB3 nanobody did not influence cell-cycle progression but inhibited the secretion of angiogenic factor CXCL1. In a xenograft model system using 22A cells, the biparatopic NB1-

NB3 effectively reduced tumor growth and decreased CD31 marker expression in tumours, while the bivalent NB2-NB2 did not. The use of ACKR3 targeting nanobodies demonstrated that ACKR3 plays a role in angiogenesis.

Fusion of single-chain variable fragments (scFv) recognising human ACKR3 with the Fc portion of IgG1 resulted in the generation of the antibody derivative X7Ab (Table 2) that inhibited CXCL12 signalling (Salazar et al., 2018). X7Ab was able to kill ACKR3-expressing glioblastoma (GBM) cells via Fc-mediated ADCC, CDC and antibody-dependent cellular phagocytosis (ADCP), and in combination with temozolomide (TMZ) significantly reduced tumour growth and improved overall survival in a mouse GBM model.

Structural analysis of antibodies/nanobodies binding to CXCR4

Antibodies are modular proteins that consist of four polypeptide chains – two light and two heavy chains. Each light and heavy chain are linked to each other through disulphide bonds, forming two identical Fabs. Each Fab consists of two variable domains VH and VL, and two constant domains CH1 and CL in the heavy and light chains, respectively. The two variable domains VH and VL represent the Fv fragment and form an antigen-binding site, also called a paratope. Two other domains CH2 and CH3 of the heavy chain form the Fc region.

A paratope results from the specific folding of six hypervariable loops of the variable domains, three in the light chain (L1, L2, L3) and three in the heavy chain (H1, H2, H3) (Putnam et al., 1979; Sela-Culang et al., 2013). The six loops mediate antigen recognition and have been named complementarity determining regions or CDRs, while the constant domains in the Fc region are responsible for inducing effector functions of an antibody. Despite the high structural similarity between antibodies, their binding capabilities are enormously diverse (Sela-Culang et al., 2013).

Nanobodies are derived from a single variable domain of heavy-chain only antibodies (VHH) found in llamas and camels, and therefore their paratope is composed only of three hypervariable loops, CDR1-3. Interestingly, the CDR3 of nanobodies is often elongated, favouring binding of conformational epitopes on a target molecule hidden from conventional antibodies (Mujic-Delic et al., 2014).

Antigen-antibody recognition involves a series of non-covalent interactions between an antibody paratope and a binding site on an antigen, or an epitope. The exact composition of the paratope is antibody-dependent and therefore represents a challenge for the study of the antibody-antigen interface. Identification of paratopes is often done through the identification of amino acid residues within CDRs, which are the most variable regions between antibodies. However, not all residues within the CDRs are involved in antigen binding, but only a small percentage according to previous analyses (Ofran et al., 2008; Padlan, 1994). The CDR residues not directly involved in antigen binding are key for forming a favourable conformation of the CDRs (Sela-Culang et al., 2013). The specific conformation of the different loops containing the paratope residues is crucial for recognising the antigen with high specificity and affinity, as proven by CDR-derived linear peptides which bind their antigens with considerably lower affinities (Polonelli et al., 2008; Saragovi et al., 1991).

CDR3 is considered to be the most important region for antigen binding, but CDR2 is longer on average and has been proven to form the same number of interactions with the antigen (Kunik and Ofran, 2013). Furthermore, CDR2 is proven to be the most solvent exposed of all loops (Hattori et al., 2008), which might relate to its longer length. Also in terms of energetic contribution of binding, CDR3 provides on average the highest contributions, but there are cases where other CDRs, including CDR2, contribute the most to the binding energy (Burkovitz et al., 2013; Kunik and Ofran, 2013; Sela-Culang et al., 2013). This fact emphasises the high variability on the antigen recognition of each antibody, and the need for a specific analysis to

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better understand the antibody-antigen interface and to attempt to pharmacologically enhance or disrupt it.

