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REVIEW ARTICLE

New paradigms in adenosine receptor pharmacology: allostery, oligomerization and biased agonism

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Adenosine receptors are a family of GPCRs containing four subtypes (A_1, A_{2A}, A_{2B} and A_3 receptors), all of which bind the ubiquitous nucleoside adenosine. These receptors play an important role in physiology and pathophysiology and therefore represent attractive drug targets for a range of conditions. The theoretical framework surrounding drug action at adenosine receptors now extends beyond the notion of prototypical agonism and antagonism to encompass more complex pharmacological concepts. New paradigms include allostery, in which ligands bind a topographically distinct receptor site from that of the endogenous agonist, homomeric or heteromeric interactions across receptor oligomers and biased agonism, that is, ligand-dependent differential intracellular signalling. This review provides a concise overview of allostery, oligomerization and biased agonism at adenosine receptors and outlines how these paradigms may enhance future drug discovery endeavours focussed on the development of novel therapeutic agents acting at adenosine receptors.

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Abbreviations

NAM, negative allosteric modulator; PAM, positive allosteric modulator

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Introduction

Adenosine is an endogenous purine nucleoside present both intracellularly and extracellularly. It is consists of an adenine group attached to a ribose sugar by a glycosidic bond. Adenosine, as both a precursor and metabolite of adenine nucleotides, provides the structural building block of **ATP** and thus plays a central role in the basic energy transfer of all living organisms (Fredholm, 2007). Adenosine also acts as a ubiquitous extracellular signalling molecule to exert a wide range of physiological actions throughout the body, predominantly reducing cellular work and restoring energy balance (Fredholm, 2007). Adenosine mediates its myriad of physiological and pathophysiological actions *via* the activation of the adenosine family of GPCRs, which comprise four subtypes, namely, the **adenosine A_1 receptors**, **adenosine A_{2A} receptors, adenosine A_{2B} receptors** and **adenosine A_3 receptors** (Fredholm *et al.,* 2001; Alexander *et al.,* 2017a).

Classically, adenosine-mediated signalling is subdivided based on the effects of adenosine receptor activation on cAMP levels. The A_1 receptors and A_3 receptors preferentially couple to *Pertussis* toxin-sensitive $G_{i/o}$ proteins to inhibit **adenylyl cyclase** whereas the A_{2A} receptors and A_{2B} receptors stimulate adenylyl cyclase through activation of G_s proteins (Fredholm *et al.,* 2001). Adenosine receptors can also modulate a variety of additional second messengers. A_1 receptor agonists activate potassium channels (including K_{ATP} channels in the myocardium and neurons), increase intracellular calcium and inositol triphosphate levels by activating **PLC** (*via* Gβγ), stimulate **PKC** activity and inhibit N-type voltage-sensitive Ca^{2+} channels in neurons (Jacobson and Gao, 2006). The A_{2A} receptors almost exclusively couples to **cAMP/PKA** signalling *via* G_s, except in the striatum where G_{olf} stimulation predominates (Jacobson and Gao, 2006). The A_{2B} receptors, however, appear to be promiscuously coupled, partnering with G_s to stimulate cAMP/PKA in most tissues but also interacting with $G_{q/11}$ to activate PLC and mobilize calcium stores in mast cells and cardiac fibroblasts (Jacobson and Gao, 2006). The A_3 receptors, *via* $G_{i/o}$ coupling, activate PLC and Ca^{2+} signalling through the Gβγ and activates K_{ATP} channel opening in the myocardium (Jacobson and Gao, 2006). Furthermore, all adenosine receptor subtypes activate MAPK pathways, including phosphorylation of **ERK1/2** *via* a variety of mechanisms (Fredholm *et al.,* 2001). More recent evidence has also emerged of interactions with β-arrestin proteins, which adds another layer of complexity to adenosine receptor signalling (Mundell and Kelly, 2011).

New paradigms in adenosine receptor pharmacology

The ability of GPCRs to transduce external stimuli into intracellular signal transduction has traditionally been explained by the ternary complex model. In the classical version, the formation of a ternary complex consisted of a single receptor, agonist and G protein for receptor activation (Lane *et al.,* 2017). While conceptually still useful, more recently identified paradigms such as dimerization, allostery and biased agonism (Figure 1) have necessitated an evolution in this theoretical framework. Importantly, exploiting the unique features of these paradigms is likely to facilitate the development of targeted therapeutic agents acting on adenosine receptors that stimulate potent therapeutic signal transduction with minimal on-target adverse effects.

