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REVIEW

Allosteric interactions at adenosine A_1 and A_3 receptors: new insights into the role of small molecules and receptor dimerization

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The purine nucleoside adenosine is present in all cells in tightly regulated concentrations. It is released under a variety of physiological and pathophysiological conditions to facilitate protection and regeneration of tissues. Adenosine acts via specific GPCRs to either stimulate cyclic AMP formation, as exemplified by G_s-protein-coupled adenosine receptors (A_{2A} and A_{2B}), or inhibit AC activity, in the case of $G_{i/o}$-coupled adenosine receptors (A_1 and A_3). Recent advances in our understanding of GPCR structure have provided insights into the conformational changes that occur during receptor activation following binding of agonists to orthosteric (i.e. at the same binding site as an endogenous modulator) and allosteric regulators to allosteric sites (i.e. at a site that is topographically distinct from the endogenous modulator). Binding of drugs to allosteric sites may lead to changes in affinity or efficacy, and affords considerable potential for increased selectivity in new drug development. Herein, we provide an overview of the properties of selective allosteric regulators of the adenosine A_1 and A_3 receptors, focusing on the impact of receptor dimerization, mechanistic approaches to single-cell ligand-binding kinetics and the effects of A_1- and A_3-receptor allosteric modulators on *in vivo* pharmacology.

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Abbreviations

ADA, adenosine deaminase

Introduction

Adenosine is a reactive metabolite that has a major role in regulating a number of physiological and pathological processes, including inflammation, pain, hypoxia and cardiovascular regulation (Fredholm *et al.*, 2011). Adenosine acts via four specific GPCRs, which have been denoted as adenosine A_1, A_{2A}, A_{2B} and A_3 receptors (Alexander *et al.*, 2013; Fredholm *et al.*, 2011). The A_1 and A_3 receptors preferentially couple to $G_{i/o}$ proteins and have an inhibitor action on AC activity, while the A_{2A} and A_{2B} receptors couple to G_s proteins and stimulate cyclic AMP formation (Jacobson, 2009; Fredholm *et al.*, 2011; Muller and Jacobson, 2011). The crystal structure of the A_{2A} receptor in both antagonist (Jaakola *et al.*, 2008) and agonist (Xu *et al.*, 2011) bound conformations has recently been solved. Numerous selective agonists and antagonists for each adenosine receptor subtype are now available for the study of receptor function (reviewed in Jacobson, 2009; Fredholm *et al.*, 2011; Muller and Jacobson, 2011). In the case of the $G_{i/o}$-coupled adenosine receptors (A_1 and A_3) reviewed here, a number of compounds are undergoing evaluation for disease indications (Muller and Jacobson,

2011). These include A_1-receptor partial agonists (e.g. capadenoson, selodenoson and tecadenoson) for paroxysmal supraventricular tachycardia, atrial fibrillation and angina pectoris, and A_3-receptor agonists (e.g. CF101, CF102) for inflammatory disease, glaucoma and cancer (Bar-Yehuda *et al.*, 2011; Cohen *et al.*, 2011; Muller and Jacobson, 2011; Albrecht-Kupper *et al.*, 2012; Tendera *et al.*, 2012).

Activation of cell surface adenosine receptors by endogenous adenosine requires it to be available at the extracellular surface of cells. Extracellular adenosine can rise as a consequence of several pathways (Fredholm *et al.*, 2011). It can be formed intracellularly following various metabolic processes and be exported from cells via membrane transporters, or it can be formed in the extracellular space from adenine nucleotides released from cells. Once ATP or ADP is released, the nucleotide is broken down by nucleoside triphosphate diphosphohydrolases (e.g. CD39) and then ecto-5′-nucleotidase (CD73) to adenosine (Fredholm *et al.*, 2011; Knapp *et al.*, 2012). It is well known that neurons and platelets can store and release ATP and ADP, respectively, in response nerve stimulation and platelet activation. However, more recently, there has been a growing awareness that the intracellular second messenger cyclic AMP may also be a source of extracellular adenosine in many cell types. Thus, intracellular cyclic AMP can be released from cells in response to receptor stimulation (McCrea and Hill, 1993; Baker *et al.*, 2004) and it is known that extracellular cyclic AMP can be rapidly converted to adenosine via the action of ecto-phosphodiesterase and ecto-5′-nucleotidase (Dubey *et al.*, 2001; Chiavegatta *et al.*, 2008; Goedeke, 2008).

The intricacies of localized extracellular release of adenine nucleotides and subsequent production of adenosine following CD73 activity has recently provided insights into the role of adenosine A_1 receptors in mediating localized analgesia in animals and humans (Goldman *et al.*, 2010; Sowa *et al.*, 2010; Street and Zylka, 2011). Thus, there is evidence that localized A_1-receptor activation may underlie the antinociceptive effects of acupuncture as manual stimulation of acupuncture needles can result in localized release of adenine nucleotides and adenosine formation, leading to an analgesia that can be mimicked by an A_1-receptor agonist (Goldman *et al.*, 2010). Furthermore, intrathecal application of recombinant CD73 (to enhance formation of adenosine) produced a long-lasting antinociceptive effect that was not observed in adenosine A_1-receptor knockout mice (Sowa *et al.*, 2010). Similarly, recent work with human neutrophils has highlighted an autocrine role for ATP, which is released from the leading edge of neutrophils during chemotaxis (Chen *et al.*, 2006; Corriden and Insel, 2012). ATP is then rapidly converted to adenosine, which then acts via adenosine A_3 receptors (recruited to the leading edge of neutrophils) to promote cell migration (Chen *et al.*, 2006; Corriden and Insel, 2012; Corriden *et al.*, 2013). In addition, there is increasing evidence that adenosine A_1 and A_3 receptors may be involved in promoting angiogenesis and the release of VEGF in response to local hypoxia and neoplasia (Clark *et al.*, 2007; Merighi *et al.*, 2009).