Mutation of residues in the binding pocket of small molecules and peptides, such as D97$^{2.63}$, Y116$^{3.32}$, D171$^{4.60}$, Q200$^{5.39}$, or E288$^{7.39}$ in CXCR4, does not affect the binding of antibodies and nanobodies (Figure 3). CXCR4 residues are annotated with the UniProt numbering, followed by the Ballesteros-Weinstein annotation, when applicable (in the Ballesteros-Weinstein annotation the first number before the dot indicates the transmembrane helix, and the value after the dot indicates the relative position of the residue in the helix with respect to the most conserved residue, randomly designated as 50). Indeed, several studies have probed the CXCR4 epitope using mutagenesis, and concluded that CXCR4 antibodies and nanobodies recognise different domains within the extracellular region of the receptor, including the N-terminus and the three extracellular loops (ECLs) (Figure 3 and 4). This explains that often small molecules are not able to displace antibodies or nanobodies. To illustrate, AMD3100 does not affect the binding of MEDI3185 to CXCR4 (Peng et al., 2016a).

As shown in Figure 4, most antibody and nanobody epitopes involve multiple residues in the CXCR4 ECL2, including e.g. E179, D181, and D187. This indicates that the most significant interactions between CXCR4 and antibodies and nanobodies might be of an electrostatic nature. However, several studies report non-significant effects from single-point mutations in ECL2, while the impact of large substitutions or multiple simultaneous mutations show a marked impairment on their binding (Peng et al., 2016a; Xu et al., 2007). These observations indicate that the epitope of these antibodies may be dispersed throughout ECL2 (Figure 4A). The same phenomena are observed in mutations of the N-terminal, where large deletions significantly affect the binding of CXCR4 antibodies 12G5 and 6H8 (Brelot et al., 2000).

Nanobodies are believed to be able to recognise more buried epitopes (e.g. binding pockets) due to their smaller size and molecular structure. The antibody-like scaffolds called i-bodies,

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AM3-114, AM3-523, and AM4-272 have been reported to bind to more buried amino acids within CXCR4 (Griffiths et al., 2016), including V112$^{3.28}$ and D262$^{6.58}$, yet rather close to the extracellular surface of the receptor. Other nanobodies, including 238D2, 238D4, VUN400, VUN401, and VUN402, show unique but overlapping epitopes mostly involving the N-terminus and ECL2 of CXCR4 (Jahnichen et al., 2010; Van Hout et al., 2018), similar to mAbs (Figure 4B).

Despite the overall common epitope, each antibody and nanobody has a specific pattern of binding, which often correlates with their unique mode of action. To illustrate this, antibodies and nanobodies binding to different epitopes are able to differentially inhibit CXCL12, block the entrance of different HIV strains, or inhibit CXCL12 induced signalling (Carnec et al., 2005; de Wit et al., 2017; Jahnichen et al., 2010; Peng et al., 2016a; Van Hout et al., 2018). However, the binding mode of these antibodies and nanobodies and their molecular mechanism of action is still poorly understood. Protein-protein interactions, such as the antigen-antibody interface, are challenging to predict and require the experimental identification of residues involved in the interaction from both proteins. As described in this section, several studies aimed to identify the epitope on the receptor side, while only one study has focused on the identification of the CXCR4-targeting antibody paratope (Peng et al., 2016a). Peng et al. selected the residues near the apex of each loop for mutagenesis, which are more likely to be solvent exposed and therefore antigen-accessible. CDR3 was found to be the most critical for binding, with smaller contributions from CDR1. With this knowledge they were able to predict the mode of interaction between CXCR4 and MEDI3185, enabling designing new specific therapeutics targeting CXCR4.

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Conclusions

CXCR4 and ACKR3 are two therapeutic targets with great potential in view of their involvement in tumorigenesis and autoimmune disorders. Their role in disease progression, expression pattern and the fact that both bind CXCL12 create an opportunity to consider them for dual targeting, which might be superior in treatment of cancer. Several anti-CXCR4 mAbs are in clinical development with many more antibodies, nanobodies and other fragments in preclinical stage. Also, some antibodies and nanobodies blocking ACKR3 function have been developed. Nevertheless, there are still no antibodies approved against CXCR4 or ACKR3, representing a clear niche for development of novel CXCR4 and ACKR3 targeting biologics. Nanobodies and nanobody-based molecules are an interesting alternative to mAbs because of their unique properties advancing GPCR targeting.

We described how antibodies and nanobodies are versatile and promising tools for in vitro GPCR studies, as well as for clinical applications. It is important to decipher the structural and molecular mechanisms by which they recognise and bind their antigens. Such understanding will be of great importance for a better prediction of epitopes but also for the engineering of new antibodies with desired binding properties.

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Authorship contributions

Wrote or contributed to the writing of the manuscript: Bobkov, Arimont, Zarca, De Groof, van der Woning, de Haard, Smit.

