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Extracellular nanobodies for GPCR modulation mechanisms, engineering, and therapeutic possibilities

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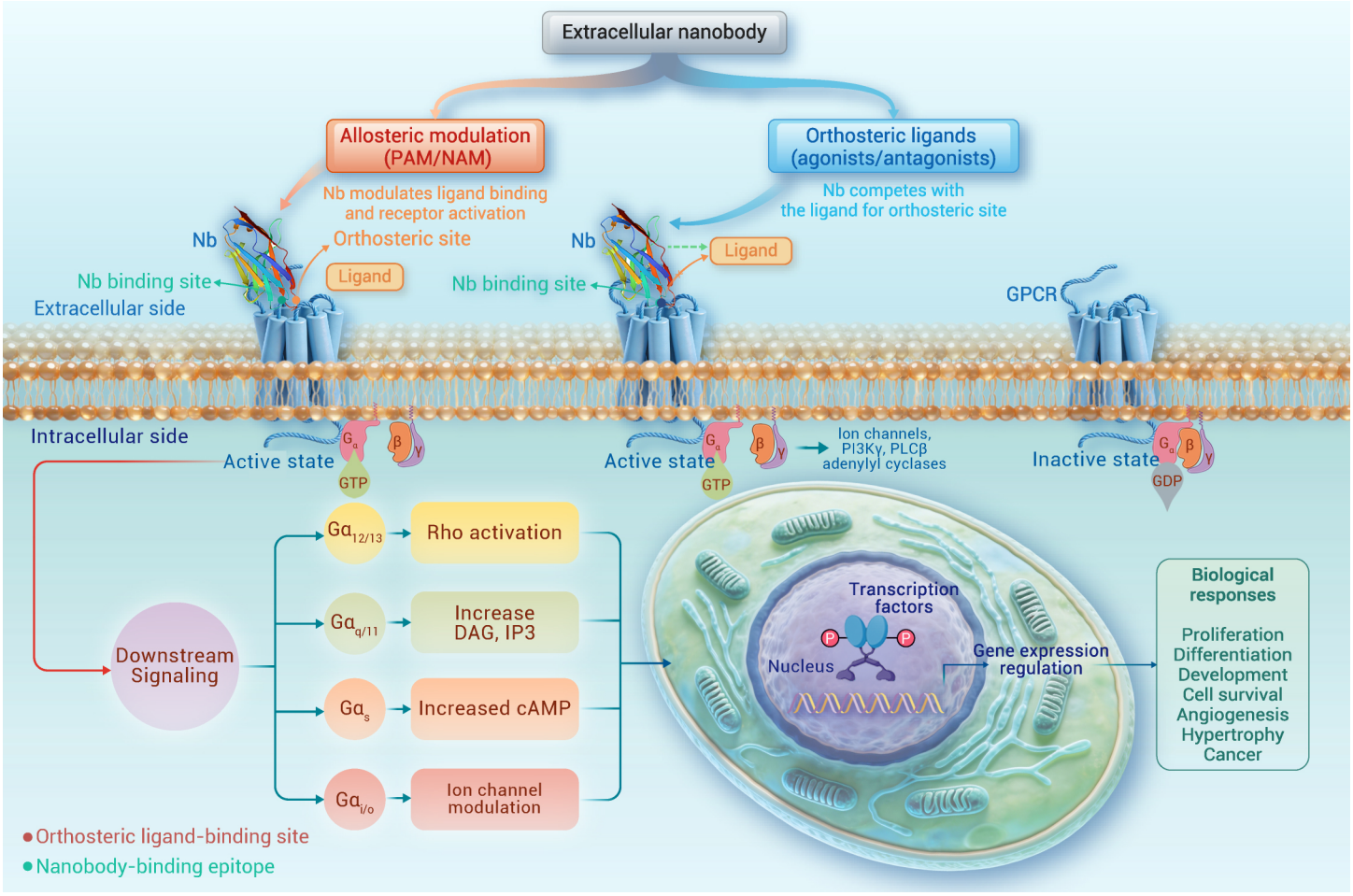
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Nanobodies (single-domain antibodies) target G protein-coupled receptors (GPCRs)，including orphan GPCRs.
- Nanobodies act as antagonists, agonists, or allosteric modulators to control GPCR signaling.
- Nanobodies improve precision and safety in treating diseases like cancer and neurological disorders.
- Nanobody engineering and AI-based design enhance drug targeting and therapeutic efficacy.

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Extracellular nanobodies for GPCR modulation mechanisms, engineering, and therapeutic possibilities

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GPCRs act as the core of cell signaling and play crucial roles in the development and progression of major diseases, including cancer, neurological disorders, and metabolic diseases. Nanobodies are single-domain antibodies originally derived from camelids. They are small in size, easy to design, highly penetrable, with a high affinity, and have become an ideal tool for controlling GPCR signaling. Nanobodies can act as agonists, antagonists or positive/negative allosteric regulators by recognizing the extracellular loops (ECLs), ligand-binding sites, or allosteric sites of GPCRs, stabilizing specific conformations and inducing biased signaling, which can significantly improve the precision of regulation and the therapeutic window. In this article, we summarize the action of nanobodies in regulating GPCRs: extracellular and intracellular targeting, ligand site blockade, biasd signaling regulation, structure-directed functional modification, and antagonistic properties of novel target GPCRs, such as CXCR4/CXCR7, APJ, and MC4R. We evaluate their potential applications and advantages in viral GPCRs, peptide-like receptors, and angiotensin receptors, among others. We also discuss the cutting-edge applications of nanobodies in orphan GPCR targeting, heterodimer modulation, transmembrane transport (e.g., crossing the blood-brain barrier), and their potentials in difficult-to-formulate GPCRs. In the future, along with AI-based structure prediction and high-throughput screening platforms, nanobodies will be able to evolve intelligently from function definition to precise construction. Especially in the context of bispecific and multivalent constructs as well as nano-delivery systems, nanobodies will be the engine of GPCR regulation strategies to provide safer, more specific, and efficient treatments for refractory neurological, metabolic, and tumor diseases.

INTRODUCTION

G-protein-coupled receptors (GPCRs) are integral membrane proteins that play a central role in cellular communication by transmitting signals from extracellular ligands through conformational changes in their transmembrane (TM) helices.[1] Due to their involvement in various physiological processes, GPCRs have become key targets for drug discovery, particularly for cancer, diabetes, immune disorders, and neurological diseases.[2] However, the isolation and characterization of biologically active GPCR antigens pose significant challenges due to their intrinsic hydrophobicity, dynamic conformational states, and complex structure, which includes seven TM helices and variable extracellular exposure.[3] While certain GPCRs, such as class C GPCRs and glycoprotein hormone receptors, possess large extracellular domains that aid in ligand accessibility, others have limited extracellular exposure and low surface expression, which complicates drug development efforts.[4]

GPCRs are a large group of proteins targeted by many medicines-30% to 40% of approved drugs act on them, mainly as small molecules or short peptides. Table 1 summarizes the main features of the different drug forms. Importantly, factors to consider include not only epitope binding and pharmacokinetic properties, but also the functional roles of different drug modes in regulating receptor conformation and biased signaling. Small molecules, for example, have favorable pharmacokinetics, absorption, and synthesis.[5] Despite these advantages, developing small-molecule drugs that can selectively and effectively modulate specific GPCR isoforms—variations of GPCRs encoded by the same or closely related genes—remains highly challenging due to the vast diversity within this protein family.[4] Lack of isoform specificity often leads to off-target effects, reduced efficacy, and adverse reactions, especially in treating complex diseases like cancer and neurological disorders. In addition, the performance of small-molecule GPCR ligands in clinical trials has often been affected by target-specific pharmacokinetics, off-target effects, and tissue distribution limitations. In some cases, frequent doses are required, or the drug fails to reach protected areas (e.g., the central nervous system). In many GPCR drug discovery projects, the focus is still on ligands and relies on known endogenous ligands or well-validated surrogate agonists/antagonists. For orphan GPCRs, the model faces challenges: the lack of known ligands makes assay design challenging and hinders progress in high-throughput chemical screening.

Antibody-based therapies have emerged as a promising and technically alternative, offering enhanced specificity, binding affinity, and prolonged serum presence. Polyclonal antibodies have been widely developed for detecting membrane GPCRs in experimental studies. Although monoclonal antibodies offer greater specificity compared to polyclonal antibodies and small molecules, their use in targeting GPCRs is hindered by several challenges. Due to their large size, monoclonal antibodies often struggle to access buried or intracellular epitopes on GPCRs, limiting their ability to fully modulate receptor function.[6] Furthermore, antibody discovery and manufacture are costly, and few agents have clinical approval, such as Mogamulizumab for C-C chemokine receptor type 4 (CCR4)-positive T-cell leukemia, and Erenumab, an antagonist of the calcitonin gene-related peptide type one receptor (CALCRL) for migraines, and the bispecific antibody Talquetamab for relapsed or refractory multiple myeloma.[7] In addition to antibody-based strategies, other modalities, such as hormones and therapeutic peptide drugs, can mimic endogenous ligands and bind to the orthosteric site, but they are usually rapidly hydrolyzed and degraded by proteases and lack favorable pharmacokinetic properties without chemical modifications. While natural ligands can bind receptors with high affinity, they are limited in their ability to regulate conformational bias and in long-term systemic exposure.

A promising alternative to GPCR-targeting drugs is nanobodies (single-domain antibodies from camelids, VHH). Their small size (12–15 kDa) allows tissue penetration and access to epitopes that are not accessible to conven-

Table 1. Comparative summary of major therapeutic modalities targeting GPCR.