The above studies suggest that localized regulation of adenosine production (e.g. by recombinant CD73), or its activity at its target adenosine receptor itself (e.g. A_1 or A_3), may have important therapeutic implications. One way in which the activity of endogenous adenosine can be subtly regulated at the level of its target receptor is via drugs that bind to an allosteric site on the receptor and act as allosteric modulators to enhance or inhibit the binding and/or function of adenosine. Here, we review the properties of various small-molecule allosteric regulators of the adenosine A_1 and A_3 receptors focusing on the impact of receptor dimerization, mechanistic approaches to single-cell ligand-binding kinetics and the effects of A_1- and A_3-receptor allosteric modulators on *in vivo* pharmacology.

Allosteric regulation of GPCRs

GPCRs comprise the largest family of transmembrane proteins and represent major targets for drug discovery (Williams and Hill, 2009; Roth and Marshall, 2012). Considerable advances in our knowledge of GPCR structure have been made recently (Jaakola *et al.*, 2008; Chien *et al.*, 2010; Chung *et al.*, 2011; Rasmussen *et al.*, 2011) and this has led to significant insights into the conformational changes that occur during receptor activation in response to agonists that act at the same site (orthosteric) as the endogenous hormone or neurotransmitter (Chung *et al.*, 2011; de Graaf *et al.*, 2011; Rasmussen *et al.*, 2011). However, over the past decade, there has been an increasing acceptance that drugs can also bind to a topographically distinct site (allosteric) on the GPCR protein and elicit a conformational change that can lead to a change in the affinity or efficacy of a ligand occupying the classical orthosteric binding site (Figure 1A; May *et al.*, 2007; Kenakin, 2009, 2012; Keov *et al.*, 2010). This suggests that GPCRs are able to bind more than one ligand simultaneously (i.e. both an allosteric and an orthosteric ligand; May *et al.*, 2007; Kenakin, 2009, 2012; Keov *et al.*, 2010). Various mathematical models have been developed to explain these phenomena, but key features of an allosteric mechanism of action are that the effect is saturable, can depend on the specific ligand occupying the orthosteric site (probe dependence) and provides scope for both positive and negative effects on ligand binding and/or function (May *et al.*, 2007; Kenakin, 2009, 2012; Keov *et al.*, 2010).

Some of the earliest allosteric modulators were discovered for the adenosine A_1 receptor (Bruns and Fergus, 1990; Bruns *et al.*, 1990; Göblyös and Ijzerman, 2011; Kimatrai-Salvador *et al.*, 2012). PD 81,723 has become a reference allosteric enhancer for the A_1 receptor. Early studies demonstrated that PD 81,723, which has A_1-receptor antagonist properties at high concentrations, was able to increase the binding of an orthosteric agonist radioligand at lower concentrations of PD 81,723 to enhance the functional activation of the A_1 receptor in the brain (Janusz and Berman, 1993) and cardiovascular tissues (Amoah-Apraku *et al.*, 1993) and to slow down the dissociation of the agonist radioligand from the A_1 receptor (Bruns and Fergus, 1990; Bruns *et al.*, 1990); the latter effect being indicative of an allosteric mechanism of action (see below; May *et al.*, 2007; Keov *et al.*, 2010; Göblyös and Ijzerman, 2011). Furthermore, recent studies using site-directed mutagenesis have indicated that the allosteric binding site for PD 81,723 may reside within extracellular loop 2 of the adenosine A_1 receptor (Peeters *et al.*, 2012).

Selective allosteric enhancers of agonist binding have also been described for the adenosine A_3 receptor (Gao *et al.*, 2001;

