Molecular basis of modulating adenosine receptors activities

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Abstract

Modulating cellular processes through extracellular chemical stimuli is medicinally an attractive approach to control disease conditions. GPCRs are the most important group of transmembranal receptors that produce different patterns of activations using intracellular mediators (such as G-proteins and Beta-arrestins). Adenosine receptors (ARs) belong to GPCR class and are divided into A_1AR, A_{2A}AR, A_{2B}AR and A_3AR. ARs control different physiological activities thus considered valuable target to control neural, heart, inflammatory and other metabolic disorders. Targeting ARs using small molecules is essentially works through binding to orthosteric and/or allosteric sites of the receptors. Although targeting orthosteric site is considered typical to modulate receptor activity, allosteric sites provide better subtype selectivity, saturable modulation of activity and variable activation patterns. Each receptor exists in dynamical equilibrium between conformational ensembles, the equilibrium is affected by receptor interaction with other molecules. Changing the population of conformational ensembles of the receptor is the method by which orthosteric, allosteric and other cellular components controls receptor signaling. Herein, the interactions of ARs with orthosteric, allosteric ligands as well as intracellular mediators are described. A quinary interaction model for the receptor is proposed and energy wells for major conformational ensembles are retrieved.

List of abbreviations

AR	Adenosine receptor
A_1AR	Adenosine Receptor subtype 1
A_{2A}AR	Adenosine receptor subtype 2A
A_{2B}AR	Adenosine receptor subtype 2B
A_3AR	Adenosine receptor subtype 3
GPCR	G-protein coupled receptor
GIP	GPCR interacting proteins
β_2AR	Beta-2 Adrenergic receptors
PAM	Positive allosteric modulator
NAM	Negative allosteric modulator
SEM	Silent allosteric modulator
7TM	Seven transmembrane helix
TM	Transmembrane helix
GRKs	G-protein coupled receptor kinases
EC_{50}	Potency measurement
E_{Max}	Efficacy measurement

1 Introduction

Adenosine receptors (ARs) belong to GPCR family of transmembranal receptors, and are classified into A_1, A_{2A}, A_{2B}, and A_3 subtypes. These subtypes produce different intracellular signals. With respect to adenylyl cyclase activity, it is inhibited by activation of A_1 and A_3 receptors and stimulated by activation of A_{2A} and A_{2B} receptors [1]. Medicinally, A_1AR controls sleep, vasoconstriction and inhibition of neurotransmitter release; A_{2A}AR controls sleep, angiogenesis and immunosuppression; A_{2B}AR controls vascular integrity and myocardial preconditioning [2]; while A_3R regulates mast cell and myocardial preconditioning. Therefore, ARs are considered valuable target to manage variety of neural, heart, respiratory, renal, inflammatory, metabolic and cellular disorders [1, 3-6].

Ligands can bind to main endogenous ligand binding site (orthosteric site) or to distinct sites (allosteric sites). Purinergic signaling involves extracellular adenosine, adenosine triphosphates and similar molecules like xanthines which bind orthosteric site. Under physiological conditions the level of adenosine is usually low, which is sufficient to activate A_1, A_{2A} and A_3. However, because of their lower affinity to adenosine, the A_{2B} receptors are activated mainly at pathophysiological conditions when the level of adenosine is highly elevated [7, 8]. Several other purinergic and non-purinergic, selective and non-selective ligands were synthesized and tested [9-12]. Many selective orthosteric ligands are either approved or under clinical trials [13].

For allosteric sites, the amino acid sequences are much variable among different GPCR subtypes compared to orthosteric site, thus offer better opportunity for designing subtype selective ligands [14]. Therapeutically, compounds act on allosteric sites provides better subtype selectivity and fine modulation of orthosteric ligands activities [15]. Frequently, the activity of allosteric ligands necessitates the presence of bounded orthosteric ligand. Therefore, the allosteric modulation follows the type of orthosteric ligand, receptor subtype as well as cellular and membranal compositions [15]. Allosteric ligands (or modulators) offer selective and saturable effect and preserve endogenous spatiotemporal signaling, and therefore, produce safer therapeutic effect compared to orthosteric ligands [16, 17]. The interaction between allosteric and orthosteric ligands on a receptor will affect efficacy (E_{max}), potency (EC_{50}) and steepness (Hill Coefficient) on the concentration-response curve for both ligands (please see section 6.4). The saturable effect of allosteric ligands is related to the modulation of a saturable parameter (which is Hill coefficient) in concentration response curve. Thus, allosteric ligands may affect the rate of response change with respect to orthosteric ligand concentration while keeping E_{max} and EC_{50} unaffected. This provides safer control of the receptor activities bounded by orthosteric ligand limits of potency and efficacy.

Although "subtype selectivity" is therapeutically recommended, it may not be sufficient to avoid unwanted effects since the same receptor propagates different intracellular signals. Moreover, simple "agonist/antagonist" properties become insufficient to describe ligand's mode of action. Therefore, "functional selectivity" or "biased signaling" is an immerging valuable therapeutic goal [18]. Biased signaling is an interesting feature of some orthosteric and allosteric ligands. Some ligands can stabilize GPCR conformations that favors particular signaling paradigm (among other possible signals) mediated by the receptor [19]. Therefore, biased ligands offers great opportunity of separating therapeutic pathways from other unwanted pathways mediated by the same receptor subtype [20]. Several examples are available for biased ligands for ARs and other GPCRs [21-24].

In an attempt to formalize the previous concepts, this review describes the molecular interaction between ARs and each of orthosteric and allosteric ligands which leads to changes in conformational ensembles of the receptors. These changes determine intensities and spectrums of the propagated signals to intracellular mediators.

2 Orthosteric pocket and ligands

Similar to other GPCRs, crystal structures are steadily resolved for ARs. Currently, crystal structures are available for A_1 and A_{2A} receptors with various orthosteric ligands and G-protein mimics [25-31]. According to crystal structures and mutagenic studies, the orthosteric pocket in ARs is allocated close to extracellular half of seven transmembrane helices (7TM) tunnel. All crystallized agonists and antagonists reach to almost similar depth within the TM tunnel with agonists carrying C5'-substituted ribose ring extends deeper ([Figure 1).

For A_{2A} receptor, the orthosteric pocket is composed of non-polar amino acids and many polar amino acids that form specific interactions with the ligand. The upper part of the pocket is formed of $N253^{6.55}$ and $E169^{ECL2}$ which interacts with nitrogen atoms of adenine, while $L249^{6.51}$, $M270^{7.35}$ and $I274^{7.39}$ form hydrophobic pad for adenine against the phenyl ring of $F168^{ECL2}$ from the opposite side. The pocket is locked from top by salt bridge between $E169^{ECL2}$ and $H264^{ECL3}$. The lower part is formed of $T88^{3.36}$, $S277^{7.42}$, $H278^{7.43}$, $H250^{6.52}$, $C185^{5.46}$ and $N181^{5.42}$ where most of them form specific interactions with ribose ring and its substitutions. The $E13^{1.39}$ is in proton transfer communication (i.e. through H-bonding) to $H278^{7.43}$ which is necessary for orienting the latter residue to interact with ribose ring of nucleoside-based analogues [32]. The residue of $W246^{6.48}$ forms the bottom of orthosteric binding pocket, contributes in receptor activation and differentiates agonists from antagonists [33, 34]. The residues of $E13^{1.39}$, $S277^{7.42}$, $H278^{7.43}$, $T88^{3.36}$ and $H250^{6.52}$ form the ribose ring binding pocket. Water molecules bridges extend from adenine and ribose heteroatoms toward other distal amino acids ([Figure 2). The subtype selectivity among ARs is maintained by residues of $H250^{6.52}$ and $M270^{7.34}$ as well as the ECL2 [27, 35] and is discussed below.

[**Figure 1 insert here**]

[Figure 1: Orthosteric ligands from 18 crystallized structures for A_{2A} receptor. All orthosteric ligands occupy same volume at extracellular opening of 7TM tunnel and surrounded by ECLs.]

[**Figure 2 insert here**]

[Figure 2: Crystal structure for NECA bound to A_{2A} orthosteric site (PDB ID 2YDV) showing a) front view for polar interactions and b) side view for hydrophobic pads. Please note that part of TM2, TM3 and ECL2 are removed for clarity. Note amino acids are numbered according to sequence in each subtype in addition to Ballesteros-Weinstein numbering scheme in superscript.]

Adenosine represent the typical endogenous orthosteric which acts as agonist for ARs. The general features of AR agonist are the presence of purine and ribose rings. Deamination of its adenine (to inosine) or phosphorylation of its ribose ring (to AMP) reduce its binding affinity, however may enhance subtype selectivity [36] and introduce signaling bias [37]. Examples of orthosteric agonists are provided in [Figure 3.

For antagonists the main feature is absence of ribose ring, therefore, the interactions with $N181^{5.42}$ and $H250^{6.52}$ are lost while the side chain at C8 of adenine make direct interactions with $N253^{6.55}$. In an antagonist, if the side chain replacing ribose are not flexible enough, it will push side chain of $H250^{6.52}$ to outside the lumen which result in better stack with each of $W246^{6.48}$ and $F182^{5.43}$. Antagonists may have adenine-based, xanthine-based or 1,2,3-triazine-based heterocyclic rings, where all occupy equivalent position within orthosteric pocket [38-40].

