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Allosteric modulation of G protein-coupled receptors: perspectives and recent developments

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Allosteric modulation of G protein-coupled receptors has recently been recognized as an alternative approach to gain selectivity in drug action. In this overview, allosteric modulators that enhance or diminish the effects of (endogenous) agonists or antagonists on a variety of G protein-coupled receptors are described. Emphasis is placed on the latest developments in this research area, including data on the first clinical studies. It appears that all three major classes of G protein-coupled receptors (A, B and C) are amenable to allosteric modulation by small molecules. This constitutes an attractive and novel means to identify new leads in the drug discovery process. However, it requires a re-engineering of the majority of current assays. Finally, it is suggested to introduce the term 'non-competitive agonism' or 'allosteric agonism' next to allosteric modulation.

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▼ G protein-coupled receptors (GPCRs) are attractive drug targets. It is estimated that approximately half of the marketed medicines act through members of this protein class [1]. These membrane-bound proteins most probably share the typical architecture of rhodopsin, that is, of a cavity with seven transmembrane (TM) α-helical domains as outer boundaries, which are ideally suited to accommodate small molecules or molecular fragments. Such molecules are the natural hormones and neurotransmitters in the first place, all acting as endogenous agonists. They act as 'primary messengers' to convey signals into the cell that give rise to, among others, the production of 'second messengers' leading to a cellular response. Synthetic agonists, antagonists and inverse agonists have been developed over the years, yielding the rich repertoire of current medicines.

However, it should be stressed that the interaction with the receptor by hormones and neurotransmitters is different from most synthetic drugs. Endogenous ligands are often synthesized *in situ* on demand. They might also be subject to rapid and extensive breakdown, often already at the site of action. Both processes of synthesis and degradation effectively cause transient receptor stimulation. By contrast, a synthetic agonist (such as morphine, isoproterenol, and so on), which is often designed to be metabolically stable, might lead to a more continuous stimulation of receptor proteins, which is not necessarily desirable. Similarly, receptor occupancy by an antagonist can yield a prolonged blockade of receptor function, which might not be compatible with the 'kinetics' of a pathological condition.

A more controlled and selective 'tuning' action on the receptor is feasible through allosteric modulation. Particularly in enzyme research, this phenomenon has long been recognized as a general mechanism for the control of protein function [2]. 'Allosteric' refers to binding sites that are different from the 'orthosteric' primary substrate or ligand binding site, to which the binding of modulators results in conformational changes that might profoundly influence protein, and hence GPCR, function. Because the endogenous ligand remains to play a key role, the overall pharmacology resembles physiology more closely than with the use of synthetic ligands (Figure 1).

The purpose of the current overview is to comment on the latest findings in this fascinating research field. This includes, a limited discussion of representatives of the current ligand repertoire, a mutational analysis of allosteric binding sites, a summary of available clinical data and a reflection on the definition of allosteric modulation. In addition, we would like to make a plea for the re-engineering

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of some of the available receptor assays to screen for allosteric modulation.

Specifically, the interaction of small molecules with the receptor macromolecule itself will be discussed, rather than addressing other allosteric interactions, such as with G proteins. Therefore, this overview will be confined to small organic molecules that have been shown to interact with the receptor protein in an allosteric manner and that might be regarded as leads for future medicines. With respect to receptor molecules the characteristic subdivision of human receptors into classes A (or 1, rhodopsin-like), B (or 2, secretin-like) and C (or 3, metabotropic glutamate-like) will be followed ([3], see also the public-domain receptor database at http://www.gpcr.org). The scope of this review is limited in that only some recent examples and literature can be examined. Therefore, interested readers are referred to several other reviews on the same subject [4–6].

Class A receptors

The rhodopsin-like receptors constitute by far the largest group of GPCRs, which is termed class A. Many representatives have been shown to be allosterically regulated. Most extensive studies have been performed on adenosine and muscarinic receptors, which will be discussed below.