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Figure legends

Figure 1. Overview of antibody formats. A) schematic representation of the structure of a conventional antibody (Conv. Ab), Heavy Chain-only antibody (HCAb) and immunoglobulin new antigen receptor (IgNAR). The antibodies consist of constant domains (CH, CL or C) and variable domains (VH, VL, VHH or VNAR) which make up the fragment crystallizable (Fc) region and fragment antigen-binding (Fab) domain (for conventional antibodies). B) Overview of commonly used fragments derived from Conv. Abs, HCAbs or IgNARs. scFv: single chain variable fragment; Nb: nanobody; Biv. Nb: bivalent nanobody; Bispec. Nb: bispecific nanobody; Nb-Fc: nanobody-Fc fusion protein.

Figure 2. Three dimensional models of a conventional antibody (left), a nanobody-Fc fusion protein (middle), and a nanobody (right). These models were produced by homology modelling in the software modeller (v9.15) (Webb and Sali, 2017) and based on the crystal structure with PDB ID: 1HZH (Saphire et al., 2001) as a template. The sequence of the Nanobody corresponds to the Nb VUN400 (Van Hout et al., 2018).

Figure 3. Differences between the reactivity[a], Kd[b] or IC_{50}[c] values of wild-type and mutant (WT value – mutant value / WT value) reported for 17 CXCR4 antibodies, nanobodies, and antibody-like scaffolds extracted from literature (Brelot et al., 1999; Brelot et al., 2000; Carnec et al., 2005; de Wit et al., 2017; Gerlach et al., 2001; Griffiths et al., 2016; Jahnichen et al., 2010; Peng et al., 2016a; Rosenkilde et al., 2007; Rosenkilde et al., 2004; Thiele et al., 2014; Van Hout et al., 2018). The effects are coloured for an easier interpretation as follows: blue for the less significant effect (reactivity difference 0-0.5, Kd/IC_{50} difference 0-3 fold units), yellow for an intermediate effect (reactivity difference 0.5-0.7, Kd/IC_{50} difference 3-8 fold units), and red for the most significant effects (reactivity difference 0.7-1, Kd/IC_{50} difference >8 fold units).

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Figure 4. CXCR4 snakeplot representations with the most relevant residues involved on the binding of each antibody and antibody-like scaffold (A), or nanobody (B), as indicated in Figure 3. Snakeplots are based on GPCRdb representations (Pandy-Szekeres et al., 2018).

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Table 1. Comparison of therapeutic antibodies and small-molecule compounds targeting GPCRs

Antibodies	Small Molecules
General properties	
Mostly antagonists	Antagonists, agonists, allosteric modulators
Preference for extracellular epitopes	Binding multiple pockets, including intracellular
Administration mostly intravenous or subcutaneous	Oral administration possible
Immunogenicity minimized by humanization	Low risk for immunogenicity
Effector functions	No effector functions
Longer serum half-life, reduced dosing frequency	Shorter serum half-life, variable dosing frequency
Restricted blood-brain barrier penetration	Good blood-brain barrier penetration
Development	
High target expression during immunization and selection needed	Production not dependent on target expression
Higher costs of development and manufacturing	Lower costs of development and manufacturing
GPCR targeting	
Possibility of targeting low-druggability GPCRs	Poor tractability, failed to target a variety of GPCRs (e.g. class B2, F)
Enhanced selectivity and specificity	Lower selectivity, often target family-conserved binding sites
Less off-target effects	Off-target effects
Applications	
Easy to label and functionalise e.g. bispecifics, fragments, conjugates	Challenging to label
Clinical development	
Lower overall rate of attrition and higher transition rates at all stages of development	Lower approval success rates

Table 2. Overview of monoclonal antibodies, nanobodies and antibody-based fragments and scaffolds directed at and modulating CXCR4 and ACKR3 function

Antibody (Company)	Target	Format	Mechanism of action	Indication	Phase	References
Ulocuplumab (Bristol-Myers Squibb)	CXCR4	hIgG4	CXCR4 inhibition, apoptosis induction	AML WM	Phase 1/2 ongoing (NCT02305563) Phase 1/2 ongoing (NCT03225716)	(Kashyap et al., 2016; Kuhne et al., 2013)
LY2624587 (Eli Lilly and Company)	CXCR4	hzIgG4	CXCR4 inhibition, apoptosis induction	Metastatic cancer	Phase 1 completed (NCT01139788)	(Peng et al., 2017; Peng et al., 2016b)
PF-06747143 (Pfizer)	CXCR4	hzIgG1	CXCR4 inhibition, apoptosis induction, ADCC and CDC	AML	Phase 1 terminated (NCT02954653)	(Kashyap et al., 2017; Liu et al., 2017; Zhang et al., 2017)
hz515H7/ F50067	CXCR4	hzIgG1	CXCR4 inhibition, ADCC and CDC	MM	Phase 1	(Broussas et al., 2016; Fouquet et al., 2018)