Dimerization and higher-order oligomerization

Traditionally, GPCRs have been depicted as monomeric units, interacting at a one-to-one ratio with their corresponding heterotrimeric G protein. However, over the last two decades, this canonical thinking has evolved, with evidence supporting the complexing of some GPCRs into dimers or higher-order oligomers (Pin *et al.,* 2007). Homodimerization describes the self-association of receptor subunits, and heterodimerization describes the association of two different receptor subunits. Class C GPCRs are known to function as obligate dimers (Pin *et al.,* 2007), whereas the presence and physiological implications of Class A GPCR oligomerization remains contentious (Felce *et al.,* 2017). However, a growing body of evidence supports the ability of at least a subset of Class A GPCRs to form complexes (Felce *et al.,* 2017), and as such, the potential to engender unique signalling profiles. Indeed, it has been proposed that oligomerization diversifies the number of receptor entities possible from the limited number of GPCR genes, adds to their pharmacological complexity and represents novel opportunities for drug discovery (Pin *et al.,* 2007). Physiologically relevant, oligomeric interactions of adenosine receptors have been identified by evidence gathered largely within the central nervous system. Assembly of adenosine receptors into heteromers are proposed as a probable mechanism underlying functional cooperativity observed in the brain and also more recently in the heart (Franco *et al.,* 2008; Chandrasekera *et al.,* 2013; Surendra *et al.,* 2013).

Adenosine receptor homomers. The ability of adenosine receptors to form homomers was first described for the A_1 receptors . The possibility of A_1 receptor dimers in the brain cortex was suggested some 20 years ago after antibody immunoprecipitation and immunoblotting revealed higher-order bands that appeared to correspond to A_1 receptor homomers (Ciruela *et al.,* 1995). More recent studies have supported the existence of A_1 receptor homomers at the plasma membrane using techniques such as bimolecular fluorescence complementation and fluorescence correlation spectroscopy (Briddon *et al.,* 2008). The assembly of A_{2A} receptors into homomeric complexes has been predominantly studied through the use of tagged receptors in resonance energy transfer assays. Resonance energy transfer between a donor and acceptor molecule in close proximity, including BRET and FRET, have demonstrated that A_{2A} receptors form dimers at the cell surface (Canals *et al.,* 2004) and may further associate into oligomers with three or more A_{2A} receptor protomers (Gandía *et al.,* 2008). To date, A_{2B} receptor homomeric interactions have not been reported. A_3 receptor homomers have recently been suggested, using fluorescent ligand binding kinetics to quantify allosteric interactions across an A_3 receptor homomeric interface (May *et al.,* 2011). Collectively, despite evidence of adenosine receptor homomers in heterologous

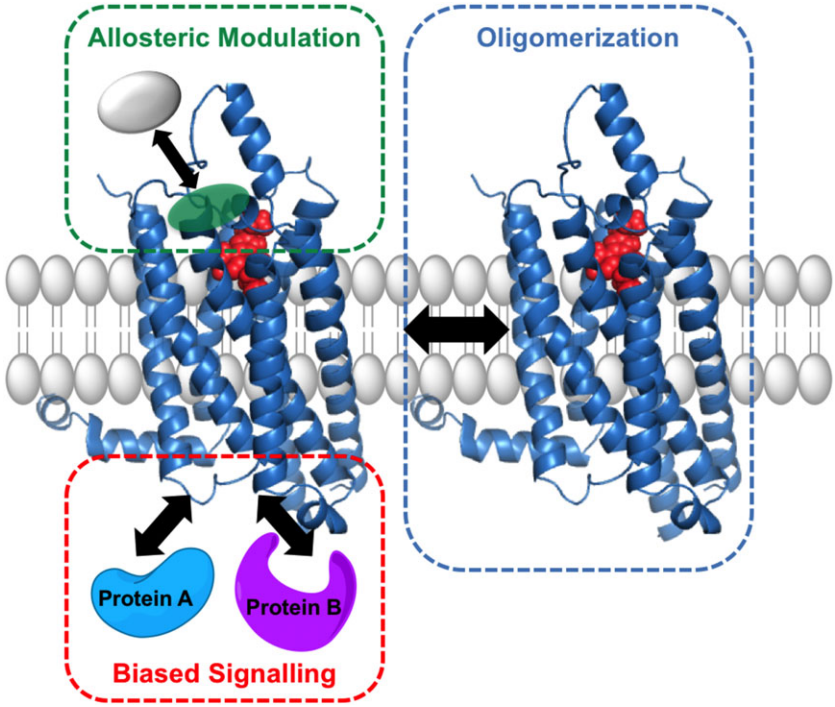

Figure 1

New paradigms in adenosine receptor pharmacology. Recent paradigms include the following: (i) allosteric modulation, the influence on ligand pharmacology observed upon the binding of a second ligand to a topographically distinct, but conformationally linked binding site on the receptor macromolecule; (ii) biased agonism, ligand-dependent stabilization of differential receptor conformations linked to distinct signalling outcomes; and (iii) oligomeric complexing of two or more GPCRs.

expression systems, the physiological consequence of endogenously expressed homomeric adenosine receptor complexes has not been elucidated.