Adenosine receptors

PD81,723, a 2-amino-3-benzoylthiophene derivative (Figure 2), was the first allosteric enhancer of the A_1 adenosine (A_1A) receptor to be reported [7,8]. The compound, at μM concentrations, selectively increases the binding and function of reference A_1A receptor agonists in various species, including human. Several groups extended these seminal studies. Dialkyl and cyclized PD81,723 analogs with an appropriately substituted benzoyl ring also show high allosteric activity [9]. The most interesting compound of this series was LUF 5484 (Figure 2), being 2.4-fold more potent than PD81,723. Similar findings were reported by Tranberg *et al.* [10]. Substitution at C-4 with a phenyl ring and at C-5 with bromine is also well tolerated [11]. The binding site of PD81,723 and its many analogs on the A_1A receptor has yet to be elucidated.

The first selective allosteric enhancers of agonist binding at human A_3 adenosine (A_3A) receptors were reported by Gao *et al.* [12]. The effects of the reference A_3A receptor agonist Cl–IB-MECA on forskolin-induced cAMP formation were significantly enhanced by several 3-(2-pyridinyl)isoquinoline derivatives. However, most compounds also display orthosteric binding affinity to the human A_3A receptor. The ratio between allosteric and orthosteric binding was most promising for VUF5455 (Figure 2), and therefore it might be a lead for the design of pure allosteric enhancers of A_3A receptors.

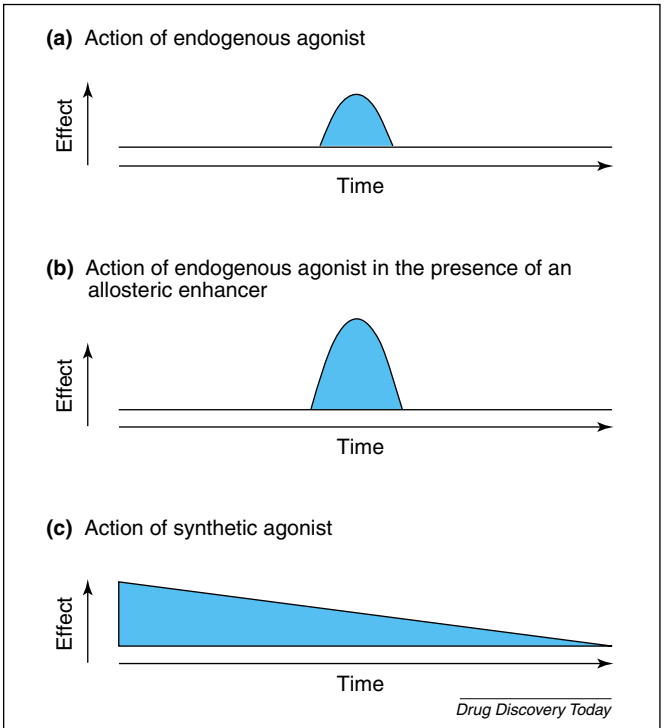

Figure 1. An allosteric enhancer offers a more physiologic alternative to synthetic agonists. An allosteric enhancer can only act in the presence of an (endogenous) agonist, which mimics the duration and intensity of action of hormone or neurotransmitter much better than that induced by a synthetic agonist. This combined action of two compounds (i.e. agonist and enhancer) might also induce significant gains in selectivity of drug action.

Another structural lead is the imidazoquinoline derivative DU124183 (Figure 2) [13]. DU124183 selectively enhances agonist binding and function at human A_3A receptors. The compound has a unique mechanism of action as it also enhances the intrinsic activity of Cl–IB-MECA in the cAMP assay by 30%. Again, the compound also displays orthosteric activity.

Mutation studies of the human A_3A receptor showed that several amino acids appear to be involved in recognition of the allosteric modulators [14]. The Phe182Ala (TM5) and Asn274Ala (TM7) mutations eliminated the allosteric effects of both modulators but had little effect on agonist binding, as was the case for Asn30Ala (TM1) and Asp58Asn (TM2). Interestingly the Asp107Asn (TM3) mutation abolished the effects of DU124183, but not VUF5455.