According to crystal structures, the heterocyclic ring for both agonist (NECA) and antagonist (ZM241385) occupies similar position within the orthosteric binding site. The binding of orthosteric agonists to A_{2A} receptor usually involves simultaneous interaction with TM3 ($T88^{3.36}$) and TM7 ($S277^{7.42}$ and $H278^{7.43}$) while the antagonist interact with either of them at a time [41]

The message-address theory is occasionally applied to design ligands for GPCR. The theory has been initially formulated to design opioid receptors antagonists [42], however it seems to work also for AR. Where the adenine heterocycle works as address to guide the molecule for AR subtypes while ribose moiety works as message for "active" conformation [43]. Up to our knowledge, no agonists are available which lacks ribose ring except the partial agonists of 7-(prolinol-N-yl)-2-bromophenylamino-thiazolo[5,4-d]pyrimidines [44], LUF5834 [45] and their derivatives. Compounds with larger side chains at adenine nucleus usually protrude outside the pocket toward the space between TM1, TM2 and TM7 and affect the conformations of ECLs [41] as well as the off-rate of dissociation. Therefore, compounds of small side-chains may have slow off-rate due to the preservation of salt-bridge between ECL2 and ECL3 that close the binding site. While compounds of larger side chains may have faster off-rate, unless the side chain make favorable interactions with the receptor. In other word, ligands which interact with

E169^{ECL2} and /or H264^{ECL3} or carrying bulky substitutions are eventually causing salt-bridge to break and show low residence time (i.e. fast dissociation rate) even if having almost similar dissociation constants. Therefore ZM24138 (antagonist) which does not break the bridge have intermediate residence time [46]. On contrary, UK-432097 (agonist) that breaks the bridge however makes strong interactions with E169^{ECL2} have higher residence time compared with CGS-21680 (antagonist) that does not break the bridge [47]. Therefore, if such ligands establish extra interactions with the receptor, the residence time may increase even the ligand breaks the bridge.

The subtype selectivity is mediated by different orientation of TM helices, ECLs and gate keepers among ARs. The selectivity for A_1 over A_{2A} is mediated by different conformation of ECL2, different gatekeeper residue T/M at position 270 as well as presence of secondary pocket in A_1 close to orthosteric binding site [27, 41]. The lower affinity of A_{2B}R to adenosine may be related to its ECL2 structure. The ECL2 in A_{2B}AR negatively regulates receptor activity, while the loop in A_1AR act as positive regulator [48]. Affinity and subtype selectivity for orthosteric ligand can be determined experimentally as well as computationally. Non-biased MD and metadynamics [49] can be used to find the pathway the ligand use to access orthosteric site. MD combined to free energy perturbation is considered a superior tool to rank ligands by affinity compared to molecular docking, especially for adenosine derivatives [50]. Recently we reviewed and reported various types of MD strategies that are currently being used for the design and delivery of drugs [51, 52].

[Figure 3 insert here]

[Figure 3: Examples of agonists whether adenosine-based or not as well as antagonists whether xanthine-based or not.]

3 Allosteric sites and ligands (modulators)

Allosteric modulators are defined as ligands that bind to an allosteric site on the GPCR to modulate the binding and/or signaling properties of the orthosteric site [17]. Allosteric modulators are classified as positive, negative and neutral (or silent) modulators; which increases, decreases and have no effect on orthosteric ligand activity, respectively. Another class includes allosteric agonist; which exerts agonistic effect even in absence of orthosteric ligand (Ago-Allosteric modulators) [53, 54]. Bitopic ligands are capable of binding to and bridging both allosteric and orthosteric binding sites [55-57]. The chemistry of allosteric modulators range from cations, small molecule, peptides and up to receptor dimerization [58-61]. For GPCRs, allosteric modulators may include lipids, ions, amino acids, small peptides as well as proteins such as autoantibodies, GPCRs homo or heterodimerization (including isolated receptor peptides

mediate dimerization) [62-67]. Interestingly, two adenosine molecules may simultaneously bind to both of orthosteric and allosteric sites resulting in one adenosine molecule behaves as allosteric ligand [68]. The allosteric modulators may interact with extracellular, intracellular and transmembranal parts of the receptor. The ability of various molecules to modulate orthosteric ligand activity through allosteric interaction with the receptor enlightens new era of GPCR controls [69]. Common allosteric ligands for ARs are shown in [Figure 4

Unlike orthosteric pockets, there are no available crystal structures for ARs with allosteric ligands. However, structures are available for other GPCRs belong to different families with different allosteric ligands ([Figure 5). According to available crystal structures, the known allosteric sites for GPCRs varied between inside to outside the 7TM tunnel and involves intra- and extra-cellular loops [70]. The muscarinic acetylcholine receptor (M2 receptor) bound to both of orthosteric agonist Iperoxo and positive allosteric modulator LY2119620 ([Figure 6) revealed allosteric site at extracellular part [71]. While for β_2-adrenoceptors, a negative allosteric ligand bound between TM helices and intracellular loops [72]. A review for crystallized GPCR structures with allosteric ligands is available [70]. Within class A GPCR, the most known allosteric sites are at domains comprising the second (ECL2) and third (ECL3) extracellular loops, as well as the top extracellular part of TM7 [17].

For ARs, allosteric sites may be distal or partially overlapped with orthosteric site. Putative sites are close to orthosteric (bounded by ECL2) or sodium ion binding sites. Compared to A_{2A} receptor, the crystal structure for A_1 receptor has different extracellular loop conformation and wider extracellular cavity next to orthosteric site, which may accommodate orhtosteric as well as allosteric ligands [27]. The same cavity may be used to design selective A_1AR orthosteric ligands by substituting adenine N6 with bulky alkyl groups [73].

Alanine scanning and mutagenic studies are used to reveal probable allosteric sites in ARs. Mutational studies are usually not sufficient to allocate allosteric sites, however, denoting possible residual involvement in mediating allosterisim (Table 1). Recent summary of site-directed mutagenic studies is available for AR to characterize effects of mutation on agonist, antagonist, partial agonist and allosteric ligands bindings and modulation of activties [35]. Computational techniques are also used to find out available as well as hidden allosteric sites, i.e. those which only appear upon orthosteric ligand binding or in meta-states of receptor [74-77]. Most of the techniques involved are related to analysis of MD trajectories that occasionally coupled to molecular docking [78] or finding void spaces that are conformationally related to orthosteric binding site [74, 77-79]. Metadynamics simulation was used to allocate allosteric sites for cholesterol in A_{2A}AR [80]. Binding studies for orthosteric/allosteric bivalent ligand of variable linker size coupled with molecular docking also helps allocating probable allosteric

sits next to orthosteric site; for example the allosteric part of divalent LUF6258 may bound at ECL2 while the adenosine part binds the orthosteric site [81].

A probable allosteric pocket is present in A_1AR within the extracellular termini of TM2, TM6 and TM7, and surrounded by ECL2 and ECL3 [27]. Another, smaller allosteric pocket was proposed to exist in A_{2A} near $Y9^{1.35}$ and $Y271^{7.36}$ [82]. ECL2 shows considerable involvement in binding allosteric ligands and in cooperativity with orthosteric ligands [83]. Site-directed mutagenesis and molecular modeling studies in A_1 receptors suggest allosteric modulators bind to a pocket in ECL2 flanked by $S150^{ECL2}$ and $M162^{ECL2}$ [84]. Orthosteric ligands may bind transiently at site near ECL2 before entering orthosteric pocket. Meta-stable states are exist for both of ligands and receptor. For ligands, meta-stable interaction with ECLs help orienting orthosteric ligand during entrance to the pocket [49]. While for receptor, meta-stable conformations provides opportunity for interaction with allosteric modulators [60] and propagation of biased signals [85]. Meta-stable interaction with ECLs has been observed for different orthosteric ligands (agonist and antagonists) binding to β2 adrenoceptor through un-biased MD simulation [86]. The transient interaction probably takes place during orthosteric ligands egress [87], and may be interrupted by presence of allosteric ligand [84]. The polar residues of $E172^{ECL2}$ and aromatic residues of $W156^{ECL2}$ and $W146^{ECL2}$ are, probably, involve in polar interactions and pi-pi stacking with each of orthosteric ligand (i.e. transiently) and allosteric modulator [48, 88]. ECL2 forms part of orthosteric pocket in both A_1AR and A_{2A}AR, yet the allosteric pocket in A_1AR is wider than that in A_{2A}AR [27]. For A_3AR, an allosteric pocket is proposed near the orthosteric site using binding assays where bulky allosteric modulators (i.e. VUF5455) showed antagonistic effect to orthosteric ligand which can be minimized by reducing bulkiness (i.e. LUF6000) [89]. ECL2 modulates receptor activities differently in different ARs subtypes. ECL2 in A_1AR positively regulate the receptor activity while the ECL2 in A_{2B}AR acts as negative regulator [48].