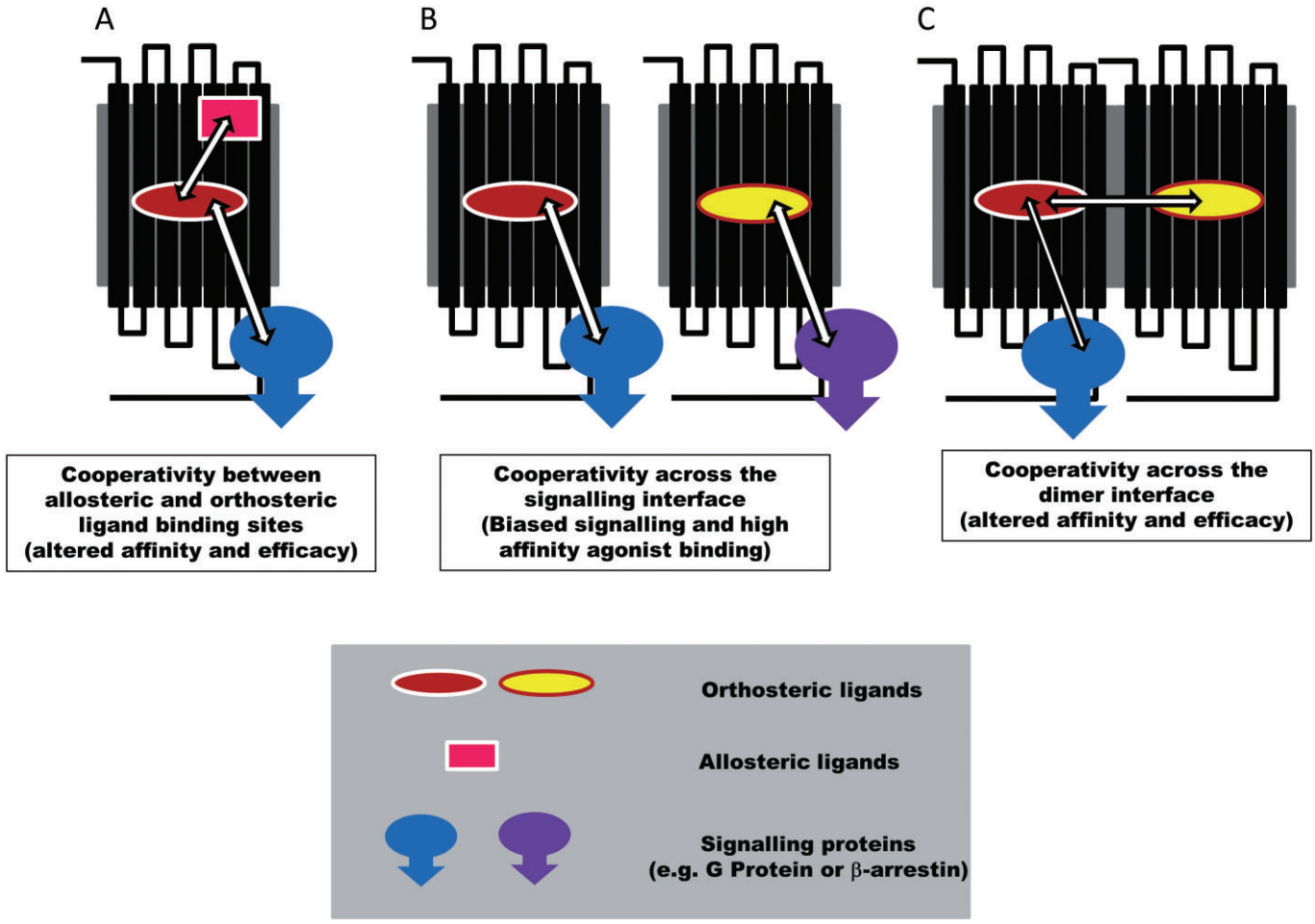

Figure 1

Schematic representation of allosteric regulation of GPCRs by (A) allosteric ligands, (B) signalling proteins or (C) GPCR dimerization. See text for further explanation.

Heitman *et al.*, 2009; Göblyös and Ijzerman, 2011). The impact of an allosteric modulator is not, however, restricted to the binding and function of orthosteric agonists. For example, the food dye Brilliant Black BN is able to act allosterically to reduce the affinity of particular adenosine A_1- and A_3-receptor antagonists (e.g. xanthine amine congener) for the orthosteric site without altering the ability of agonists to interact with these two receptors (May *et al.*, 2010a). This is a good example of probe dependence where the effect observed differs, depending on the nature of the ligand occupying the orthosteric site. In addition to small molecules exerting allosteric influences on GPCRs, there is considerable evidence that sodium ions can also mediate allosteric effects on a range of GPCRs, including both the adenosine A_1 and A_3 receptors (Liu *et al.*, 2012). In the case of A_1 and A_3 receptors, a highly conserved aspartate residue in transmembrane region 2 of each receptor ($Asp^{2.50}$) has been implicated in the allosteric actions of sodium ions. Mutation of this residue to alanine or asparagine largely abolishes the effect (Barbhaiya *et al.*, 1996; Gao *et al.*, 2003). In a recent high-resolution crystal structure of the adenosine A_{2A} receptor, the precise location of the sodium ion and its associated water cluster has been identified and shown to interact with $Asp^{2.50}$ (Liu *et al.*, 2012).

Recent studies with imidazoquinolinamine allosteric enhancers (e.g. LUF5999, LUF6000 and LUF6001) of the adenosine A_3 receptor have shown that they have differing effects on the affinity and efficacy of a selective A_3-agonist Cl-IB-MECA (Gao *et al.*, 2011). This illustrates the independence of allosteric actions on binding affinity and efficacy. Furthermore, the allosteric modulation of orthosteric agonist efficacy was dependent on the intracellular signalling response being measured. This suggests that the allosteric modulation of agonist efficacy may be functionally biased (Gao *et al.*, 2011). Functional selectivity of orthosteric and allosteric ligands has also been investigated for the adenosine A_1 receptor (Cordeaux *et al.*, 2004; Valant *et al.*, 2010; Langemeijer *et al.*, 2013). The magnitude of positive allosteric modulation of the 2-amino-3-benzoylthiophene adenosine A_1-receptor allosteric enhancer, VCP520, varied between pathways (Valant *et al.*, 2010). This is an example of an

allosteric modulation engendering functional selectivity in the actions of orthosteric ligands (Valant *et al.*, 2010). These studies highlight the ability of allosteric ligands to further 'fine-tune' orthosteric ligand responses. Signalling bias from GPCRs is a concept that has developed considerably over the last few years as knowledge that GPCRs can regulate signalling pathways independently of heterotrimeric G protein has become available (e.g. β-arrestin pathways; Kenakin, 2012; Whalen *et al.*, 2011). Thus, it is clear that activation of β-arrestin pathways are not only associated with desensitization and receptor internalization but can also change the signalling pathways that are activated. Furthermore, specific agonists appear to be able to direct signalling to different pathways via the same cell surface receptor. Some of the best evidence for this has come from the β_2-adenoceptor field, where certain β-blockers (e.g. propranolol) can have an inverse agonist effect of G_s-mediated signalling pathways, but an agonist action on MAP kinase (Azzi *et al.*, 2003; Baker *et al.*, 2003). The concept of biased signalling, however, is a natural extension of allosterism (Figure 1B). The intracellular signalling proteins (e.g. heterotrimeric G proteins of β-arrestin) bind to the GPCR at a site distinct from the orthosteric binding site. As a consequence, they can be considered as allosteric regulators (in this case, proteins) that can have a reciprocal effect on ligand binding (or coupling in the case of the protein) and lead to altered affinity and efficacy for particular agonists (Kenakin, 2012). In many ways, therefore, biased signalling is a natural consequence of a key feature of allosterism, namely probe dependence (Figure 1B).