Feature/Criterion	Small Molecules	Nanobodies	Conventional Antibodies (mAb)	Hormones/Peptides
Molecular Size	~ 300-700 Da	~ 12-15 kDa	~ 150-160 kDa	~ 0.5-5 kDa
Manufacturing	Mature chemical synthesis	Recombinant expression	Complex biologic production	Peptide synthesis/ recombinant
Target Epitope	Orthosteric pocket	Cryptic/allosteric & recessed epitopes	Surface-exposed only	Natural orthosteric sites
Accessibility	Broad; limited CNS	Access hidden sites	Restricted by size	Depends on exposure
Subtype Selectivity	Often limited	High	High for accessible epitopes	High
Biased Signaling Control	Limited	Strong potential via conformational stabilization	Limited	Depends on ligand nature
Pharmacokinetics	Short half-life; oral possible	Rapid clearance without engineering; engineerable	Long	Often short due to proteolysis
BBB Penetration	Often limited	Better than mAb; engineering strategies exist	Poor	Limited
Off-Target Effects	Moderate-high	Low	Very low	Variable
Clinical Experience	Extensive	Emerging	Established in some fields	Established in endocrinology
Development Cost	Relatively low	Moderate-high	High	Moderate

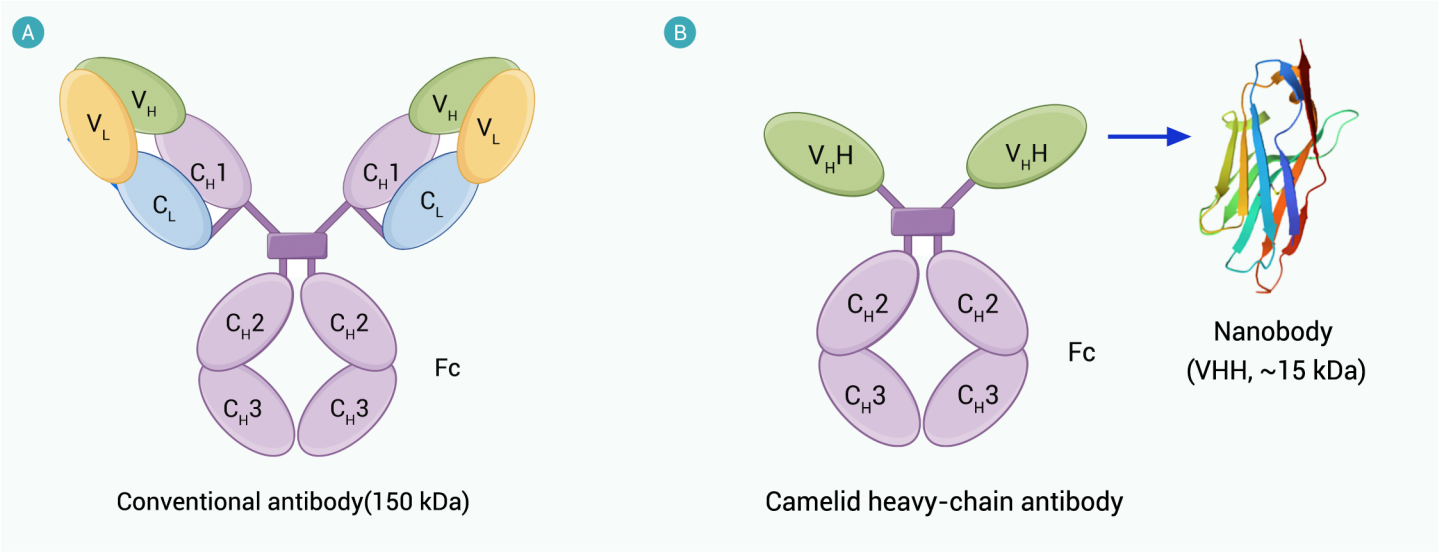

Figure 1. Schematic representation of conventional antibodies and camel heavy-chain antibodies' variable domain (V_HH)/nanobody (Nb) (A) Conventional antibody (~150 kDa) includes two light chains (V_L), two heavy chains (V_H), and constant regions (CH_1, CH_2, CH_3, C_L). (B) Camelid heavy-chain antibody (HCAb) lacks light chains and consists of a single variable domain (V_HH, nanobody, ~15 kDa) with constant regions (CH_2, CH_3). The right panel depicts the nanobody's 3D structure.

tional antibodies(Figure 1).[6] A large CDR3 region allows binding to hidden epitopes on GPCR receptors, such as the β2-adrenergic receptor and the μ-opioid receptor(μOR), thereby enhancing affinity and selectivity and reducing off-target effects.[4,8] Nanobodies are not a universal substitute for small molecules, especially for well-known targets (e.g., CXCR4, μOR). Rather, nanobodies excel where other molecules fail. Nanobodies distinguish subtypes or states more sharply than small molecules by engaging ECLs or recessed conformation-selective epitopes, as with MC4R-selective orthosteric nanobody antagonist pN162. For therapies aimed at stabilizing a specific receptor conformation or biasing signaling, nanobodies act as conformational clamps, a function absent in other ligands. Nanobodies also provide a modular platform (multivalency, bispecificity, ligand-nanobody conjugates) to tune avidity, residence time, pathway preference, and can modulate biased signaling by stabilizing specific conformational states of GPCRs, which is not possible with small molecules or conventional antibodies, and can control receptor biased signaling pathways more precisely (Figure 2). For example, for the type 1 parathyroid hormone receptor (PTHR1, Table S1), nanobodies can convert a standard agonist into a highly selective ligand that activates specific signaling pathways for therapeutic benefit while avoiding off-target effects.[9] While small molecules and other GPCR ligands can induce biased signaling, nanobodies uniquely stabilize specific GPCR conformations that are often inaccessible to conventional ligands. This characteristic is particularly beneficial for conditions that require selective modulation of signaling pathways. In chronic pain management, for instance, nanobody-induced biased signaling at opioid receptors (ORs) has been shown to alleviate analgesic side effects by avoiding the activation of harmful signaling pathways.[8] Engineering can extend the serum half-life of nanobodies, supporting sustained effects and low immunogenicity for chronic diseases.[10,11] In summary, despite the complexity of GPCR drug targets, nanobodies provide unparalleled specificity and affinity, precise control of signaling bias, and enable advanced modulation strategies, including targeting of orphan GPCRs, across a wide range of diseases.[12]

NANOBODIES: EXTRACELLULAR VS. INTRACELLULAR

Nanobodies targeting GPCRs can be classified by binding site into two types: intracellular nanobodies (intra-VHHs, also known as intrabodies) and extracellular nanobodies (extra-VHHs), the stabilities of which can be influenced by disulfide bonds, but the reducing environment of the cytoplasm

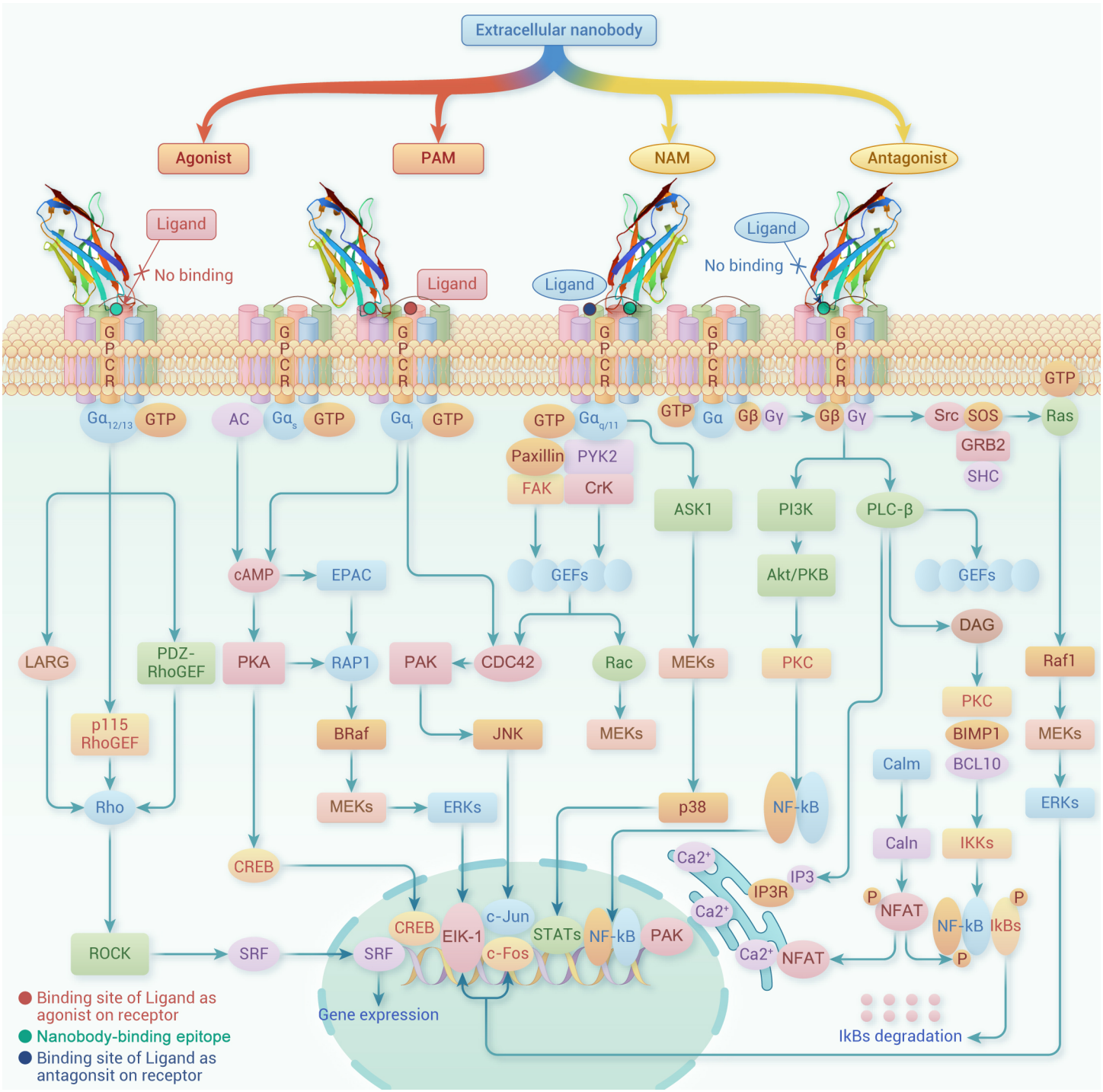

Figure 2. Modulation of GPCR signaling by extracellular nanobodies Extracellular nanobodies can influence GPCR function in multiple ways. Some nanobodies act as agonists, directly activating the receptor; others act as positive allosteric modulators (PAMs) to enhance ligand-induced signaling, negative allosteric modulators (NAMs) to reduce receptor activation, or antagonists to block ligand binding. These different modes of action reshape downstream signaling networks, including G protein-mediated pathways, second messenger production (cAMP, IP_3/DAG), kinase cascades (PKA, PI3K/Akt, MAPKs), and transcriptional responses (e.g., NF-κB, NFAT, CREB). Colored markers in the diagram indicate the respective binding sites of ligands and nanobodies on the receptor.

