Review

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GPCR-targeting nanobodies: attractive research tools, diagnostics, and therapeutics

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G-protein-coupled receptors (GPCRs) represent a major therapeutic target class. A large proportion of marketed drugs exert their effect through modulation of GPCR function, and GPCRs have been successfully targeted with small molecules. Yet, the number of small new molecular entities targeting GPCRs that has been approved as therapeutics in the past decade has been limited. With new and improved immunization-related technologies and advances in GPCR purification and expression techniques, antibody-based targeting of GPCRs has gained attention. The serendipitous discovery of a unique class of heavy chain antibodies (hcAbs) in the sera of camelids may provide novel GPCR-directed therapies. Antigen-binding fragments of hcAbs, also referred to as nanobodies, combine the advantages of both small molecules (e.g., molecular cavity binding, low production costs) and monoclonal antibodies (e.g., high affinity and specificity). Nanobodies are gaining ground as therapeutics and are also starting to find application as diagnostics and as high-quality tools in GPCR research. Herein, we review recent advances in the use of nanobodies in GPCR research.

Towards nanobody-based targeting of GPCRs

GPCRs (see Glossary) are seven transmembrane-spanning proteins that detect a vast repertoire of stimuli (ranging from light to large glycoproteins) and activate various intracellular signaling pathways. Upon activation, GPCRs undergo a conformational change that enables the recruitment and activation of proteins that relay signals from the plasma membrane to the cell interior. These include several classes of G proteins that modulate effector proteins and the scaffolding protein β-arrestin, which has been implicated in both termination of G protein-mediated GPCR signaling as well as initiation of distinct GPCR-dependent signaling cascades [1].

GPCR-induced signaling is a virtually universal means of intercellular communication, and deregulated GPCR signaling has been implicated in a multitude of diseases. An estimated 30% of marketed drugs exert their effect through modulation of GPCR function [2]. Considering that only a small proportion of these receptors are targeted by current therapies [3], the GPCR family provides a large untapped source of potential therapeutic interventions. Not surprisingly, many pharmaceutical companies have active programs aimed at identifying novel GPCR-interacting molecules. Low molecular weight molecules have proven to be very successful as therapeutics. Yet, in the past decade the number of small new molecular entities targeting GPCRs that were approved as therapeutics has

Glossary

Affinity: a biochemical parameter representing the intrinsic ligand–target binding strength, generally described by the equilibrium constant (K_d) of association (K_{on}) and dissociation rates (K_{off}).
Avidity: functional affinity, representing the combined binding strength of multiple ligand–target interactions.
Complementarity-determining region (CDR): the variable domains of antibodies contain three hypervariable CDR loop regions, which primarily form the antigen-binding paratope.
Chemokine receptors: a family of GPCRs that upon activation by chemokines orchestrate directional migration of immune cells in homeostatic and inflammatory conditions.
Clearance: a pharmacokinetic parameter depicting the rate at which a substance is excreted from the body.
Crystallization chaperone: a molecule that binds the crystallization target and increases the probability of high-quality crystallization potentially through restriction of conformation diversity.
Epitope: the antigen surface that is recognized by an antibody (fragment).
Formatting: the procedure of multimerization to improve the pharmacokinetic and/or pharmacodynamic properties of an antibody fragment.
G protein-coupled receptors (GPCRs): a superfamily of seven transmembrane domain cell surface receptors, which are key modulators of signal transduction and well-established drug targets.
Heavy chain antibody (hcAb): a fully functional antibody devoid of light chains naturally expressed in camelids.
Nanobody (Nb): an antibody fragment consisting of the recombinant 12–15 kDa VHH domain. Nbs are the smallest functional antibody-based biologics known to date.
Paratope: the interacting surface of the antibody (fragment) that binds the epitope.
Positron emission tomography (PET): an indirect high resolution molecular imaging technique using a radiolabeled tracer.
Single photon emission computed tomography (SPECT): a direct molecular imaging technique using a radiolabeled tracer.
Tissue penetration: a pharmacokinetic parameter describing the propensity of a molecule to penetrate deep into the tissue.
Valency: the number of paratopes of an antibody (fragment).
VHH: the variable domain of a heavy chain antibody, which is fully capable of binding an antigen.

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been limited. The high attrition rate in preclinical and clinical trials, ascribed to, for example, toxicity, insufficient efficacy, or inadequate selectivity, is leading to an enormous increase in drug discovery costs [4]. In the meantime, the implementation of biologics as therapeutics has gained considerable attention (Box 1). Monoclonal antibodies (mAbs) are attractive therapeutics as they are highly selective and display extended half-lives compared with small molecules. With new and improved immunization-related technologies and advances in expression and/or purification methods of GPCRs, there are ongoing efforts directed at antibody-based targeting of GPCRs. Recently, mogamulizumab, a mAb targeting the chemokine receptor CCR4, was approved for treatment of adult T cell leukemia in Japan [5]. Currently, several therapeutic mAbs targeting GPCRs for the treatment of various indications (e.g., inflammation, oncology) are under investigation in clinical trials [3,6,7].

Although proven effective for various indications, mAbs are large (150 kDa) heteromultimeric glycoproteins, which are associated with the inability to target intracellular components and molecular cavities, limited drug administration routes, and high production costs. Nanobodies (Nbs) represent a potential novel class of antibody-based therapeutics with some favorable characteristics. They have already been successfully developed against several drug targets. Recently, we identified the first Nbs targeting GPCRs *in vivo*. Furthermore, the Nb platform has led to significant breakthroughs in basic research on GPCR structure and signaling. In this review, we will focus on the exciting progress in the field of GPCR-targeting Nbs.

