Nanobodies: New avenues for imaging, stabilizing and modulating GPCRs

Timo W.M. De Groof\textsuperscript{a}, Vladimir Bobkov\textsuperscript{a,b}, Raimond Heukers\textsuperscript{a,c}, Martine J. Smit\textsuperscript{a,*}

\textsuperscript{a} Amsterdam Institute for Molecules, Medicines and Systems (AIMMS), Division of Medicinal Chemistry, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1108, 1081 HZ, Amsterdam, the Netherlands
\textsuperscript{b} Argenx BVBA, Industriepark Zwijnaarde 7, 9052, Zwijnaarde, Belgium
\textsuperscript{c} QVQ Holding B.V., Yalelaan 1, 3484 CL, Utrecht, the Netherlands

\section*{ARTICLE INFO}

Keywords:
G protein-coupled receptors  
Nanobodies  
Antibodies  
Imaging  
Crystallization  
Modulation

\section*{ABSTRACT}

The family of G protein-coupled receptors (GPCRs) is the largest class of membrane proteins and an important drug target due to their role in many (patho)physiological processes. Besides small molecules, GPCRs can be targeted by biologicals including antibodies and antibody fragments. This review describes the use of antibodies and in particular antibody fragments from camelid-derived heavy chain-only antibodies (nanobodies/VHHs/sdAbs) for detecting, stabilizing, modulating and therapeutically targeting GPCRs. Altogether, it becomes increasingly clear that the small size, structure and protruding antigen-binding loops of nanobodies are favorable features for the development of selective and potent GPCRs-binding molecules. This makes them attractive tools to modulate GPCR activity but also as targeting modalities for GPCR-directed therapeutics. In addition, these antibody-fragments are important tools in the stabilization of particular conformations of these receptors. Lastly, nanobodies, in contrast to conventional antibodies, can also easily be expressed intracellularly which render nanobodies important tools for studying GPCR function. Hence, GPCR-targeting nanobodies are ideal modalities to image, stabilize and modulate GPCR function.

\section*{1. Introduction}

G protein-coupled receptors (GPCRs) are a superfamily of receptors involved in the regulation of a wide variety of physiological processes such as growth, homeostasis and behavioral regulation (Rosenbaum et al., 2009; Koblika, 2013). Because of this regulatory role, GPCRs have been involved in multiple diseases, including metabolic and cardiovascular diseases, neurological disorders and cancer (Salazar et al., 2007; Capote et al., 2015; Du and Xie, 2012; Huang et al., 2017; Bar-Shavit et al., 2016). GPCRs have been a major drug target with approximately 30–40% of the FDA-approved drugs mediating their effects via these receptors (Santos et al., 2017; Sriram and Insel, 2018; Hauser et al., 2017). Small molecules, which make up around 80% of the FDA approved GPCR-targeting drugs, are most commonly used to target and modulate these receptors (Sriram and Insel, 2018; Hauser et al., 2017). However, obtaining small molecules with high affinity, potency and selectivity is still a major challenge in the drug discovery process (Sriram and Insel, 2018; Kola and Landis, 2004). Moreover, GPCRs are known to be highly dynamic and exist in multiple conformations which also poses a big challenge to find drugs targeting a specific conformation (Latorraca et al., 2017; Marti-Solano et al., 2016). To resolve some of these challenges, research has also been focusing on GPCR-targeting antibodies and antibody fragments in the last decade. In this review, we will discuss the different applications of antibodies and antibody-fragments ranging from the use as diagnostics and imaging probes, conformational stabilizers, (therapeutic) competitors and modulators as well the use of intrabodies (Fig. 1).

In general, antibodies (Abs) consist of 2 identical heavy and 2 identical light chains, which are organized as one fragment crystallizable (Fc) region (consisting of CH2 and CH3 domains) and two variable Ag-binding fragments (Fab, consisting of CH1-VH and CL1-VL, κ or λ domains) (Fig. 2)( Schroeder and Cavacini, 2010). As a consequence of a highly intricate system of gene recombination, mutations and in vivo selection, the human immune system generates conventional antibodies with particular high affinity for their target. This is mainly the consequence of the complementarity-determining regions (CDRs) that lie within the variable domains (VH, VL) of the Fab fragments, which are responsible for the interaction with the epitope (Schroeder and Cavacini, 2010; Rajewsky, 1996; Jolly et al., 1996). The Fc-region, which makes up the tail region of the Ab, can interact with Fc receptors or C1q molecules which leads to the activation of the immune system by different pathways such as antibody-dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Although different antibody formats exist in the human body, most therapeutic
formats involve antibodies from the IgG isotype (Schroeder and Cavacini, 2010). Next to their high affinity and specificity, monoclonal Abs (mAbs), allow less frequent dosing and show less variability in pharmacokinetics between patients as compared to small molecules (Hutchings et al., 2017). Nevertheless, the use of Abs is also accompanied by some disadvantages such as a high production cost, limited tissue penetration and the inability to therapeutically target intracellular targets or epitopes (Chames et al., 2009).

Interestingly, while there are many FDA/EMEA approved therapeutic antibodies, only a few of these target GPCRs. At present, Mogamulizumab (targeting CCR4) and Erenumab (targeting calcitonin gene-related receptor (CGRPR)) are the only GPCR-targeting mAbs on the market. CCR4 is a chemokine receptor and is expressed on multiple immune cells and plays an important role in the immune response (Yoshie and Matsushima, 2015). However, CCR4 is overexpressed on multiple lymphomas and is believed to be involved in the suppression of the antitumor immune response (Ishida and Ueda, 2006). Mogamulizumab is currently approved in Japan and the United States for clinical use for the treatment of multiple lymphomas, such as adult T cell lymphoma and cutaneous T cell lymphoma. Upon binding to CCR4+ lymphoma cells, Mogamulizumab induces cell lysis (ADCC) of CCR4+ lymphoma cells by activation of the natural killer cells through binding of its defucosylated Fc-tail to Fcγ receptors (Niwa et al., 2004; Ishii et al., 2010; Duvic et al., 2016; Kaplon and Reichert, 2018). Erenumab is an antagonistic Ab which targets the CGRPR and is recently approved in the USA and EU for the prevention of migraine. Treatment with Erenumab resulted in a reduction of migraine frequency, migraine effects and use of acute migraine-specific medication (Kaplon and Reichert, 2018; Shi et al., 2016; Goadsby et al., 2017). Next to Mogamulizumab and Erenumab, a few other mAbs targeting GPCRs (e.g. CCR5, CXCR4) are currently in phase III clinical trials, while multiple other GPCR-targeting mAbs are in phase I-II clinical trials or still in preclinical development (Hutchings et al., 2017).