may not consistently maintain disulfides. Thus, the functionality of intra-VHHs depends on their intrinsic sequence and folding stability, with specific amino acid modifications used to enhance resilience while maintaining binding specificity.[13] Conversely, the oxidizing extracellular conditions promote disulfide bond formation and improve structural stability in extra-VHHs.[4] Intra-VHHs have been shown to function as biosensors, capable of detecting conformational changes in GPCRs, or as direct regulators of intracellular processes. By altering interactions with signaling molecules such as G proteins and β-arrestins, intra-VHHs can support pathway-specific (biased) signaling. When fused to fluorescent markers, intra-VHHs enable visualization of receptor activation, subcellular signaling, and GPCR dynamics in live cells.[4,6]

Extra-VHHs bind to extracellular regions of GPCRs and can act as antagonists, inverse agonists, or positive/negative allosteric modulators (PAMs/NAMs) with high specificity (Figure 2).[14] These nanobodies modulate the actions of endogenous ligands by interacting with orthosteric or allosteric sites, promoting receptor conformations that selectively engage G proteins or β-arrestins (Figure 3). As a result, they can enhance therapeutic effects, minimize off-target activity, and reduce adverse signaling.[8,14] Owing to their versatility, extra-VHHs are valuable as therapeutic leads and as tools for investi-

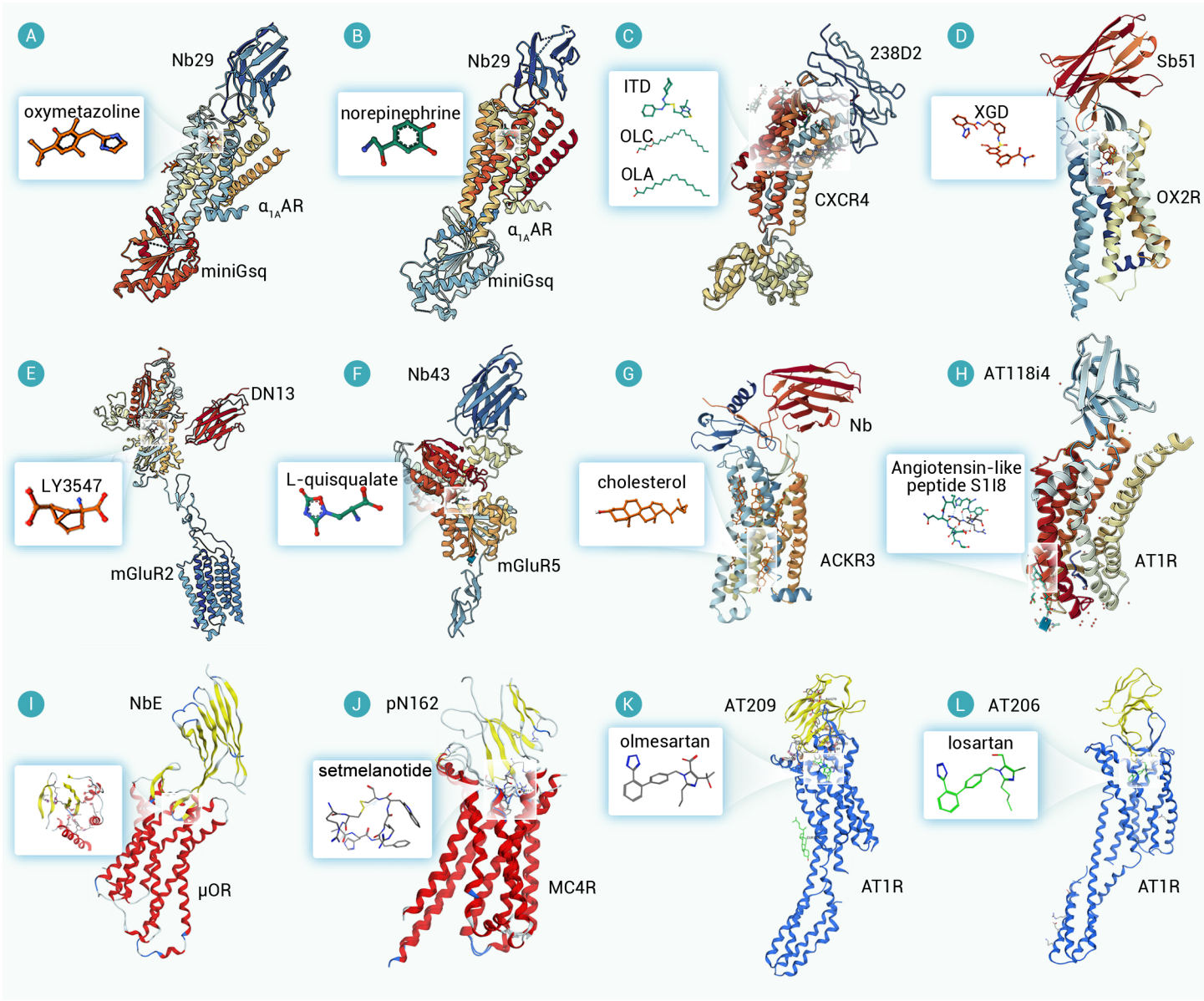

Figure 3. Schematic representation of GPCRs in a complex with VHH and various ligands (A) Cryo-EM structure of the Nb29-α_{1A}AR-miniGsq complex bound to the agonist oxymetazoline, illustrating selective modulation of receptor activation and nanobody-stabilized conformations (PDB ID: 7YM8). (B) Cryo-EM structure of the Nb29-α_{1A}AR-miniGsq complex interacting with noradrenaline, highlighting nanobody-mediated stabilization of receptor conformation and miniGαq coupling (PDB ID: 7YMH). (C) X-ray crystallographic structure of the CXCR4 chemokine receptor bound to the antagonist ITD, with OLC and OLA ligands, accompanied by an extracellular nanobody, showcasing ligand-binding versatility in nanobody-stabilized receptor states (PDB ID: 3ODU). (D) Cryo-EM structure of the OX2R receptor complexed with nanobody Sb51 and the small-molecule agonist XGD, demonstrating allosteric stabilization and structural modulation (PDB ID: 7L1V). (E) Cryo-EM structure of the mGluR2 receptor in complex with the agonist LY3547407 and nanobody DN13, illustrating nanobody binding's role in biased signaling modulation (PDB ID: 7EPB). (F) X-ray crystallographic structure of the mGlu5 receptor bound to nanobody Nb43 and the orthosteric endogenous agonist L-glutamate, highlighting nanobody interactions at the apex of the VFT domain and their contribution to receptor activation and ligand selectivity (PDB ID: 6N51). (G) Cryo-EM structure of the ACKR3/CXCR7 receptor in complex with cholesterol and an extracellular nanobody, highlighting receptor conformation regulation by lipid-like molecules (PDB ID: 7SK8). (H) X-ray crystallographic structure of the AT1R receptor in complex with the angiotensin-like peptide S1I8, stabilized by nanobody AT118i4, illustrating nanobody-facilitated biased signaling (PDB ID: 6DO1). (I) Cryo-EM structure of the μ-opioid receptor (μOR) in complex with nanobody NbE, showing nanobody stabilization of the active receptor state (PDB ID: 8QOT). (J) Cryo-EM structure of the melanocortin-4 receptor (MC4R) with nanobody pN162 and ligand Setmelanotide, highlighting dual orthosteric and allosteric interactions (PDB ID: 8QJ2). (K) Cryo-EM structure of AT1R with nanobody AT209, bound to the small-molecule antagonist olmesartan, demonstrating probe-dependent allosteric modulation of ligand dissociation kinetics (PDB ID: 9EAH). (L) Cryo-EM structure of AT1R bound to losartan and nanobody AT206, representing another example of small-molecule/nanobody synergy in receptor modulation (PDB ID: 9EAI).These structures demonstrate diverse GPCR-nanobody interactions and were sourced from the Protein Data Bank (PDB, https://www.rcsb.org) The blue boxes indicate the binding sites of the nanobodies and ligands.

gating GPCR structure, function, and allosteric mechanisms.

Extracellular nanobody acting as antagonist

Nanobodies targeting the extracellular structural domain of GPCRs (extra-VHHs) are currently focused on chemokine receptors for research and translation and show significant therapeutic potential in oncology, immune diseases, and HIV. The core mechanism is to bind to the receptor's ECLs and/or orthosteric sites, disrupting ligand-receptor interactions and inhibiting downstream signaling, thereby reducing pathological processes such as cell migration, proliferation, and immune escape. With high epitope specificity, nanobodies enable more precise pathway modulation and reduce the risk of off-targeting.[4,8]

A major therapeutic objective is to inhibit CXCR4 and CXCR7 signaling, given their roles in cancer progression, immune regulation, HIV pathogenesis, embryogenesis, hematopoiesis, and immune-cell trafficking. Dysregulation of these pathways is associated with enhanced tumor proliferation, metastasis, and survival.[15] CXCR4 and CC chemokine receptor 5(CCR5) are also central to HIV entry into $CD4^+$ T cells , making them key targets for antiviral intervention.[16] Nanobodies 238D2, 238D4, and 10A10 are highly selective monovalent neutral antagonists that recognize extracellular-loop epitopes