Sodium ion (Na+) binding site is another allosteric site and is composed of $D52^{2.50}$, $W246^{6.48}$, $N280^{7.45}$ and $S91^{3.39}$ [90]. The increased dynamic flexibility of $W246^{6.48}$ and $N280^{7.45}$ in the absence of the sodium ion suggests their involvement in communication with sodium [91]. Amiloride and derivatives are allosteric modulators for AR (as well as for other GPCRs) which interact with Na binding site [92-94]. Although amiloride act as negative allosteric modulator, its binding modes at agonist-occupied and antagonist-occupied ARs differ markedly, and consequently, its modes of interaction with orthosteric ligands [94]. Binding to sodium ion pocket is mediated by $D52^{2.50}$ and may causes dislodging of orthosteric ligands especially if it carries side chain that extends to orthosteric pocket [92].

Non-specific allosteric binding sites are available to interact with chemical modifiers to modulate ARs activity. For example, 2,3,5-substituted thiadiazoles may catalyze formation of disulfide bridge between cysteine residues of the receptor [95].

[Figure 4 insert here]

[Figure 4: Examples of allosteric modulators for adenosine receptors whether NAM or PAM.]

[Figure 5 insert here]

[Figure 5: Multiplicity of allosteric sites across GPCR subfamilies. The structures are superimposed using β2-adrenergic receptor as representative crystal structure. 7TM are shown in ribbon and allosteric modulators in stick. (obtained from [70] with permission).]

[Figure 6 insert here]

[Figure 6: Crystal structures for a) Muscarinic acetylcholine receptor (M2 receptor) simultaneously bound to the orthosteric agonist iperoxo and the positive allosteric modulator LY2119620 (PDB ID 4MQT), b) for beta2-adrenoceptor bound to allosteric antagonist (PDB ID 5X7D). The van der Walls surfaces of allosteric ligands are shown.]

[Table 1 insert here]

4 Types of intracellular mediators

GPCR binds intracellular mediators to propagate the signal intracellularly. The common mediators are G-proteins and beta-arrestins .The heterotrimeric complex of G proteins (α, β and γ) mediates the signal by the α subunit which has a Ras-like GTPase domain involved in exchanging ADP for ATP leading to dissociation of the complex from GPCR. The Gα subunit has many classes such as G stimulatory ($G_s\alpha$), G inhibitory ($G_i\alpha$), G other ($G_o\alpha$), $G_{q/11}\alpha$, and $G_{12/13}\alpha$. Each of these Gα proteins share a similar mechanism of activation, however, mediates different signaling pathways. While free α_s-subunit activates adenylyl cyclase, βγ dimer activates potassium channels or phospholipase for subsequent downstream signaling [96]. Beta-arrestins are responsible for receptor internalization results in fading the intensity of the downstream signals of the active receptor [97-99]. Beside G-proteins and beta-arrestins, large number of intracellular mediators are being discovered and classified as GPCR interacting proteins or GIPs [100]. In a manner similar to the receptor, mediators also exist in a population of conformational states and consequently mediates different intracellular signals [101].

5 The interaction model between modulators, receptor and mediators

Binding of ligands to allosteric sites affects the activity mediated by orthosteric site in different ways. Allosteric modulators can be classified as positive (PAM), negative (NAM) or silent (SAM) modulators

which increases, decreases or have no effect on orthosteric ligand activities but competes other modulators activities, respectively [102]. All the previously mentioned allosteric modulators activities require presence of orthosteric ligand. Other classes include allosteric modulators which exerts agonistic effect per se, in other word, it need no orthosteric ligand (Ago-Allosteric modulators) [53, 54, 103]. Although the previous classification is not precise; as the same allosteric ligand may affect the receptor interaction with intracellular mediators differently, i.e. acts as PAM on certain pathways and NAM on others, this is defined as biased signaling [17, 104]. Accordingly, whatever binds the receptor (orthosteric ligands, allosteric ligands or both) the type and strength of activation of intracellular mediators depends on population pattern of receptor conformational ensembles (please see section 6.4).

Since both of G-proteins and beta-arrestins share same binding pocket within GPCR [105], an exclusive binding of G-protein and beta-arrestin to AR was suggested which provide a quaternary interaction model [66]. However, recent studies show that simultaneous partial binding and activation of both of them is possible [106]. Beta-arrestin can initially get anchored to phosphorylated C-terminal and ICLs of GPCR -or probably without phosphorylation [107]- before being dragged toward the shared binding pocket (next to DRY motif) through at least two steps binding [105, 107, 108]. Therefore, quinary interaction model is possible which is composed of receptor, orthosteric ligand, allosteric ligand, G-protein and beta- arrestin ([Figure 7). This interaction assumed only two conformational states for the receptor (active and inactive states) and a single conformation for each of the other molecules. However, it is known that each of orthosteric ligand, allosteric ligand, receptor, G-protein and beta-arrestin has a population of conformational states and the pattern of which is changed upon interaction with each other. For example, beta-arrestin has a population of conformational ensembles, which regulates different downstream signaling. Pattern of the population is affected by phosphorylation pattern of GPCR C-terminal i.e. the phospho-barcode [99, 109, 110]. The phospho-barcode is related to the mode of GRKs (G-Protein Coupled Receptor kinases) binding that is controlled by receptor conformational ensembles populated due to orthosteric/allosteric ligand binding [96, 111]. In addition to conformational diversity, the structural diversity of α, β and γ G-protein subunits as well as beta-arrestins and GIPs further increases the diversity of possible signaling pattern propagated by the receptor.

[Figure 7 insert here]

[Figure 7: The quinary complex model of allosteric interactions at ARs; a thermodynamically complete, extended model taking into account the concomitant binding of orthosteric ligand, O, allosteric ligand, L, and G protein, G, Beta-arrestin, B, on a receptor that can exist in two conformational states (R and R*). Where the conversion of R to R* is governed by isomerization constant; the binding of each of O,

L, G and B to R is governed by dissociation constants; the conversion of R to R* in presence of O, L, G and B is governed by activation cooperativity constants; binding of each one of O, L, G and B to R in presence of another is governed by binding cooperativity constants. Binding of B to R and R* is considered to occur without initial phosphorylation of receptor according to Storme et al 2018 (ref 105)]

6 Molecular basis of adenosine receptor activation, allosterism and biased signaling

6.1 Conformational states of the receptor.

The model which describe the mode of action of GPCRs says that the receptors exists in equilibrium of different conformational states [112] and that orthosteric and/or allosteric ligands stabilizes particular sets of conformations [113]. The conformations either stabilized by induced-fit or conformational selection, depending on ligand affinity to the GPCR. Higher affinity ligand like covalently bound ligand (e.g. retinal for rhodopsin) is able to induce conformational change while low affinity ligand (e.g. isoproterenol for β_2AR and NECA for A_{2A}AR) is just enough to change conformational equilibrium [114].

The GPCR conformational states are usually captured using X-ray crystallography, NMR and molecular dynamic simulations. Several strategies are followed to crystallize ARs at particular conformational state using ligands [115] and mutational traps [116]. X-ray crystal structures are available for A_1 and A_{2A} in inactive [26, 28], intermediate active (Meta) [29] and fully active states [25, 30]. Fully active states requires presence of an agonist as well as a G-protein or its mimics [31].

The pattern of distribution for receptor conformational states is affected by interaction with other molecules. Therefore, orthosteric ligands, allosteric ligands, intracellular G-proteins, Beta-arrestins, GIPs and even cell membrane components such as steroids, all may contribute to the pattern. The analysis of binding thermodynamics for AR ligands suggests that the conformational changes needed to produce the pharmacological effect are relatively small in this class of receptors [117]. The binding of agonists to AR are shown to be entropy-driven which can be explained by the disorganization of water molecules [117] probably close to Na binging site. Thus, the formation of internal water channel due to conformational changes of $W246^{6.48}$ and $Y288^{7.53}$ can be regarded as a sign for receptor activation [118, 119].

Upon activation, the transmembrane helices 5−7 rearrange to accommodate G protein insertion typically by inward movement of TM5 accompanied by rotatory outside movement of TM6 and rotatory inward movement of TM7 [41, 120]. The ribose sugar of the agonist do the trigger by pushing indole group of $W246^{6.48}$ which is accompanied by a twisted outward movement of half of TM6 below $P248^{6.50}$, such twist is facilitated by preserved anchoring of the above residues of $H250^{6.52}$ and $N253^{6.55}$ to the agonist [121] as shown in [Figure 8. The rotameric switches in TM5 ($Y197^{5.58}$ and $F201^{5.62}$) change conformation to outside the TM core, allowing intracellular part of TM7 to go in (most importantly

NPxxY motif) and residues of S277$^{7.42}$ and H278$^{7.43}$ to interact with ribose sugar of the agonist. The ionic lock between R102$^{3.50}$ and E228$^{6.30}$ which is often engaged in inactive GPCR structures, is broken by the reorientation of TM6 [33, 41]. The interaction between AR and G-protein is mediated mainly by intracellular part of TM3, TM5 and TM6 as well as the ICL2, which form a cytoplasmic pocket that interact with finger loops of arrestin and finger helix of G-protein [105]. Two motifs play a major role in interaction between AR and intracellular mediators namely; NPxxY (TM7) and DRY motif (TM3). DRY motif binds the C-terminal of G-protein [35] and beta-arresin [107]. The DRY motif in intracellular part of TM3 represented by the residues; D101$^{3.49}$, and R102$^{3.50}$ and Y103$^{3.51}$ is coordinated with either Na ion and G-protein. By interaction of D101$^{3.49}$ with the dislodged Na ion, the conformation of R102$^{3.50}$ is changed to interact with G-protein. Sodium also interacts with D52$^{2.50}$ and affects the NPxxY motif conformation [122]. NPxxY motif is involved in receptor activation mechanism and interaction with intracellular mediators. Both Na and NPxxY cooperate to form the continuous water pathway which is a sign of active GPCR [118]. Protonation of D52$^{2.50}$ is associated with extrusion of Na from its coordination [123] toward the acidic groups of NPxxY during its passage to intracellular compartment. This passage is energetically favorable as it follow down the Na electrochemical gradient; which lead to stabilize the active-state conformation of the receptor. [123, 124].