Besides the development of humanized antibodies that target GPCRs for therapeutic purposes, functional GPCR-targeting autoantibodies have also been found and react with the individual’s own GPCRs. Functional autoantibodies have been found in the sera of healthy individuals in low levels and might play an important physiological role. However, upregulation of GPCR-autoantibody levels has been found in patients with several disorders including heart failure, asthma, glaucoma, autoimmune encephalitis and Graves’ disease (Segovia et al., 2012; Zuo et al., 2011; Venter et al., 1980; Turki and Liggett, 1995; Junemann et al., 2018; Minich et al., 2004; Dalmau and Graus, 2018; Chefdeville et al., 2016). Examples of GPCRs targeted by autoantibodies in autoimmune disorders include β1 adrenergic receptor, β2 adrenergic receptor (β2AR), muscarinic acetylcholine receptor M3, γ-aminobutyric acid (GABA) type B receptor, metabotropic glutamate receptors and thyrotropin receptor (Dalmau and Graus, 2018; Chefdeville et al., 2016; Cabral-Marcues and Riemekasten, 2017). Further research on the mechanism of GPCR-targeting autoantibodies in these different autoimmune diseases could lead to new insights and potential therapeutic targets. Functional autoantibodies targeting GPCRs have recently been extensively addressed by Cabral-Marcues et al. and will not be part of this review (Cabral-Marcues and Riemekasten, 2017).

Besides conventional GPCR-targeting Abs, research is currently also focusing on receptor-targeting Ab fragments (Fig. 2). One of the main advantages of using fragments is their small size, which makes it possible to target cryptic epitopes (De Genst et al., 2006; Muyldeermans, 2013). Some commonly used, mAb-derived fragments, include Fab fragments or single-chain variable fragments (scFvs) which are respectively 3 and 6 times smaller than conventional Abs. However, these Ab fragments are more prone to aggregate and generally show reduced affinity for their target, compared to their full-length counterparts (Nelson, 2010). Single domain Ab fragments (sdAbs) are a special class of Ab fragments which are derived from heavy chain only-Ab which are unique to camels or cartilaginous fishes. These sdAbs are called VHIs (variable domain of heavy chains of heavy chain-only antibodies) or nanobodies® (Nbs, for camels) and VNAR fragments (for cartilaginous fishes) (Muyldeermans, 2013). Since these Ab fragments are derived from naturally-occurring antibodies that lack light chains, their CDR regions were optimized during in vivo selection and maturation in these animals, resulting in a relatively high stability, solubility and affinity for their target. Due to a relatively long CDR3, as compared to those found on conventional antibodies, sdAbs have a more convex...
Fig. 2. Overview of different antibody formats. Representation of both (A) conventional antibodies, (B) camelid-derived single domain antibodies, (C) cartilaginous fishes/shark-derived single domain antibodies and their different derived antibody-fragments.

Fig. 3. Binding paratope and GPCR binding of a Fab fragment and nanobody. A) Fab fragments have relatively larger and more concave paratopes compared to the smaller, more convex paratopes of nanobodies. The Fab fragment and nanobody have been generated using PDB ID 2XWT, PDB ID 3P0G and PyMOL software. B) Schematic representation of a VH/VL domain of a Fab fragment and VHH domain/nanobody (Nb). Typically nanobodies have relatively long CDR3 region compared to Fab fragments. C) The shape of the binding paratope of the Fab fragment favors binding of linear epitopes (e.g. the N-terminus of a GPCR) while nanobodies are able to bind cryptic epitopes such as GPCR ligand binding pockets.
2. Nanobodies for in vivo imaging

The recent development of various GPCR-targeting Ab-(fragments) provide new opportunities to visualize endogenous GPCR expression, which has been challenging due to low expression levels. While receptor expression and ligand-receptor ratio are often studied using overexpression of tagged receptors, endogenous receptors can now be detected using labeled Abs or Ab-fragments. This regards detection of GPCRs in for example immunohistochemistry or in vitro/vivo diagnostics. Ab(fragments) can be conjugated to multiple type of tracers such as radionuclides, fluorophores, quantum dots, iron oxide particles and microbubbles (i.e. phospholipid shell containing low solubility complex gas) to allow visualization via techniques like postriton emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance (MR) imaging, optical imaging (fluorescence and bioluminescence), and photoacoustic (PA) imaging (England et al., 2016). To our knowledge, only two GPCR-targeting Abs have been used for in vivo imaging. These involve mAbs recognizing either CXCR4 or ACKR3/CXCR7. In both studies, the Abs were labeled with the radionucleide 90Zr and could visualize GPCR expression in xenograft tumors by means of PET imaging. Additionally, the ACKR3-mAb was also labeled with 125I to allow visualization using SPECT (Behnam Azad et al., 2016; Azad et al., 2016).