The ability of receptor-associated proteins to act as allosteric modulators of ligand binding and efficacy can be extended to neighbouring receptors that form homo- or heterodimers or higher order oligomers (Figure 1C). For example, we have recently provided evidence for negative cooperativity across the dimer interface of an adenosine A_3-receptor homodimer (May *et al.*, 2011). In this case, binding of an orthosteric ligand to one protomer (monomeric component) of the homomeric complex can markedly alter the affinity of a ligand binding to the second protomer (May *et al.*, 2011). Evidence is also accumulating that the adenosine A_1 receptor can form heterodimers with P_{2Y} receptors, adenosine A_{2A} receptors, β_1- and β_2-adrenoceptors to influence orthosteric ligand binding and/or intracellular signalling (Suzuki *et al.*, 2006; Chandrasekera *et al.*, 2013; Cristovao-Ferreira *et al.*, 2013; Franco *et al.*, 2013). In addition to partner receptors within oligomeric complexes, other extracellular proteins can also bind to GPCRs and mediate allosteric influences. For example, adenosine deaminase (ADA), which is a key enzyme catalysing the deamination of adenosine, can be released from cells and bind to cell surface proteins and act as an ectoenzyme (Gracia *et al.*, 2013). One of the proteins that bind ADA is the adenosine A_1 receptor (Ciruela *et al.*, 1996; Gracia *et al.*, 2013). The association of ADA with the adenosine A_1 receptor can lead to enhanced agonist affinity and efficacy (Gracia *et al.*, 2008; 2013). The consequence of a metabolic enzyme for the endogenous activator being associated with the cell surface adenosine receptor, which can enhance affinity and efficacy, is therefore likely to amplify local signalling while limiting the duration of action (and spread of activity) due to its local metabolic activity.

Overview of small-molecule allosteric regulators acting on the adenosine A_1 and A_3 receptors

The therapeutic potential of allosteric regulators that can amplify or modulate the local actions of adenosine is clear and efforts are in progress to develop these reagents for a wide range of GPCRs. The development of allosteric modulators targeted at the adenosine receptor family has recently been comprehensively reviewed (Göblyös and Ijzerman, 2011; Jacobson *et al.*, 2011). For brevity, we will focus our discussion on the medicinal chemistry of allosteric modulators specifically mentioned within this review (Figure 2). As stated earlier, PD 81,723 was one of the key compounds originally described in back-to-back papers from the Parke-Davis Pharmaceutical Research Division (Bruns and Fergus, 1990; Bruns *et al.*, 1990) recounting the identification of the first allosteric regulators of adenosine A_1-receptor binding. The chemical series was originally identified from the Parke-Davis compound bank via a 300-ligand adenosine A_1 binding screen. While the 2-amino-3-benzoylthiophene chemical scaffolds had been originally synthesized as intermediates for benzodiazepine-like compounds (Tinney *et al.*, 1974), recognition of their adenosine antagonist activity prompted a more thorough analysis of this privileged chemical template (Bruns and Fergus, 1990). From this medicinal chemistry study, second-generation compounds were unexpectedly found to increase the specific binding of [^{3}H]N^6-cyclohexyladenosine to rat brain membranes. This, in turn, resulted in the synthesis of further compounds to identify pertinent structure–activity relationships and, in so doing, identified PD 81,723 as a key analogue displaying a significantly improved allosteric profile (Bruns *et al.*, 1990). This core structure has been further modified by a number of groups thereby developing a robust structure activity relationship profile and a series of ligands with comparable or more favourable allosteric activity (van der Klein *et al.*, 1999; Kourounakis *et al.*, 2000; Baraldi *et al.*, 2003, 2004; Nikolakopoulos *et al.*, 2006; Romagnoli *et al.*, 2008; Valant *et al.*, 2010). One particularly successful manipulation centred on removal of the 4- and 5-methyl groups and installation of substituted phenyl rings back into these positions of PD 81,723 (Aurelio *et al.*, 2008). It was interesting to note that the most efficacious compound possessed no substituent in the 5-position and this lead to further exploitation of this observation through the synthesis and evaluation of the next generation of ligands, which identified VCP520 as a potent allosteric enhancer of A_1-receptor-mediated signalling (Aurelio *et al.*, 2009).

In a similar fashion as the discovery of PD 81,723, the lead compounds recognized as allosteric modulators of the adenosine A_3 receptor were identified from screening diverse chemical libraries in binding assays at this receptor subtype (Jacobson *et al.*, 2011). In this instance, certain lead molecules were shown to increase the level of binding of [^{125}I]AB-MECA (Gao *et al.*, 2001; 2002). Key molecular scaffolds that supported allosteric modulation at the adenosine A_3 receptor were identified as 3-(2-pyridinyl)isoquinolines (e.g. VUF5455) and 3*H*-imidazo-[4,5-*c*]quinolin-4-amines (e.g. LUF5999, LUF6000 and LUF6001). With regard to the former, further exploration of the 3-(2-pyridinyl)isoquinoline scaffold