TM helices of AR undergo different levels of movements upon activation. While TM1, 2, 3 and 4 undergo minimal conformational changes, TM5, 6 and 7 have substantial movements [121]. The dynamical movements of TM1, TM2 and TM3 are affected by extracellular loops (ECLs) conformations. The ECLs in ARs may affect basal conformational states, transition between conformations, activity of allosteric modulators and cooperativity with orthosteric ligands [48, 88]. Most importantly, ECL2 and residue of T/M270$^{7.35}$ form gatekeepers for ligands access to orthosteric site [27]. The conformation of ECL2 is affected by the distribution of cysteine residues able to make disulfide bridges. The ECL2 in A_{2A}AR has three cysteine residues while A_1AR has only one. Therefore, ECL2 in both subtypes adopt different conformations and consequently different interaction with orthosteric and allosteric ligands [48, 84, 88, 125].

For A_{2A}AR, two conformations were observed for each of inactive and active states regarding integrity of ionic lock between TM3-TM6 and the docking pocket of G-protein. For inactive state, the predominant conformation follows the integrity of ionic lock between TM3 and TM6, while for active state it follows the docking of G-protein [126]. According to previous comparison between rhodopsin and β_2AR [114] as well as NMR studies [127], A_{2A}AR appears to have different energy landscape compared to β2–adrenergic receptor [31]. The conformation of A_{2A}AR bound to agonist is in an active-intermediate state, whereas for β_2AR the agonist binding may fluctuate between active and inactive states. For β_2AR it seems that energy well for R″L complex is shallower and/or coupling between orthosteric

pocket and G-protein docking pocket is weaker (i.e. agonist easily detached from orthosteric pocket or has lower ability to stabilize receptor conformation at G-protein docking pocket, respectively) ([Figure 9).

[Figure 8 insert here]

[Figure 8: The interaction of ribose sugar of agonist with residues of TM3, TM6 and TM7 through H-bonds (red lines). All of the residues described pull ribose sugar into contact with W246$^{6.48}$ so that the latter residue changes its conformation and tilt the lower part of TM6 to outside the tunnel of the receptor. Please note that TM1 and TM5 are removed for clarity.]

[Figure 9 insert here]

[Figure 9: The energy value over reaction coordinates of the inactive receptor (R), over Meta states (R′, R″, R‴), until active state (R*), and the concomitant interactions with ligand (L) and G-protein (G). The TM helices which change conformation are noted, although it may have some differences between the two receptors [56]. The β_2AR have shallower energy well for R″L complex compared to A_{2A}AR. In addition, the fluctuation of receptor to active state in absence of ligand in β_2AR is higher than A_{2A}AR due to higher energy barrier for the latter.]

6.2 Induction/stabilization of a particular set of conformational states by Modulators: Mechanistic overview

For adenosine receptor (and other GPCRs), it is proposed that different compounds (orthosteric or allosteric ligands) stabilize different conformational states by affecting the population of macro-states that are governed by micro-switches [34]. A conformational coupling exists between extracellular part (i.e. orthosteric site) and intracellular part (i.e. mediators site) of the receptor. For some GPCRs as β_2AR, the conformational coupling between orthosteric pocket and G-protein pocket is weak. Thus, the GPCR may adopts active, intermediate active or inactive conformations at its intracellular part even in presence of agonist or inverse-agonist at its orthosteric site [128]. The case is different for A_{2A}AR, where A_{2A}AR bound to agonists have G-protein pocket exists as either intermediate-active or fully active states, therefore stronger coupling exists between orthosteric pocket and G-protein pocket [31, 129]. Stronger coupling ensures strict control of the signal and lower receptor basal activity. The coupling depends also on type of orthosteric ligand, for example lower coupling is observed with adenosine compared to UK432097 agonist during MD simulations of A_{2A}R [130].

At certain extent of coupling, the GPCR can be –practically- divided in two semi-independent domains; the orthosteric binding site and mediator binding site [129, 131]. Studies showed that the coupling is lower for intermediate states, followed by active, then inactive states of GPCRs [132]. More precisely, receptor bound to agonist has more dynamical fluctuation at its G-protein binding site if

compared to inverse-agonist [133]. The population of conformational stats of receptor depends on type of orthosteric/allosteric ligands. For example, the population of inactive states for $A_{2A}AR$ increases in presence of inverse agonist (ZM241385), however, the population is then reduced if non-selective allosteric modulator (Amiloride) is added [126]. Therefore, although amiloride acts as NAM, it restores the basal activity of receptor probably by restoration of conformational fluctuation.

6.3 Receptor-mediator interaction

Although mediators are responsible for mediating the conformational signal from intracellular part of receptor, mediators can also induce or stabilize particular conformational states and affect probability of orthosteric/allosteric ligands binding. It was stated that G-protein can induce or stabilize particular GPCR conformation [66] and the fully active conformation of adenosine receptor (and other GPCRs) could not be obtained merely by agonist binding; without the availability of G-protein or its mimics [120, 134, 135]. Therefore, G-protein binding affects the dissociation rate of orthosteric/allosteric ligands from GPCR [136]

6.4 Allosterisim and biased signaling

An orthosteric ligand interacts with receptor with particular affinity (determined by its dissociation constant) and stabilizes (or induces) receptor conformational states to particular extent (determined by its efficacy). Allosteric modulator may interfere with orthosteric ligand binding affinity, efficacy and steepness of dose-response curve ([Figure 10). Therefore, allosteric modulator may inhibit or potentiate orthosteric ligand binding. For example amiloride and its derivatives decrease dissociation rate of antagonists and occasionally agonists from ARs [94] while DU124183 decrease dissociation of agonists [137]. On contrary, PD81723 [138] increases the dissociation rate for antagonists [139]. In addition to affinity, allosteric modulator may affect the efficacy of orthosteric ligands. For example, LUF6000 can enhance the efficacy of low (but not high) efficacious agonists on A_3AR, such enhancement could convert nucleoside-based antagonists into agonist [140].

The allosteric modulation can be attributed to direct effect of allosteric ligand on the population of receptor conformational states as observed for Na^+ which acts as NAM and Ca^{+2} which acts as PAM on $A_{2A}AR$, these modulators stabilize particular conformational state as observed by NMR and MD simulations [60, 91]. Otherwise, allosteric ligand may act locally on the receptor as the interaction with ECL2 that contributes or caps the orthosteric pocket, e.g. LUF6000 interaction with adenosine on A_3AR [141], and PD81723 interaction with NECA on A_1AR and $A_{2A}AR$ [142]. Therefore, allosteric ligand may modulates conformational ensembles of orthosteric ligand which in-turn modulates receptor conformations [143]. In such mode of action, allosteric ligands may increase the activity for the agonist

within limited concentration, however, it turns into competitive antagonist at higher concentration [138, 144]. Moreover, the degree of modulation of allosteric ligand depends on type of orthosteric ligands; which is observed as biased signaling for those GPCRs that have many endogenous orthosteric ligands i.e. “Probe Dependence Allosterisim” [145, 146]. With respect to ago-allosteric ligand, probably it binds at orthosteric site in absence of an agonist and stabilizes particular conformational state of GPCR which is very close to that required for the agonist to bind, in addition, it may give its seat for agonist without competition [103].

Due to the multilevel activation of GPCR, the proper study for allosterisim requires known magnitude of both agonist-induced functional response and allosteric modulation of that response. Using concentration-response curve, the change in orthosteric ligand efficacy (E_{max}), potency (EC_{50}) and Hill coefficient (H) can be assessed in presence of allosteric modulator. The value of H measures the cooperativity between orthosteric and allosteric sites on receptor activity; and can be above, blow or equal to unity for PAM, NAM and SAM, respectively. The value can be above or below unity for PAM and NAM, respectively. than one for PAM and less than one for NAM and H has value below 1 if As shown in [Figure 10c, the

Kinetic models can be used to represent allosteric effect and competition on ARs [147]. The approaches used to measure allosterisim for ARs include: evaluation of the inhibitory activity of forskolin-stimulated cAMP accumulation or phosphorylation of ERK1/2 in CHO cells stably expression ARs, binding of radiolabeled ligands and competition by antagonists [148]. However, the kinetic assays have limitations in measuring allosterism, for example in a system of receptor pre-equilibration with orthosteric ligand it is neither suitable to measure effect of allosteric modulator that has low affinity for receptor occupied with orthosteric ligand nor to measure changes in efficacy [104]. Moreover, kinetic assays requires properly equilibrated system which might not be easily achieved in some cases [149]. Efficacy can be measured from rate of dissociation of mediator (e.g. G-protein) from receptor as well as rate of dissociation of secondary messenger (e.g. GDP) from mediator [101].