Nanobodies in particular are very interesting tools for in vivo imaging due to their relative homogenous distribution through tissues, high tumor penetration, rapid tumor accumulation and fast clearance from the blood circulation (De Groef et al., 2010; Cortez-Resamotaz et al., 2002; Oliveira et al., 2012). Nanobodies, displaying high affinity for their target, allow for a faster and efficient uptake of the nanobody-based probes into tumors compared to conventional Abs. This results, together with a fast clearance in the blood, in an optimal tumor-to-background contrast (Oliveira et al., 2013; Bannas et al., 2015). To date, no nanobodies have been used for in vivo imaging of GPCRs. However, they have been successfully used to visualize for example receptor tyrosine kinases. Examples include the tumor-related epidermal growth factor receptor (EGFR) and its family member HER2 (Vosjan et al., 2011; van Driel et al., 2014; Xavier et al., 2013; D’Huyvetter et al., 2012; Massa et al., 2014; Pruzynski et al., 2013; Pruzynski et al., 2014; Debbie et al., 2017; Xavier et al., 2016; Vaidyanathan et al., 2016; Zhou et al., 2017; Zhou et al., 2018). In different studies, nanobodies targeting these receptors were conjugated to radionuclides or fluorophores (Vosjan et al., 2011; van Driel et al., 2014; Xavier et al., 2013; D’Huyvetter et al., 2012; Massa et al., 2014; Pruzynski et al., 2013; Pruzynski et al., 2014; Debbie et al., 2017; Xavier et al., 2016; Vaidyanathan et al., 2016; Zhou et al., 2017; Zhou et al., 2018). Currently, the 68Ga-labeled HER2-targeting nanobody (2Rs15d) is in clinical trials for imaging of HER2 in breast cancer patients (Keyaerts et al., 2016). These studies demonstrate the potential of nanobody-based in vivo imaging for the GPCR-family. For example, the overexpression of the chemokine receptors CXCR4 and ACKR3/CXCR7 in some tumors, which are associated with tumorigenesis, metastasis and poor prognosis, would form a good target for such an approach (Behnam Azad et al., 2016; Philipp-Abbrederis et al., 2015; Sun et al., 2010). In addition, overexpression of both receptors is found in a subset of therapeutically resistant cancer (stem) cells (Roccaro et al., 2014; Wurth et al., 2014; Wu et al., 2015; Scala, 2015). Various studies, including ours, already described the development of nanobodies targeting these receptors. In addition to in vivo imaging, radio- or fluorescent labeled nanobodies (and other Ab formats) can be used as therapeutics when labeled with β-emitting radioisotopes or photosensitizers. For example, the potential of radiolabeled (125I and 131I) HER2-targeting nanobodies and photosensitizer-coupled EGFR-targeting nanobodies as therapeutics has recently been shown (D’Huyvetter et al., 2014; D’Huyvetter et al., 2017; van Driel et al., 2016). To which extend this is a feasible approach for GPCRs remains to be investigated. Taken together, with the rise of an increasing number of GPCR-targeting nanobodies, in vivo imaging and potentially targeted therapy might be promising next steps for these new tools.

3. Nanobodies as conformational stabilizers

GPCRs can activate a wide variety of signal transduction routes and are dynamic molecules that can adopt multiple conformations upon ligand binding. Interestingly, agonists, antagonists or inverse agonists induce different conformational changes (Manglik and Kobila, 2014). In addition, biased ligands can selectively activate one pathway over another (Rankovic et al., 2016). The binding of these ligands and the way this binding is translated into activation of intracellular signaling pathways is complex and not yet fully understood. Understanding the molecular mechanisms and consequences of specific GPCR conformations is important to gain a better insight in the signaling mechanisms of GPCRs, which could aid structure-based drug design (Congreve and Marshall, 2010). To obtain more information on different conformations, crystal structures have proven to be important. However, GPCR-conformations are dynamic and conformational heterogeneity is found between GPCRs at the same time in a cell. This heterogeneity is one of the major obstacles in obtaining high resolution crystal structures of GPCRs, a problem commonly solved by introducing mutations, binding of high affinity antagonists or crosslinking ligands to the receptor (Kobila, 2013; Warne et al., 2008; Dore et al., 2011, 2014; Hollenstein et al., 2013; Tan et al., 2013; Cherezov et al., 2007; Kuferova et al., 2014). Binding of small molecule antagonists, most often with low affinity and rapid association and dissociation rates, increases the conformational heterogeneity. Most GPCR crystal structures to date are therefore from inactive conformations (Manglik and Kobila, 2014; Ghanouni et al., 2001). Another way to reduce the conformational heterogeneity and obtain crystal structures of active GPCRs, is by applying antibody-fragments and especially nanobodies during co-crystallization.

Due to their size and three dimensional structure, nanobodies are known to bind conformational epitopes more easily than conventional Abs (De Genet et al., 2006; Lauwerys et al., 1998). Multiple studies have shown the successful use of nanobodies in stabilizing different GPCR conformations which enabled the formation of GPCR crystals and the elucidation of multiple new GPCR structures (Rasmussen et al., 2011; Ring et al., 2013; Che et al., 2018; Huang et al., 2015; Burg et al., 2015; Kruse et al., 2013). One of the first examples of a stabilized structure involved the β2AR. In 2007, the first crystal structure of the β2AR bound to an inverse agonist carazolol was published (Rasmussen et al., 2007). In an attempt to obtain the inactive conformation of β2AR, the receptor was stabilized with a Fab fragment binding to the intracellular loop 3. However, crystallization of the receptor-Fab complex in combination with agonists did not prove to be successful. In order to stabilize the active agonist-bound β2AR conformation, nanobodies were selected and screened against an agonist-bound receptor. Finally, one lead hit (Nbx80) specifically recognized the agonist-bound receptor and displayed G-protein like characteristics (Rasmussen et al., 2011). A co-crystal structure of the β2AR in complex with the agonist BI-167107 and Nb80 showed that Nb80 bound intracellularly to β2AR with the CDR3 region binding into the core of the receptor while the CDR1 region provided additional stabilizing interactions (Rasmussen et al., 2011).

Although the use of Nb80 proved to be successful in obtaining the crystal structure of β2AR in complex with a high-affinity ligand, crystallizing the β2AR in complex with low-affinity ligands was more difficult. This required affinity maturation of Nb80 to obtain the new
N6b89, which displayed a 10-fold improved affinity (Ring et al., 2013). This increase in affinity decreased receptor-heterogeneity in the sample and this facilitated the crystallization of the receptor in complex with the low-affinity ligand adrenaline. By obtaining crystals of the different agonists binding to the β2AR-N6b89 complex, it was observed that different agonists, by means of different interactions, stabilized same conformational rearrangements (Ring et al., 2013).

Nanobodies, functioning as G-protein mimetics, stabilized the agonist-bound active conformation of both the M2 Muscarinic acetylcholine receptor (M2R), μ-opioid receptor (μ-OR), κ-opioid receptor and the viral chemokine receptor US28 and helped solving their (crystal) structure (Che et al., 2018; Huang et al., 2015; Burg et al., 2015; Kruse et al., 2013). The combination of different nanobody-stabilized GPCR conformations even led to a new model compromising of multiple conformational states (Staus et al., 2016). This was demonstrated by using both the previously described Nb80 and Nb60, which stabilized the agonist bound and inverse-agonist bound conformation of the β2AR respectively (Staus et al., 2016).