Muscarinic receptors

Diallylcaracurine V (Figure 2), which is structurally closely related to alcuronium, showed a nanomolar allosteric activity on [^{3}H]-*N*-methylscopolamine (NMS)-occupied human muscarinic-2 (M_2) receptors [15] and a high selectivity versus

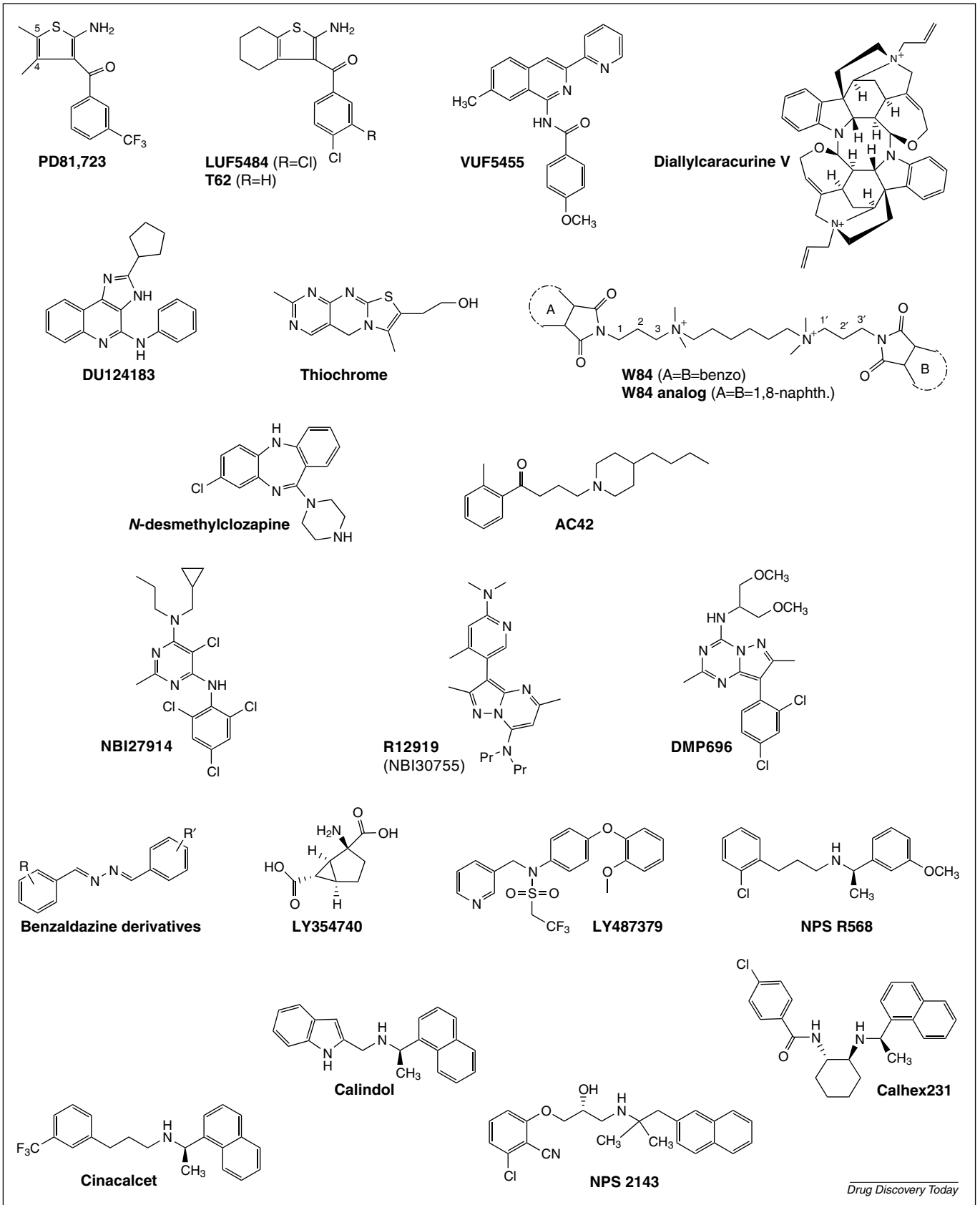

Figure 2. Allosteric modulators of G protein-coupled receptors.

human M_5 receptors [16]. This latter feature was investigated in receptor mutation studies by the same authors, in which they identified a threonine residue in TM7 of the M_2 receptor (Thr423) to be largely responsible for the favorable selectivity ratio. In a later study, Voigtländer *et al.* demonstrated that in fact yet another amino acid, Tyr177 in the second extracellular loop of the M_2 receptor, also influenced this selectivity ratio [17]. The combined mutations (Tyr177Gln and Thr423His, from the wild-type M_2 receptor to an engineered one with two 'M_5' residues) abolished the M_2 over M_5 selectivity of diallylcaracurine with respect to affinity for [^{3}H]NMS-occupied receptors.