(e.g., ECL2) of CXCR4, compete with CXCL12, and selectively inhibit CXCL12-CXCR4 signaling and suppress CXCR4-dependent chemotaxis and CXCR4-tropic HIV entry.[17] Constructing 238D2 and 238D4 into bivalent forms significantly enhances their affinity and efficacy while mobilizing $CD34^+$ stem cells. Their efficacy is comparable to that of the classic CXCR4 antagonist plerixafor (AMD3100).[18] Notably, the monovalent form exhibits neutral antagonism, whereas the biparatopic form can display inverse agonist activity against the constitutively active mutant CXCR4-N3.35A , further enhancing the inhibition of pro-tumor signals.[17] Additionally, monovalent or bivalent nanobodies such as VUN401 and VUN403 can bind partially overlapping epitopes of CXCR4, exhibit broad-spectrum anti-HIV activity, and, to a certain extent, separate the anti-HIV effect from broader CXCR4 inhibition, thereby enabling more precise targeting.[19] While VUN402 shows limited impact on CXCR4 internalization and chemotaxis, it remains a candidate for selective anti-HIV strategies.[19] Furthermore, VUN400-HiBiT acts as a living-cell conformational probe, complemented by LgBiT to form NanoLuc, which reports real-time binding to CXCR4 ECL2 in HEK293 and Jurkat T cells, detecting extracellular conformational changes induced by agonists/antagonists. For example, binding of AMD3100 to the transmembrane region displaces VUN400-HiBiT from ECL2, facilitating monitoring of receptor expression and distinguishing orthosteric/allosteric ligand effects.[20] A variety of ACKR3/CXCR7 nanobodies exhibit high selectivity and potency at different sites on the cell surface.[21] Among them, Nb2 and Nb3 act as competitive antagonists to inhibit ACKR3-induced β-arrestin2 recruitment; in contrast, Nb1 behaves as an ACKR3 agonist, achieving biased regulation by competitively substituting for ACKR3 and reducing CXCL11/CXCL12 clearance.[4] Building on these findings, researchers fused Nb1 and Nb3 to create Nb4, which further increases affinity, blocks β-arrestin2 signaling, boosts CXCL11-driven angiogenesis, and limits tumor growth. The ACKR3-specific antagonist VUN701 blocks the upward movement of TM7 by steric inhibition and prevents the conserved NPxxY motif. The key is to insert the extended CDR3-hairpin into the orthosteric pocket of ACKR3. CDR1/CDR2 interacts with the N-terminal and ECL domains. Thus, they differ from similar receptors such as CXCR3 or CXCR4. VUN701 sterically blocks TM7 movement, stopping NPxxY motif activation, and blocks CXCL11 signaling (IC_{50} = 105 nM, KB = 23.3 nM, pKB = 8.64) and CXCL12 activation (IC_{50} = 157 nM, KB = 9.8 nM, pKB = 8.01) , while controlling CXCL12 potency by concentration (K_D = 10 nM, pK_D = 8.00). At the same time, it can regulate the potency of CXCL12 in a concentration-dependent manner (KB = 18.2 nM, pKB = 7.74) and selectively acts on the biased pathway without changing the efficacy of β-arrestin2 recruitment.[22] Modifications to the CDR1 region of VUN701 enable it to show antagonist activity against CXCR4 while maintaining its inhibitory effect on ACKR3. Compared with small molecules such as ACT-1004-1239 (IC_{50} = 3.2 nM, Table S1), nanobodies exhibit similar or better site specificity. In the β-arrestin2 competition assay, its IC_{50} is 0.2–20 nM (pIC_{50} = 7.7–9.7), and through CDR modifications, it can be reprogrammed as an agonist, partial agonist, or inverse agonist, thus enhancing therapeutic potential.[22]

Another important target for extra-VHHs is CXCR2, a chemokine receptor associated with inflammatory disorders.[23,24] Nanobodies 2B2 and 127D1 bind to different epitopes at the N-terminal and can strongly inhibit CXCR2 signaling.[25] For instance, nanobodies such as 97A9, 163E3, and 163D2 function as inverse agonists, inhibiting CXCR2 signaling induced by CXCL1 and CXCL8, especially bivalent nanobodies.[26,27] While their efficacy in *vitro* assays may be lower than that of small molecules, they provide distinct advantages in receptor modulation.[28,29] Small molecules, such as navarixin (IC_{50} = 0.97 nM, Table S1), are very effective but often face off-target effects, short serum half-lives, and a limited therapeutic window. Since the immune response may neutralize or alter the drug's efficacy, the long-term stability of nanobodies and the risk of anti-drug antibodies (ADA) should be emphasized.[30,31] Nonetheless, engineering approaches like PEGylation or sequence optimization can mitigate immune-related concerns, enhance stability, and preserve selectivity, which is particularly beneficial for managing chronic diseases.[32]

The viral GPCR US28, encoded by human cytomegalovirus (HCMV), binds multiple human chemokines, activates proliferation/inflammatory signaling, and nanobodies targeting the extracellular region of US28 with nanomolar affinity can inhibit chemokine binding and US28 signaling.[33] Its bivalent form can partially inhibit $US28^+$ glioblastoma (GBM) cells by targeting both ligand-dependent and constitutive activities.[33,34] VUN100 targets Tyr16 and ECL3, can be conjugated with photosensitizers, and induces cytotoxicity in US28-positive GBM cells.[35] The bivalent form, with enhanced affinity, has a partial inverse agonist effect (inhibiting about 50% of signaling). Additionally, VUN100 can selectively activate latent HCMV in primary $CD14^+$ monocytes to regulate replication and clear viral reservoirs, and may synergize with antiviral therapy.[36] In comparison to small molecules like VUF2274, nanobodies such as VUN100 can be tuned to further increase target specificity and minimize off-target activity through receptor-tailored interactions.[10,34]

Novel nanobodies targeting Chemerin Receptor 23 (ChemR23) and the angiotensin II type 1 receptor (AT1R) have shown potential for cardiovascular and inflammation treatment. This is achieved using a general epitope-guided approach, which relies on yeast display synthetic libraries and fluorescence-activated cell sorting/magnetic-activated cell sorting (FACS/MACS) screening. Notably, molecular structures such as CA4910 and CA5183 offer high specificity and potential for engineering. For instance, CA4910 inhibits partial signals of chemerin (half-maximal inhibitory concentration, IC_{50} = 60 ± 10 nM, Table S1) and exhibits even stronger inhibition of chemerin (149–157)(IC_{50} = 17 ± 2 nM, Table S1), whose large CDR3 region further enables access to cryptic epitopes.[37] This high specificity minimizes off-target effects, addressing a critical limitation of small-molecule antagonists like CHEMR23-IN-2 (IC_{50} = 3.2 nM, Table S1), which cannot exploit allosteric probe dependence or conformation-selective binding, as seen in AT206/AT209 nanobodies (Figures 3K-L).[38,39] Nanobodies exhibit engineering flexibility, as demonstrated by bivalent CA4910 , which displayed increased binding affinity (K_D = 95 ± 4 nM, Table S1) and competitive binding potency (IC_{50} = 18 ± 4 nM) compared to its monovalent form, leading to nearly complete inhibition of chemerin-induced intracellular calcium release (IC_{50} = 17 ± 2 nM, Table S1). Similar to the AT118-AT206 chimaera, multivalency amplifies functional efficacy while preserving target specificity.[39] Unlike small molecules, nanobodies uniquely combine compact size, evolvable scaffolds(e.g., single-point mutations converting AT209 from an allosteric to a competitive antagonist; Figure 3K), and compatibility with bispecific formats.[39] For AT1R-targeted therapeutics, nanobodies such as AT118 and AT118i4 demonstrate precise pharmacological reprogramming.[40] AT118i4 acts as a high-affinity inverse agonist (K_D = 48.4 nM) with signaling bias—modulating Gq activation (EC_{50} = 89 nM) and β-arrestin recruitment (EC_{50} = 130 nM, Table S1), which mirrors the structure-guided engineering of AT206, where CDR1 substitution converted allosteric modulation to competitive antagonism.[39] In vivo, AT118i4 reduces arterial pressure by 28.5 ± 4.2 mmHg in hypertensive models, achieving efficacy comparable to losartan while avoiding non-selective receptor perturbation.[41,42] Critically, nanobodies exploit ECLs for isoform discrimination, as seen in AT209's ECL2 β-sheet interaction (Figure 3K) and AT206's direct contact with losartan (Figure 3L).[26,43-45]

In the realm of μOR analgesic target, conventional opioids like morphine and fentanyl have limitations in use due to adverse effects, including respiratory depression and dependency. Building on this approach, the antagonistic nanobody NbE—targeting the extracellular domain of μOR with its CDR3—binds aromatic residues Tyr^{106}, Phe^{107}, Tyr^{108}, as well as crucial sites in the transmembrane region (e.g., His^{297}, Asp^{147}), forming a β-hairpin structure that deeply penetrates the orthosteric pocket of μOR.[46] NbE establishes hydrogen bonds and hydrophobic interactions with these residues to inhibit μOR activity effectively (Figure 3I). Selective targeting of μOR by NbE relies on interactions with ECL2 (Lys^{209} ,Arg^{211}, and Gln^{212}) and ECL3 (Glu^{310}), demonstrating specific binding preferences(Figure 3I). Mutational analysis indicated that KRQL-A mutations decreased binding affinity, underscoring the importance of the ECLs in isoform recognition. NbE effectively blocks DAMGO/morphine-induced cAMP inhibition and β-arrestin recruitment with high affinity in the nanomolar range (K_D=56 nM, Table S1) and maintains its antagonistic properties when expressed on the membrane via GPI anchoring. The cyclic peptide 8 based on the CDR3 structure (with a D-Pro-L-Pro rigid scaffold) reproduced the function of NbE (K_i = 39 nM), completely reversing the DAMGO effect at 10 μM and showing no cross-activity against other ORs, thus achieving the molecular miniaturization of the active centre of the nanobody. The insertion-type binding mechanism of the μOR family nanobody complex structure provides a new paradigm for non-addictive

analgesics, and the CDR3 peptide inherits the targeting ability of nanobodies.

Among class A GPCRs, only orexin receptor 2 (OX2R) and apelin receptor (APJ) have formed complex structures with extra-VHHs. The anti-OX2R nanobody Sb51 binds atop a small-molecule agonist, partially overlapping the binding site of the natural peptide orexin B (Figure 3D), potentially stabilizing conformations linked to biased signaling.[47] JN241, a nanobody antagonist targeting APJ, binds with K_D = 83 pM via extensive ECL interactions and CDR3 insertion; it inhibits cAMP (IC_{50} = 5.9 nM) and β-arrestin recruitment (IC_{50} = 7.0 nM).[48] Small-molecule APJ antagonists (MM54, IC_{50} = 93 nM) and peptide antagonists (ALX 40-4C, IC_{50} = 2.9 μM) are less potent and lack the precision of nanobody-receptor engagement,[49,50] highlighting the potential of nanobody-based antagonists to offer enhanced receptor modulation in selectively biased signaling pathways, using their high specificity and structural precision, positioning them as promising candidates for therapeutic development in complex GPCR systems.[47,48]

Extracellular nanobody acting as agonist or PAM

Beyond antagonism through blockade of endogenous ligand binding, extracellular nanobodies can also activate GPCRs as agonists or potentiate signaling as positive allosteric modulators (PAMs). For most class A GPCRs, the relatively short N-terminus and limited ECL surface make it difficult to generate conventional monoclonal antibodies that recognize native conformations, in contrast to many class B GPCRs, which have larger, more antigenic N-terminal domains. As a result, agonistic or PAM-like nanobodies for class A targets are typically obtained through structure-guided selection/engineering rather than classical immunization.