Due to pluridimensional efficacy (multiple efficacies) of GPCR and the concept that a ligand may shows different efficacies, the conventional classification of compounds acting on the receptor into agonist and antagonist is eventually going into break [150]. Activation of particular intracellular mediators (or pathways) on the expense of others leading to what is known as biased signaling, differential efficacy or functional selectivity ([Figure 11). Biased signaling can be due to orthosteric ligands [151-154] or allosteric modulators [155], and offers a therapeutic potential to separate wanted from unwanted pathways upon receptor activation by drug [156, 157].

[Figure 10 insert here]

[Figure 10: Effect of PAM (red) and NAM (blue) on orthosteric ligand a) potency, b) efficacy and c) steepness of dose-response curve.]

[Figure 11 insert here]

[Figure 11: Web chart show pluridimensional biased signaling.]

7 Conclusions

Targeting ARs have high therapeutic potentials for treating myocardial, inflammatory and neuronal disorders; however, receptor subtype or functional selectivity is required. Since ARs belong to GPCR family, each receptor subtype has a population of different conformational ensembles. Each ensemble has different energy well depth that is proportional to its stability. In absence of ligands, this population has a pattern that mediates basal activity. Orthosteric ligands change the pattern of distribution for those ensembles and consequently intracellular signaling. Allosteric ligands affect the pattern of distribution by direct interaction with orthosteric ligand, receptor or both. A quinary complex model is proposed to describe interaction between orthosteric ligand, allosteric ligand, receptor and two intracellular mediators of G-protein and beta-arrestin. Therefore, according to receptor conformational ensembles stabilized, different patterns of activations can be obtained for intracellular signaling pathways. Allosteric ligand provides opportunities to tune potency, efficacy, steepness of dose-response curve for each signaling pathway among pathways activated by orthosteric ligand. Therefore, allosteric modulation of receptor activity gathers higher attention for future design of adenosine receptor drugs.

8 Remarks

Authors declare that theory of biological evolution and its related terms mentioned in this article and in references are not considered per se by them.

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Table 1: Mutational studies performed on adenosine receptors which shed light on residues important for allosteric modulators binding and/or involved in their modulation mechanism.

Receptor	Mutation	Effect on orthosteric and allosteric ligands activities	Reference
A_1	G14T$^{1.37}$	constantly active, no effect of allosteric PD81723 (PAM)	[158]
	T277A$^{7.42}$	Decrease agonist activity, abolish effect of allosteric PD81723 (PAM)	[139]
	D55A$^{2.50}$	allosteric regulation of binding by sodium (NAM)	[159]
	W156A^{ECL2}	Abolish effect of PD81723 (PAM) on increasing orthosteric CPA binding	[48]
	MGE(162–164)AAA^{ECL2}	Increase effect of PD81723 (PAM) on increasing orthosteric CPA binding	
	S150A^{ECL2}	Reduce allosteric activity of 1-277	[84]
A_{2A}	E13Q$^{1.39}$ and H278Y$^{7.43}$	Weakly affect allosteric regulation of binding by sodium	[160]
A_3	F182A$^{5.43}$ N274A$^{7.45}$	Reduce allosteric effect of DU124183, VUF5455 and HMA “amiloride analog 5-(N,N-hexamethylene)-amiloride” (all NAMs) and have no valuable effect on agonist binding	[161]
	N30A$^{1.50}$ D58N$^{2.50}$	Abolish effect of DU124183 and VUF5455 but not HMA	
	D107N$^{3.49}$	abolished the effects of DU124183, but not VUF5455 or HMA.	
	T94A$^{3.36}$ H95A$_3$.37 K152A^{ECL2} W243A$^{6.48}$	did not influence allosteric effects of the modulators.	

	L244A$^{6.49}$ S247A$^{6.52}$		

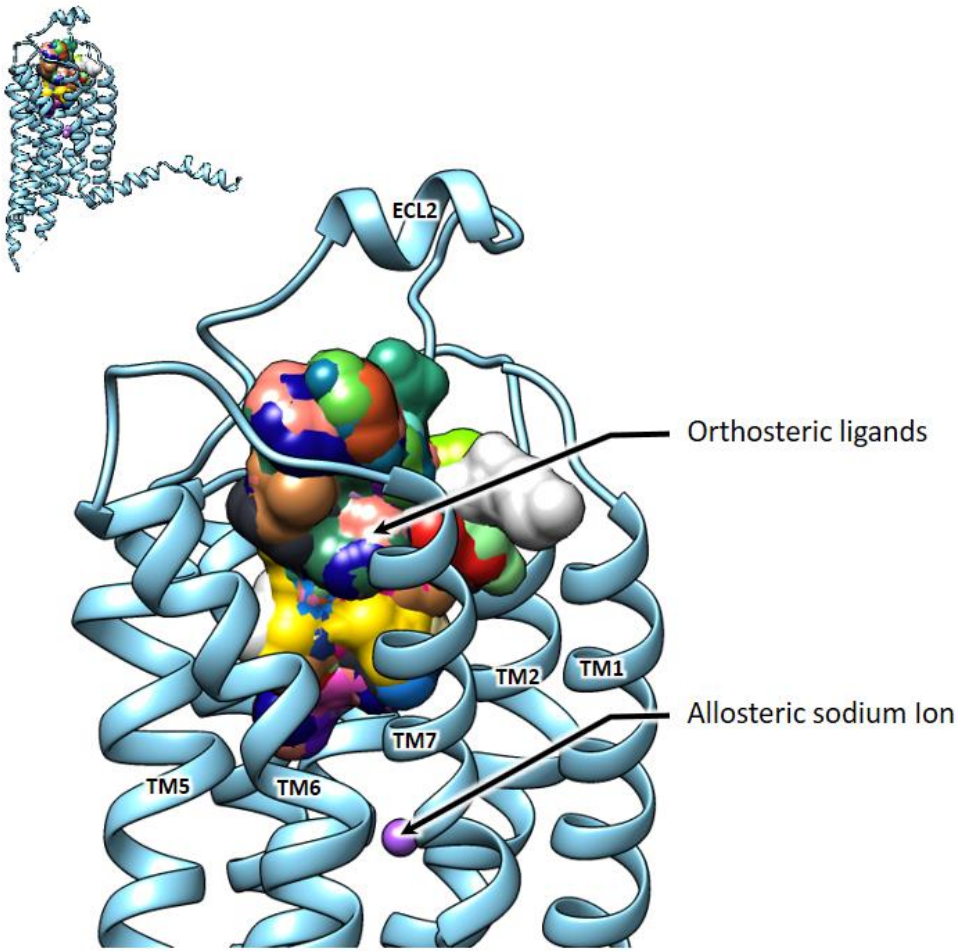
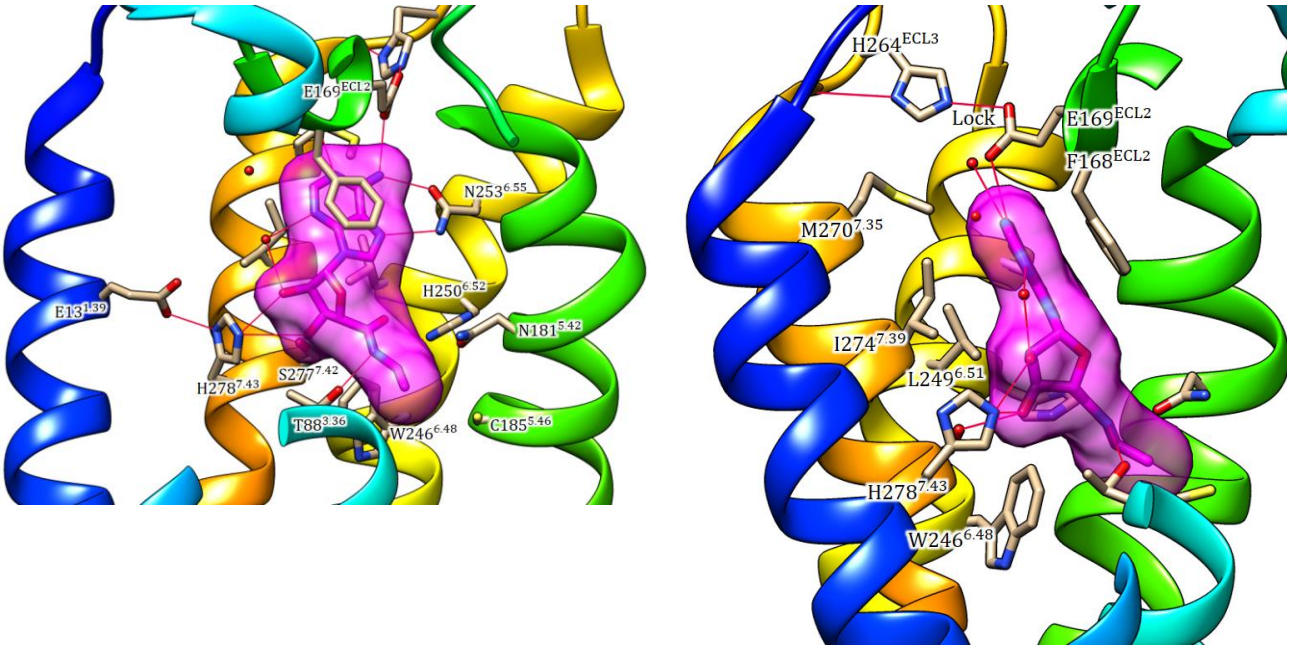


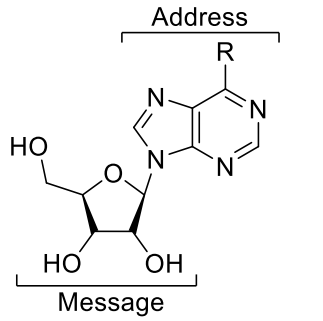
Figure 1: Orthosteric ligands from 18 crystallized structures for A_{2A} receptor. All orthosteric ligands occupy same volume at extracellular opening of 7TM tunnel and surrounded by ECLs.



