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Opportunities for Functional Selectivity in GPCR Antibodies

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Abstract

Monoclonal antibodies (mAbs) have been used for decades as tools to probe the biology and pharmacology of receptors in cells and tissues. They are also increasingly being developed for clinical purposes against a broad range of targets, albeit to a lesser extent for G protein-coupled receptors (GPCRs) relative to other therapeutic targets. Recent pharmacological, structural and biophysical data have provided a great deal of new insight into the molecular details, complexity and regulation of GPCR function. Whereas GPCRs used to be viewed as having either “on” or “off” conformational states, it is now recognized that their structures may be finely tuned by ligands and other interacting proteins, leading to the selective activation of specific signaling pathways. This information coupled with new technologies for the selection of mAbs targeting GPCRs will be increasingly deployed for the development of highly selective mAbs that recognize conformational determinants leading to novel therapeutics.

Keywords

GPCR; ligand induced conformation; biased signaling; monoclonal antibody; therapeutic antibody

Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of membrane receptors in the mammalian genome with approximately 850 members [1]. Moreover, it has been estimated that individual cell types, including cancer cells express, > 100 of the approximately 370 non-chemosensory GPCRs [2]. It is not surprising then, that GPCRs have long been targeted for drug discovery and development such that in 2012, it was estimated that 40–50% of marketed drugs act on GPCRs [3].

Another important family that constitutes a large fraction of the mammalian proteome is the immunoglobulin/antibody superfamily [4]. A unique feature of antibodies is their ability to adapt their ligand-binding domain in response to antigenic stimulation, making them a versatile framework for binding to virtually any macromolecule. In 1975, Kohler and Milstein published one of the most remarkable papers in the history of modern immunology that described an immunization and cell culture technique for the generation of antigen specific monoclonal antibodies (mAbs) [5]. As a result, mAbs have become powerful research tools and the most common form of biologics for therapeutic purposes [6].

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Although the cost of making mAbs is higher than that for small molecule drugs, mAbs are more selective and therefore tend to have fewer non-specific or off target toxicity issues. Due to their long half-life in plasma, mAbs also have a longer duration of action than small molecule drugs. Hence, they have become a very attractive class of therapeutics.

With protein therapeutics on the rise coupled with the importance of GPCRs as pharmaceutical targets, it seems obvious that there should be some therapeutic mAbs that act on GPCRs (Figure 1). However, according to the IMGT Repertoire (www.imgt.org/mAb-DB/index#Human), there are currently thirty-seven mAbs on the market, but the first mAb targeting a GPCR was only recently approved in Japan, and currently there are no GPCRs targeting mAbs in late stage clinical development. These statistics reflect a 2008 statement by Gupta et al. who described the use of GPCR mAbs in clinical applications as “underdeveloped” but with “great potential” [7]. Fortunately, the situation is changing, and there are now several GPCR mAbs in early clinical trials, mostly targeting receptors with large peptide/protein ligands such as the chemokine receptor family, or the glucagon-like peptide receptor (GLP1R) (Table 1). Since two years have elapsed since the last major review of this topic [8], it is the intent of this brief commentary to provide an update on where the field stands. In particular, we highlight new information about GPCR structure and pharmacology that has emerged regarding conformational specificity and biased signaling, and focus on methods for mAb selection that may allow more efficient generation of mAbs that recognize or stabilize specific conformational/pharmacological states of GPCRs.

Antibody selection

Traditional immunization-based approaches

One of the fundamental reasons that mAbs against GPCRs have been slow to populate therapeutic portfolios is that they are frequently difficult to make by traditional immunization-based methods [7]. GPCRs are membrane proteins that are generally expressed at low levels in cells, have relatively small mass exposed outside of the membrane, have low stability and require detergents or phospholipid membranes for solubility. Furthermore GPCRs purified and solubilized in detergents, the most common membrane mimetic, are generally unstable. For these reasons, they are difficult to purify and obtain in sufficient amounts for immunization compared to soluble proteins [7, 8].