A representative example is the apelin receptor APJ (a Gαi-coupled class A GPCR) with endogenous ligands Apelin and Apela, which regulates humoral homeostasis and cardiovascular function; an agonistic antibody format could therefore have therapeutic value in chronic heart failure.[51] Guided by the APJ-ligand structural framework and inspired by the antagonist JN241 that binds ECL2, the agonistic nanobody JN241-9 was engineered: insertion of a tyrosine residue between E104 and S105 converted JN241 into the full agonist JN241-9, illustrating that targeted, structurally guided mutations—particularly in CDR3—can switch pharmacology from antagonism to agonism. Notably, the engineered nanobody showed pathway selectivity, favoring G protein signaling over β-arrestin recruitment, consistent with biased modulation.[48]

In class C GPCRs, the first reported PAM nanobodies for mGlu2—DN10 and DN13—enhance the agonistic potency of LY379268.[52] They engage partially overlapping epitopes within the orthosteric ligand-binding region and potentiate the inhibitory effects of the orthosteric mGlu2/mGlu3 agonist DCG-IV in hippocampal slices. DN10 displays intrinsic agonist activity (partial mGlu2 activation), whereas DN13 alone has no effect; yet, in the presence of DN13 (Figure 3E), a low concentration of DCG-IV nearly reaches the maximal effect seen with saturating drug concentrations (30.5 ± 1.9%). Given DN10's partial agonism, DN13 was considered more selective and was preferred for in vivo studies. When infused into the CA3 region of the hippocampus, DN13 alone did not affect contextual fear memory consolidation, but it significantly enhanced the effect of low-dose DCG-IV, further disrupting contextual fear memory without altering fear memory consolidation. Together, these data underscore the selective role of mGlu2 homodimers in memory modulation and illustrate how nanobody PAMs can fine-tune receptor responses via biased signaling. VPAC1 (vasoactive intestinal peptide receptor 1) monovalent nanobodies targeting the extracellular N-terminal domain are highly specific and have moderate affinity for the receptor, but lack functional activity. Their binding is enhanced by VPAC1 agonists (VIP, PACAP-27) and weakened by antagonists, suggesting weak allosteric regulation under these conditions.[53] After fluorescent conjugation, they can detect VPAC1 on human leukocyte surface at levels comparable to mouse monoclonal antibodies, and can be used for immunohistochemical staining of human gastrointestinal tissue sections.

Nb29 and other nanobodies targeting the α_{1A} adrenergic receptor (α_{1A}AR) have shown their versatility as PAMs.[54] Nb29 selectively binds to the extracellular vestibule of α_{1A}AR, distinct from the orthosteric pocket, with a preference for the oxymetazoline-bound state. This binding either stabilizes the receptor in an inactive conformation or prevents ligand access to the orthosteric pocket, thereby inhibiting receptor activation (Figures 3A-B). Although Nb29 does not act as an agonist and does not significantly affect the EC_{50} values of oxymetazoline or norepinephrine, it moderately reduces the receptor's maximal signaling efficacy. This suggests that Nb29 may function as a weak PAM or neutral allosteric modulator, with its CDR3 loop forming charge networks with $E180^{ECL2}$ and $E305^{7.32}$, and a cation-π interaction with $F308^{7.35}$, stabilizing the inward conformation of TM7; residues at 7.35 are also known to be important for PAM/NAM binding in muscarinic acetylcholine receptors. Nb29 shifts agonist competition curves more strongly for oxymetazoline than for noradrenaline, due to a preference for the oxymetazoline-bound state, and emphasizes the conformation dependence of its function.

Similar to monoclonal antibodies, screening directly for nanobodies that function as agonists or PAMs remains challenging. However, targeted GPCR nanobodies acting as agonists can be obtained through direct structural or genetic engineering techniques, or by coupling them to natural peptide agonists or their analogs.

Extracellular nanobody targeting MC4R: full agonist development

MC4R is a class A GPCR that is important for appetite and energy balance via the leptin-melanocortin system, an established target for obesity treatment.[55,56] However, current drugs lack selectivity. Peptide agonists such as α-MSH (EC_{50}: 1.9 nM, 0.9 nM, and 30.0 nM, Table 2) or setmelanotide (EC_{50}: 11.5 ± 4.1 nM, 2.1 nM, 451.0 nM, Table 2) for MC1R, MC3R, and MC5R can cause off-target, pigmentation-related side effects. Table 2 summarizes representative MC4R agonists from nanobody, peptide, and small-molecule modalities, outlining differences in potency, signaling, selectivity, and safety profiles. To address subtype specificity, a fully activating orthosteric nanobody agonist, pN162, was isolated from an immunized camelid repertoire using a screening strategy with an MC4R-β2AR chimeric receptor and a conformational binder (ConfoBody, Cb80).[57] Notably, cryo-EM at ~3.4 Å reveals that the elongated CDR3 of pN162 inserts deeply into the MC4R orthosteric pocket and occupies the ligand-binding space. Unlike melanocortin peptides, pN162 uses the CDR3 residue R101 and mimics the Ca^{2+} binding site by forming salt bridges with $E100^{2.60}$ and $D126^{3.29}$, both of which are required for Ca^{2+}-dependent peptide activation. This enables pN162 to activate MC4R by driving key conformational changes, such as TM6 movement, DRY motif remodeling, and CWxP/toggle-switch shifts needed for downstream signaling (Figure 3J).[57]

As a structural mechanism, pN162 has high selective power both in binding and functionally. At 1 μM, it binds MC4R but not other human melanocortin receptor homologs. Functionally, it can activate MC4R significantly (EC_{50} = 12.5 ± 5.8 nM) but does not activate MC1R/MC3R/MC5R, and thus selectively agonistically targets MC4R in these systems. The structural basis for this selectivity is further supported by three MC4R residues—$V103^{2.63}$ (contacting CDR1 F29), $F184^{4.60}$ (contacting I^{102}), and $Q273^{ECL3}$ (contacting framework Y^{37}) -which form exclusive contacts with pN162 not conserved or only partially conserved in other melanocortin receptor subtypes. Definitive attribution, however, will require reciprocal cross-subtype mutagenesis.[57]

At the signaling level, pN162 can activate Gs and arrestin, and partially activate Gq pathways. Its Gq signaling activity(EC_{50}=121.3 nM, Table 2) is much lower than that of setmelanotide (EC_{50}=9.9 nM, Table 2).[57] Given that Gq-related pathways can affect cardiovascular function, this difference may be important for safety discussions. Notably, previous studies have shown that setmelanotide does not affect blood pressure or heart rate. Together, these findings indicate that pN162 leverages both 'orthosteric occupancy-driven activation' and 'subtype-specific interface determinants.' This dual mechanism compensates for some off-target effects of peptide agonists and supports the use of the nanobody strategy to target precise interventions in MC4R-associated CNS and metabolic diseases.

THERAPEUTIC APPLICATIONS OF NANOBODY AND FUTURE PROSPECTS FOR GPCRS

Nanobodies for Orphan GPCRs

Orphan GPCRs (oGPCRs) are a class of GPCRs without clearly identified endogenous ligands.[64] The absence of natural ligands and limited signal annotation restricts traditional small-molecule R&D, which relies on endogenous clues. Small molecules also have difficulty achieving subtype selectivity

Table 2. Pharmacological properties of representative MC4R agonists across different molecular modalities.

Drug	Type	Action	Disease	Status	IC_{50}	EC_{50}	Affinities	Pharmacodynamic features	Safety / Tolerability
pN162[57]	Nanobody	Agonist	Obesity, MC4R deficiency	Preclinical	-	hMC4R:12.5±5.8 nM; hMC1R/MC3R/MC5R: no effect;	K_d(hMC4R)=2.3nM	Subtype-selective full MC4R agonist; stabilizes theorthosteric active conformation via CDR3 insertion; strong Gs signaling with attenuated Gq activation, consistent with biased signaling	No detectable activity at MC1R/MC3R/MC5R; potentially reduced pigmentation-related and off-target risks; in vivo safety remains to be established
Setmelanotide (RM-493)[56]	Peptide	Agonist	Monogenic obesity (POMC, PCSK1, LEPR)	Approved	-	hMC4R: 2.0±1.1 nM; hMC1R: 11.5±4.1nM; hMC3R: 2.1nM; hMC5R: 451.0 nM; rMC4R:0.28 nM;	K_i (hMC4R)=2.1nM, K_i (rMC4R)=2.7nM	Potent MC4R agonist activating Gs and β-arrestin pathways; limited clinically relevant Gq signaling	Generally well tolerated; nausea and injection-site reactions common; pigmentation effects reported due to partial MC1R activation
α-MSH [57]			Obesity due to POMC, PCSK1, or LEPR deficiency		-	hMC4R: 14.1±7.7 nM; hMC1R: 1.9 nM; hMC3R: 0.9 nM; hMC5R: 30.0 nM	K_i(hMC1R)=0.12nM; K_i(hMC3R)=31nM; K_i(hMC4R)=660nM; K_i(hMC5R)=5700nM	Endogenous non-selective melanocortin agonist; activates multiple receptor subtypes without signaling bias	Short half-life; pigmentation and systemic off-target effects limit chronic use
Bremelanotide Acetate[58]			Sexual dysfunction		-	Concentration of radioligand: hMC3R:0.4 nM; hMC4R:0.2 nM	K_d(hMC4R)=10 nM	Central melanocortin activation; non-selective receptor engagement	Transient hypertension and nausea; cardiovascular monitoring recommended
Tetracosactide (Tetracosactrin)[59]			ulcerative colitis, Crohn's disease, Rheumatoid arthritis, Osteoarthritis	Preclinical	-	hMC4R:0.65 nM	-	Broad melanocortin receptor activation with limited subtype selectivity	Systemic endocrine effects limit long-term metabolic application
RO27-3225 TFA[60]			Neurological disease Inflammation/Immunology		-	hMC4R: 1nM; hMC1R: 8 nM	-	Partial subtype selectivity; limited conformational and signaling bias control	Preclinical stage; off-target profile under evaluation
PF-00446687[61]	Small molecule	Selective agonist	Endocrinology	Preclinical	-	hMC4R: 12±1nM; hMC1R: 1.02±0.30μM; hMC3R: 1.16±0.35μM; hMC5R: 1.98±0.20μM	K_i(hMC4R)=27±4nM; K_i(σR)=330nM; K_i(NaV channel) =690nM; K_i (M2 mAChR) =730nM	Selective MC4R activation with reduced activity on other melanocortin receptors	Off-target binding observed at σ receptors and ion channels at higher concentrations
THIQ[62,63]			Eliciting erectile activity in rodents		hMC4R: 1.2nM; rMC4R: 0.6nM	hMC4R: 2.1nM; rMC4R: 2.9nM	-	MC4R agonist and pharmacoperone; improves surface expression of misfolded MC4R mutants	CNS-related effects require further evaluation