Adenosine receptor heteromers. As for many other rhodopsin-like Class A GPCRs, there is growing recognition of adenosine receptor heterodimeric interactions with other receptors, in particular with members of the **dopamine** receptor family (Franco *et al.*, 2008; Fredholm *et al.*, 2011). Heterodimerization of adenosine receptors was first suggested as the basis of the negative functional crosstalk displayed between the A_{2A} receptor and **dopamine D_2 receptor** in the striatum on locomotor activity, with implications in the treatment of Parkinson's disease (Fuxe *et al.*, 2015). The heteromer, recently suggested to comprise A_{2A} receptor and D_2 receptor homodimers assembled into a heterotetramer, represents the most widely studied and accepted adenosine receptor heteromer to date (Casadó-Anguera *et al.*, 2016). These A_{2A}-D_2 receptor heteromers have also been suggested to participate in higher-order oligomeric complexes, interacting with both the **cannabinoid CB_1 receptor** and the **metabotropic glutamate $mGlu_5$ receptor** as determined by sequential BRET-FRET techniques (Fredholm *et al.*, 2011). The A_1 receptor was reported to form a functional dimer with the **dopamine D_1 receptor** but not the D_2 receptor in co-transfected mouse fibroblasts (Gines *et al.*, 2000). Heteromeric interactions within the adenosine receptor family have also been identified. A_1 and A_{2A} receptor heteromers, detected in recombinant cells and human brain tissue, have been implicated in the presynaptic control of glutamatergic neurotransmission (Ciruela *et al.*, 2006). The A_{2A} receptor has additionally been proposed to complex with the A_{2B} receptor, providing the dominant forward transport signal for efficient cell surface expression of the A_{2B} receptor, the importance of which was highlighted in splenocytes from A_{2A} receptor knockout mice (Moriyama and Sitkovsky, 2010).

While the potential involvement of adenosine receptor heteromers in neurotransmitter signalling in the brain is well studied, the role of such complexes in other systems including the heart is only recently being realized. Interactions between the A_1 receptor and **δ** and **κ** opioid receptors have been detected using co-immunoprecipitation and hypothesized to be involved in cardioprotection by remote ischaemic preconditioning (Surendra *et al.*, 2013). Similarly, A_1 receptor dimers with **β_1** and **β_2** adrenoceptors demonstrate novel heteromers with altered ligand binding affinity and ERK1/2 phosphorylation (Chandrasekera *et al.*, 2013).

Dimerization or receptor crosstalk?. It must be acknowledged that evidence of receptor interactions occurring at downstream signalling pathways does not confirm the presence of direct interactions at a receptor level nor does evidence of direct receptor association in recombinant cells constitute proof of physiological relevance. According to the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology, in order for an oligomeric interaction to be considered physiologically significant, it must have evidence of physical association in native tissue

or primary cells and demonstrate specific pharmacological properties unique to the dimer that is altered in the absence of one of the subunits, preferably validated with the use of knockout animals or RNA interference technology (Pin *et al.,* 2007). Although not all of the examples of adenosine receptor heteromers mentioned above fulfil the complete criteria for oligomeric classification, the increasing recognition of the importance of GPCR complexing to physiology and pathophysiology is likely to provide novel opportunities for adenosine receptor drug discovery (Franco *et al.,* 2008; Fuxe *et al.,* 2015).

Allostery

Allosteric ligands recognize a topographically distinct, yet conformationally linked, receptor binding site to that of the orthosteric endogenous ligand (May *et al.,* 2007). Importantly, recent advances in GPCR crystallography have enabled, for the first time, direct visualization of the discrete nature of orthosteric and allosteric ligand binding at Class A GPCRs (Figure 2) (Kruse *et al.,* 2013; Zheng *et al.,* 2016). Upon binding, allosteric ligands have the capacity to stabilize active and/or inactive receptor conformation(s), thereby modulating receptor activity in the absence of orthosteric ligand. Furthermore, allosteric ligands can modulate the kinetics, affinity and/or efficacy of the ligand bound within the orthosteric site (May *et al.,* 2007; Lane *et al.,* 2017). Allosteric ligands are typically classed into a number of categories. Positive and negative allosteric modulators (PAMs or NAMs) increase and decrease, respectively, the affinity and/or efficacy of an orthosteric ligand, whereas neutral allosteric ligands exhibit neutral cooperativity with the orthosteric ligand (May *et al.,* 2007). It is important to note that the classification of an allosteric ligand is also dependent upon the orthosteric counterpart. That is, allosteric modulators can demonstrate differential cooperativity depending on the co-bound orthosteric ligand, a phenomenon termed probe dependence (Valant *et al.,* 2012b). Moreover, the effect of an allosteric modulator on both orthosteric ligand efficacy and affinity is not always unidirectional, in that a modulator can increase the affinity of an orthosteric ligand while decreasing the efficacy and *vice versa* (May *et al.,* 2007).