A new series of allosteric modulators, consisting of W84 (Figure 2) and analogs was described by Muth *et al.* [18]. The affinity of the modulators for free M_2 receptors was compared with [^{3}H]NMS-occupied receptors. W84 showed negative cooperativity (allosteric inhibition) with [^{3}H]NMS in this experimental setup. As an example from this study, replacement of both phthalimido groups in W84 by 1,8-naphthalimido groups (Figure 2) yielded a more potent allosteric modulator, again negatively cooperating with [^{3}H]NMS binding. Disubstitution by methyl groups at position C-2 yielded a compound with modest positive cooperativity (allosteric enhancement). A further increase in positive cooperativity was obtained by the introduction of a second double-methyl substitution at position C-2′. Hence, it appeared that subtle structural variations resulted in distinct pharmacological profiles.

A new enhancer of acetylcholine binding to and activation of M_4 receptors was recently reported by Lazareno *et al.* [19]. Thiochrome (Figure 2) at 100 μM increased the affinity of acetylcholine fourfold for inhibiting [^{3}H]NMS binding to this receptor subtype. Similarly, it reduced acetylcholine release from rat striatal slices containing autoinhibitory presynaptic M_4 receptors but not from hippocampus (M_2 receptors).

An interesting new development is the discovery of 'allosteric agonists' for muscarinic receptors, that is, compounds that appear to activate the receptor at sites distant from the acetylcholine-binding site. Please note that such compounds are not modulatory, in that their activity does not require the presence of the endogenous agonist. AC42 (Figure 2), which is a lead compound resulting from a broad screening program, is a typical example, being an agonist for M_1 receptors interacting among others with parts of the N-terminus and the upper region of TM1 [20]. Similarly, *N*-desmethylclozapine (Figure 2) was identified as an allosteric M_1 receptor (partial) agonist. Its binding site was explored through site-directed mutagenesis of the receptor in which Tyr381 (TM6) appeared to play an important role. When this residue was mutated to alanine the receptor largely lost its capability of recognizing acetylcholine, whereas *N*-desmethylclozapine turned into a highly potent full agonist [21]. This finding led the authors to conclude that the *N*-desmethylclozapine binding site does not fully overlap with the one for acetylcholine.

Class B receptors

The receptors in this family are activated by large peptides with high amino acid identity. The secretin receptor was the first one to be cloned in this family, hence the often-used term of 'secretin-like' receptor class. The receptor N-terminus, with a length of 60 to 80 amino acids, contains conserved cysteine bridges and is particularly important for the binding of the cognate ligands. Other receptors with far longer N-terminal tails (up to several times the size of the TM domain) might be regarded as a subclass of class B receptors. These tails contain several well-characterized protein modules with, for example, immunoglobulin motifs [22]. In these cases the endogenous ligands and details of their binding sites are often unknown.

There is limited, although convincing evidence for allosteric modulation of class B receptors, of which we will give one example.

Corticotropin-releasing factor-1 receptors

The non-peptide antagonists (NPAs) NBI35965 (chemical structure not disclosed), NBI27914, DMP696 (Figure 2) and antalarmin are strong allosteric inhibitors of peptide agonist binding to the cloned human corticotropin-releasing factor (CRF_1) receptor [23]. NBI35965 slowed the dissociation of [^{125}I]CRF and [^{125}I]sauvagine binding from the receptor with an EC_{50} value of 52 and 130 nM, respectively. The NPAs bind to the same site on the CRF_1 receptor and display similar potency in displacing [^{125}I]sauvagine binding: antalarmin (K_i = 0.38 nM), NBI27914 (K_i = 0.95 nM), NBI35965 (K_i = 1.45 nM) and DMP696 (K_i = 2.34 nM).