Abbreviations: h, human; r, rat; σR, σreceptor; NaV, voltage-gated sodium channel; mAChR, muscarinic acetylcholine receptor. Values are reported as presented in the original studies. -, data not available.

among homologous GPCRs, raising developmental challenges. Still, oGPCRs are linked to various diseases, presenting significant drug discovery opportunities (Table 3).[65]

Nanobodies are not a new idea: they target cell-surface epitopes, stabilizing specific receptor conformations and pathway states, activating or inhibiting receptors in the absence of endogenous ligands, and inducing biased regulation.[66,67] Nanobodies frequently exhibit higher subtype selectivity among homologous receptors, which reduces the risk of off-target effects.[13] When combined with engineering methods that extend half-life and enhance stability, nanobodies become more feasible for long-term treatment of chronic diseases.[68,69]

oGPCRs such as GPR6 and GPR37 are linked to neurodegenerative diseases, including Parkinson's disease (PD). GPR6 regulates the dopamine pathway, and GPR37 regulates the dopamine transporter. Imbalanced GPR37 can worsen disease progression and symptoms. Thus, nanobodies targeting GPR6/GPR37 may be promising signal reprogramming tools.[70,71] In metabolism, protective variants of GPR75 are linked to anti-obesity effects, suggesting that nanobodies targeting GPR75 could address obesity and metabolic syndrome.[72] In cancer, GPR161 is considered pro-tumor in adult neural tube-derived tumors. Selective GPR161 inhibition may suppress tumor growth while sparing normal tissues.[73] In cardiovascular and inflammatory diseases, GPR17 functions as a receptor for tissue damage and plays a role in the response to brain injury and inflammation. Nanobodies targeting GPR17 can modulate its activity, promoting repair mechanisms to enhance recovery following stroke or traumatic brain injury.[74,75] GPR183 controls immune cell behavior, and GPR183 nanobodies offer targeted immunomodulation in chronic inflammation, such as inflammatory bowel disease (IBD).[76]

oGPCRs offer targets for neurological, metabolic, oncological, cardiovascular, and inflammatory diseases. Nanobodies—with small size, high affinity, and conformational selectivity—regulate oGPCR signaling, enhancing treatment specificity and effectiveness to address unmet clinical needs.

Bispecific and multivalent nanobodies for GPCR heterodimers

Nanobody engineering provides a practical approach to studying complex interactions among GPCR heterodimers. It also presents a novel therapeutic approach for these interactions.[125] Unlike monomeric or homodimeric GPCRs, heterodimers exhibit distinct pharmacological properties, including altered binding, receptor activation, signaling, and trafficking, which regulate metabolic functions, cardiovascular health, immune response, and neural responses.[126,127] Recent studies have shown that heterodimerization of GPCRs can affect binding affinity, downstream signaling, and receptor translocation, suggesting that we need more precise tools to control signaling and optimize pharmacodynamics in therapeutic interventions.[128]

Bispecific formats can bind simultaneously to both protomers of a heterodimer, achieving dual-site occupancy/dual-channel regulation.[129] This mode of engagement enhances selectivity and improves pharmacodynamics while potentially reducing the risk of off-target effects. The multivalent format can bind to multiple sites or receptor complexes, increase affinity and conformational stability, and prolong functional duration.[10,130]

In the central melanocortin system, MC3R and MC4R can form heterodimers that alter how the receptors regulate energy balance and appetite. As a result, bispecific or trivalent nanobody formats could, in principle, be designed to target these interactions more precisely than single-target ligands.[126,131] Similar approaches can also be applied in oncology (where heterodimers play a role in tumorigenesis and metastasis) and cardiovascular/inflammatory diseases (e.g., the pathways of GPR17 and GPR183).[76,100] In general, bispecific, trivalent, and multivalent nanobodies provide an extensible toolkit for targeting GPCR heterodimers and should better balance safety and efficacy.

NANOBODIES IN GPCR MODULATION: MECHANISMS, CHALLENGES, AND FUTURE DIRECTIONS

Nanobodies that target the extracellular regions of GPCRs (extra-VHHs) recognize specific sites on the receptor surface, with ECL2 being a common binding site. In class A GPCRs, ECL2 is often the longest and most structurally distinct loop, shaping the channel for the entry and exit of molecules and influencing drug recognition, activation, and selectivity.[132] However, ECL2's flexibility makes it difficult for traditional drugs to bind effectively. Nanobodies, with their small size and flexible structure, can access cryptic and conformation-dependent epitopes and modulate these extracellular regions. Supporting this, the anti-CXCR4 nanobody VUN400 can bind to ECL2 and modulate receptor activity,[20] while nanobodies targeting ChemR23 and AT1R also use key surface regions (including areas near ligand-binding sites) to achieve selectivity and regulate function.[37,40]

Functionally, extra-VHHs can block or compete with natural ligands at their binding sites, acting as antagonists or agonists, or they can act as NAMs or PAMs to alter the effects of antagonists or agonists (Figure 3). It should be emphasized that NAM/PAM effects are highly dependent on the receptor-ligand context: a NAM may still reduce signaling output even when the receptor is already bound to an agonist, and a PAM may also disrupt the cooperative allosteric mechanism in an amplifying manner, highlighting the need for careful balancing of allosteric effects in therapeutic design.[133] A key benefit of extra-VHHs is their ability to recognize specific receptor shapes: generating nanobodies from camelids using cells that display GPCRs with native post-translational modifications, molecules that recognize authentic, conformation-dependent receptors are often produced.[134] This increases specificity, reduces unwanted side effects, and helps guide signals toward the desired response, fitting the goals of precise drug targeting.[135]

A representative technological advancement is CLAMP (Conjugation of Ligands and Antibodies for Membrane Proteins), which links short GPCR ligands to the C-terminus of nanobodies via chemical coupling, forming a ligand-antibody conjugate that can accurately target receptors and regulate signal transduction.[136] By tethering a ligand close to the receptor, CLAMP can transform weak or poorly selective ligands into potent, functionally biased modulators. For example, coupling the weak PTH(1-11) peptide to a high-affinity anti-PTHR1 nanobody (Nb_{PTHR1}) yields a conjugate with EC_{50} = 3 nM, matching the activity of full-length PTH (1-34, EC_{50} > 500 nM) in Gs/cAMP signaling while abolishing β-arrestin and Gq/Ca^{2+} pathways (Table S1), thus functioning as a highly selective PTHR1 agonist with > 5000-fold enhanced signaling bias—an attractive direction for separating beneficial from harmful signaling in diseases such as osteoporosis. Furthermore, CLAMP enables transactivation of neighboring GPCRs: the nanobody binds one receptor, while the linked peptide activates another, thus extending understanding of GPCR signaling. Additionally, CLAMP's flexibility is demonstrated by its use with GLP1R (glucagon-like peptide-1 receptor)—attaching inactive GLP1 (glucagon-like peptide-1) mutants to nanobodies restores Gs activity and blocks β-arrestin signaling, suggesting new strategies for treating metabolic disorders.[9] In essence, nanobodies' dual role as inhibitors and precise allosteric carriers, highlights their potential as therapeutic interventions.

Overcoming physiological barriers, such as the blood-brain barrier (BBB), is a major hurdle for biologics targeting central nervous system (CNS) indications. The tightly joined endothelial cells and selective transport systems restrict entry of large biomolecules into the CNS, and only a small proportion of peripherally administered proteins reach the brain. Nanobodies have emerged as promising tools for CNS delivery due to their relatively small size, structural simplicity and often cationic charge, which enables interaction with the negatively charged cell membrane and promotes brain uptake via adsorptive-mediated transcytosis. Notably, nanobodies may overcome biological barriers, such as the BBB more efficiently than conventional antibodies. Engineered nanobody constructs have demonstrated the potential to exploit these pathways; however, achieving consistent and efficient BBB penetration remains a formidable task. To address these delivery challenges, receptor-mediated transcytosis (RMT) systems—such as Trojan Horse strategies using nanobody fusions with ligands targeting the transferrin receptor (TfR), which is highly expressed on brain endothelial cells—have been explored to enhance CNS uptake of biologics.[137-139] For instance, TfR-targeting nanobody constructs can deliver linked proteins, such as neurotensin (NT), into the brain, demonstrating functional RMT transport.[139] Optimizing receptor engagement, trafficking, and transport efficiency to favour transcytosis and minimize intracellular degradation remains an ongoing challenge. Furthermore, nanobodies' simple structure facilitates humanization and generally reduces immunogenicity, while their versatility as orthosteric antagonists/agonists or allosteric modulators enables precise control of signaling pathways—an advantage for developing targeted thera-

Table 3. Representative GPCRs targets associated human diseases.

GPCR Classification	Orphan Receptor Names	Associated Diseases	References
A	GPR6	Parkinson disease	71
	GPR17	Stroke, Brain and spinal cord trauma, Multiple sclerosis	76
	GPR20	Gastrointestinal stromal tumor	77
	GPR26	Obesity, Neurodegenerative diseases	78
	GPR27	Hepatocellular Carcinoma, Gastric cancer	79,80
	GPR37	Parkinson disease, Inflammation, Pain, Autism, Brain tumors.	70
	GPR37L1	Familial progressive myoclonus epilepsy	81
	GPR39	Neurodiseases, Cardiometabolic disease	82
	GPR45	Obesity	83
	GPR52	Schizophrenia, Psychiatric disorders	84
	GPR61	Obesity, Cachexia	85
	GPR64	X-linkedcongenital bilateral absence of the vas deferens (CBAVD); Male infertility	86
	GPR75	Metabolic syndrome, Obesity	72 87
	GPR84	Inflammation	88,89
	GPR85	Schizophrenia	90
	GPR101	X-linked acrogigantism	91
	GPR171	Anxiety and mood disorder	92
	GPR139	Neuropsychiatric and behavioural disorders	93
	GPR141	Autoimmune disease	94
	GPR155	Autism spectrum disorders	95
	GPR160	Epithelial to mesenchymal transition (EMT)	96
	GPR161	Pediatric Medulloblastoma.	73
	GPR176	Profibrotic, Colorectal Cancer	97,98
	GPR182	Melanoma	99
	GPR183	Inflammatory bowel diseases (IBD)	100
B	CALCR	Osteoporosis	101
	CRHR1	Depression, Anxiety disorders	104,105
	SCTR	Diabetes	106
	LGR4	Cancer	107,108
	LGR5	Cancer	109,110
	GCGR	Diabetes	111
	GLP1R	Diabetes	112
	GLP2R	Obesity	113
	PTH1R	Osteoporosis, Cardiovascular disease, Homeostasis	114,115
	VIPR1	Hepatocellular carcinoma, Inflammatory diseases	116,117
	VIPR2	Eye diseases	118
Adhesion	ADGRE5	Rheumatoid arthritis	119
	GPR56	Brain malformation, Myelination defects	120
	GPR110	Cancer, osteoporotic	121-123
	GPR133	Glioblastoma	124