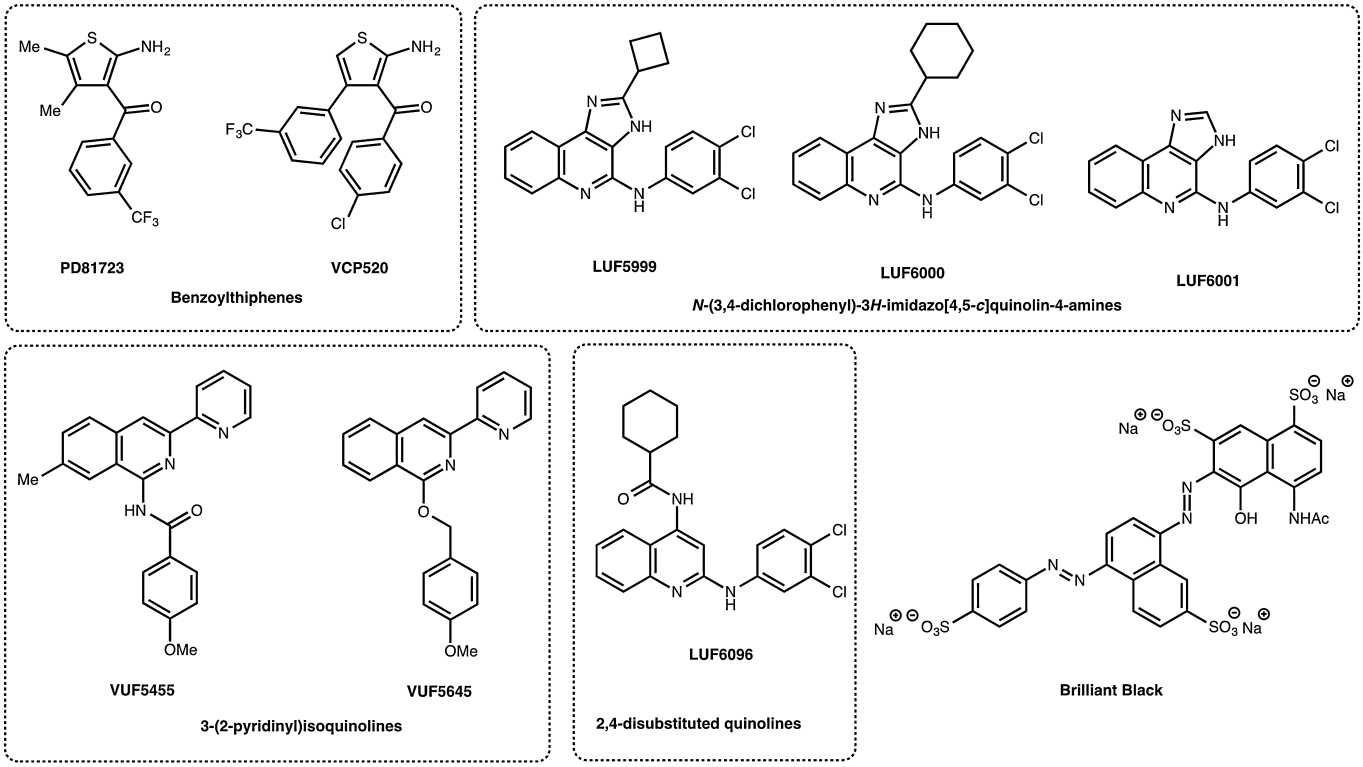

Figure 2
A selection of adenosine receptor allosteric modulators.

revealed a complex situation where some members were pure antagonists of the orthosteric binding site, for example, VUF5455 itself (Heitman *et al.*, 2009), consequently rendering them not particularly useful as future therapeutics.

However, the imidazoquinolinamines fared better in this respect; the original molecule DU124183 (Gao *et al.*, 2002) was further modified at the 2- and 4-positions and numerous resultant derivatives displayed potentiation of the maximum efficacy of Cl-IB-MECA at the A_3 receptor (Göblyös *et al.*, 2006). Indeed, LUF6000 was shown to enhance agonist efficacy in a functional assay and decrease agonist dissociation rate without influencing agonist potency. This was postulated to be a result of its experimentally observed decreased interaction with the orthosteric binding site on the adenosine A_3 receptor. As previously mentioned, a more thorough analysis of this and related ligands (LUF5999 and LUF6001) identified that these imidazoquinolinamine allosteric enhancers displayed differing effects on the affinity and efficacy of Cl-IB-MECA at the A_3 receptor (Gao *et al.*, 2011). In a related study, with the intention of overcoming the issues associated with the orthosteric antagonism shown by the 3-(2-pyridinyl)isoquinolines, a series of ring opened imidazoquinolinamines were synthesized to afford a range of 2,4-disubstituted quinolines as a new class of allosteric enhancers at the A_3 receptor (Heitman *et al.*, 2009). Rewardingly, the best compound (LUF6096) was not only able to allosterically enhance the binding of Cl-IB-MECA to a similar level as LUF6000 but it also displayed negligible orthosteric affinity for any of the adenosine receptor subtypes. These compounds have begun to be used in mechanistic studies to identify the basis of these allosteric effects on efficacy and affinity and the extent to which these two effects are related.

Mechanistic insights from single-cell ligand-binding kinetics

Allosteric interactions are a mode of communication between distal binding sites. Intra- and intermolecular GPCR allosterism with transmembrane proteins and allosteric small molecules can generate a unique spectrum of resting and/or active distribution of GPCR conformations, which, in turn, can significantly influence the pharmacology of orthosteric and/or allosteric ligands. Typically, GPCR allosterism changes the properties of conformationally linked binding sites and therefore the association and/or dissociation kinetics of the cognate orthosteric ligands (May *et al.*, 2007; Smith and Milligan, 2010). Orthosteric ligand affinity is described by the ratio of the association to dissociation rates, and as such, an allosteric interaction that alters orthosteric ligand affinity does so by mediating a change in one or both of these parameters. Dissociation kinetic assays can be used as a powerful mechanism to validate an allosteric mechanism of action of a ligand since orthosteric and allosteric ligands must interact with the receptor simultaneously to change the dissociation kinetics of a labelled orthosteric ligand. Plotting the dissociation rate of labelled orthosteric ligand in the presence of

a range of interacting ligand concentrations provides a concentration–response relationship of a purely allosteric effect (Kostenis and Mohr, 1996). Furthermore, the midpoint of this curve provides an estimate of affinity of the orthosteric ligand for the allosteric modulator occupied receptor.