Besides stabilizing receptor conformations merely for structural analysis, an interesting additional feature of GPCR-stabilizing nanobodies is their use as tools for finding new conformational-specific compounds. By linking the stabilizing nanobodies to the C-tail of the β2AR, M2R or μ-OR, these GPCRs could be locked into an active ligand-bound state (Pardon et al., 2018). Fragment-based screens were performed on the β2AR-Nb80 fusion protein and multiple hits were identified that preferentially bound the active conformation of the β2AR. Iterative growing of these fragments led to new compounds with higher affinity and selectivity for the active conformation indicating that these stabilizing nanobodies can be used to specifically find agonists but can also help in distinguishing neutral antagonists from inverse agonists (Chevillard et al., 2018).

4. Nanobodies modulating GPCR function

4.1. Antibodies targeting extracellular epitopes

Multiple therapeutic GPCR-targeting Abs have been generated and were tested in (pre)clinical studies (Hutchings et al., 2017). In addition, several studies have reported the generation of conventional antibodies targeting other GPCRs such as the sphingosine 1-phosphate receptor 3, metabotropic glutamate receptor 7 (mGLUR7), glucagon receptor, CC chemokine receptor 4 formyl-peptide receptor 1 and β1 adrenergic receptor (Harris et al., 2012; Ullmer et al., 2013; de Wit et al., 2017; Bradley et al., 2015; Maussang et al., 2013; Heukers et al., 2018; Peyrassol et al., 2016; Scholler et al., 2017). The use of nanobodies is a very interesting approach to modulate GPCRs, especially since you can use them in different formats ranging from mono- to multivalent formats (Fig. 2). Some of the more prevalent formats include bivalent (linking two nanobodies binding to the same epitope), bispecific (linking two nanobodies binding to different targets) and biparatopic nanobodies (linking two nanobodies targeting different epitopes on the same target) and trivalent nanobodies (linking three nanobodies). Different nanobody formats can lead to an increase in apparent affinity as well differences in potency and pharmacological effects. Studies have reported nanobodies acting as antagonists, inverse agonists or allosteric modulators and will be described further in this paragraph.

As example of antagonistic nanobodies are the nanobodies targeting the extracellular part of CXCR4 that we generated in 2010 (Jahnichen et al., 2010). Two nanobody clones, 238D2 and 238D4, bound different but partially overlapping epitopes. Nevertheless, the nanobodies were able to fully inhibit CXCL12 binding and CXCL12-induced signaling and migration as well as HIV entry. Biparatopic nanobodies, constructed via a fusion with a short 15-20 Gs linker, demonstrated increased apparent affinities and potencies. Inverse agonistic properties were observed for the biparatopic nanobodies but not their monovalent counterparts. Moreover, the biparatopic nanobodies induced stem cell mobilization in vivo similarly to AMD3100 (Jahnichen et al., 2010). In the same way, another nanobody 10A10 targeting CXCR4 and CXCR4 WHIM mutants, displaying gain of function, was generated. 10A10 was able to fully inhibit CXCL12 binding to the WT and mutated receptors. As a bivalent construct 10A10 showed increased binding affinity and potency in reducing CXCL12-mediated G protein activation (de Wit et al., 2017).

Another example of bivalent/biparatopic nanobodies functionally inhibiting a chemokine receptor (CXCR2) was reported by Bradley et al. (2015). Two different classes of nanobodies were identified which bound distinct non-overlapping epitopes. Generated nanobodies of each class (127D1 and 163E3) were able to inhibit CXCL1 and CXCL8-mediated [35S]-GTP;S binding but showed differences in the percentage of inhibition as well their mode of action. Additionally, the biparatopic nanobody 127D1-35 GS-163E3 constructed with a glycerine-serine linker possessed superior potency and efficacy comparing to their monovalent and bivalent counterparts. Since the monovalent nanobodies bound non-overlapping epitopes and did not compete with each other, the biparatopic nanobody could bind only one CXCR2 molecule. However, binding studies with CXCR2-mutants revealed that the biparatopic nanobody could also bind across two receptors, proposing that the nanobody could inhibit the function of monomeric and/or homodimeric forms of CXCR2 (Bradley et al., 2015).

In another study, several nanobodies binding different epitopes on the extracellular part of ACKR3 (CXCR7) were generated by our group. Unlike small-molecule compounds directed to ACKR3, which are agonists and thereby inhibiting scavenging of CXCL11/12, the monovalent nanobodies (Nbs 2-3) exhibited antagonistic properties by displacing CXCL12 and inhibiting CXCL12-dependent β-arrestin2 recruitment, whereas Nb1 only displaced CXCL12 binding (Maussang et al., 2013; Wijtmans et al., 2012). Coupling of the Nb1 and Nb3, binding distinct epitopes, resulted into a biparatopic Nb4 with increased apparent affinity and potency. Most importantly, Nb4 was able to significantly reduce tumor growth and angiogenesis in head and neck xenograft in vivo model (Maussang et al., 2013).

Recently, we described another example of a therapeutic nanobody targeting the chemokine receptor US28. US28 is a viral chemokine
receptor, which is encoded by the humane cytomegalovirus, and activates multiple oncogenic pathways both in a ligand-dependent and ligand-independent manner. In this study, we identified an US28-Nb which displayed a moderate affinity and antagonistic properties as a monovalent nanobody. A bivalent format, generated by linking two US28 nanobodies with a 35 GS linker, led to an increase of apparent affinity around a hundred fold and also provided the nanobody with partial inverse agonistic properties. This bivalent nanobody could inhibit US28 signaling up to 50% and showed reduced US28-enhanced tumor growth both in vitro and in vivo (Heukers et al., 2018).

In contrast to the described examples, a bivalent nanobody CA4910 against ChemR23 showed only a slight increase in apparent affinity compared to the monovalent nanobody, but a strong increase in efficacy of inhibition of chemerin-induced Ca2+ release and chemotaxis. The authors stated that this could be explained by the increased size of the bivalent nanobody providing more efficient steric hindrance to chemerin binding (Peyrassol et al., 2016).