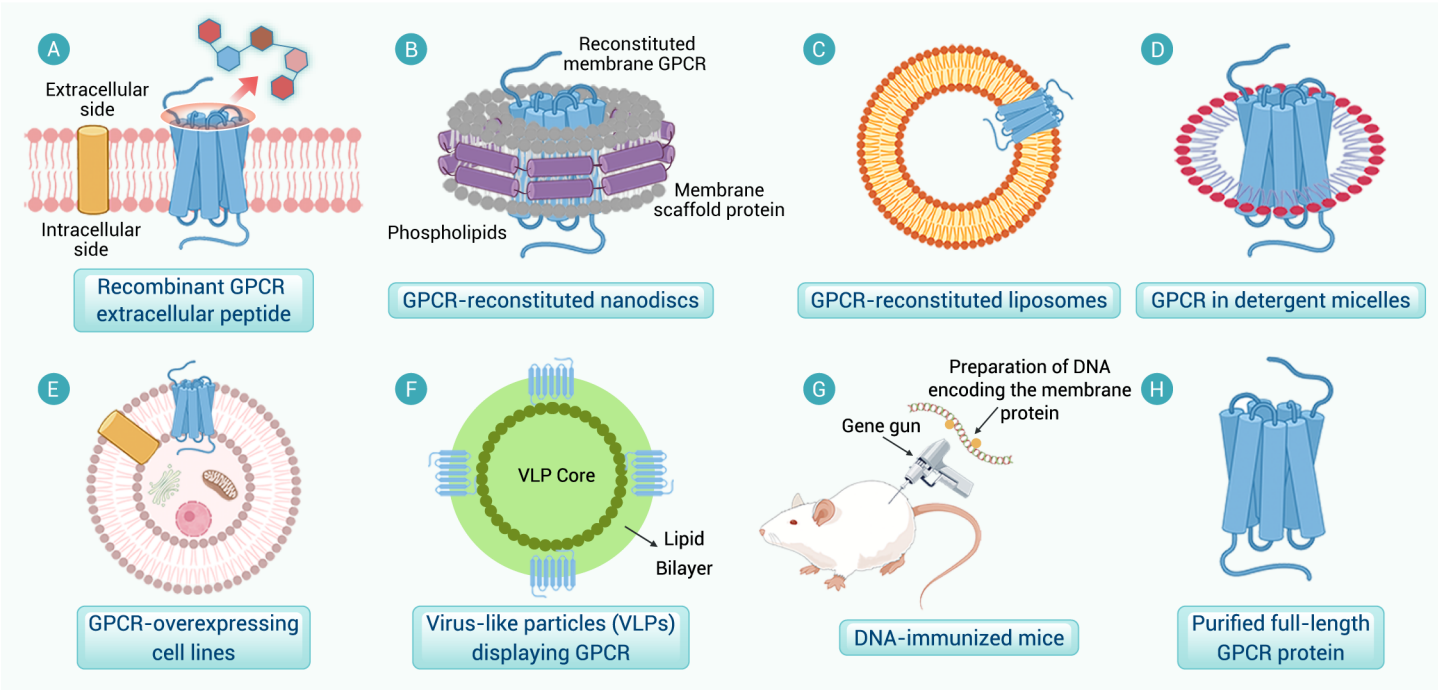

Figure 4. Schematic overview of GPCR expression strategies Various methods are used to prepare GPCR antigens. These include recombinant peptides from the N-terminal or loop regions of GPCR (A), nanodiscs with GPCR (B), liposomes carrying GPCR (C), cell membrane fragments containing GPCR (D), cells with enhanced GPCR expression (E), and virus-like particles (VLP) presenting GPCR (F). Other methods use DNA immunisation in mice (G) and purified full-length GPCR proteins (H).

pies for neurological and immune disorders.[10]

Nanobody drugs targeting GPCRs begin with library selection: naïve libraries are broad but often require significant optimization to achieve high affinity and potency. Immunized libraries are higher-affinity binders but entail additional time and logistical challenges. Furthermore, synthetic libraries eliminate the need for animal immunization but rely heavily on library design and selection conditions (Figure 5). Once the appropriate library is selected, antigen preparation is crucial, particularly given the conformational diversity of GPCRs arising from their seven transmembrane segments. Techniques such as protein fusion, virus-like particles (VLPs), and membrane-mimetic models maintain the native target structure during selection (Figure 4). By combining efficient GPCR antigen preparation with advanced antibody screening, teams generate diverse therapeutic nanobodies with high specificity and affinity for their targets, which provides a solid foundation for targeted GPCR drugs and accelerates advancements toward new and effective therapeutic solutions.

Advancements in nanobody technology have greatly expanded their therapeutic potential, enabling applications beyond the conventional. Developing bivalent and bispecific nanobodies with two identical or different paratopes improves their binding avidity is improved through dual-paratope binding.[140,141] As a result, such nanobodies are valuable for treating complex diseases involving multiple signaling pathways, such as cancer and neurological disorders.[142] For example, Fc-fused nanobodies can trigger antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[32] CXCR4-targeting nanobodies show increased potency in inhibiting CXCR4-mediated signaling and HIV entry;[143] they also induce ADCC- and CDC-mediated cell death in CXCR4-overexpressing cells, highlighting the flexibility of nanobody-based approaches in addressing complex disease mechanisms.[19] Nanobodies can also promote biased signaling by selectively stabilizing specific GPCR conformations, thereby avoiding detrimental effects and demonstrating superior receptor targeting and therapeutic performance compared with monovalent nanobodies.[144,145] Recent developments include PAGERs (Programmable Antigen-Responsive Cellular Behavior GPCRs), which combine high-affinity nanobodies and conditional self-inhibitors at the extracellular N-terminus of GPCRs, triggering receptor activation and allowing control of downstream responses such as transgene expression, real-time fluorescence signaling, and endogenous G-protein activation.[146] Importantly, these systems also highlight emerging challenges in controllability, durability, and in vivo performance that must be addressed before broader therapeutic translation.

Nanobody-based therapeutics targeting GPCRs show promise but face challenges, including rapid renal clearance, proteolytic degradation, and limited tissue residence. Approaches encompassing protein engineering and nanoparticle-based delivery seek to address these challenges by enhancing half-life and biodistribution but introduce trade-offs in terms of size, activity, and manufacturability, underscoring the need for optimized design and delivery mechanisms that ideally combine experimental and computational strategies to enhance stability and efficacy.[147] Artificial intelligence (AI) will fundamentally address key challenges in nanobody-based GPCR therapy (Figure 5). By analyzing large datasets, AI can early improve nanobody binding, stability and selectivity early, minimising trial-and-error.[148,149] Machine learning models can further advance molecular design by predicting nanobody-GPCR interactions and enabling new techniques such as bispecific nanobodies.[147,150] AI supports manufacturing by pre-selecting molecules for stability and production efficiency.[151] For GPCRs in particular, AI will be most powerful when coupled with experimentally resolved structures and/or computationally sampled conformational ensembles, enabling prioritization of conformation-selective epitopes, screening of CDR3 geometries compatible with recessed pockets and ECL topologies, and early filtering for manufacturability and polyreactivity risks.[149] A realistic near-term roadmap would focus on AI-guided VHH design using structural and conformational ensembles combined with iterative functional selection, rather than simply replacing experimental screening with in silico predictions.[147] The JAM (Joint Atomic Modelling) platform enables rapid, large-scale generation of functional anti-GPCR VHHs—producing hundreds of highly selective, picomolar-affinity nanobodies targeting CXCR4 and CXCR7, including the first reported CXCR7 antibody agonists (EC_{50} = 63 nM) with potencies close to the endogenous ligand SDF-1 (EC_{50} = 22.5 nM).[152] JAM's experiment-guided targeted generation system can generate over 300 ideal CXCR7 agonists in a week with a single sample, achieving more than 7500-fold enrichment in functional success rate, expression yield, and stability, and can design complex functional nanobodies without iterative experimental screening.[152] Future advances, including test-time computational scaling (e.g., JAM multi-round self-optimization) and predictive modeling, will enable the modeling of conformational changes and signaling pathways, and generate antagonistic antibodies simultaneously to direct and control signaling bias in the GPCR