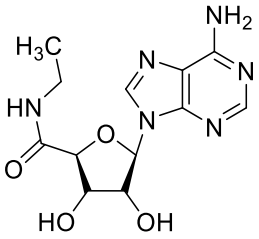
a) b)

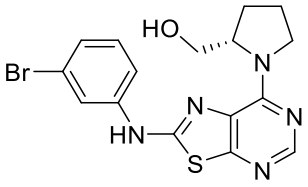
Figure 2: Crystal structure for NECA bound to A_{2A} orthosteric site (PDB ID 2YDV) showing a) front view for polar interactions and b) side view for hydrophobic pads. Please note that part of TM2, TM3 and ECL2 are removed for clarity. Note amino acids are numbered according to sequence in each subtype in addition to Ballesteros-Weinstein numbering scheme in superscript.

Agonists /partial agonists

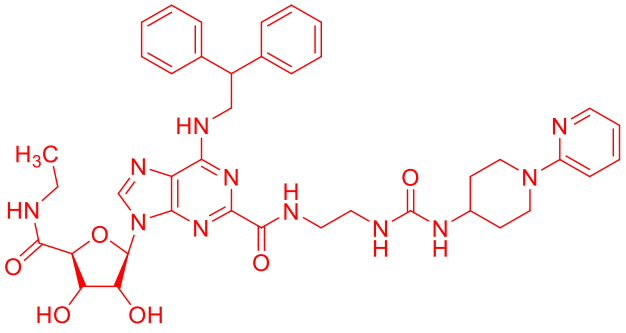


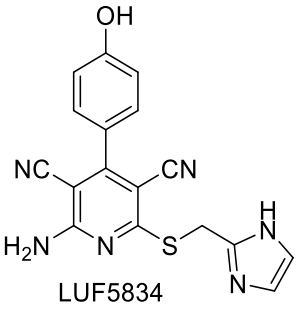
R= NH_2, adenosine
R= OH, inosine



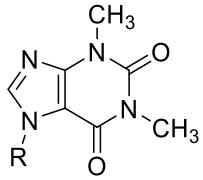


7-(prolinol-N-yl)-2-bromophenylamino-thiazolo[5,4-d]pyrimidines

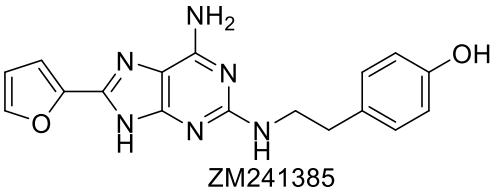




Antagonists



R=H, theophylline
R=CH_3, caffeine



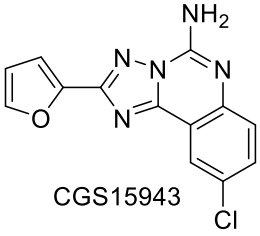


Figure 3: Examples of agonists whether adenosine-based or not as well as antagonists whether xanthine-based or not.

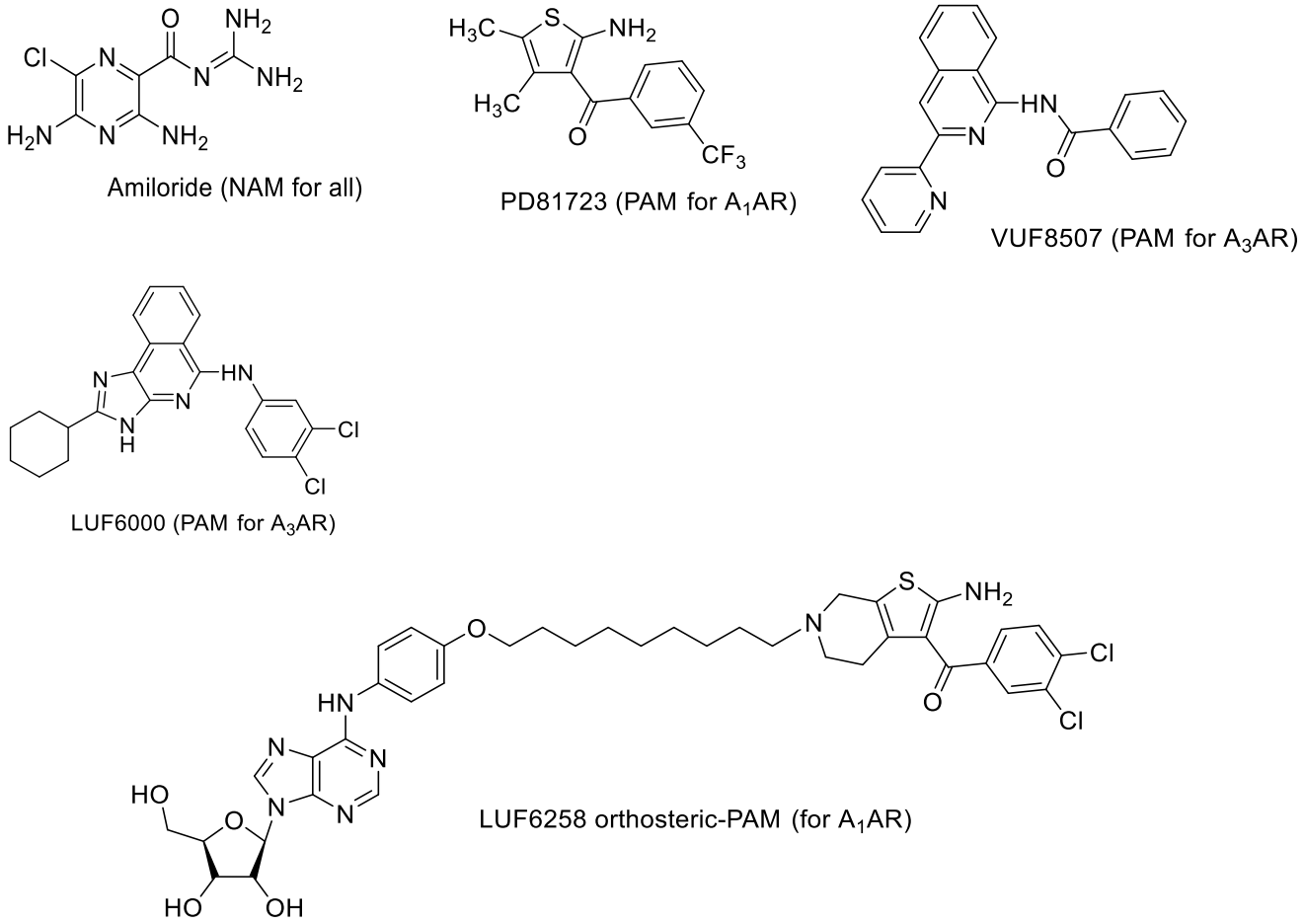


Figure 4: Examples of allosteric modulators for adenosine receptors whether NAM or PAM.