Recently, positive allosteric modulating nanobodies (DN10 and DN13) targeting the mGlu2 receptor were identified which increased the potency of mGlu2 agonist LY379268. In addition, DN10 (but not DN13) could also partially activate the mGlu2 receptor on its own and demonstrated intrinsic agonist activity. Both nanobodies were shown to bind different but (partially) overlapping epitopes and could potentiate mGlu2 agonists in hippocampal brain slices. In addition, DN13 was also tested in vivo and showed the same effect by potentiating the disruption of contextual fear memory (Scholler et al., 2017).

I-bodies, which are based on the shark VNAR fragments, have also been used to target CXCR4. VNAR fragments are structurally similar to the i-set domain of immunoglobulins (Streltsov et al., 2004). This resulted in the engineering of a human i-body scaffold by using the Ig domain of human neural cell adhesion molecule 1 and incorporating two binding loops which mimic the CDR1 and CDR3 of the VNAR. Antagonistic CXCR4-targeting i-bodies were selected and inhibited HIV entry as well cell recruitment in vivo (Griffiths et al., 2016). However, the i-bodies could not mobilize stem cells although they could bind to these cells. In a follow-up study, the CXCR4-specific i-body AD-114 was further tested in a pulmonary fibrosis setting. AD-114 displayed anti-fibrotic effects and reduced lung injury in vivo (Griffiths et al., 2018).

As inverse agonistic activities were observed with bivalent nanobodies targeting the chemokine receptors CXCR4, ACKR3/CXCR7, CXCR2 and the viral chemokine receptor US28, there might be a common mechanism involved, which could be therapeutically exploited. Currently, the mechanism behind this observation is not well understood and might be interesting for further investigation.

4.3. Nanobodies as intrabodies

Most of the published GPCR-targeting Abs or Ab-fragments target the extracellular side of the receptor and can modulate receptors by acting as agonists, antagonists or inverse agonists. Intrabodies however, which is the term used upon intracellular expression of Ab(−fragments), have proven to be an interesting approach for targeting intracellular epitopes of GPCRs or GPCR-signaling proteins. Since the cytoplasm is a reducing environment, especially nanobodies, by possessing less (exposed) disulphide bonds, are particularly suited being more stable in this reducing environment (Marshall and Dubel, 2016; Moutel et al., 2016).

Nanobodies recognizing specific GPCR-conformations have been used as conformational biosensors to investigate GPCR-signaling. As an example, Nb80 was fused to GFP (Nb80-GFP) and visualized the colocalisation (on the plasma membrane and endosomes) of the biosensor with β2AR after receptor activation by the agonist isoprenaline in a time-dependent manner (Irannejad et al., 2013). Further experiments using the nanobody-biosensor showed the important role of endocytosis of β2AR on receptor signaling and the presence of endosomal signaling. Genetically encoded nanobodies targeting the active conformation of opioid receptors (Nb33-GFP), revealed localization bias of opioid receptors upon drug action (Stoeber et al., 2018). Both receptor signaling in different compartments (plasma membrane, endosomes and Golgi apparatus) was shown as well as the kinetics of receptor activation at the different subcellular compartments using this biosensor. Another nanobody-biosensor that provided more information regarding internalization and activation of GPCRs was Nb37-GFP that recognizes that (active) guanine-nucleotide-free form of Gαo. This nanobody was used as biosensor to demonstrate the activation of β2AR and glucose-dependent insulinotropic receptor in endosomes (Irannejad et al., 2013; Ismail et al., 2016).

Besides the use as biosensors, intrabodies have also been used to modulate GPCR-signaling. Different classes of nanobodies, targeting intracellular epitopes of the β2AR, were identified and were shown to be stabilize either the active or inactive conformation of the receptor (Staus et al., 2014). Both families of nanobodies stabilizing the active and inactive conformation showed reductions in G-protein signaling and β-arrestin recruitment upon expression. However, the inactive conformation stabilizing intrabodies (such as Nb60) showed reduction of receptor expression while this was not the case for the active conformation stabilizers (Staus et al., 2014). These nanobodies (Nb60 and Nb80) have proven to be important tools to demonstrate the ligand-specific effects of a wide array of ligands to the β2AR and provided more insight in receptor activation resulting in the development of a new multiple receptor state model (Staus et al., 2016).

Although GPCR-specific intrabodies can inhibit receptor signaling, more generic intrabodies have also proven to be interesting tools to investigate GPCR signaling. In a recent study, multiple Fab fragments were identified that targeted β-arrestin1 or β-arrestin2. These Fab fragments also potentiated the β-arrestin2 and/or β-arrestin-clathrin interaction resulting in a panel of different β-arrestin modulating tools. One of the Fab fragments (Fab5) inhibited β-arrestin-clathrin interaction while having no effect on the binding of (phospho-)ERK2. Intracellular expression of a ScFv format of this antibody fragment resulted in inhibition of GPCR endocytosis while having no effect on β-arrestin mediated ERK-MAP kinase activation. This effect was shown on multiple receptors including V1R, M5R and multiple dopamine receptors proving that the intrabody (ScFv5) is a generic GPCR endocytosis inhibitor (Ghosh et al., 2017).

In addition, a nanobody (Nb5) targeting Gβγ was generated that could bind various Gβγ subtypes and competed with the binding of Gβγ regulatory proteins. The nanobody could inhibit Gβγ-mediated GIRK signaling in striatum neurons, originating from D2R or D4R, and influence the human apelin receptor-mediated PI3K-AKT pathway. Interestingly, the nanobody did not have any effect on Gαq or Gαs-mediated signaling (Gulati et al., 2018).