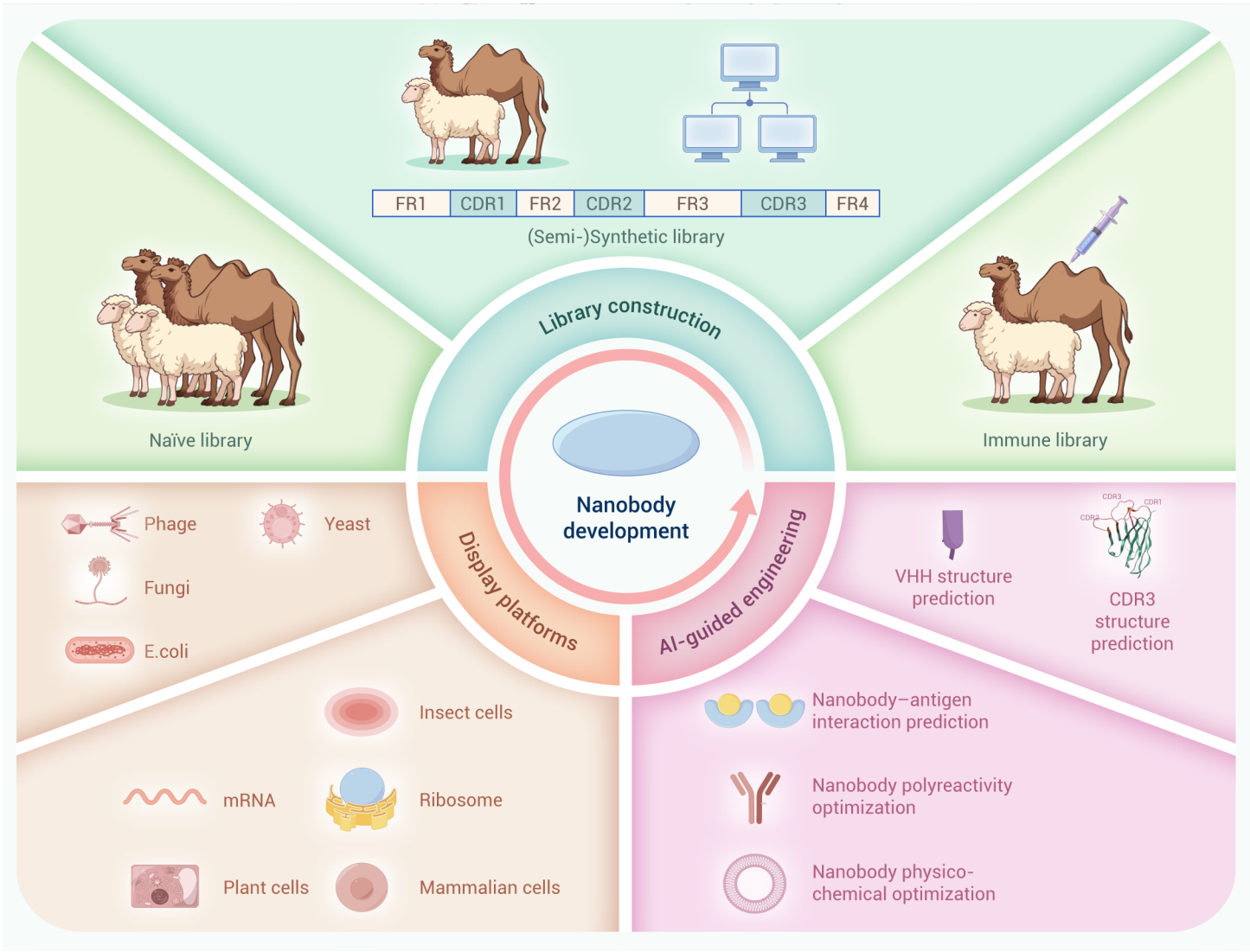

Figure 5. Schematic overview of the process for nanobody (Nb) production and optimization Nanobody production begins with the construction of libraries, which can be immune libraries from immunized camelids, naïve libraries from non-immunized animals, or semi-synthetic libraries designed in silico with targeted CDR diversification. Once constructed, these libraries are screened using display platforms such as phage, yeast, E. coli, fungi, mammalian cells, plant cells, insect cells, ribosome display, and mRNA systems. Advanced artificial intelligence engineering techniques are then applied to optimize nanobody properties, including predicting nanobody-antigen interactions, reducing polyreactivity, improving physicochemical properties, and predicting the structures of the VHH and CDR3 regions.

pathway. AI models will also take into account early factors, such as expression levels, monomer stability (> 90%), low multispecificity (e.g., a BVP score significantly lower than that of Ustekinumab), and high yield (> 75 mg/L) to ensure the product is suitable for large-scale industrial development and optimization. Additionally, the compact, flexible JAM-generated nanobodies seamlessly integrate with next-generation delivery platforms, improving targeting, half-life, and biodistribution. Future JAM developments, including multi-round computational optimization and ligand-induced conformational modeling, aim to predict epitopes for complex multi-transmembrane conformations and non-templated scaffold generation (with deviations from natural antibodies exceeding 7Å), providing access to previously unreachable GPCR states and assemblies. Together, these advantages position JAM as a distinct leader in translating nanobody-based modulation of GPCRs into viable clinical therapies.

Multifunctional nanobodies link immune cells with targeted therapies, including antibody-drug conjugates (ADCs), CAR-T cell therapies, and bispecific antibodies targeting GPCRs expressed in tumors, thus enhancing therapeutic precision. When conjugated with drugs or imaging agents, nanobodies form nanobody-drug conjugates (NDCs), which specifically target cancer cells, minimizing collateral damage to healthy tissues.[153,154] Nanobodies can also serve as antigen recognition domains in CAR-T cells, enhancing T-cell recognition and facilitating the elimination of GPCR-expressing tumor cells. As these technologies advance, future clinical trials will be crucial for assessing their safety, long-term efficacy, and potential for broader applications. Their success could establish nanobodies as a transformative mainstream therapy for complex GPCR pathway-related diseases.

SUMMARY

Extra-VHHs, also referred to as nanobodies, are emerging as a feasible tool for GPCR drug discovery, as traditional small molecules or peptides often struggle to achieve subtypes selectivity or to stably achieve predictable signal outputs. By recognizing ECL regions of receptors or more cryptic conformation-sensitive epitopes, extra-VHHs can act as antagonists/agonists to modulate receptors or as allosteric modulators, stabilizing specific receptor conformations, and fine-tuning signaling bias. Nanobodies thus offer greater therapeutic value in disease states that require wider therapeutic windows and reduced off-target effects within a given drug class. A number of challenges must be addressed when translating the clinical use of nanobodies, including but not limited to tissue distribution, low in vivo exposure due to renal clearance, protease degradation, the risk of immune response, and production and formulation constraints. Potential solutions involve strategies such as extending half-life (Fc/albumin binding, PEGylation), multivalent/bispecific design, and enhanced delivery methods (especially in CNS scenarios). In the future, AI and structure-guided processes will likely

function as experiment-driven accelerators: on the one hand, by characterizing conformational ensembles of GPCRs and identifying extracellular epitopes relevant to the desired drug, on the other hand, by suggesting CDR structure designs geometrically matched to the ligand entry/vestibule regions and excluding potential development risks (e.g., aggregation, polyreactivity, lack of stability). A near-term approach will not let AI replace traditional screening. Instead, it will focus on defining structures and conformational ensembles, creating AI-generated candidate designs, building focused libraries, performing functional screening for bias and potential, using iterative optimization, and generating GPCR nanobody therapeutic candidates with conformational selectivity and improved delivery and developability, all with greater efficiency.

ABBREVIATIONS

Abbreviation	Full term
GPCR(s)	G-protein-coupled receptor(s)
TM	Transmembrane (helix)
FDA	U.S. Food and Drug Administration
BBB	Blood-brain barrier
VHH/Nb	Variable domain of heavy chain-only antibody/Nanobody
extra-VHH	Extracellular nanobody
intra-VHH	Intracellular nanobody
CDR	Complementarity-determining region
ECL/ECL1/2/3	Extracellular loop(s)
PAM/NAM	Positive/Negative allosteric modulator
GPI	Glycosylphosphatidylinositol (anchor)
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium ion
Gs/Gq	Gs-protein/Gq-protein
β-arrestin	Beta-arrestin
KD/Ki	Equilibrium dissociation constant/Inhibition constant
IC_{50}/EC_{50}	Half-maximal inhibitory/effective concentration
$pKD/pKB/pIC_{50}$	Negative log KD/KB/IC_{50}
IF	Immunofluorescence (context-dependent)
FRET	Förster resonance energy transfer
cryo-EM	Cryogenic electron microscopy
HEK293	Human embryonic kidney 293 cells
ADA	Anti-drug antibody
RMT	Receptor-mediated transcytosis
TfR	Transferrin receptor
ADCC/CDC	Antibody-dependent cellular/complement-dependent cytotoxicity
NDC/ADC	Nanobody-drug/Antibody-drug conjugate
CAR-T	Chimeric antigen receptor T cell
VLP	Virus-like particle
MACS/FACS	Magnetic-/Fluorescence-activated cell sorting
NGS	Next-generation sequencing
AI	Artificial intelligence
DRY motif	Asp-Arg-Tyr motif
CWxP (toggle)	CWxP toggle switch motif
PAGERs	Programmable Antigen-Responsive Cellular Behavior GPCRs
CLAMP	Conjugation of Ligands and Antibodies for Membrane Proteins
APJ	Apelin receptor
OX2R	Orexin receptor type 2
μOR	μ opioid receptor
PTHR1	Parathyroid hormone receptor type 1
VPAC1	Vasoactive intestinal peptide receptor 1
$α_{1A}AR$	$α_{1A}$ adrenergic receptor
AT1R	Angiotensin II type 1 receptor
ChemR23	Chemerin receptor 23
GLP1R	Glucagon-like peptide-1 receptor
MC4R/MC3R/MC1R/MC5R	Melanocortin receptor 4/3/1/5
CCR4/CCR5	CC chemokine receptor 4/5
CXCR2/CXCR3/CXCR4	C-X-C chemokine receptor 2/3/4
ACKR3 (CXCR7)	Atypical chemokine receptor 3
US28	HCMV-encoded chemokine receptor US28
HCMV	Human cytomegalovirus
GBM	Glioblastoma
HIV	Human immunodeficiency virus
VIP/PACAP-27	Vasoactive intestinal peptide/Pituitary adenylate cyclase-activating polypeptide-27
PTH(1-34)/(1-11)	Parathyroid hormone fragments
DCG-IV/LY379268	mGlu2/3 orthosteric agonists
NbE, Sb51, JN241, JN241-9, DN10/DN13, Nb29, AT118/AT118i4/AT206/AT209, CA4910/CA5183, VUN100/VUN401-403/VUN701	(Study-specific nanobody names)
JAM	Joint Atomic Modeling
GLP-1	Glucagon-like peptide-1

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AUTHOR CONTRIBUTIONS

Y.X.，Z.L., J.X., Z.F. and T.W. contributed equally. Conception and supervision: C.Z., J.L., L.J.J. Project administration and funding acquisition: J.L., L.J.J., C.Z. Writing - original draft and visualization: Y.X., Z.L. Methodology and investigation: Z.L., Z.F., S.L., F.T., H.T., L.L. Data curation and analysis: J.X., F.T., D.J.H.S., H.T. Formal analysis and visualization: S.L., D.J.,H.S., T.W., W.S.C.. Resources and validation: Z.F., S.L., L.L., C.Z.. Writing - review and editing: Y.X., L.J.J., C.Z. Final approval of the manuscript: All authors. Y.X., Z.L., J.X., Z.F. and T.W. are listed as co-first authors as they both made substantial contributions to the design, execution, and writing of this study. All authors contributed to the manuscript and approved the final version. Figures were designed with Adobe Illustrator software and modified by Figdraw and MedPeer (https://www.medpeer.cn/).

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Not applicable.

DATA AND CODE AVAILABILITY

No new data were created or analyzed in this study.

SUPPLEMENTAL INFORMATION

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