Allostery at adenosine receptors. Allostery has been detected and quantified at all four adenosine receptors, although the majority of allosteric ligands have been identified for the A_1 and A_3 receptors (Göblyös and IJzerman, 2011). The A_1 receptor was the first adenosine receptor, and in fact the first GPCR, for which PAMs of orthosteric agonists were identified (Bruns and Fergus, 1990). These compounds were centred around a 2-amino-3-benzoylthiophene scaffold and include the now well-characterized A_1 receptor allosteric modulator, **PD 81,723**. These early studies identified that PD 81723 pharmacology displayed hallmarks of allostery, particularly the positive allosteric modulation of orthosteric

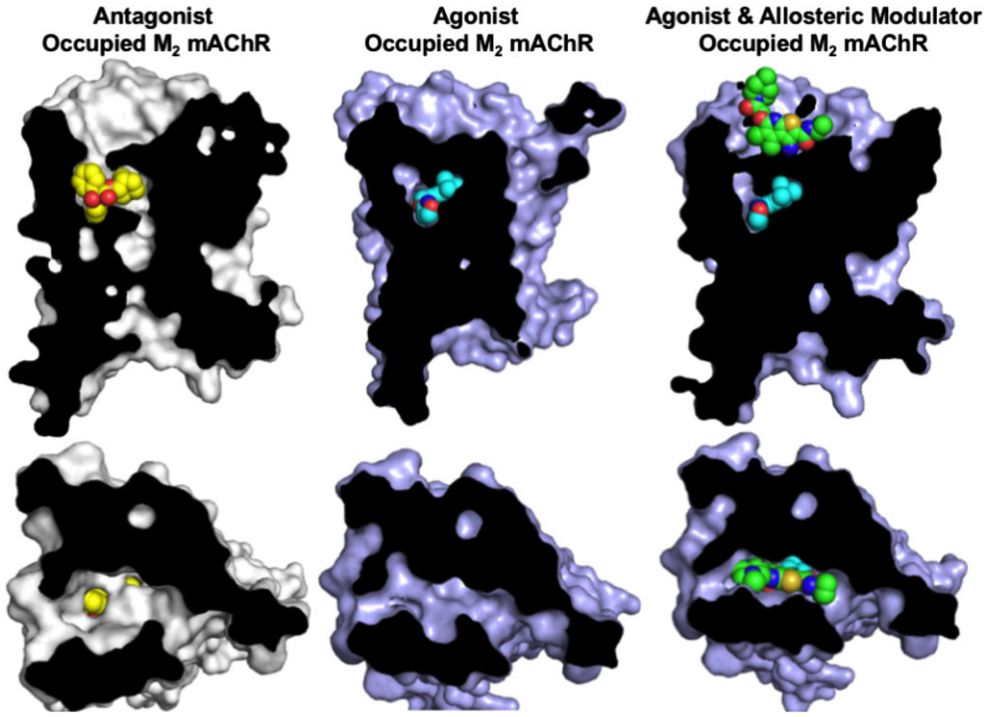

Figure 2

Allosteric ligands bind to a topographically distinct receptor site to orthosteric ligands. The phenomenon of topographically distinct binding of orthosteric and allosteric ligands has been directly demonstrated for Family A GPCRs. For example, **M_2 muscarinic acetylcholine receptor** crystal structures clearly show that the orthosteric antagonist, **3-quinuclidinyl benzilate** (QNB; yellow; PDB ID 3UON), and the orthosteric agonist, **iperoxo** (cyan; PDB ID 4MQS), bind overlapping binding sites, whereas the PAM, **LY2119620** (green; PDB ID 4MQT), recognizes a spatially discrete binding site within the extracellular vestibule. Top panel: side view; Bottom panel: top view.

agonists (PAM behaviour) and the ability to retard orthosteric agonist dissociation kinetics (Bruns and Fergus, 1990). To date, while the structure–activity relationships of allosteric modulators of A_1 receptors has been extensively investigated, the vast majority of active compounds still contain the 2-amino-3-benzoylthiophene scaffold, with these derivatives typically encompassing substitutions at the 3-, 4- and 5- position of the thiophene ring (Göblyös and IJzerman, 2011). Recent studies employing molecular modelling, mutagenesis and pharmacological analysis suggest that A_1 receptor allosteric ligands recognize a common allosteric pocket within the extracellular vestibule, bounded by the second and third extracellular loops and the top of transmembrane domains 2, 6 and 7, a region also suggested to be employed as a transit pocket for orthosteric ligands (Peeters *et al.,* 2012; Kennedy *et al.,* 2014; Nguyen *et al.,* 2016a, 2016b). High-resolution crystal structures of antagonist-bound A_1 receptors have recently been solved (Cheng *et al.,* 2017; Glukhova *et al.,* 2017). Compared to inactive A_{2A} receptor crystal structures (Cheng *et al.,* 2017), the A_1 receptor has a wider binding cavity, potentially capable of accommodating both orthosteric and allosteric ligands. As such, the more open binding pocket of A_1 receptors provides insight into why, in contrast to the A_{2A} receptor, numerous A_1 receptor allosteric ligands have been identified. Interestingly, docking of allosteric ligands into the inactive A_1 receptor crystal structure suggests that 2-amino-3-benzoylthiophene PAMs of A_1 receptor orthosteric agonists may interact with the A_1 receptor orthosteric site in the inactive state (Glukhova *et al.,* 2017). These findings support an earlier suggestion of a mixed allosteric/orthosteric mode of action of the 2-amino-3-benzoylthiophene A_1 receptor allosteric modulators depending on the activation state of the receptor (Bruns and Fergus, 1990).