5. GPCR-targeting nanobody-fusion constructs as future perspective

A large benefit of nanobodies is related to their small size. This small size is achieved by removing the two constant domains that would otherwise constitute the Fc-domain of an intact antibody. However, this also means that nanobodies lack effector function that are generally ascribed to conventional antibodies. Presence of the Fc domain is associated with longer retention in the blood and activation of the immune system via antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis and complement-dependent cytotoxicity (CDC) (Nelson, 2010). In order to restore the antibody-mediated effector functions, nanobodies can be re-fused to Fc-domains or optimized Fc-domains. This approach has been shown for other targets such as the human epidermal growth factor receptor 2 (HER2/ErbB2/Neu) (Qasemi et al., 2016; Wu et al., 2018). This resulted in ADCC mediated cell death and inhibition of tumor growth in vivo (Wu et al., 2018). More recently, CXCR4-targeting nanobodies have also been fused to an Fc-domain to obtain nanobody-Fc fusion proteins.
US28-binding CX3CL1 chemokine-mutant to PE38 to specify the toxins but other nanobody-based immunotoxins have been described. To date, no GPCR-targeting nanobodies have been fused with tubulin polymerization (Auristatins) or the bacterial toxin derived from Pseudomonas exotoxin A (PXA, PE38) to bind with enhanced tumor penetration could prove to be important advantages in immunotoxin therapy (Oliveira et al., 2013). Examples of such toxic moieties include toxic chemical compounds that interfere with tubulin polymerization (Auristatins) or the bacterial toxin derived from Pseudomonas or plants (PE38, Ricin, Cucurmosin) (Dosio et al., 2011). To date, no GPCR-targeting nanobodies have been fused with toxins but other nanobody-based immunotoxins have been described. The first nanobody-toxin described was an anti-vascular endothelial growth factor receptor 2 (VEGFR2) nanobody, which was fused to PE38 and inhibited cell proliferation at subnanomolar concentrations (Behdani et al., 2013). In addition, an anti-EGFR nanobody was fused to the glycoprotein cucurmosin and also induced cell toxicity, by inhibiting protein synthesis, when incubated with high expressing-EGFR cell lines while no cell toxicity was seen for a low expressing-EGFR cell line in vitro (Deng et al., 2017). In addition, nanobody-based immunotoxins have also shown their antitumor activity in vivo with an anti-CpD7 (humanized) nanobody which was fused to PE38 and treatment of xenograft mice with the nanobody-toxin showed prolonged survival (Tang et al., 2016; Yu et al., 2017). Recently, an US28-targeting toxin was generated by fusion of an US28-binding CX3CL1 chemokine-mutant to PE38 to specifically kill US28-expressing HCMV-infected cells both in vitro and in vivo (Spiess et al., 2015; Krishna et al., 2017). Similar approaches could be followed using GPCR-specific nanobodies which would encompass enhanced selectivity.

6. Concluding remarks and future perspectives

GPCRs are known to play an important role in a wide array of physiological processes and thus are important both for fundamental and translational research. Although these receptors have successfully been targeted by small molecules, challenges involving selectivity and potency during the drug discovery process are encountered. GPCR-targeting biologicals have been proposed as alternative and have already shown their potential with two GPCR-targeting Abs on the market. Especially nanobodies have garnered quite some attention lately due to their size and 3D conformation. Due to these characteristics, GPCR-targeting nanobodies can be used as versatile tools both for therapeutic and diagnostic applications as well as research tools for fundamental questions involving GPCR biology and structure. Even though nanobodies have already quite some potential in the GPCR field, there are still some possibilities to further expand. The use of nanobodies as imaging tools and guiding effector molecules to (cancer-) cells has already been shown but not for GPCR-targeting nanobodies. Since GPCRs play quite an important role in cancer biology, it is only a matter of time before GPCR-targeting nanobodies will be used for these purposes (Dosam and Gurtkind, 2007; O’Hayre et al., 2013). In addition, the use of nanobodies as research tool can also be further exploited. Both their use as conformational stabilizers and intrabodies will most likely lead to further insights in GPCR mediated signaling and GPCR conformations. In addition, nanobodies could also prove to be interesting tools to further investigate topics such as homo- and heterodimerization of GPCRs, biased signaling as well further exploring their possible uses in small molecule drug discovery. To conclude, GPCR-targeting nanobodies have shown to be interesting tools in the GPCR field and their potential will further be exploited in the future.

Conflicts of interest

The authors declare no competing interests.

Acknowledgments

This work was supported by The Netherlands Organization for Scientific Research (NWO VICI grant 016.140.657); EU horizon’s 2020 MSA programme (ONCORNET 641833).

References

Aazd, B.B., Chatterjee, S., Lesniak, W.G., Lisok, A., Pullambhatla, M., Baniahmad, F., Azad, B.B., Chatterjee, S., Poizier, J.T., Pullambhatla, M., Laker, G.D., Pomper, M.G., Nimmagadda, S., 2016. A fully human CXCR4 antibody demonstrates diagnostic utility and therapeutic efficacy in solid tumor xenografts. Oncotarget 7, 12344–12358.

Bannas, P., Lezn, A., Kunick, V., Fume, W., Risine, B., Schmid, J., Haag, F., Leingartner, A., Trepel, M., Adam, G., Koch-Nothe, F., 2015. Validation of nanobody and antibody based in vivo tumor xenograft NIRF-imaging experiments in mice using ex vivo flow cytometry and microscopy. JoVe, e52462.

Bannas, P., Hambach, J., Koch-Nothe, F., 2017. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. Front. Immunol. 8, 1603.

Bar-Shavit, R., Mazo, M., Kanchalar, A., Nas, J.K., Agronovich, D., Grisaru-Granovsky, S., Uziel, B., 2016. G protein-coupled receptors in cancer. Int. J. Mol. Sci. 17.

Behdani, M., Zeinali, S., Karimipour, M., Khanamad, H., Schoonnoogh, E., Asemzar, A., Shey, N., Mozami-Golrizi, R., Baniamad, F., Habibi-Anboushi, M., Hassanudeh-Glassabe, M., Muyldermans, S., 2013. Development of VEGFR2-specific Nanobody Pseudomonas exotoxin A conjugated to provide efficient inhibition of tumor growth. J. Biotechnol. 30, 205–209.

Behnem Azad, B., Lisok, A., Chatterjee, S., Poizier, J.T., Pullambhatla, M., Laker, G.D., Pomper, M.G., Nimmagadda, S., 2016. Targeted imaging of the atypical chemokine receptor 3 (ACKR3/CXCR7) in human cancer xenografts. J. Nucl. Med. 57, 981–988.