Unique characteristics and therapeutic potential of Nbs

Discovery and advantages of Nbs

In the early 1990s, Hamers-Casterman *et al.* discovered that camel sera contain a unique class of antibodies devoid of light chains and composed only of heavy chains [8]. Besides camels, other closely related members from the

Box 1. Conventional mAbs and fragments

The immune system contains a diverse repertoire of tools to protect the host organism against harmful pathogens and xenobiotics. Antibodies, which are glycoprotein complexes secreted by B cells, are one of these tools that provide humoral (i.e., antibody-mediated) immunity by binding and subsequent neutralization of target molecules. The most abundantly expressed antibodies are the immunoglobulin-γ (IgG) proteins, which are the origin of conventional mAbs. The overall structure of these 150 kDa molecules is very complex, consisting of four peptides made up from two identical heavy (H) and two identical light (L) chains together assembling in a tetrameric Y-shaped structure (see Figure 1 in main text). Both chains of conventional mAbs consist of several different functional domains. Whereas the heavy chains consist of one variable domain (VH) and three constant domains (CH1–3), the light chains consist of only two domains, one variable (VL) and one constant domain (CL). The constant domains CH1 and CL together with the variable domains comprise the two Fab regions, both able to bind one antigen each. The bivalent structure of an antibody is believed to contribute to increased avidity, thereby conferring high retention times. The remaining part of the antibody complex consisting of the CH2–CH3 homodimer is termed the Fc region [86]. The specific antigen-binding characteristics of a mAb are dependent on the complementarity-determining regions (CDRs) encoded by both variable domains (VH and VL), which together constitute the variable fragment (Fv). The constant domains in the Fc region are not directly involved in binding but are involved in mediating additional immune responses upon target binding.

To improve the pharmacokinetics of mAbs, new formats of conventional antibody-based therapeutics were developed. Through removal of the Fc region and multimerization of the antigen-specific (VH and VL) domains, several types of antibody-based fragments of relatively small size were produced. The 55 kDa monovalent Fab fragments consist, as the name suggests, solely of one functional Fab region and thus also contain, besides the variable domains, the CH1 and CL constant domains. This is in contrast to the single chain Fv (scFv) fragments, which are the two variable domains directly connected by a peptide linker resulting in a 30 kDa size. Furthermore, several modulatory approaches are applied to these two functional antibody-based building blocks to generate multivalent constructs [34,87]. With the design of conventional IgG-based antibody fragments, tissue penetration is increased, whereas immunogenicity is decreased; however, this comes with the cost of increased blood clearance [88].

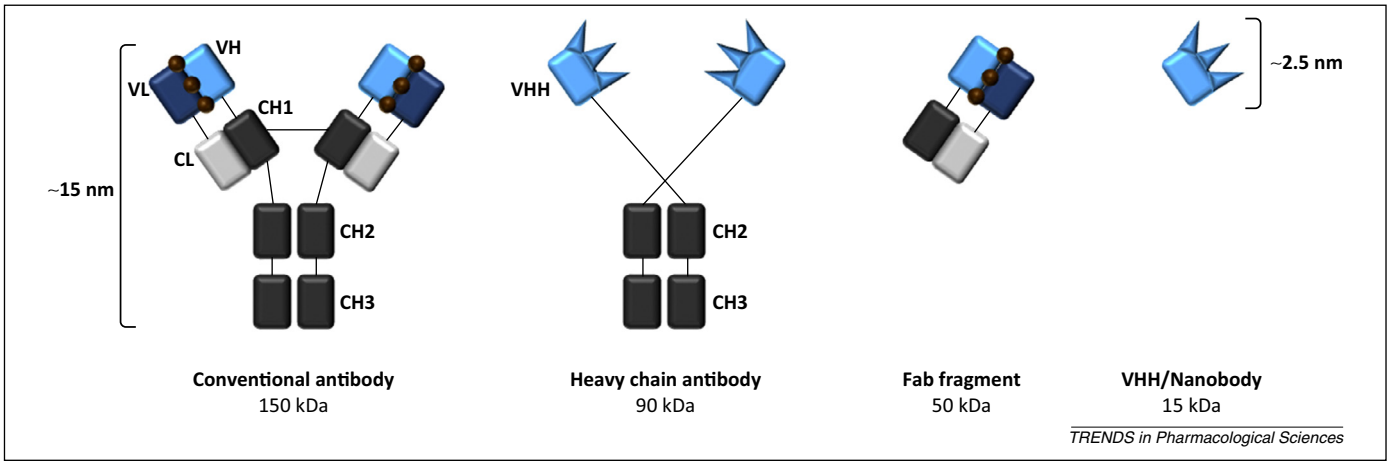

Figure 1. Structural features of different antibodies. Conventional γ-immunoglobulins consist of two heavy chains, each containing three invariant (constant) domains (CH1–CH3) and a single variable region (VH), and two light chains composed of a constant region (CL) and a variable region (VL). The VH and VL regions together form the paratope, and it is this antigen-recognizing portion of the antibody that is retained in Fab fragments. By contrast, camelid heavy chain antibodies are composed of a homodimer composed of two heavy chains containing two constant regions (i.e., they are lacking the CH1 domain) and a single variable (VHH) domain harboring three complementarity determining regions (rectangles). Nanobodies are the smallest functional single chain antigen-recognizing polypeptides known to date, with a molecular weight of 12–15 kDa and 1.5–2.5 nm dimensions.