Because of the challenges in preparing intact GPCRs for immunization [8, 9], the most common approach has been to take synthetic peptides derived from the N-terminal domains and to a lesser extent the extracellular loops (ECLs), and attach them to larger, highly immunogenic carrier proteins such as ovalbumin [9]. The resulting antibodies have unpredictable specificity, avidity and pharmacological activity, necessitating considerable screening efforts to identify those with desired properties. Therefore, they have been used primarily as probes for detection of expression and/or to understand aspects of GPCR structure/pharmacology rather than as therapeutics [7, 8]. It is particularly difficult to generate mAbs using N-terminal peptides from the class A GPCRs as immunogens, as these receptors generally have small extracellular N-termini, compared to class B and C receptors, limiting their immunogenic epitopes [10]. Furthermore, even if mAbs can be generated against N-termini, they might not show the therapeutically desired functional properties or they might recognize linear epitopes increasing the risk of cross-reactivity with unrelated proteins. It is therefore noteworthy that anti-GPCR mAbs that have entered clinical development are largely directed against the ECLs, or a combination of the N-terminus and ECLs. Other issues with the use of peptide antigens are that correct folding and the presence of post-translational modifications (PTMs, e.g. tyrosine sulfation and glycosylation) that may be present in the intact GPCRs, are generally absent in peptides [11].

Because GPCRs often have high sequence and structural homology between species, they also tend to be poorly immunogenic; for example, the chemokine receptor, CXCR4, has 90% sequence identity between humans and mice. This high degree of conservation impacts the ability of the immune system of the immunized host to produce antibodies against such GPCRs, as they are seen as “self”. B-cells that target self proteins are either deleted during embryonic development or are under very tight regulatory control, so that such ‘forbidden clones’ are never allowed to expand into antibody producing plasma cells [12].

The final issue is that one can never be certain of obtaining the types of mAbs that are desired with this approach [7, 8]. For example, following a typical immunization protocol, mAbs may or may not be generated that recognize inactive, active with agonist, constitutively active or other specific structural/conformational determinants. However, this latter issue highlights one of the reasons that GPCRs may be such good mAbs targets-- because of the possibility for “state-specific” recognition [13].

New Immunization Methods

Recently, new and improved immunization technologies have emerged that should increase the success rate over classical peptide immunization [8] and enhance the potential for the resulting mAbs to be used as therapeutics, rather than simply as pharmacological reagents. These approaches include GPCRs over-expressed on whole cells and cell membranes that allow recognition of the GPCR extracellular domains in their native conformation including non-contiguous epitopes and PTMs, as well as DNA immunizations. The downside is that the abundance of other potentially more immunogenic cell surface components can result in a high background of antibodies that recognize unwanted targets, making selection akin to finding a needle in a haystack unless the background can be eliminated by depletion steps [14]. One very recent approach that addresses the issue of background head-on involves the use of purified receptors. As a pre-requisite to the flurry of GPCR structures that have been solved since 2007 [15], there have been major advances in the ability to express GPCRs in heterologous systems, and to purify and reconstitute them *in vitro*. As described later, this technology will allow purified receptors to be used in selections, enabling one to manipulate conditions to enhance the selection of mAbs with desired properties.

State-specific recognition of GPCRs

Despite the fact that immunization approaches as generally practiced have no inherent bias, mAbs with desirable properties, such as specificity for GPCRs in agonist, antagonist and inverse agonist conformational states, have been identified using these methods [7, 13, 14, 16] (Table 2). The earliest examples came from studies of rhodopsin where a mAb to a peptide from the N-terminus recognized the activated receptor more avidly than the inactive form [17]. Gupta reported anti-N-terminal peptide mAbs or anti-sera, first to the μ opioid receptor and later to a variety of GPCRs (α_{2A} and β_2 adrenergic receptors, the cannabinoid receptor, CB₁, and the angiotensin AT₁ receptor) that recognized the agonist activated state of the receptor [7, 13]. Furthermore, the authors could follow increases in antibody binding to the μ opioid receptor following ligand binding *in vivo*. As another example, Goetzl et al., reported the generation of rat mAbs to the human sphingolipid S1P₁ receptor using transfected whole cells, one of which recognized a conformational determinant [18]. Mancina et al., were also able to isolate mAbs that recognized conformational determinants of the 5HT_{2c} receptor only when bound to ligand [14]. From these and other studies (Table 2) it is apparent that it is possible to generate mAbs that recognize specific ligand-bound conformational states (Figure 2). As well, some mAbs recognize specific conformations independent of the presence of agonists or antagonists [7, 19]. That it is possible to have mAbs with state-specific recognition is not surprising because it has been known for a long time that natural agonists generally have a preference for the active G-protein coupled state