At the A_3 receptor, the first allosteric modulators were based on a series of 3-(2-pyridinyl)isoquinoline derivatives, which, interestingly, were previously characterized as A_3 receptor antagonists (Göblyös and IJzerman, 2011). One such derivative is the allosteric modulator, VUF5455, which can not only modulate orthosteric agonist behaviour in both binding and functional assays but can also exhibit modest orthosteric antagonist properties (May *et al.,* 2010b; Göblyös and IJzerman, 2011). In light of these antagonistic properties, future studies aimed to modify the scaffold to enhance the allosteric properties of the ligands but mitigate orthosteric antagonism. This led to the development of a series of imidazoquinoline and 2,4-disubstituted quinolone derivatives, which exhibited improved allosteric properties and minimal orthosteric antagonism, including **LUF6000** and **LUF6096** (Göblyös and IJzerman, 2011).

The identification of selective allosteric modulators at the A_{2A} and A_{2B} receptors has proven less fruitful. At the A_{2A} receptor, 1-[4-(9-benzyl-2-phenyl-9H-purin-6-ylamino)-phenyl]-3-phenyl-urea derivatives and 1-[4-(9-benzyl-2-phenyl-9H-8-azapurin-6-ylamino)-phenyl]-3-phenyl-urea derivatives have been suggested to act as PAMs for both orthosteric agonists and antagonists (Giorgi *et al.,* 2008; Göblyös and IJzerman, 2011). A fragment-based drug discovery approach has also identified putative PAMs and NAMs of the A_{2A} receptors, thereby offering additional insights into potential allosteric scaffolds (Chen *et al.,* 2012). At the A_{2B} receptor, a series of 1-benzyl-3-ketoindole derivatives have been investigated, with some derivatives bearing PAM activity and others NAM activity (Trincavelli *et al.,* 2013).

In addition to the selective adenosine receptor allosteric modulators mentioned above, it should also be noted that numerous non-selective compounds can also allosterically bind to and modulate some or all of the adenosine receptors. Examples include the promiscuous allosteric modulator SCH-202676, various **amiloride** analogues, **adenosine deaminase**, sodium ions, the food dye brilliant black BN and the endocannabinoid **2-arachidonylglycerol** (May *et al.,* 2010a; Göblyös and IJzerman, 2011; Gracia *et al.,* 2011). Importantly, advances in GPCR structural biology will almost certainly facilitate the discovery of new subtype-selective allosteric modulators of adenosine receptors. Indeed, recent antagonist-bound A_1 receptor and A_{2A} receptor crystal structures have identified potential allosteric pockets that could be targeted for future development of novel adenosine receptor allosteric ligands (Glukhova *et al.,* 2017; Sun *et al.,* 2017).

Potential therapeutic advantages of adenosine receptor allostery. GPCR allostery offers numerous advantages over prototypical orthosteric agonists and antagonists, including the potential for increased subtype selectivity, preservation of endogenous spatiotemporal signalling profiles, saturability of modulation and probe dependence (May *et al.,* 2007). The potential for greater subtype selectivity arises due to the allosteric binding site typically having greater sequence variation compared to the highly conserved endogenous agonist binding site (May *et al.,* 2007). The degree of allosteric modulation of orthosteric affinity and/or efficacy is contingent upon the cooperativity between the allosteric modulator and orthosteric ligand and as such can be saturated and probe dependent (May *et al.,* 2007). The saturability of effect can avoid on-target adverse effects, such as over-stimulation or complete inhibition of receptor activity. Probe dependence may be advantageous for GPCRs targeted by multiple endogenous ligands (or metabolites), but when the desired therapy aims to only modulate one (Wootten *et al.,* 2012). In these cases, a desirable modulator would exhibit positive or negative cooperativity with the ligand of interest but neutral cooperativity with all others (or *vice versa*).

A particularly important advantage of adenosine receptor PAMs is their ability to maintain endogenous spatiotemporal patterns of signalling (May *et al.,* 2007). Unlike orthosteric agonists which, theoretically, when present, will promote sustained stimulation of signalling, PAMs have the capacity to remain quiescent in the absence of endogenous ligand, thereby modifying signalling with spatiotemporal specificity, that is, when and where the endogenous agonist is present. In the case of adenosine receptors, the metabolism and generation of adenosine is a dynamic process, and numerous disease states, such as ischaemia and inflammation, are associated with alterations in the level of endogenous adenosine. Accordingly, it can be envisaged that an adenosine receptor allosteric modulator could enhance or limit adenosine signalling predominantly in tissues where and when the pathophysiology is occurring, thereby affording

spatiotemporal control and consequently reducing the risk of adverse (on-target) effects.