(Pierre Fabre)						
MEDI3185 (Medimmune)	CXCR4	hIgG1^{mut}	CXCR4 inhibition, apoptosis induction	Hematologic malignancies	Preclinical	(Kamal et al., 2013; Peng et al., 2016a; Schwickart et al., 2016)
IgGX-auristatin	CXCR4	IgG, ADC	Auristatin-mediated cytotoxicity	Metastatic cancer	Preclinical	(Kularatne et al., 2014)
238D2, 238D4 (Ablynx)	CXCR4	Nb	CXCR4 inhibition, anti-HIV activity, HSCs mobilization	-	Preclinical	(Jahnichen et al., 2010)
10A10	CXCR4	Nb	CXCR4 inhibition	WHIM syndrome	Preclinical	(de Wit et al., 2017)
VUN400-402	CXCR4	Nb	CXCR4 inhibition, anti-HIV activity	-	Preclinical	(Van Hout et al., 2018)
VUN400-402	CXCR4	Nb-Fc	CXCR4 inhibition, anti-HIV activity, ADCC and CDC	-	Preclinical	(Bobkov et al., 2018b)

AD-114 (AdAlta)	CXCR4	i-body	CXCR4 inhibition, anti-HIV activity	IPF	Preclinical	(Griffiths et al., 2016; Griffiths et al., 2018)
bAb-AC1, bAb-AC4	CXCR4	antibody scaffold	CXCR4 inhibition	-	Preclinical	(Liu et al., 2014)
NB1-3 (Ablynx)	ACKR3	Nb	ACKR3 inhibition	Head and neck cancer	Preclinical	(Maussang et al., 2013)
X7Ab	ACKR3	scFv-Fc	ACKR3 inhibition, ADCC, CDC and ADCP	GBM	Preclinical	(Salazar et al., 2018)

Abbreviations: hIgG, human IgG; hzIgG, humanized IgG; $IgG1^{mut}$, triple mutant lacking ADCC and CDC; ADC, antibody-drug conjugate; mIgG, murine IgG; Nb, nanobody; Nb-Fc, nanobody fused with Fc domain from IgG1; scFv, single chain variable fragment fused with Fc domain from IgG1; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; HSCs, hematopoietic stem cells; AML, acute myeloid leukaemia; WM, Waldenström's macroglobulinemia; ESCC, esophageal squamous cell carcinoma; WHIM, warts, hypogammaglobulinemia, infections, and myelokathexis; IPF, idiopathic pulmonary fibrosis; GBM, glioblastoma

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Figure 1.

