GPCR Drug Discovery Reaches New Heights of Stability

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The development of mutagenesis methodology to produce GPCRs stabilised in a specific conformation has opened the door to further high resolution structural studies, and several other applications relevant to drug discovery.

Keywords

G-protein coupled receptors (GPCRs)

GPCR-directed drug discovery

GPCR stability

G-protein coupled receptors (GPCRs) are the targets for approximately 25 per cent of approved drugs (1), including some long established and easily recognisable classes of therapies in the neurosciences (for example, anti-psychotics such as clozapine), cardiovascular (beta blockers such as carvedilol) and respiratory diseases (beta agonists such as ventolin). Their prevalence in medicine has continued in more recent times: since the year 2000, GPCR-directed therapies have represented 20-25 per cent of launched drugs, but many of these have been against well-established targets, and the breakthroughs promised from advances in several areas of drug discovery technology have had little impact for GPCRs. Consequently, many members of this family, with good evidence linking them to disease, have remained shelved as intractable for drug discovery. One widely acknowledged reason for this is that GPCR drug discovery has until recently been heavily dependent on working with the targets presented in cells or membrane extracts, with little structural knowledge of the interactions between protein and ligands. The instability of GPCRs when removed from their natural environment in membranes is the primary reason for their loss of ligand binding activity when isolated and pure, precluding the use of many of the techniques routinely deployed for soluble proteins such as kinase domains and proteases.

While the general GPCR topology of seven membrane-spanning helices has been known since the 1980s, and was visualised by structural studies in the 1990s, the resolution obtained then was too low to be of great use for the understanding of interactions with ligands and for drug discovery. An indication that this was changing came from the publication in 2000 of the successful isolation and purification and solution of the crystal structure of bovine rhodopsin (2). Rhodopsin, however, is by no means a typical GPCR, with a covalently attached ligand and a sufficient level of expression in the retina to preclude the need for recombinant protein production, and while several structures of other forms of rhodopsin followed, it was not until 2007 that the structure of a non-rhodopsin GPCR was reported (3). In this case, the human beta 2 receptor was expressed using baculovirus and insect cells, and complexed with a Fab to assist crystallisation.

Several features of the structure were poorly resolved, however, and shortly afterwards two different approaches yielded higher resolution structures of beta receptors. The first involved the generation of proteins in which T4 lysozyme is fused into an intracellular loop of the receptor; as with the antibody complexes, the lysozyme assists crystallisation (4). In the second, a limited number of mutations are introduced to the receptor to increase its stability and allow it to be purified and crystallised outside a membrane environment (5). The increase in stability is measured from the unfolding temperature of the protein, which can be monitored from the loss of binding of a radioligand in a thermal titration. Since the initial structures of the beta receptors, utilisation of complexes with antibody fragments, lysozyme fusions and receptor stabilisation by mutagenesis have accelerated the rate at which novel structures have appeared; the list of distinct proteins for which structures have been reported numbers 12, all from family A (the most common), with structures of five of these being reported in the first three months of 2012 (see Figure 1).

Using the Structural Information

GPCRs exist in structurally distinct but inter-convertible active and inactive states, with the former being able to signal through the G-proteins bound to the intracellular

face of the GPCR. While most structures reported so far are of inactive states, cases where both forms of a receptor have been crystallised are proving insightful for understanding the mechanism of activation (6). Rearrangements of the ligand binding pockets are observed, typically leading to a contraction in the active states, and are accompanied by changes to the overall conformation of the receptor, with the largest being a movement of over 10 angstroms of the cytosolic end of the sixth trans-membrane helix (see Figure 2, page 16).

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The growing body of structural information is now enabling meaningful conclusions to be drawn concerning the similarities and differences between GPCRs, and the means by which they interact with ligands (7,8). It is also proving valuable in understanding the selectivity that some compounds display for closely related receptors (6). Beyond this, comparisons of their ligand binding sites with kinases and proteases (in terms of the size of their pockets, the nature of protein-ligand interactions, and the way in which water fills the empty binding sites) suggest that the structure-based approaches to drug discovery common for these targets are readily applicable to GPCRs (9).