The therapeutic utility of allosteric modulators at adenosine receptors has been established in various preclinical animal models and some preliminary clinical trials. Adenosine mediates anti-nociceptive effects in models of neuropathic pain, with studies suggesting a role for the A_1 receptors. As such, the potential for A_1 receptor PAMs with spatiotemporal selectivity represents a therapeutically attractive approach to enhance anti-nociception, without stimulating on-target side effects, such as A_1 receptor-mediated bradycardia. Animal models of neuropathic pain have shown A_1 receptor PAM-mediated anti-nociceptive effects with minimal adverse effects (Pan *et al.,* 2001; Imlach *et al.,* 2015). The A_1 receptor PAM, T62, progressed into clinical trials (Phase II) in patients with chronic postherpetic neuralgia but, this study was terminated due to a subset of patients experiencing transient elevated liver transaminases. In addition to neuropathic pain, allosteric modulation of the A_1 receptor has been shown to be beneficial in other animal models of disease, including cardiac and renal ischaemia–reperfusion injury (Park *et al.,* 2012; Butcher *et al.,* 2013).

The potential therapeutic benefit of A_3 receptor PAMs, such as LUF6096 and LUF6000, has also been demonstrated. LUF6096 promoted cardioprotection with no haemodynamic side effects in a model of myocardial ischaemia–reperfusion injury (Du *et al.,* 2012). LUF6000 has been shown to inhibit inflammation in models of arthritis, osteoarthritis and liver inflammation (Cohen *et al.,* 2014). Furthermore, LUF6000 (also known as CF602), is currently under preclinical assessment for the treatment of inflammation by Can-Fite BioPharma. An A_{2B} receptor PAM KI-7, a 1-benzyl-3-ketoindole derivative, was shown to promote human mesenchymal stem cell differentiation to osteoblasts under *in vitro* settings, suggesting a potential therapeutic utility in diseases with disordered bone formation, such as osteoporosis (Trincavelli *et al.,* 2014). Collectively, these studies highlight that allosteric modulation represents a promising current and future approach for adenosine receptor therapies.

Bivalent and bitopic ligands

Bivalent ligands are hybrid ligands comprising two adjoined pharmacophores, typically targeting two sites within a single GPCR and/or across a homodimeric/heterodimeric interface (Valant *et al.,* 2012c). Bivalent ligands can be classed according to their pharmacophore composition, whereby a homobivalent ligand comprises two of the same pharmacophore and a heterobivalent ligand comprises two distinct pharmacophores. Similar to bivalent ligands, bitopic ligands also incorporate two pharmacophores; however, these are explicitly composed of an orthosteric pharmacophore bridged to an allosteric pharmacophore *via* a linker region (Valant *et al.,* 2012c). Bitopic ligands simultaneously bind both the orthosteric and allosteric binding sites (bitopic binding) in a single GPCR. Some bitopic ligands, in addition to simultaneously engaging two sites, may also have the capacity to bind dynamically in a 'flip-flop' manner, whereby the ligand interchangeably engages with both the orthosteric and allosteric sites (Valant *et al.,* 2012c).

Bivalent ligands targeting adenosine receptors. A series of bivalent ligands at adenosine receptors have been synthesized, including heterobivalent A_1-A_3 receptor agonists (Jacobson *et al.,* 2000), β_2 adrenoreceptor-A_1 receptor agonists (Karellas *et al.,* 2008), μ-opioid receptor-A_1 receptor antagonists (Mathew *et al.,* 2009), D_2 receptor agonist-A_{2A}A receptor antagonists (Jörg *et al.,* 2015) and D_1 receptor agonist-A_1 receptor antagonists (Shen *et al.,* 2013). From a drug discovery perspective, such bivalent ligands may afford the selective targeting of adenosine receptor homodimers and heterodimers and thus may be therapeutically useful in diseases where proposed dimeric interactions have been implicated. Currently, however, bivalent ligands have remained tool compounds to interrogate dimerization and have not progressed into the clinic.

Bitopic ligands targeting adenosine receptors. In contrast to bivalent ligands, there has been a relative paucity of adenosine receptor bitopic ligands. Bitopic ligands may possess a number of advantages, including greater subtype selectivity, due to binding to a topographically distinct region of the receptor and improvements in affinity due to the simultaneous engagement of two sites. The first reported adenosine receptor bitopic agonist, LUF6258, was synthesized by linking the A_1 receptor PAM, PD 81723, to the N^6 substituent of an orthosteric adenosine-derived agonist by the means of a nine-carbon chain (Narlawar *et al.,* 2010). In these studies, a combination of radioligand binding and functional assays was used to validate a bitopic mechanism of action. Although LUF6258 exhibited increased efficacy compared to the parent orthosteric pharmacophore, it surprisingly did not demonstrate an increase in affinity, which conceptually should be expected. A rationally designed A_1 receptor bitopic ligand, VCP746, was similarly synthesized by attaching the A_1 receptor PAM, VCP171, to the N^6 substituent of adenosine *via* an aromatic linker unit and a six-carbon alkyl linker (Valant *et al.,* 2014). In these studies, VCP746 displayed a 100-fold higher affinity than either the parent orthosteric or allosteric pharmacophore and also maintained efficacy. Interestingly, in the aforementioned studies, both VCP746 and LUF6258 exhibited an atypical signalling profile at the A_1 receptors, whereby they demonstrated preferential [^{35}S]GTPγS binding over ERK1/2 phosphorylation when compared to their parent orthosteric pharmacophore (Narlawar *et al.,* 2010; Lane *et al.,* 2013; Valant *et al.,* 2014). This suggests that bitopic ligands have the capacity to engender complex modes of pharmacology. It is evident that the scope for bitopic ligands at adenosine receptors is broad. Although bitopic ligands are often not particularly 'drug-like' (due to their inherently large structure), the potential generation of novel bitopic ligands as chemical probes at all four adenosine receptor subtypes is nonetheless promising.