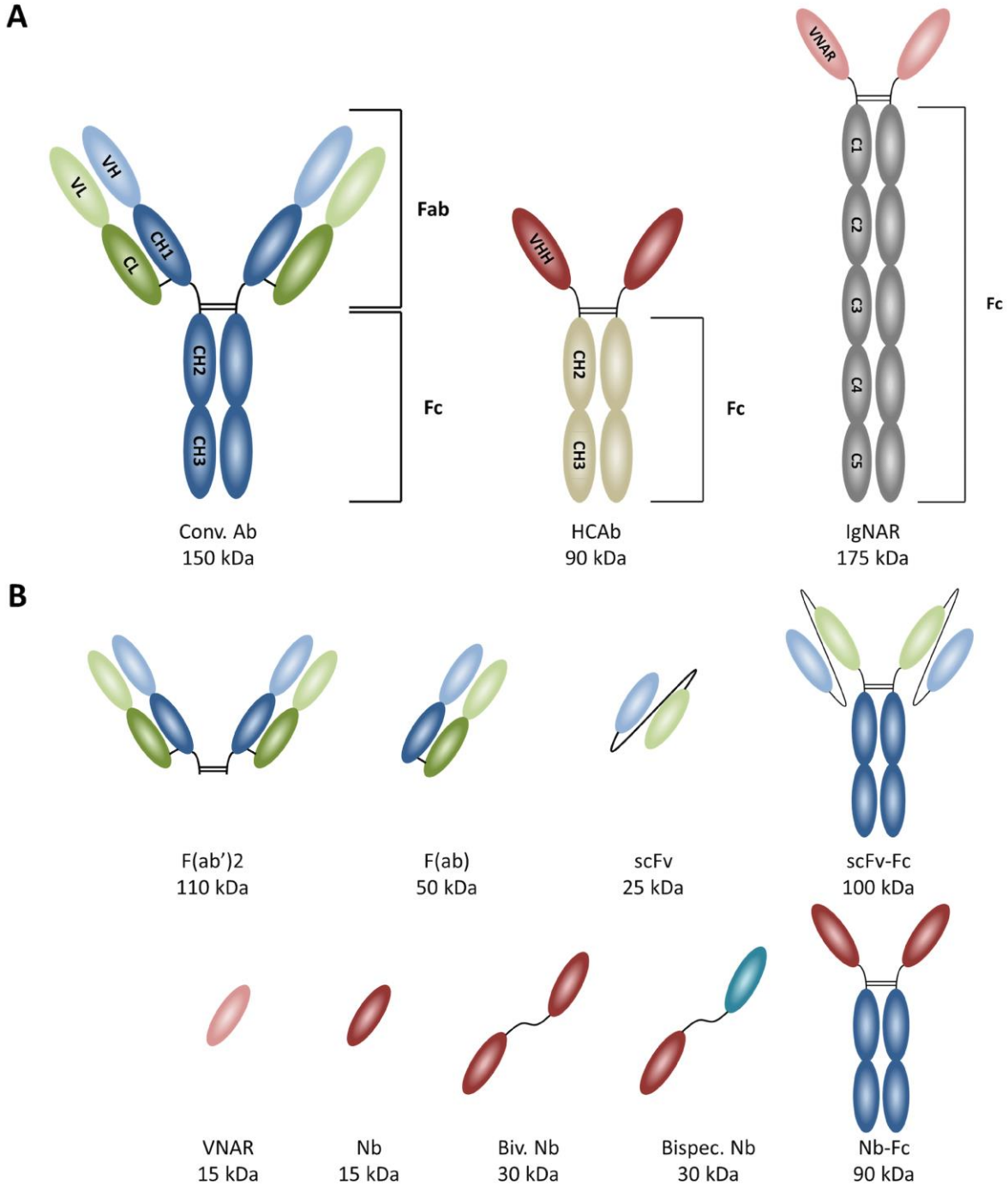

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Figure 2.

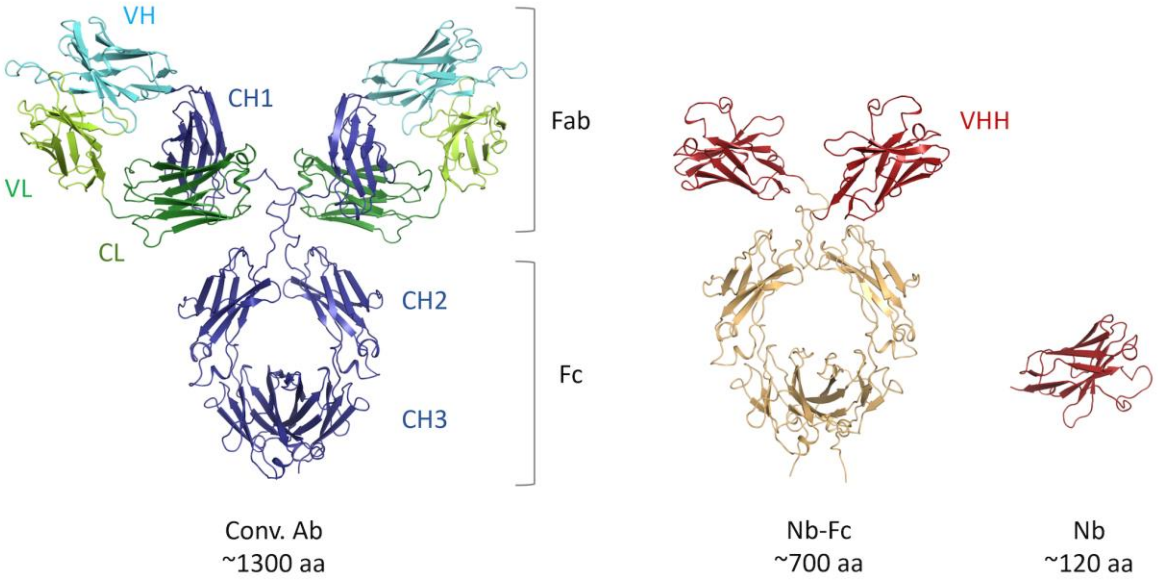

Figure 3.