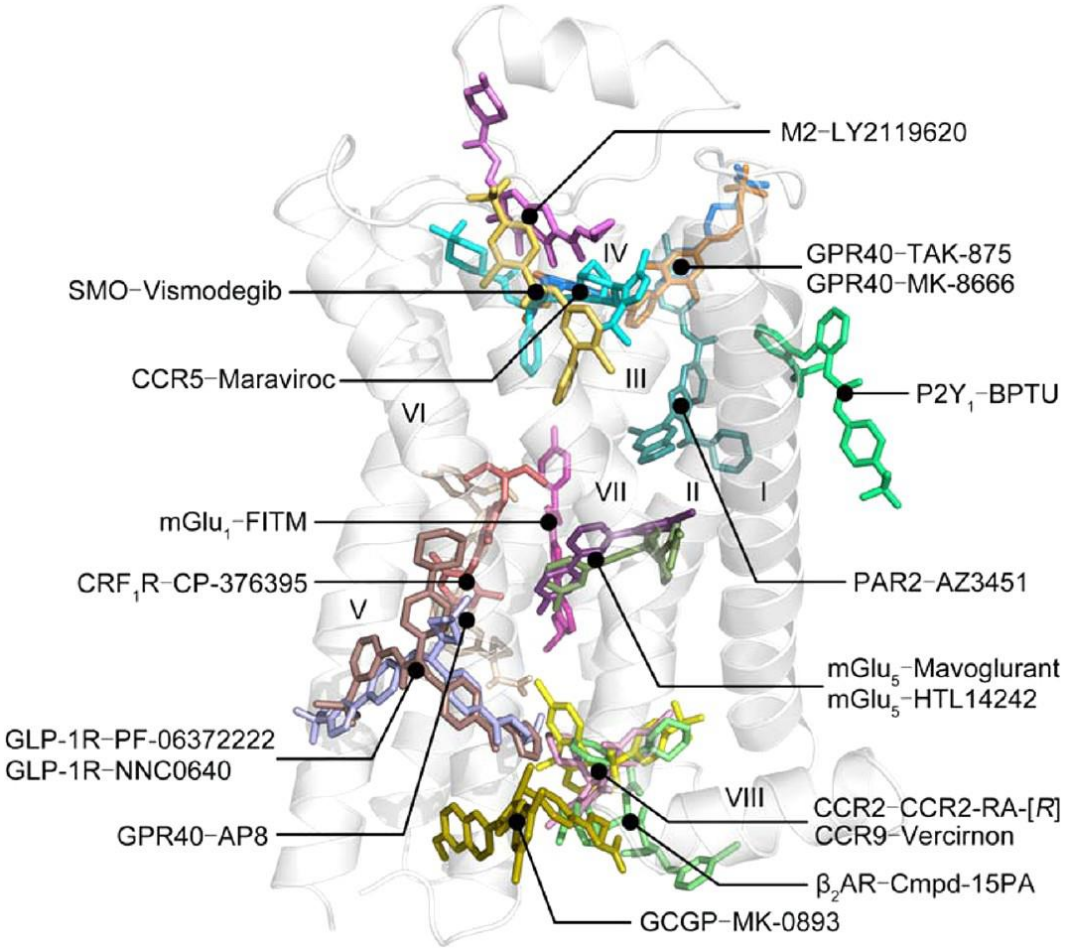
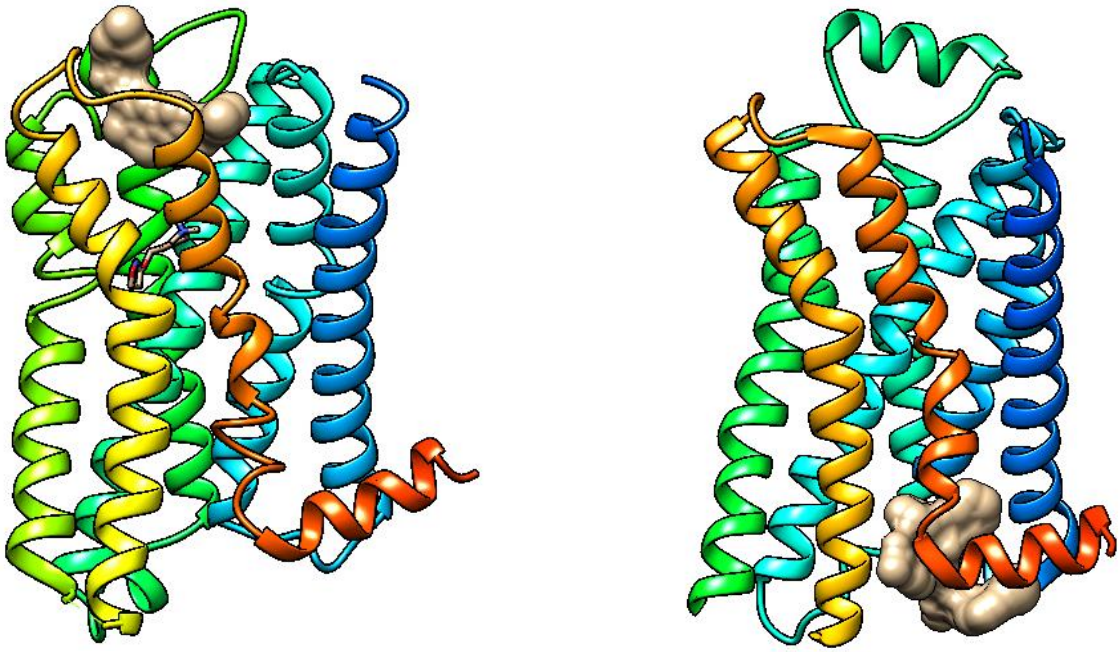


Figure 5: Multiplicity of allosteric sites across GPCR subfamilies. The structures are superimposed using β2-adrenergic receptor as representative crystal structure. 7TM are shown in ribbon and allosteric modulators in stick. (obtained from [70] with permission).



a) b)

Figure 6: Crystal structures for a) Muscarinic acetylcholine receptor (M2 receptor) simultaneously bound to the orthosteric agonist iperoxo and the positive allosteric modulator LY2119620 (PDB ID 4MQT), b) for beta2-adrenoceptor bound to allosteric antagonist (PDB ID 5X7D). The van der Walls surfaces of allosteric ligands are shown.

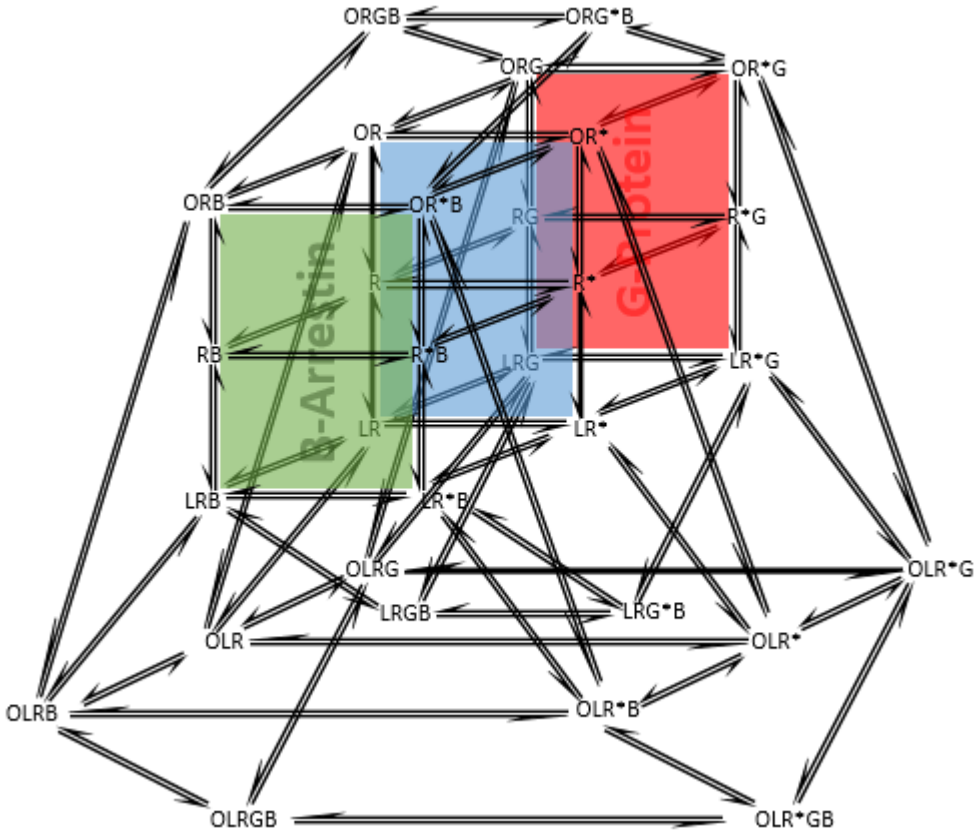


Figure 7: The quinary complex model of allosteric interactions at ARs; a thermodynamically complete, extended model taking into account the concomitant binding of orthosteric ligand, O, allosteric ligand, L, and G protein, G, Beta-arrestin, B, on a receptor that can exist in two conformational states (R and R*). Where the conversion of R to R* is governed by isomerization constant; the binding of each of O, L, G and B to R is governed by dissociation constants; the conversion of R to R* in presence of O, L, G and B is governed by activation cooperativity constants; binding of each one of O, L, G and B to R in presence of another is governed by binding cooperativity constants. Binding of B to R and R* is considered to occur without initial phosphorylation of receptor according to Storme et al 2018 (ref 105)