Bobbok, V., Zarca, A.M., Van Hout, A., Arimoto, M., Doijen, J., Bialkowski, M., Toffoli, E., Klarenbeek, A., van der Woning, B., van der Vlieg, H.J., Van Loy, T., de Haard, H., Schols, D., Heukers, R., Smit, M.J., 2018. Nanobody-Fe construct targeting chemokine receptor CXCR4 potentially inhibit signaling and CXCR4-mediated HIV-entry and induce antibody effector functions. Biochem. Pharmacol. 158.

Bradley, M.E., Dombrocki, B., Manini, J., Willis, J., Vlomic, D., De Tarcy, S., Van den Heede, K., Roobrouck, A., Grot, E., Kent, T.C., Laermans, T., Steffensen, S., Van den Bergh, G., Brown, Z., Charlton, S.J., Cromie, K.D., 2015. Potent and efficacious inhibition of CXCR2 signaling by biaffropic nanobodies combining two distinct modes of action. Mol. Pharmacol. 87, 251–262.

Burg, J.S., Ingram, J.R., Venkatakrishnan, A.J., Jude, K.M., Dukkipati, A., Feinberg, E.N., Angelini, A., Washag, D., Dror, R.O., Ploegh, H.L., Garcia, K.C., 2015. Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. Science 347, 1113–1117.

Cabrera-Marcues, O., Iriekekenins, G., 2017. Functional autoantibodies targeting G protein-coupled receptors in rheumatic diseases. Nat. Rev. Rheumatol. 13, 648–656.

Capote, I.A., Mondezes Peres, R., Lympemopolous, A., 2015. GPCR signaling and cardiac function. Eur. J. Pharmacol. 763, 143–148.

Chames, P., Van Regenmortel, M., Weiss, E., Baty, D., 2009. Therapeutic antibodies: successes, limitations and hopes for the future. Br. J. Pharmacol. 157, 220–223.

Che, T., Majumdar, S., Zaidi, S.A., Onodachi, P., McEvoy, J.D., Wang, S., Mosier, P.D., Uprety, R., Vardy, E., Krumm, B.E., Han, G.W., Lee, M.Y., Pardon, E., Steyaert, J., Huang, X.P., Strachan, R.T., Tribo, A.R., Pasternak, G.W., Carroll, F., Stevens, R.C., Cherezov, V., Katrich, V., Wacker, D., Roth, B.L., 2018. Structure of the nanobody-stabilized active state of the kappa opioid receptor. Cell 172, 55–67 e15.

Chevedichev, A., Honnorat, J., Hampe, C.S., Desert, V., 2016. Neuronal central nervous system syndromes probably mediated by autoantibodies. Eur. J. Neurosci. 43, 1533–1552.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Koblika, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Koblika, B.K., Stevens, R.C., 2007. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318, 1258–1265.

Chevillard, F., Rimmer, H., Betti, C., Parson, E., Ballet, S., Van Hilten, N., Steyaert, J., Diederich, W.E., Kolb, P., 2018. Binding-site compatible fragment growing applied to the design of beta2-adrenergic receptor ligands. J. Med. Chem. 61, 1118–1129.

Congreve, M., Marshall, F., 2010. The impact of GPCR structures on pharmacology and
Latorraca, N.R., Venkatkrishnan, A.J., Dror, R.O., 2017.GPCR dynamics: structure in motion. Chem. Rev. 117, 139–155.
Lauwersy, M., Abhari Ghahroudi, M., Desmyter, A., Kinne, J., Holzer, W., De Genst, E., Wyns, L., Myllymäki, S., 1998. Potent enzyme inhibitors derived from drosophila homeotic genes. J. Med. Chem. 41, 17, 3521–3524.
Manglik, A., Kobliha, B.K., 2014. The role of protein dynamics in GPCR protein function: insights from the beta2AR and rhodopsin. Curr. Opin. Cell. Biol. 27, 136–143.
Marshall, A.L., Dubel, S., 2016. Antibodies inside of a cell can change its outside: can intracellular antibodies provide a new therapeutic paradigm? Comput. Struct. Biotechnol. J. 14, 304–308.
Martí-Solano, M., Schmidt, D., Selent, J., 2016. Drugging specificity of monovalent antibodies (nanobodies) behaving as antagonists of the human heavy-chain antibodies. EMBO J. 17, 3512–3520.
Massa, S., Xavier, C., De Vos, J., Caveliers, V., Lahoute, T., Myllymäki, S., Devogdt, N., 2014. Site-specific labeling of cysteine-tagged cameld single-domain antibody. Fragments for use in molecular imaging. Angew. Chem. Int. Ed. 57, 797–798.
Maussang, D., Mudej-Delic, A., Descamps, J.F., Stortelers, C., Vanlandelocht, P., Stigter, van Walsum, M., Vischer, H.F., van Roy, M., Voronj, M., Gonzalez-Pajuelo, M., van Dongen, G.A., Merchiers, P., van Rompaey, P., Smit, M.J., 2013. Llama-derived single variable domains (nanobodies) directed against chemokine receptor CXCR7 reduce head and neck cancer cell growth in vivo. J. Biol. Chem. 288, 29562–29572.
Minich, W.B., Lenzner, C., Morgenhalter, N.G., 2004. Antibody fragments to TSH-receptor in thyroid autoimmune disease interact with monomeric antibodies whose epitopes are broadly distributed on the receptor. Clin. Exp. Immunol. 136, 123–139.
Moutel, S., Bery, N., Bernard, V., Keller, L., Klemere, E., de Marco, A., Lizet, L., Rain, J.C., Favre, G., Olicon, A., Perez, F., 2016. NaI-H1: a universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. Elle S. Myllymäki, S., 2015. Nanobodies: natural single-domain antibodies. Annu. Rev. Biochem. 82, 775–797.
Nelson, A.L., 2010. Antibody fragments: hope and hype. Mabs 2, 77–83.
Niwa, R., Shoji-Hosaka, E., Sakurada, M., Shinkawa, T., Uchida, K., Nakamura, K., Matsunaga, K., K., Hartmann, N., Shiina, K., 2014. Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced anti-inflammatory antibody-dependent cellular toxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res. 64, 2127–2133.
O’Hayre, M., Vazquez-Prado, J., Kufareva, I., Stawiski, E.W., Handel, T.M., Seshagiri, S., Ring, A.M., Manglik, A., Kobilka, B.K., 2014. The role of protein dynamics in GPCR function: insights from the viral G protein-coupled receptor US28 potently inhibits cytomegalovirus infection in vivo. Proc. Natl. Acad. Sci. U. S. A. 112, 8427–8432.
Philipp-Abbrederis, K., Herrmann, K., Knop, S., Schottelius, M., Eiber, M., Luckerath, K., Wang, X., van Diest, P.J., van Bergen en Henegouwen, P.M., 2012. Rapid visualization of tumor targeting tumors with nanobodies for cancer imaging and therapy. J. Contr. Release. 172, 607–617.
Pardon, E., Betti, C., Laeremans, T., Chevillard, F., Guillemyn, K., Kolb, P., Ballet, S., Roccaro, A.M., Sacco, A., Jimenez, C., Maiso, P., Moschetta, M., Mishima, Y., Aljawai, Y., O’Hayre, M., Vazquez-Prado, J., Kufareva, I., Stawiski, E.W., Handel, T.M., Seshagiri, S., Ring, A.M., Manglik, A., Kruse, A.C., Enos, M.D., Weis, W.I., Garcia, K.C., Kobilka, B.K., T.W.M. De Groof et al.
M., Chatalic, K.L., Casters, H., Janssen, E., de Graaf, C., Smit, M.J., de Esch, I.J., Leurs, R., 2012. Synthesis, modeling and functional activity of substituted styrene-amides as small-molecule CXCR7 agonists. Eur. J. Med. Chem. 51, 184–192.