Biased agonism

Classical GPCR signalling assumed that agonists stabilize a single active receptor conformation to stimulate downstream signal transduction. According to the classical theoretical framework, agonist efficacy was simply based on the strength of the imparted signal, and as such, relative agonist potency ratios should be independent of the influence of

stimulus–response coupling and receptor density (Kenakin and Christopoulos, 2013). Recent evidence from pharmacological, biophysical and biochemical experiments have demonstrated that structurally distinct ligands occupying the same GPCR in the same cellular background can generate different functional outcomes in a manner that cannot be explained by simple differences in stimulus–response coupling (Kenakin and Christopoulos, 2013). Biased agonism describes the ability of ligands to differentially influence receptor behaviour in a pathway-dependent manner (also referred to as 'functional selectivity' or 'signalling bias'). At the molecular level, biased agonism is thought to arise due to the stabilization of different active receptor conformations, leading to the engagement of an alternative subset of intracellular effectors, and in turn, the activation of differential signalling pathways (Figure 3). Much of the early work on GPCR bias examined G protein-dependent versus G protein-independent β-arrestin signalling (Kenakin and Christopoulos, 2013); however, it is also recognized that ligand bias can be detected within G protein-dependent pathways (Baltos *et al.*, 2016a).

The discovery that clinically efficacious drugs targeting the **μ-opioid receptor** (Sternini *et al.*, 1996) and β-adrenoceptors in particular (Wisler *et al.*, 2007; Kenakin and Christopoulos, 2013) impart distinct physiological outcomes *via* unique biased signalling profiles has revealed the novel opportunities for biased ligands in drug discovery (Violin *et al.*, 2014). The ability of distinct GPCR-agonist complexes to differentially activate intracellular signals provides a new avenue for the development of drugs that are not only 'receptor subtype-selective' but also 'pathway-selective'. Biased agonism thus allows the opportunity to specifically design pathway-selective drugs that will separate on-target side effects from therapeutic effects mediated by the same receptor and is actively being pursued in drug discovery programmes (Violin *et al.*, 2014). While biased agonism offers great clinical potential, it also presents challenges. For example, the screening of ligands across multiple signalling endpoints is essential. However, the selection of appropriate endpoints is complicated by the fact that the desirable signalling profile for most drug targets has not yet been established (Violin *et al.*, 2014). In addition, biased agonism can be dependent on the cellular background in which it is detected, such that a particular bias profile in a heterologous system does not infer the same signal bias profile will be observed in endogenous systems or indeed *in vivo*. The recognition that observed bias is influenced by cellular context also gives rise to the idea of context-dependent bias, whereby, conceivably, the receptor bias can change with alterations in membrane composition and intracellular signalling complement, for example, as a consequence of disease progression. However, the generation of bias fingerprints does provide the opportunity to screen and identify compounds that display a distinct profile from the endogenous ligand and are therefore more likely to engender different pharmacological outcomes, presenting