of GPCRs over other states. Similarly, ligands can have “functional selectivity” or “ligand bias”, meaning that they can activate different signaling pathways (e.g. G protein- versus β -arrestin-dependent pathways) [20–22] or even change the trafficking behavior of receptors [23]. With the recent explosion in structural information on GPCRs, it has become clear that these functional consequences are due to the ability of the ligands to stabilize different conformational states of the receptors as illustrated in Figure 2. These findings have generated great excitement in the GPCR community because of the concept that biased ligands may prove to be superior therapeutics by minimizing signaling events that cause adverse side effects in certain diseases [24, 25]. As an example, G protein biased ligands that do not activate β -arrestin mediated desensitization are predicted to avoid β_2 adrenergic receptor induced tachyphylaxis in asthma, and μ opioid receptor mediated tolerance to analgesics [24].

The realization that it is possible to generate mAbs that recognize specific ligand-induced conformational states of GPCRs, suggests that it may be possible to develop antibodies that are sensitive to other parameters that affect GPCR function; these include association with other receptors and interaction with different intracellular proteins (i.e. G protein coupling), PTMs etc. For example, in addition to agonist and antagonist bound states, GPCRs may form homo- and hetero-oligomers (Figure 2). Rios and coworkers showed agonist mediated increases in the recognition of μ opioid receptors by a mAb when the cells were exposed to a CB_1 receptor agonist [26]. These results suggest the existence of heteromers between the two GPCRs and that the agonist induced structural changes in one partner of the heteromer could be transmitted to the other partner and detected by the mAb. Extrapolating state-specific recognition further, one could envision a mAb that recognizes a GPCR in a cell-context dependent manner -- e.g. only in cancer cells but not in normal cells. One could also imagine mAbs that induce specific conformational states and ligand bias as they may have unique therapeutic value.

In vitro selections and immunization-free approaches to mAb generation

The problem with immunogen-based approaches for generating antibodies with conformation-specific recognition is that extensive screening is required; and even then, antibodies with the desired properties may not be represented in the pool. This is because there is little or no control over the conformational state of the receptor *in vivo*, there is no selection present in the *in vivo* setting to enrich for the antibodies of interest, and because of the generally poor immunogenicity of GPCRs. To get around these issues and realize the full potential of mAbs with respect to recognition of defined states, new strategies are being developed. These strategies capitalize on the power of *in vitro* selections of recombinant antibody libraries. Clever library designs from naïve, immune and synthetic antibody repertoires combined with selection methods using phage display, yeast display, mammalian display, ribosome or cis display enable selection of high affinity antibodies with desired functional properties [27–33]. Furthermore, enhancement of screening approaches such as the use of next generation sequencing [34] empowers selection of superior mAbs.

As described above, the reliance on *in vivo* selection through classical immunization approaches largely constrains one to a “you take what you can get” result [35]. By contrast, phage display of antibody Fabs (antigen-binding fragments) or scFvs (single-chain variable fragments) coupled with *in vitro* selection allows one to manipulate the system in a variety of ways such that antibodies can be identified with specificities that would be difficult to find by traditional immunization techniques [27]. Purified, homogeneous antigens can be used to enhance the probability of identifying antibodies with pre-defined properties and can facilitate the use of anti-selection/depletion steps to further ensure specificity. Since *in vitro* antibody display technologies links the phenotype (protein) with genotype (cDNA), one also

has the opportunity to conduct rounds of mutagenesis following initial selections for affinity or specificity maturation.