Studies with independently expressed N-terminal and 'rest of the receptor' domains (J-domain) of the CRF_1 receptor demonstrate that the NPAs bind only to the J-domain, whereas the peptide agonists bind mainly to the N-terminal domain [24]. NPAs for the CRF_1 receptor might be useful in the treatment of CRF-associated disorders such as stress and depression.

Class C receptors

Class C receptors are characterized by a large N-terminal domain with specialized motifs (Venus flytrap) that contain the actual neurotransmitter or hormone binding sites, as is the case for γ-aminobutyric acid ($GABA_B$ receptors), glutamate (metabotropic glutamate receptors) and calcium ions (calcium-sensing receptors). We will discuss the latter two in more detail, also in view of recent clinical data.

Metabotropic glutamate receptors

There is a large similarity in the overall structures of metabotropic glutamate and $GABA_B$ receptors. Both receptors are dimers with a large N-terminal containing a bilobal-structured Venus fly trap, seven TM helices and generally a large carboxyl terminal. However, the metabotropic glutamate receptors (m GluRs) are homodimers [25], whereas the $GABA_B$ receptor is a heterodimer.

The positive, negative and neutral allosteric effects of benzaldazine derivatives (Figure 2) on mGlu5 receptors (orthosteric ligand: glutamate) were described by O'Brien *et al.* [26]. Shifting fluoro atoms from the 3,3′ position in difluorobenzaldazine (DFB) to the 2,2′ position did not change the positive allosteric activity but there was a 6.5-fold loss in potency. A shift to the 4,4′ position changed the allosteric activity from positive to negative with a large (>40-fold) loss in potency. Substituting the 3,3′ difluoro atoms in DFB for 3,3′ dimethoxy groups yielded a compound displaying negative allosterism (DMeOB) with a potency similar to that of DFB. Replacing the 3,3′-difluoro atoms in DFB by 3,3′-dichloro atoms yielded a neutral allosteric compound (DClB) displaying neither positive nor negative modulation.

Schaffhauser *et al.* [27] reported on LY487379 (Figure 2) as a selective and positive allosteric modulator of mGlu2 receptors. The mGlu2 receptor agonists glutamate, DCG-IV [(2*S*, 2′*R*, 3′*R*)-2-(dicarboxycyclopropylglycine)] and LCCG1 (2*S*, 1′*S*, 2′*S*)-2-(carboxycyclopropylglycine) stimulated [^{35}S]GTPγS binding to a recombinant human mGlu2 receptor preparation in the absence or presence of 3 μM LY487379. LY487379 enhanced potency (EC_{50}) and efficacy (E_{max}) for the three orthosteric agonists glutamate, DCG-IV and LCCG-1. Mutagenic analysis showed that amino acids Ser688 and Gly689 in TM4 as well as Asn735 in TM5 of the mGlu2 receptor are important for the binding of this allosteric enhancer.

Calcium-sensing receptors

Cinacalcet (AMG073, Figure 2) is a metabolically stable analogue of the reference allosteric enhancer of calcium-sensing receptors (calcimimetic), NPS R568 (Figure 2). Cinacalcet inhibits the secretion of parathyroid hormone (PTH) and causes a dose-dependent long-lasting reduction in serum PTH levels in rats (≥8 hours at 10 mg kg^{-1} po) [28]; it is currently in clinical trials. A novel calcimimetic is calindol [29]. Calindol (Figure 2) is equipotent to NPS R568 in increasing the Ca^{2+}-induced [^{3}H]inositol phosphates (IP) accumulation in HEK 293 cells expressing the human calcium-sensing receptor (EC_{50} values of 0.31 μM and 0.50 μM, respectively).