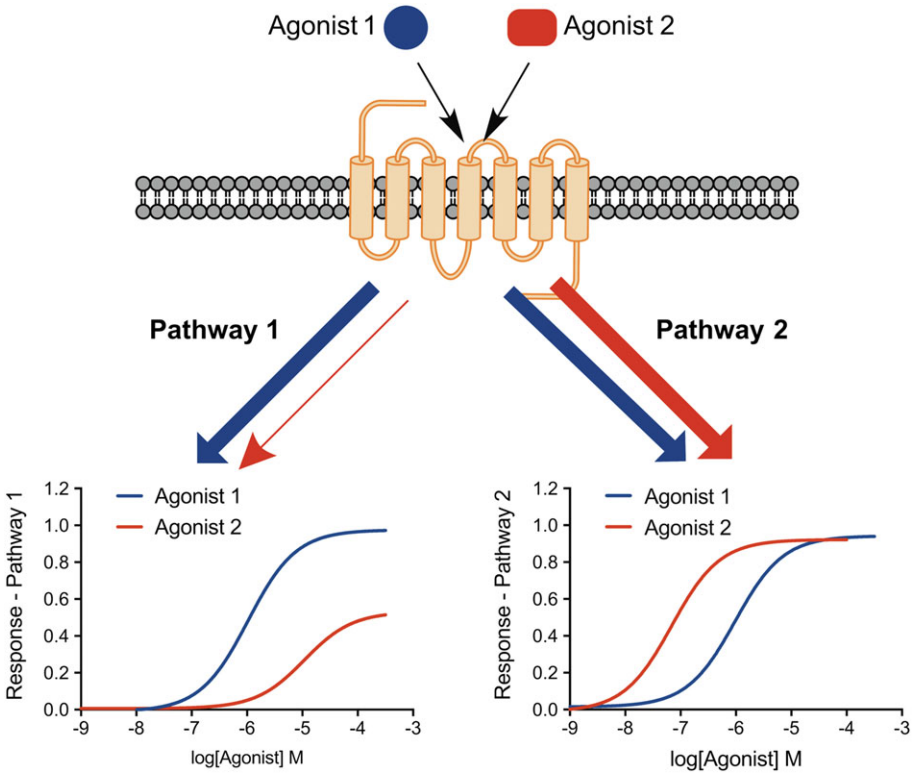

Figure 3

Schematic representation of biased agonism. Relative to Agonist 1 (blue), Agonist 2 (red) is biased towards stimulation of Pathway 2 over Pathway 1. The relative bias of Agonist 2 is shown by the reversal in potency between the two pathways.

a promising starting point with which to move lead compounds into more physiologically relevant *in vitro* and *in vivo* models (Baltos *et al.,* 2016a).

Biased agonism at adenosine receptors. Despite the increasing interest of GPCR-biased agonism (Kenakin and Christopoulos, 2013), relatively few studies have investigated the pharmacological phenomenon of signalling bias at the adenosine receptor family. An initial screen of over 800 compounds at the A_1 receptor identified only one ligand, LUF5589, that appeared to bias G protein-dependent signalling over β-arrestin recruitment (Langemeijer *et al.,* 2013). This study suggested that biased agonism at the A_1 receptor was most likely to be a rare phenomenon. However, A_1 receptor-biased agonism has recently been shown to arise from differences within G protein-dependent pathways, potentially due to differential coupling to the various $G_{i/o}$ proteins in particular (Valant *et al.,* 2014; Baltos *et al.,* 2016a). A_1 receptor-biased agonism was identified using the rationally designed bitopic agonist VCP746, which was shown to be significantly biased away from Ca^{2+} mobilization compared to other G protein-dependent pathways. The ability of VCP746 to stimulate A_1 receptor-biased agonism, relative to the reference agonist, was postulated to underlie its novel cytoprotective actions in the heart in the absence of typical A_1 receptor-mediated bradycardia (Valant *et al.,* 2014; Baltos *et al.,* 2016a). Similarly, capadenoson, an adenosine receptor agonist that has previously entered clinical trials for angina and atrial fibrillation (Bayer, 2010; Tendera *et al.,* 2012), was also shown to be an A_1 receptor-biased agonist within G protein-dependent pathways (Baltos *et al.,* 2016a). These findings highlight that the observed bias profile is highly dependent on the choice of pathways investigated. Allosteric modulators promote a conformational change in GPCR structure and as such have the capacity to stimulate biased agonism, either alone or by modulating the actions of the orthosteric ligand in a pathway-biased manner (May *et al.,* 2007). It was through the investigation for potential adenosine receptor allosteric modulators that within a series of 2-amino-3-benzoylthiophene derivatives, novel compounds that promoted pathway-biased allosteric modulation at the A_1 receptor were identified (Valant *et al.,* 2012a).

Biased agonism has also been reported at other adenosine receptor subtypes. A recent study characterizing the structure-efficacy relationship of a diverse range of A_{2B} receptor agonists identified **BAY60-6583** as a biased A_{2B} receptor agonist with a unique signalling profile (Gao *et al.,* 2014). Capadenoson (Baltos *et al.,* 2017) and VCP746 (Vecchio *et al.,* 2016), which had previously been characterized as A_1 receptor agonists, have recently been shown to also stimulate A_{2B} receptor-biased agonism. The ability of capadenoson and VCP746 to stimulate potent A_{2B} receptor-mediated cAMP accumulation in particular may lead to a desirable activity profile within cardiac cells (Vecchio *et al.,* 2016; Baltos *et al.,* 2017; Vecchio *et al.,* 2017). Studies at the A_3 receptor have detected bias both within G protein-dependent pathways (Baltos *et al.,* 2016b) and also with respect to β-arrestin translocation (Gao and Jacobson, 2008). Moreover, biased allosteric modulation has also been demonstrated with respect to the efficacy modulation mediated by the PAM LUF6000 (Gao *et al.,* 2011). Collectively, these findings demonstrate that biased agonism can indeed be detected at multiple adenosine receptor subtypes. It is hoped that the further understanding of biased agonism and the identification of novel ligands that selectively stimulate therapeutically beneficial pathways will offer exciting opportunities for targeting adenosine receptors in pathophysiology.

Conclusions

Oligomerization, allostery and biased agonism are important paradigms that increase the pharmacological complexity of drug action at GPCRs. As shown in this review, these paradigms have the potential to transform adenosine receptor drug discovery, as they posit numerous advantages that are unattainable through classical agonism and antagonism. Given the therapeutic importance of adenosine receptors, we anticipate that exploiting receptor complexing, allostery and biased agonism has the potential to improve the specificity, safety profile and therefore translational success of future therapeutic agents acting on adenosine receptors.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.,* 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.,* 2017a,b).

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Conflict of interest

The authors declare no conflicts of interest.

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