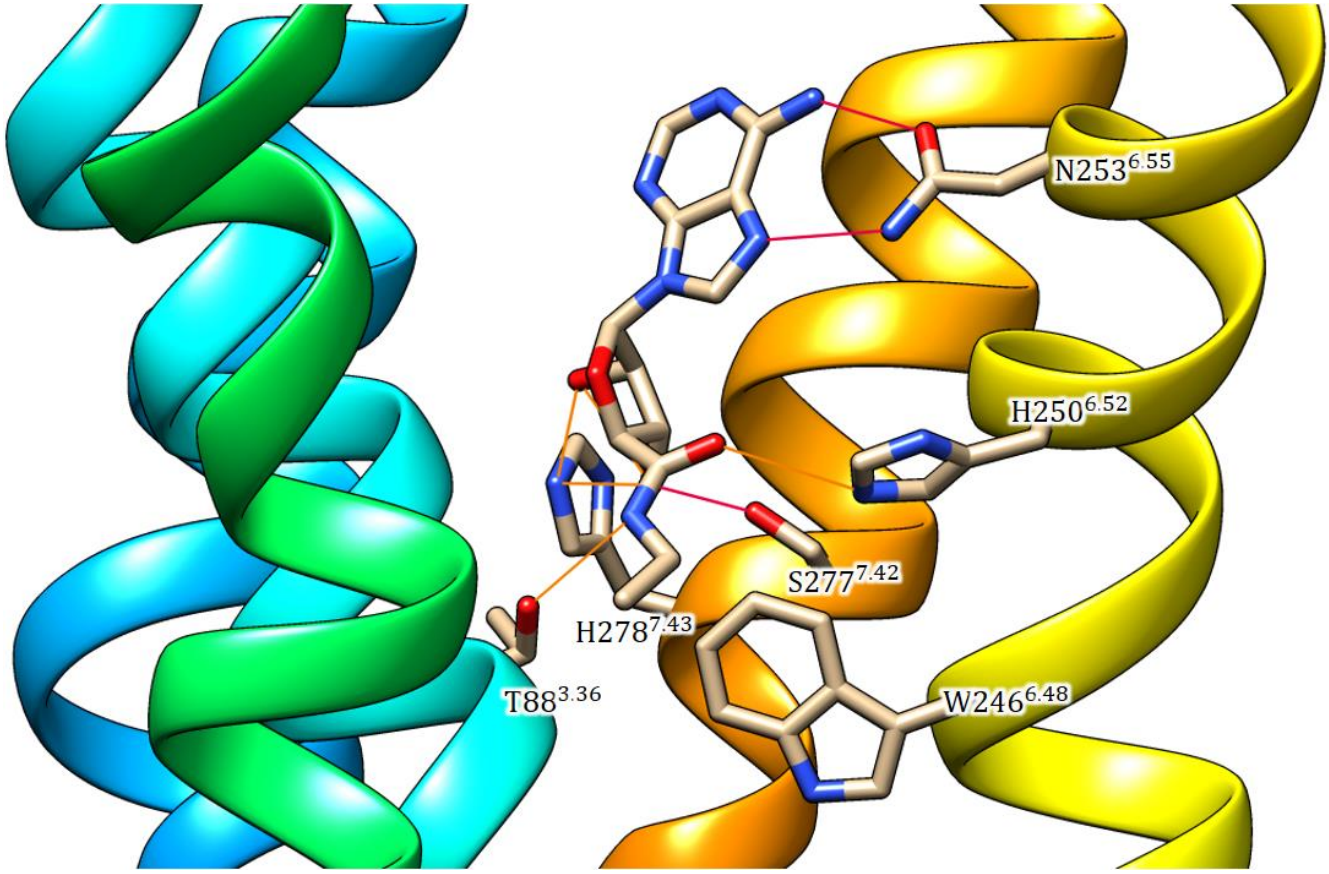


Figure 8: The interaction of ribose sugar of agonist with residues of TM3, TM6 and TM7 through H-bonds (red lines). All of the residues described pull ribose sugar into contact with W246$^{6.48}$ so that the latter residue changes its conformation and tilt the lower part of TM6 to outside the tunnel of the receptor. Please note that TM1 and TM5 are removed for clarity.

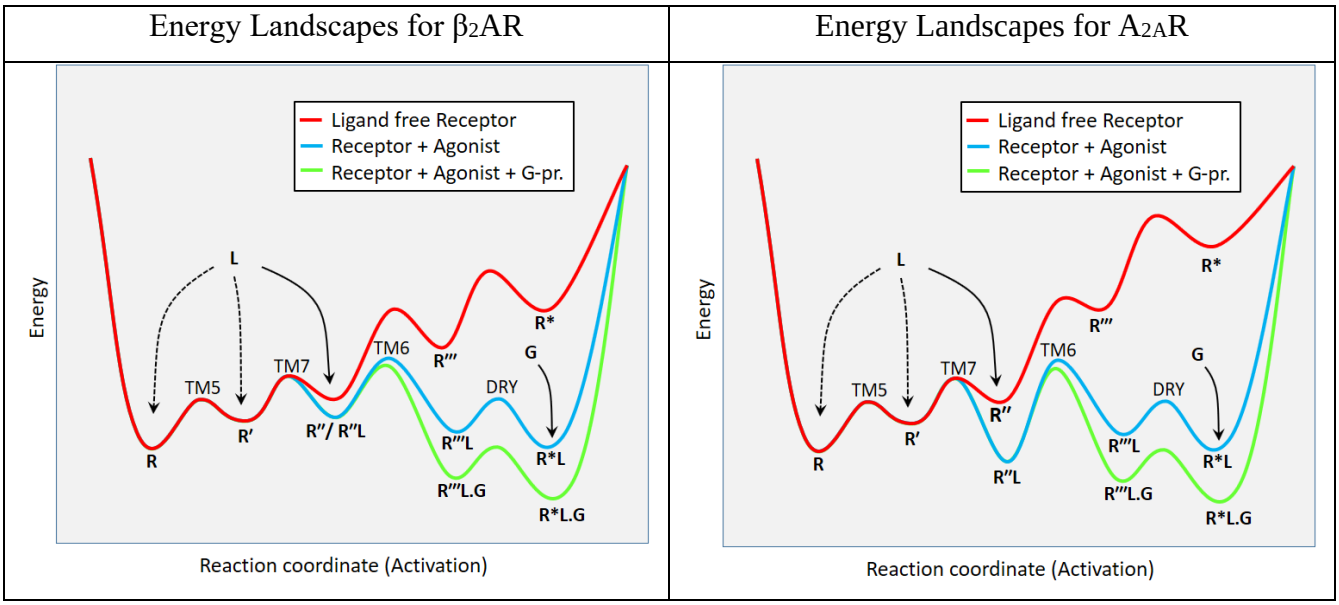
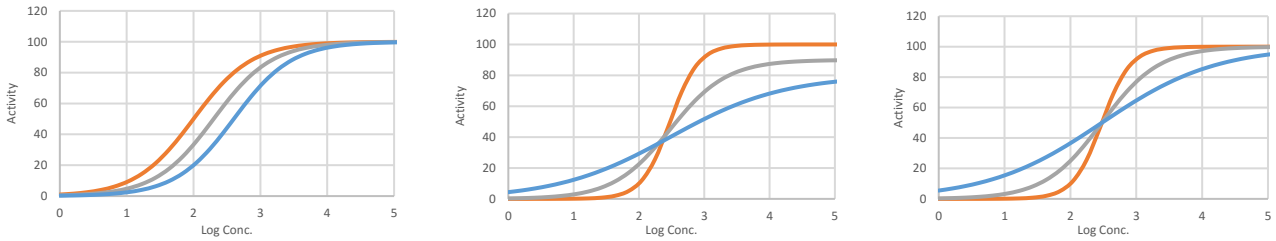


Figure 9: The energy value over reaction coordinates of the inactive receptor (R), over Meta states (R′, R″, R‴), until active state (R*), and the concomitant interactions with ligand (L) and G-protein (G). The

TM helices which change conformation are noted, although it may have some differences between the two receptors [56]. The β_2AR have shallower energy well for R″L complex compared to A_{2A}AR. In addition, the fluctuation of receptor to active state in absence of ligand in β_2AR is higher than A_{2A}AR due to higher energy barrier for the latter.



a b c

Figure 10: Effect of PAM (red) and NAM (blue) on orthosteric ligand a) potency, b) efficacy and c) steepness of dose-response curve.

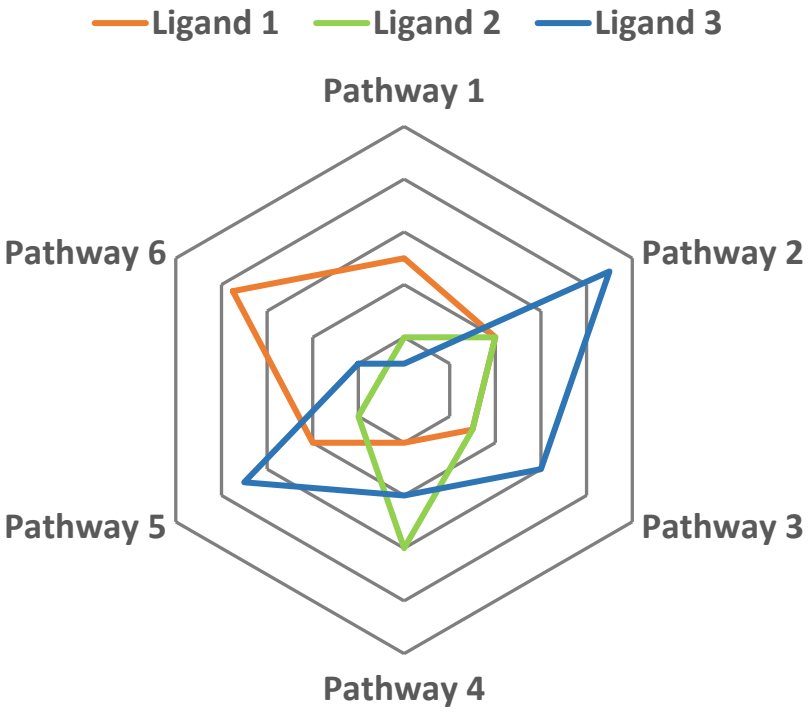


Figure 11: Web chart show pluridimensional biased signaling.