Ligand	Type	WT	N-ter	N-ter	N-ter	N-ter	N-ter	N-ter	N-ter	N-ter	N-ter	1.26	2.63	3.26	3.28	3.29	3.32	4.59	4.60	4.63	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	ECL2	5.32	5.34	5.35	5.39	5.42	5.46	6.51	6.55	6.58	6.59	6.62	ECL3	7.28	7.32	7.35	7.39	7.45	
			Deletion																																																				
CXCR4 mutants			10-20	2	7	10	11	20	21	26	28	30	32	97	110	112	113	116	170	171	174	175	176	178	179	180	181	182	183	184	187	188	189	190	191	192	193	195	196	200	204	207	255	259	262	263	266	274	277	281	284	288	294	
				E	Y	D	N	D	Y	E	C	R	E	D	K	V	H	Y	P	D	F	A	N	S	E	A	D	D	D	R	Y	D	R	F	Y	P	N	D	W	D	Q	H	G	Y	I	I	D	S	L	C	G	E	H	
				A	A	A	Q	A	A	A	A	S	K	N	A	A	A	A	L	N	A	F	D	C	A	D	A	G	A	A	S	A	V	A	L	V	S	A	C	K	A	A	W	R	W	A	A	W	N	G	A	H	E	A	A	
12G5	mAb	1[a]									0.8														0.9		0.7	0.8						0.8																							Carnec et al., 2005
		1.6[b]														0			0.4	0.7	0																			0.4		0.7			0.4	0.1	0.8	0.3	0.3		0.8				0.5	0	0.4			Rosenkilde et al., 2004
		1.9[b]														0.1			0.5	0.7	0.2		0.7					0.4		0.4								0.5		0.4	0.7	0.5	0.6	0.3	0.8	0.5	0.4	0.8			0.6	0.2		0.5			Rosenkilde et al., 2007
		4.7[c]														0.4	0.9		0.5												2.4																						0.5		0.6			Thiele et al., 2014
6H8	mAb	1[a]	0.7	0	0.1	0.1	0.1	0.2	0.5	0.2				0.5	0.2										0.1		0.1	0	0.1			0.3	0.4																			0.4			Brelot et al., 2000
701	mAb	1[a]																							0.9		0.7																													Carnec et al., 2005
708	mAb	1[a]																							0.9		0.9			0.6				0.7																					Carnec et al., 2005
716	mAb	1[a]																							0.9		0.8			0.8																									Carnec et al., 2005
717	mAb	1[a]																							0.9		0.7																													Carnec et al., 2005
718	mAb	1[a]																							0.9		0.7																													Carnec et al., 2005
MEDI3185	mAb	0.3[b]																							0.2	0	0		0																											Peng et al., 2016
10A10-10A10	Nb	1[a]																														0.9		0.2							0.5													de Wit et al., 2017
238D2	Nb	1[a]																							0.6							0.1		1		0.8	0.9		0.9		0.8								0.8					Jähnichen et al., 2010
238D4	Nb	1[a]																							0.7							1		1		0.2	0.4		0.4		0.1								0.5					Jähnichen et al., 2010
VUN400	Nb	1[a]										0.6						0.5														0.9		1		0				0.2	0.1													van Hout et al., 2018
VUN401	Nb	1[a]										0.8						0.6														0.3		0.4		0.2				0	0.1													van Hout et al., 2018
VUN402	Nb	1[a]										0.2						0.8														0.4		0.8		0.9				1	1													van Hout et al., 2018
AM3-114	i-body	1[a]											0.4			0.4														0			0.7				0.6	0.7							0.7		0.8					0.5		Griffiths et al., 2016
AM3-523	i-body	1[a]											0.9			0.9															0.9		0.5				0.2	0.4							0.9		0.4					0.1		Griffiths et al., 2016
AM4-272	i-body	1[a]											0.6			0.1															0.4		0.8				0.2	0.2							0.7		0.4					0.2		Griffiths et al., 2016

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Figure 4.