de Wit, R.H., Heukers, R., Brink, H.J., Arsova, A., Maussang, D., Cutolo, P., Strubbe, B., Vischer, H.F., Bachelerie, F., Smit, M.J., 2017. CXCR4-Specific nanobodies as potential therapeutics for WHIM syndrome. J. Pharmacol. Exp. Therapeut. 363, 35–44.

van der Woning, B., De Boeck, G., Blanchetot, C., Robkov, V., Klarenbeek, A., Saunders, M., Waselbroeck, M., Laeremans, T., Steyaert, J., Hubert, A., De Haard, H., 2016. DNA immunization combined with scFv phage display identifies antagonistic GCGR specific antibodies and reveals new epitopes on the small extracellular loops. Mabs 8, 1126–1135.

Wu, W., Qian, L., Chen, X., Ding, B., 2015. Prognostic significance of CXCL12, CXCR4, and CXCR7 in patients with breast cancer. Int. J. Clin. Exp. Pathol. 8, 13217–13224.

Wu, X., Chen, S., Lin, L., Liu, J., Wang, Y., Li, Y., Li, Q., Wang, Z., 2018. A single-domain-based anti-her2 antibody has potent antitumor activities. Transl Oncol 11, 366–373.

Wurth, R., Bajetto, A., Harrison, J.K., Barbieri, F., Florio, T., 2014. CXCL12 modulation of CXCR4 and CXCR7 activity in human glioblastoma stem-like cells and regulation of the tumor microenvironment. Front. Cell. Neurosci. 8, 144.

Xavier, C., Vaneycken, I., D’Huyvetter, M., Heemskerk, J., Keysaerts, M., Vincke, C., Devoogdt, N., Muyldermans, S., Lahoutte, T., Cavéliers, V., 2013. Synthesis, preclinical validation, dosimetry, and toxicity of 68Ga-NOTA-anti-HER2 Nanobodies for iPET imaging of HER2 receptor expression in cancer. J. Nucl. Med. 54, 776–784.

Xavier, C., Blykers, A., Vaneycken, I., D’Huyvetter, M., Heemskerk, J., Lahoutte, T., Devoogdt, N., Cavéliers, V., 2016. (18)F-nanobody for PET imaging of HER2 overexpressing tumors. Nucl. Med. Biol. 43, 247–252.

Yan, H., Gu, W., Yang, J., Bi, V., Shen, Y., Lee, E., Winters, K.A., Komorowski, R., Zhang, C., Patel, J.J., Caughey, D., Elliott, G.S., Lau, Y.Y., Wang, J., Li, Y.S., Boone, T., Lindberg, R.A., Ho, S., Venianti, M.M., 2009. Fully human monoclonal antibodies antagonizing the glucagon receptor improve glucose homeostasis in mice and monkeys. J. Pharmacol. Exp. Therapeut. 329, 102–111.

Yoshibe, O., Matsushima, K., 2015. CCR4 and its ligands: from bench to bedside. Int. Immunol. 27, 11–20.

Yu, Y., Li, J., Zhu, X., Tang, X., Yao, Y., Sun, X., Huang, Y., Tian, F., Liu, X., Yang, L., 2017. Humanized CD7 nanobody-based immunotoxins exhibit promising anti-T-cell acute lymphoblastic leukemia potential. Int. J. Nanomed. 12, 1969–1983.

Zhou, Z., Vaidyanathan, G., McDougald, D., Kang, C.M., Balyasnikova, I., Devoogdt, N., Ta, A.N., McNaughton, B.R., Zalutsky, M.R., 2017. Fluorine-18 labeling of the HER2-targeting single-domain antibody 2RÎ15d using a residualizing label and preclinical evaluation. Mol. Imag. Biol. 19, 867–877.

Zhou, Z., Chitneni, S.K., Devoogdt, N., Zalutsky, M.R., Vaidyanathan, G., 2018. Fluorine-18 labeling of an anti-HER2 VHH using a residualizing prosthetic group via a strain-promoted click reaction: chemistry and preliminary evaluation. Bioorg. Med. Chem. 26, 1939–1949.

Zuo, L., Bao, H., Tian, J., Wang, X., Zhang, S., He, Z., Yan, L., Zhao, R., Ma, X.L., Liu, H., 2011. Long-term active immunization with a synthetic peptide corresponding to the second extracellular loop of beta1-adrenoceptor induces both morphological and functional cardiomyopathic changes in rats. Int. J. Cardiol. 149, 89–94.