The first selective allosteric antagonist of calcium-sensing receptors (calcilytic) is NPS 2143 (Figure 2, [30]). The compound stimulates PTH secretion from bovine parathyroid cells (EC_{50} = 41 nM) and enhances the plasma PTH levels in normal rats after intravenous infusion (0.1 μmol kg^{-1} min^{-1}). Shorter-acting calcilytics, intermittently administered, will induce transient increases in plasma PTH levels, more closely mimicking the physiology of PTH release. This goal was achieved by replacing the 2-naphthyl moiety of NPS 2143 by a 4-methoxyphenyl group. A novel calcilytic is Calhex231 (Figure 2) [29]. Calhex231 blocks the Ca^{2+}-induced [^{3}H]IP accumulation in HEK 293 cells transiently expressing the human calcium-sensing receptor. The compound is equipotent to NPS 2143 with IC_{50} values of 0.39 μM and 0.35 μM, respectively. Mutagenesis studies in HEK 293 cells transiently expressing the human calcium-sensing receptor showed that the binding pocket of calcimimetics (NPS R568 and calindol) and calcilytics (NPS 2143 and Calhex231) are partially overlapping but not identical [29]. The calcimimetics NPS R568 and calindol bind to Glu837 and Ile841 on TM7. Furthermore, NPS R568 interacts with Phe821 and calindol with Trp818 on TM6. The calcilytics NPS 2143 and Calhex231 bind to Glu837 on TM7 and Phe684 on TM3. The amino acids Ile841 on TM7 and Phe688 and Arg680 on TM3 are also involved in the binding of NPS 2143.

Clinical studies

With the emerging interest in allosteric modulation, it does not come as a surprise that some chemical entities have now progressed from the preclinical to clinical phases of investigation. With respect to class A receptors, the allosteric enhancer of adenosine A_1 receptors T62 (Figure 2) has been tested on a limited number of healthy volunteers to facilitate dose finding (http://www.kingpharm.com/news_details.asp?id_news=258)

One particular CRF_1 receptor (class B) nonpeptide antagonist, R121919 (Figure 2), entered into Phase IIa studies. These have been discontinued, although the compound ameliorated depressive symptoms without undesired endocrine effects [31].

In a more advanced stage of clinical testing are both metabotropic glutamate and calcium-sensing receptor modulators (both class C receptor ligands). LY354740, an allosteric enhancer of the mGluR2 and mGluR3 receptors (Figure 2), was studied for its anxiolytic effect in several protocols, including fear-potentiated startle tests [32], carbon dioxide-induced panic disorder [33] and in generalized anxiety disorder [34]. The study investigating generalized anxiety disorder involved over 600 patients. The compound is currently 'on hold' after this Phase II testing.

The calcium-sensing receptor agonist cinacalcet (Figure 2) has also been studied in several clinical protocols [35,36]. The most recent and extended study involved patients receiving

hemodialysis who had inadequately controlled secondary hyperparathyroidism. Two identical randomized, double-blind, placebo-controlled trials were performed in over 700 patients, showing the benefit of the treatment. The compound lowered parathyroid hormone levels and improved the calcium-phosphorous homeostasis in the patients [36].

Concluding remarks

The specific examples discussed above raise the question of whether all GPCRs can be allosterically modulated, and, at present, there is no proof that this is not the case. This hypothesis could then be the more general starting point for a search for new chemical entities that are modulators of GPCR activity, unlike directly acting (orthosteric) ligands such as agonists and antagonists. It would, however, require a different set-up of biological screens in which, besides equilibrium studies, the dissociation and association kinetics of the receptor–ligand interaction are also taken into account. Rather than a two-component screening [i.e. an assay with (labeled) receptor preparation and compound], a three-component system with a potential allosteric modulator present, should be considered in high-throughput approaches. This experimental set-up might be used in both radioligand binding studies and assays with fluorescent probes, either addressing interaction with the receptor itself or through post-receptor signaling events.

Unfortunately, ligands are too often described as allosteric in nature without appropriate experimental evidence, such as the effect on dissociation kinetics. This stems from the classical definition of an allosteric modulator as a compound that does not have receptor activity of its own. It can only be active in the presence of an (orthosteric) agonist or antagonist and does so by influencing the dissociation from or association to the receptor of the agonist or antagonist (however, please note that the association kinetics are also influenced by competitive – orthosteric – ligands). Within this strict framework it is obvious that many ligands mentioned in this review do not qualify as 'true' allosteric modulators. AC42 and *N*-desmethylclozapine (Figure 2) are typical examples of ligands that independently activate the muscarinic M_1 receptor through sites that are distant from the acetylcholine-binding site. Particularly for the class B and C receptors, it appears to be the rule rather than the exception that the allosteric modulators can be highly active in the absence of the endogenous agonist. Most striking in this respect is the observation that the mGlu5 receptor with a deleted Venus flytrap containing N-terminal domain (the glutamate binding site) can be fully activated by 3,3′-difluorobenzaldazine (Figure 2) [37].