Typically, dissociation kinetic studies investigating intramolecular allosterism use isotopic dilution. That is, the influence of an allosteric ligand on the dissociation kinetics of an orthosteric radiolabelled probe is assessed in the presence of a saturating concentration of a second competitive orthosteric ligand (Bruns and Fergus, 1990a; Ellis *et al.*, 1991; Lee and el-Fakahany, 1991; Lazareno and Birdsall, 1995; Christopoulos *et al.*, 1997; Gao *et al.*, 2001; Avlani *et al.*, 2004; Dowling and Charlton, 2006). A key assumption required for interpreting such dissociation kinetic studies, however, is that the second ligand does not alter the rate of radioligand dissociation. This assumption is consistent within a theoretical framework describing competitive interactions between compounds at a monomeric receptor; however, more complex interactions resulting from multistep ligand binding (Swaminath *et al.*, 2004; Ilien *et al.*, 2009) or receptor dimerization (Christopoulos and Kenakin, 2002; Springael *et al.*, 2006; Han *et al.*, 2009; May *et al.*, 2011) could lead to a change in the radioligand dissociation rate. Recently, the binding kinetics of a fluorescent adenosine derivative was determined in the absence and presence of allosteric modulators at the adenosine A_1 and A_3 receptor in live single cells (May *et al.*, 2010b). Importantly, these studies were performed using a closed perfusion system that enabled rapid removal of free ligand (May *et al.*, 2010a) and therefore assessed the dissociation kinetics under 'infinite dilution' conditions in the absence of a saturating concentration of competitive orthosteric ligand. Similar to the previous studies, which used isotopic dilution to promote orthosteric radioligand dissociation, PD 81,723 significantly retarded the dissociation of the fluorescent adenosine derivate from the adenosine A_1 receptor (May *et al.*, 2010b). In contrast, VUF5455, which has previously been demonstrated to decrease the rate of agonist dissociation from the adenosine A_3 receptor (Gao *et al.*, 2001), was found to significantly enhance the fluorescent agonist dissociation rate (May *et al.*, 2010b). This discrepancy may reflect the different orthosteric agonist probes used in the different studies and therefore the ability of allosteric modulators to be highly probe-dependent (May *et al.*, 2007). Alternatively, the difference could reflect a more complex receptor arrangement than a non-interacting monomer, that is, a dimer or higher order oligomer (see below).

Traditionally, GPCRs have been considered to exist and function as monomeric proteins. However, it is now known that GPCRs can form homodimers, heterodimers and/or higher order oligomers (Smith and Milligan, 2010). Non-visual GPCRs can be classified into three families, A–C. Family C GPCRs are known to function as obligate dimers (May *et al.*, 2007; Smith and Milligan, 2010). In contrast, the extent, stability and physiological consequence of dimerization remains highly controversial for family A GPCRs, which are the largest family and include the well-characterized adrenoceptors, dopamine receptors, adenosine receptors and muscarinic ACh receptors (Smith and Milligan, 2010). Monomeric family A GPCRs reconstituted in high-density lipoprotein phospholipid bilayer particles can couple to G proteins, suggesting that this family does not need to function as obligate dimers (Kuszak *et al.*, 2009). However, evidence suggests that cell surface complexes of family A GPCRs may display a distinct profile of functional properties relative to their monomeric counterparts. For example, dimerization and/or oligomerization may influence signal transduction efficiency, receptor desensitization and/or the ligand preference for coupling to particular downstream signalling cascades (May *et al.*, 2007; Smith and Milligan, 2010; Franco *et al.*, 2013). Furthermore, a recent study provided evidence for communication between simultaneously bound orthosteric sites on homodimeric dopamine D_2 receptors (Urizar *et al.*, 2011). As such, a ligand bound to one protomer can modulate ligand function and/or affinity at a second interacting protomer. This may lead to complex pharmacology and/or the potential for dimeric species to elicit specific signalling events with unique pharmacological properties.

The fundamental premise of intra- and intermolecular allosteric modulation is based on conformational rearrangements; therefore, a wealth of information can be gained through assessing ligand-binding kinetics under different conditions. Dissociation kinetic studies provided the first evidence for homodimerization of a family A GPCR, the β-adrenergic receptor. This study used an 'infinite dilution' approach to detect a change in the dissociation kinetics of the radiolabelled orthosteric ligand, [^{3}H](-)alprenolol, in the absence and presence of unlabelled (-)alprenolol. The increased dissociation rate in the presence of unlabelled orthosteric ligand, (-)alprenolol, was suggestive of negatively cooperative interactions across a β-adrenergic homodimeric interface (Limbird *et al.*, 1975). Intermolecular cooperativity between orthosteric binding sites has since been established for a number of additional GPCRs, including adenosine and muscarinic ACh receptor subtypes (Briddon *et al.*, 2008; Casadó *et al.*, 2010; Hern *et al.*, 2010; Pisterzi *et al.*, 2010; Hu *et al.*, 2012; May *et al.*, 2011). At the adenosine A_1 and A_3receptors, dissociation kinetic analysis has been employed as a powerful method to detect intermolecular allosterism, that is, cooperative interactions across a homodimeric interface (May *et al.*, 2011). In contrast to the adenosine A_1 receptor, highly cooperative interactions were observed under 'infinite dilution' conditions between the fluorescent adenosine derivative and orthosteric agonists and antagonists at the adenosine A_3 receptor. Figure 3 shows an example of the effect of increasing concentrations of the endogenous orthosteric ligand adenosine on the dissociation kinetics of a fluorescent adenosine analogue from the human adenosine A_3 receptor. In marked contrast, adenosine had a much less marked effect on the dissociation kinetics of the fluorescent ligand from the human adenosine A_1 receptor (May *et al.*, 2011). Importantly, the intermolecular allosterism was significantly decreased upon co-expression of a non-binding adenosine A_3-receptor mutant, supporting the suggestion of cooperative interactions across the dimeric interface of cell surface adenosine A_3 receptors (May *et al.*, 2011). These studies add strength to the suggestion that the discrepancy observed between the influence of the allosteric modulator, VUF5455, in dissociation kinetic studies using isotopic dilution as compared to infinite dilution may reflect the ability of adenosine A_3 receptors to form interacting homodimers and/or higher order oligomers. In keeping with this hypoth-