Key to the success of the antibody display approaches is the library composition and diversity. Antibody libraries randomly combine heavy and light chain cDNAs obtained through RT-PCR from mRNA of B cell pools into expression vectors that afford a compartmental or physical linkage of phenotype and genotype. Large antibody libraries can mimic natural human antibody repertoires of $>10^{10}$ members in terms of molecular diversity [36, 37]. For therapeutic purposes, the strategy of using human naïve libraries alleviates the need for humanization, since the scaffold is already fully humanized, but might require affinity maturation. Libraries derived from immunized mice can also be highly useful by yielding a larger choice of high affinity immunogen-specific antibodies that can be selected by *in vitro* display methods. Multiple antibodies derived from such libraries have been humanized and have entered the clinic.

In an even more dramatic departure from traditional approaches, synthetic or semi-synthetic libraries have been engineered to introduce additional sequence diversity; these are based on mAb frameworks that are already fully human or humanized, and that have been demonstrated to have high stability and to express well [28, 38–40]. Diversity in the range of 10^{10} can be achieved from single scaffolds by introducing different amino acids through degenerate codon usage at specific sites in the hypervariable regions (CDRs) of the Fab. The synthetic nature of these libraries may have some advantages over natural libraries in that they are less biased against self-antigens. Interestingly, severe restriction of CDR diversity by designing libraries limited to as few as two (Ser and Tyr) to four (Ser, Tyr, Ala, Asp) amino acid types in the CDRs can also yield single digit nanomolar affinity binders [41, 42]. However, while providing insight into antibody/target interactions and providing great research tools, it has proven difficult to further affinity mature mAbs with severely restricted amino acid usage in the CDRs, rendering this approach less suitable for development of therapeutic mAbs. Other approaches restrict amino acid usage, but also add rational design principles against specific target classes such as carbohydrates. For example, semisynthetic libraries, have been designed by introducing basic amino acid residues at specific locations in hCDR3 to better interact with negatively charged carbohydrates, thereby expanding options to select mAbs against self antigens [43].

To our knowledge, antibodies generated against GPCRs using exclusively *in vitro* technologies have yet to be reported in the primary literature, but they are on the horizon. However, the successful results of antibodies targeting other protein classes provide useful illustrations of the power of these new technologies. To generate antibodies against a specific conformational state, one can use compounds to stabilize the purified antigen in that particular conformation (Figure 2). For example, antibodies were generated to different “on” and “off” states of caspase-1 using covalent small molecules that stabilized the respective states [44]. After rounds of selection and anti-selection coupled with affinity maturation, Fabs were identified that stabilized the two states with nanomolar affinities and selectivities of 20–500-fold over the alternate conformer. Similarly, linkage specific antibodies that recognize polyubiquitin through either K48 or K63 linked chains were generated by phage display and selection against surface immobilized polyubiquitin containing the linkage of interest; anti-selection against monoubiquitin or the alternative linkage was achieved by adding these proteins to the solution [45]. These antibodies were based on a synthetic Tyr/Ser library; and notably, attempts to generate such antibodies using traditional immunization methods did not work, a result that the authors attributed to the inability of the naturally occurring antibody repertoire to recognize such highly conserved epitopes. Similar issues may contribute to difficulties with GPCRs and, thus, these new technologies may be highly amenable to these and other membrane proteins. Consequently, GPCRs may be well suited

to *in vitro* selections so that they can be maintained under conditions that favor structural integrity, and in the presence of additives that stabilize specific conformations.

Additional emerging technologies

There are also additional technologies that may be applied to GPCRs. To stabilize GPCRs for use with phage display and *in vitro* selections of mAbs or for immunization based approaches, Heptares developed a proprietary StaR® (Stabilized Receptor) technology to engineer mutations into GPCRs that stabilize them into specific pharmacological conformations. They have applied it to the development of mAbs with agonist activity (www.heptares.com). This approach has and will be greatly facilitated by structural data on GPCRs.

Advances have also been made with constrained peptides. Pepscan's Clip Technology (www.pepscan.com) allows expression of large number of constrained peptides mimicking epitopes in the loops of receptors. The risk of such an approach is that the conformation may not completely replicate the loop structure, but a number of mAbs have been selected using this approach.