Heptares Therapeutics has industrialised the use of mutations to stabilise receptors, to generate

Figure 1: The emerging body of GPCR structural information. Solved structures of receptors are arranged by the year in which the information appeared, with inactive forms in red and active forms in green. Each four character code refers to a separate entry at www.rcsb.org, where details of each structure may be found

2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012 (Jan-Mar)	
												4dkl	Mu opioid
												4djh	Kappa opioid
												4daj	Muscarinic M3
												3v2w 3v2y	S1P1
												3uon	Muscarinic M2
											3rze		Histamine H1
										3pbl			Dopamine D3
										3odu 3oe0 3oe6 3oe8 3oe9			CXCR4
								3eml			2ydo 2ydv 3pwh 3rey 3rfm 3qak	3uza 3uzc 3vga 3vg9	Adenosine A2a
								2vt4			2y00 2y01 2y02 2y03 2y04 2ycw 2ycx 2ycy 2ycz		Beta 1 Adrenergic
							2r4r 2r4s 2rh1	3d4s		3ny8 3ny9 3nya 3kj6	3p0g 3pds 3sn6		Beta 2 Adrenergic
1f88	1hzx	1l9h	1gzm	1u19		2g87 2hpy 2i35 2i36 2i37	2j4y 2ped	2z73 2ziy 3c9l 3c9m 3cap 3dqb		3oax	2x72 3pqr 3pxo	4a4m	Rhodopsin/ Opsin

Images: Heptares Therapeutics

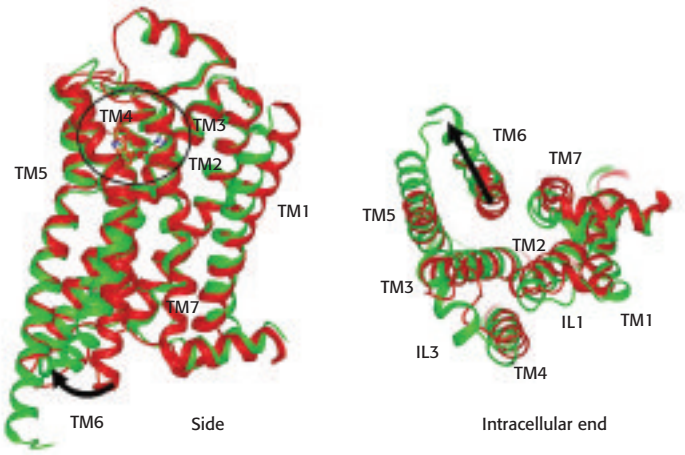

Figure 2: Ribbon views from the side (parallel to the membrane) and from the intracellular end of the β2 adrenergic receptor in the inactive (red) and active (green) conformations (see references 6 and 9). Transmembrane (TM) helices 1-7 and intracellular loops (IL) 1 and 3 are indicated. The binding site for agonists and inverse agonists is circled, and arrows indicate the shift upon activation of the intracellular end of TM6

StaR®s which enable structure-based drug discovery for GPCRs. A key feature of this is selecting which conformation of the receptor to stabilise. Most GPCR drugs bind preferentially to one form of a receptor: full and partial agonists favour the active state, while inverse agonists favour the inactive state and prevent activation. Less common are neutral antagonists (binding equally well to both states). Depending on the nature of the disease and the function of the target, a drug could be required to have any of these pharmacologies, and selection of the right form of the receptor to support drug discovery is important. Using a ligand with the desired pharmacology, StaRs can be generated in the conformation appropriate for recognition by a candidate drug. An additional benefit from using StaRs is the ability to generate crystals and structures with compounds of modest affinity, which are common in the early stages of a drug discovery programme.

It is important to note that, while the growth in structural information means there is an increasing number of opportunities for direct support of drug discovery programmes with structures of receptor-ligand complexes, the proportion of GPCRs for which structures have been solved is still low (of nearly 400 non-olfactory GPCRs in the human genome, structures are available for 12). Not surprisingly, greater impact has so far been seen in the ability to generate homology models of GPCRs where, particularly for the trans-membrane regions, the growing body of experimental data has improved their quality, with consequent insights into the mechanisms of activation and how selectivity can be achieved. Significant limitations still exist, in particular with the extracellular loops, which can contribute to the ligand binding pocket, and whose conformations vary considerably and unpredictably between receptors. However, the improvements in modelling of trans-membrane regions, combined with examination of mutagenesis studies to reveal residues contributing to ligand binding, have led to the development of models of sufficient quality for virtual screening of compound libraries to be undertaken with a reasonable degree of confidence in the output, as exemplified in the discovery and optimisation at Heptares of a series of adenosine A2a antagonists (10,11).