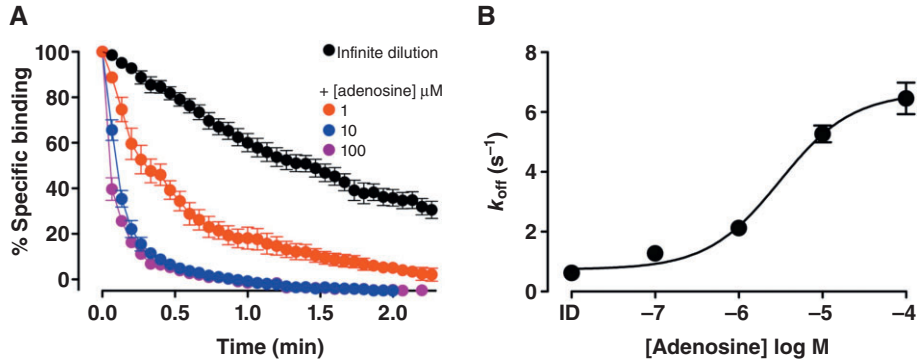

Figure 3

Adenosine mediates a significant enhancement in the dissociation of 30 nM ABA-X-BY630 from the human adenosine A_3 receptor. (A) Dissociation of a fluorescent adenosine analogue, ABA-X-BY630 (30 nM), from CHO-A_3 cells in the absence or presence of adenosine (1 μM; 10 μM; 100 μM). (B) Concentration dependence of the changes in k_{off} of 30 nM ABA-X-BY630 from CHO-A_3 cells in the absence and presence of adenosine. Data points are expressed as mean ± SEM from 3–11 separate experiments; each replicate represents the average fluorescence at the plasma membrane of 10 individual cells. Data taken from May *et al.* (2011).

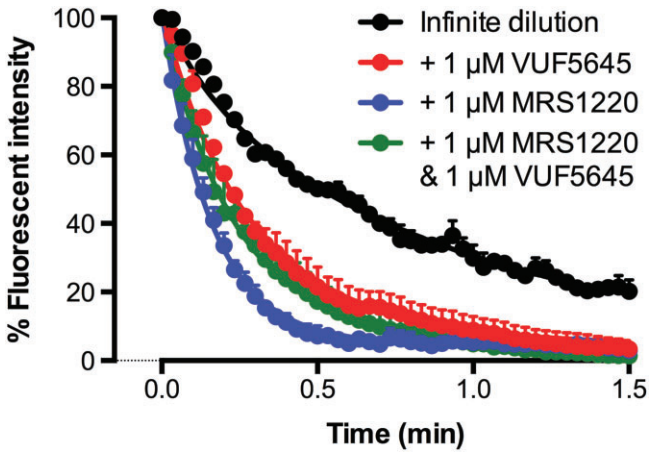

Figure 4

The influence of the competitive antagonist, MRS1220, and/or the allosteric ligand, VUF5645, on the dissociation kinetics of the fluorescent adenosine derivative, ABA-X-BY630. ABA-X-BY630 (30 nM) dissociation in the absence and presence of a 1 μM MRS1220, 1 μM VUF5645, or 1 μM MRS1220 and 1 μM VUF5645. Representative data performed in duplicate; each replicate represent the average fluorescence at the plasma membrane of 10 individual cells.

esis, if a non-fluorescent orthosteric ligand (e.g. MRS1220) is added simultaneously with a derivative of VUF5455 (VUF5645), then this allosteric compound then produces a slowing down of the dissociation kinetics of the fluorescent adenosine analogue (Figure 4).

Impact of A_1- and A_3-receptor allosteric modulators on *in vivo* pharmacology

Adenosine A_1- and A_3-receptor ligands (both agonists and antagonists) have been developed for a number of potential therapeutic indications (Muller and Jacobson, 2011). These are summarized in Table 1. The best developed indications appear to be for agonists where A_1-receptor agonism may have utility in angina, neuropathic pain, paroxysmal supraventricular tachycardia and ischaemia (Griffin *et al.*, 2003; Morrison *et al.*, 2006; Albrecht-Kupper *et al.*, 2012; Tendera *et al.*, 2012), and A_3-receptor agonists may have benefit in liver cancer, rheumatoid arthritis, autoimmune inflammatory disease, dry eye and cardiac ischaemia (Table 1; Bar-Yehuda *et al.*, 2011; Cohen *et al.*, 2011; Fishman *et al.*, 2012). With regard to cancer, it is interesting that A_3 receptors appear to be overexpressed in certain cancers (e.g. breast and colon cancer) compared to normal cells (Gessi *et al.*, 2004; Madi *et al.*, 2004; Bar-Yehuda *et al.*, 2008; Fishman *et al.*, 2012).

Studies with genetically altered mice have also suggested a role of A_1 receptors in pain (Sowa *et al.*, 2010) and ischaemia (Matherne *et al.*, 1997) and for A_3 receptors in cardiac ischaemia (Ge *et al.*, 2006) and mast cell degranulation (Salvatore *et al.*, 2000). In the latter case, it is worth pointing out that functional A_3 receptors appear to be absent from human mast cells (Fredholm *et al.*, 2011). However, although selective A_3-receptor activation is cardio-protective in wild-type mice and those overexpressing the A_1 receptor, adenosine A_3-receptor gene deletion generates an ischaemia-tolerant phenotype that might be indicative of compensatory changes (Harrison *et al.*, 2002).