GPCRs have been expressed in liposomes and magnetic nanoparticles [46, 47]. These technologies have the advantage of generating high levels of receptor compared to cellular expression, which is highly desirable for *in vitro* selections. However, this approach does not orient the receptor, resulting in internal epitopes being exposed to mAb selection. This limitation has been overcome using virus like particles.

Nanodiscs are a new class of model membranes that consist of a single phospholipid bilayer surrounded by a protein belt derived from apolipoprotein A-I [48–50]. They have been used to functionally reconstitute several membrane proteins including the β_2 adrenergic receptor and μ opioid receptor [51, 52]. Superior stability and functionality with respect to G protein coupling has also been observed for nanodisc solubilized β_2 adrenergic receptor compared to detergent solubilized receptor and, thus, nanodiscs may prove useful for *in vitro* selections [53].

In addition, advances have been made with regard to increasing expression of GPCRs at the cell surface such as using ZFP transcription factor technology (www.sangamo.com), Selexis SureTechnology (www.selexis.com) among others. Cells with high level of GPCR expression are amenable to cell panning of mAb libraries. However, this methodology is still substantially more cumbersome than selecting on purified protein, but ensures selection of mAbs against conformationally correct epitopes.

Beyond technologies involved in the generation of mAbs, more attention is being given to the incorporation of effector functions such as antibody-dependent cellular cytotoxicity (ADCC) via binding to Fc receptors. One example is a glyco-engineered mAb (mogamulizumab, POTELIGEO®) that targets the chemokine receptor CCR4 for the treatment of T cell leukemias and lymphomas [54]. This mAb was developed with Kyowa Hakko Kirin's Potelligent® technology to eliminate fucose from the glycan PTMs and thereby enhance ADCC and anti-tumor activity. Similarly, their Complegent® Technology enhances complement mediated cytotoxicity (CDC). Xencor has also developed XmAb® and Xtend™ mAb technology for enhancing ADCC and extending half-life, respectively, although to our knowledge, they have not applied these technologies to mAbs against GPCRs (www.xencor.com).

Summary

The recognition that small molecule ligands can selectively modulate signaling properties of GPCRs by conformational stabilization has tremendous significance for the development of therapeutic mAbs that do the same. In addition, the fact that the conformations of GPCRs can be allosterically modulated by a variety of factors such as hetero-oligomerization, and that these states can be distinguished by antibodies also bodes well for the development of more finely tuned therapeutic mAbs. In the antibody field, the increased emphasis on in vitro assays that select for specific conformational/functional features of targets, the ability to bypass immunization methods and the development of methods for engineering effector functions should all contribute to the more efficient development of mAbs with precisely defined properties. As these two fields come together, we can anticipate an acceleration in the pace at which GPCR mAbs enter clinical trials. The rate limiting step for the success of such therapeutics should remain at the level of fully understanding the underlying biology of specific diseases, and here, GPCR mAbs should accelerate the validation of specific targets in pre-clinical studies.

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Figure 1. Model representation of an Fab (blue, PDB ID 3KJ6) bound to the extracellular domain of a GPCR (red, PDB ID 4DJH).

Figure 2. State-specific recognition of GPCRs

Functionally selective ligands can stabilize GPCRs in conformations that confer unique signaling properties. In principle, it should be possible to generate mAbs that recognize specific ligand-bound conformational states (A), as well as conformations induced by homo- or hetero-dimerization (B).