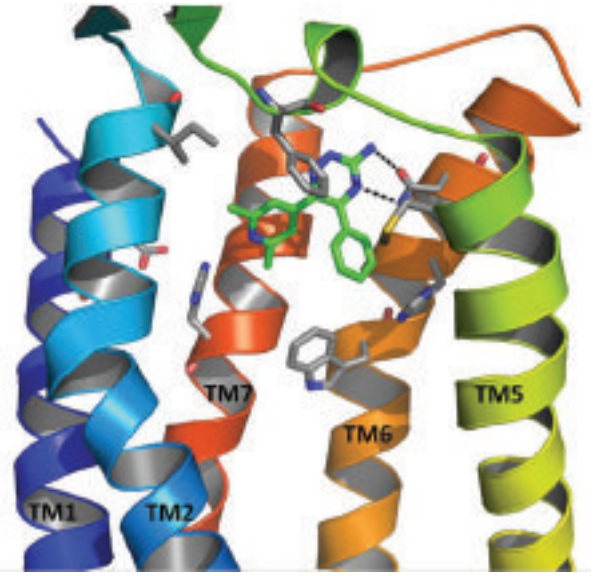

Figure 3: An x-ray structure of an Adenosine A2a receptor antagonist bound to the A2a antagonist StaR (11). Helices 3 and 4 are omitted for clarity. Amino acid side chains (grey carbons) which contribute to binding of the antagonist (green carbons) are shown

Looking Beyond the Structure

The ability to generate large quantities of pure, correctly folded proteins has applications beyond that of crystallisation and structural studies. As the receptors are now stable outside a membrane environment and can be purified while retaining ligand binding, they can be used as biochemical reagents in a similar fashion to normally soluble proteins. For example, ligand binding can be detected by biophysical methods such as nuclear magnetic resonance (NMR), capillary electrophoresis or surface plasmon resonance (SPR), enabling high concentration screening of compound libraries where the affinities may be too low to be detected by other means. Screening of fragment libraries is thus possible, offering the opportunity to steer drug

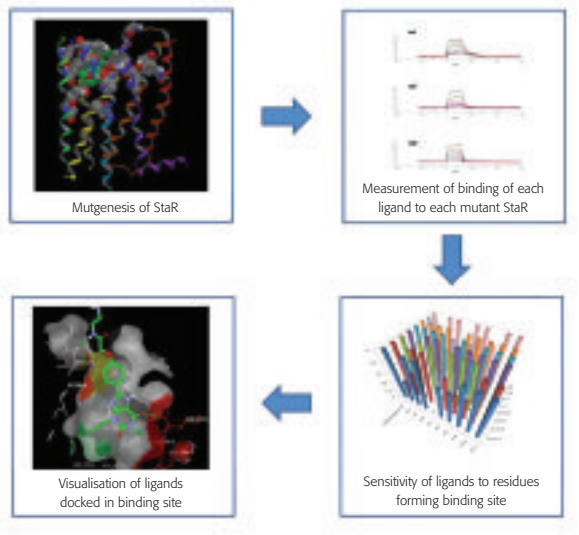

Figure 4: The Biophysical Mapping™ method (13)

discovery efforts into new areas of chemical space, potentially avoiding the liabilities associated with previous efforts. Enabling more than one form of biophysical screening is an important factor, as the potential for detecting false positives is elevated at higher compound concentrations, and confirmation of binding by orthogonal assays is required. SPR offers additional benefits in being able to measure the kinetics of association and dissociation, allowing selection and optimisation of drug candidates with specific *in vivo* kinetic properties in mind (12).

SPR has also been found to be a very powerful tool in visualising the binding modes of ligands to GPCRs, through a technique known as Biophysical Mapping™ (13). Mutations are individually introduced around the ligand binding site of a StaR to generate a panel of proteins, each differing by only one or two residues. The binding behaviours of a range of compounds are monitored against the panel of mutants, and the sensitivity of each compound to each mutation is used to guide docking of the compound in the binding site. With careful selection of mutations, the location and orientation of ligands can be predicted with sufficient confidence to guide medicinal chemistry, in the absence of x-ray structures of the complexes, or even of the receptor itself.

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Future Directions

The development of mutagenesis methodology to produce GPCRs stabilised in a specific conformation has opened the door to further high resolution structural studies, and several other applications relevant to drug discovery. Looking beyond small molecule therapies, the ability to produce pure receptors retaining native folding and ligand binding opens up new possibilities for the generation of therapeutic antibodies, where efforts have previously been hindered by working with unpurified antigens. However, for both small molecule and antibody discovery the use of these approaches is still in its infancy, with structures available for approximately three per cent of the human GPCR complement. Many more will follow, including those in families B and C which, while fewer than in family A, also include well-validated drug targets, heralding a new age of GPCR-directed drug discovery.

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