As mentioned in the introductory remarks, the ubiquitous distribution of adenosine receptors and the potential for serious side effects via the target receptor in a different organ or cell type can limit their utility. For example, in many non-cardiac therapeutic applications of A_1-receptor agonists, the potential for major side effects due to A_1-receptor actions in the heart will be seriously limiting. This may be particularly true in the case of adenosine A_1-receptor agonists that may have potential utility in the treatment of CNS diseases, such as epilepsy (Mares, 2010; Klaft *et al.*, 2012). This has led to the development of partial agonists (e.g. capadenoson; Albrecht-Kupper *et al.*, 2012; Tendera *et al.*, 2012) that may

Table 1

Therapeutic indications for selective A_1 and A_3 adenosine receptor ligands

Adenosine receptor	Changes following genetic deficit or overexpression	Potential therapeutic indications		Example drugs
A_1	Analgesic effects of adenosine abolished in A_1-KO mice. Overexpression of cardiac A_1 receptors caused increased resistance to ischaemia.	Agonist	Atrial fibrillation, angina, hyperlipidaemia, neuropathic pain, paroxysmal supraventricular tachycardia, cardiac ischaemia	Capadenoson, Tecadenoson, RPR749, GR79236
		Antagonist	Acute renal failure, heart failure (renal function)	FK-453, SLC320
A_3	Enhanced antigen-stimulated mast cell degranulation by A_3-agonists lost in A_3-KO mice. Studies with congenic A_3-/- mice indicate a cadioprotective effect of A_3-receptor activation.	Agonist	Liver cancer, rheumatoid arthritis, autoimmune inflammatory disease, dry eye, cardiac ischaemia, dry eye	Cl-IB-MECA (CF102) MRS3558 (CF502) IB-MECA (CF101)
		Antagonist	Asthma, glaucoma	KF26777, OT-7999

KO, knockout.

have less severe off-target profiles and are less prone to receptor desensitization. The potential to overcome these limitations with allosteric enhancers is obvious, particularly if the advantages offered by instilling bias into the final signalling outcome can be exploited (as a consequence of the allosteric impact of partner receptor-interacting proteins). However, although *in vitro* studies have provided convincing evidence for allosteric mechanisms of action for a number of ligands, therapeutic application of these mechanisms relies on their successful translation into whole animal physiology. Indeed, the *in vivo* actions of allosteric regulators have not been extensively investigated and there is a need to evaluate the potential for these small molecules to augment specific actions of adenosine in particular organs and cell types in a whole animal setting.

Some success has been achieved *in vivo* with two allosteric ligands. Adenosine receptor activation has been implicated in the mechanism of ischaemic pre-conditioning (Carr *et al.*, 1997; Uematsu *et al.*, 1998). Ischaemic pre-conditioning is where an organ (normally the heart) is subjected to brief periods of ischaemia and reperfusion, resulting in a resistance to infarction. For example, in human atrial muscle, both adenosine A_1- and A_3-receptor activation can mimic ischaemic pre-conditioning (Carr *et al.*, 1997). Activation of adenosine A_1 receptors has been shown to protect against renal ischaemia/reperfusion injury (Lee and Emala, 2000; Lee *et al.*, 2004). However, extra-renal side effects (e.g. bradycardia, hypotension) may limit the use of A_1-receptor agonist therapy for acute ischaemic kidney injury (Park *et al.*, 2012). Interestingly, the A_1-receptor allosteric enhancer PD 81,723 produced a dose-dependent protection against ischaemia/reperfusion injury in the kidneys of wild-type mice but not in adenosine A_1-receptor-deficient mice (Park *et al.*, 2012). This was achieved in the absence of significant effects on heart rate and BP, which suggests that renal A_1-receptor selectivity had been achieved with PD 81,723 as a consequence of amplifying the restricted increase in adenosine in the kidney following local ischaemia (Park *et al.*, 2012). A similar outcome has been reported in the CNS where administration of PD 81,723 can lead to a reduction in hippocampal injury following hyperglycaemic ischaemia in the rat (Meno *et al.*, 2003).

A positive allosteric modulator of the adenosine A_3 receptor (LUF6096) has also been shown to have benefit in an *in vivo* model of myocardial ischaemia/reperfusion injury in the dog (Du *et al.*, 2012). Thus, LUF6096 had no effect on baseline haemodynamic parameters, but pre-treatment with LUF6096 prior to coronary occlusion and during reperfusion produced a marked reduction in infarct size (ca. 50% reduction; Du *et al.*, 2012). An equivalent reduction in the infarct size could also be demonstrated if LUF6096 was administered immediately before reperfusion (Du *et al.*, 2012). These studies collectively indicate that allosteric enhancers of the adenosine A_1 and A_3 receptors may have great utility as therapeutic strategies to provide selective augmentation of the actions of adenosine released locally in conditions of disease and stress.

Concluding remarks

It is clear that allosteric mechanisms of action provide unique ways to regulate receptor function at a local level to 'fine-tune' intracellular signalling. This can be achieved by small molecules (allosteric regulators) or by protein–protein interactions involving signalling proteins (leading to biased signalling) or oligomeric partners (e.g. as a consequence dimerization). In all cases, these mechanisms provide the potential to exploit the unique pharmacology provided by allosterism to achieve both better cell and tissue selectivity of drug treatments and also interventions with more physiologically relevant kinetic profiles. Novel fluorescent techniques have been able to unravel some of the intricacies involved at the single cell level. However, the therapeutic potential of these actions awaits the clear demonstration of these mechanisms in an *in vivo* setting.

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Conflict of interest

None.

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