This and similar findings raises two questions. First, can these observations be rationalized? In Figure 3, three GPCRs

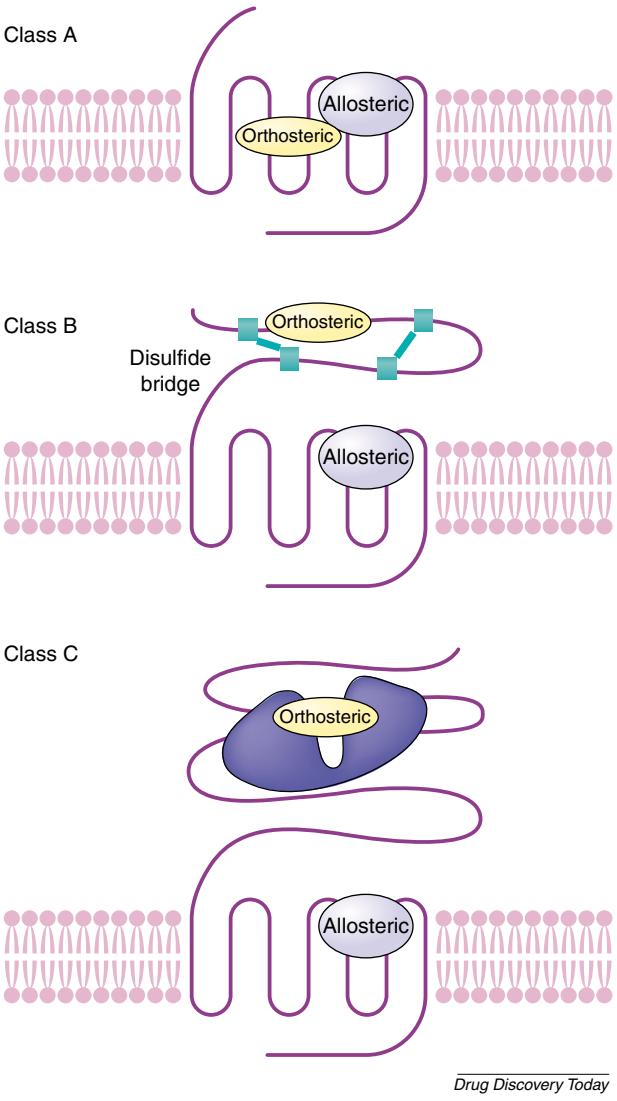

Figure 3. Schematic representations of G protein-coupled receptors. There are three classes of G protein-coupled receptors – class A (or 1, rhodopsin-like), class B (or 2, secretin-like) and class C (or 3, metabotropic glutamate-like). The putative orthosteric binding site might (class A) or might not (class B, C) be close to the allosteric binding site.

from classes A, B and C are depicted in a cartoon-like fashion. For most known class A receptors the endogenous ligand (neurotransmitter or hormone) is believed to bind and activate the receptor from within the cavity formed by the seven TM domains. The situation is different for the class B and C receptors, where the endogenous ligands generally bind to the N-terminal domains, leaving the TM cavity essentially unoccupied with substantial room for other (allosteric) ligands to bind and even activate the receptor.

The second question regards the definition of an allosteric modulator. If the strict definition of an allosteric modulator

is maintained, then we propose to use the term 'non-competitive agonism' or 'allosteric agonism' for compounds such as AC42, *N*-desmethylclozapine and DFB. That might take away some confusion, and, more importantly, provide a rational dichotomy for the classification of novel compounds.

In conclusion, because of the wide tissue distribution of G protein-coupled receptors, directly acting (endogenous) agonists or antagonists often exhibit side effects associated with the activation of receptors in tissues other than the therapeutic target. Allosteric modulators, by contrast, might induce tissue selectivity. They enhance or diminish the effects of (endogenous) agonists that are often confined to or produced in the afflicted tissue only. Hence, allosteric modulators might in fact be safer drugs. The recent surge in clinical trials with allosteric modulators might be a reflection of this assumption.

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