Table 1A

Development status of therapeutic mAbs targeting GPCRs

Target	Name	Company	Therapeutic Indication	Clinical Status
Humanized				
CCR4	Mogamuli zumab	Amgen/Kiowa Hakko Kirin	Leukemia, Lymphoma/Asthma	Phase I/II Approved in Japan for relapsed or refractory T cell leukemia and lymphoma
Human				
C3aR		Human Genome Sciences	Asthma	Preclinical
C5aR	NN-8209	G2 Therapies/Novo Nordisk	Autoimmune Disease	Phase I
CCR2	MLN1202	Takeda	Inflammation	Phase II- Discontinued
CCR4	AT0009	Affitech	Cancer	Pre-clinical
CCR5	PRO140	Progenics	HIV	Phase 2- Discontinued
CCR5	HGS004	Human Genome Sciences	HIV	Phase I- Discontinued
CCR5	HGS101	Human Genome Sciences	HIV	Discontinued
CCR8	X	Lilly	Inflammation	Undisclosed
CCR9	MLN3126	Takeda	Inflammatory Bowel Disease	Discontinued
CRTH2		Sosei/Abgenix	Inflammation	No Information
CXCR3	AT0010	Affitech	Inflammation	Discovery
CXCR4	MDX 1338	Medarex/BMS	Leukemia	Phase I
CXCR4	ALX-0651	Ablynx	Cancer	Phase I- Discontinued
CXCR4	LY2624587	Lilly	Cancer	Phase I- Discontinued
GCG-R	AMG477	Amgen	Diabetes	Phase I- Discontinued
GLP-1R	X	Abbott/Human Genome Sciences	Diabetes/Neurologic/Metabolic Disease	No information
LGR5	KM4056	Kiowa Hakko Kirin	Cancer	Pre-clinical
VPAC-1		Thrombogenics	Thrombocytopenia	Pre-clinical

The list in the table above should not be considered exhaustive; we have used the databases listed below to update and add to information published in [8]. They are the following: www.imgt.org/mAb-DB/index#Human; www.Alxn.com; www.kyowa-kirin.co.jp/english; www.gsk.com; www.janssenrmd.com; www.lilly.com; www.amgen.com; www.hgsi.com; www.abott.com; www.tpna.com; www.rosei.com; www.affitech.com; clinicaltrials.gov; <https://eudract.ema.europa.eu>

Table 1B

Development status of therapeutic mAbs targeting GPCR ligands

Target	Name	Company	Therapeutic Indication	Clinical Status
Humanized				
C5 (C5aR ligand)	Soliris™	Alexion	Paroxysmal Nocturnal Hemoglobinuria	Approved
Human				
IL-8	ABX-IL-8	Abgenix	Autoimmune Disease	Phase IIb
CCL2	Carlumumab	Janssen	Pulmonary Fibrosis	Phase II

Table 2

mAb that recognize specific conformations of GPCRs

Receptor	mAb-Source/composition	Epitope Recognized	Receptor State Detected	Pharmacology	Ref
CCR5	MC-4, MC-5, MC-7	N-terminal domain	Agonist conformation with multiple ligands bound	Agonist Inhibition	[19]
CCR5	MC-1	ICL-2	Agonist conformation	Multiple agonist inhibition	[19]
CCR5	MC-6	ECL- conformation determinant	Active conformation	Stabilizes active conformation	[19]
CXCR4	12G5	ECL-1,2 conformational	Cell type and donor specific conformations	Inhibition of chemotaxis	[55]
CXCR4	44717.111 (12G5 also examine in this paper)	Proteoliposome (PMPLs) containing CXCR4	Binds to SDF-1 bound or unbound R	Abolished binding of GP120	[56]
CXCR4		Llama immunization with CXCR4- expressing HEK293; followed by phage library selection		Antagonist and inverse agonist, binds to ECL2 and ECL3	[57]
CCR5	3A9	Phage Displayed CCR5 epitopes inferred by alanine mapping to extracellular loops and computer modeling	Not indicated	Not Indicated	[58]
β_2 AR	Camelid Nanobody Nb80	Cytoplasmic end of receptor projecting into the lattice	By modeling and Crystallography -the agonist active state	Intracellular loop binding no pharmacological activity noted	[59]
Rhodopsin	K42-41L antibody selected by reactivity with a peptide in a phage display library	Epitope found in dark-adapted rhodopsin and metarhodopsin I not in metarhodopsin II and is a sequence in C3 loop	Inhibits for formation of metarhodopsin II while stabilizing metarhodopsin I- locks C3 loop into an extended conformation	Not studied	[60]