# Non-ribose ligands for the human adenosine A1 receptor

Klaasse, E.C.

**Citation**

Klaasse, E. C. (2008, June 10). *Non-ribose ligands for the human adenosine A1 receptor*. Retrieved from https://hdl.handle.net/1887/12936

| | |
|---|---|
| Version: | Corrected Publisher's Version |
| License: | Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden |
| Downloaded from: | https://hdl.handle.net/1887/12936 |

**Note:** To cite this publication please use the final published version (if applicable).

# CHAPTER 1

## General introduction

## General Introduction

Within the mammalian body, communication is essential for the regulation of all physiological functions. Signaling from the extracellular environment to the cell's interior is conducted in most cases by cell surface receptors. The largest class of membrane bound receptors consists of the Guanylyl-nucleotide-binding Protein-Coupled Receptors, also known as the G protein-coupled receptors or GPCRs. These GPCRs can be activated by a wide variety of ligands, such as ions, peptides, hormones[1], neurotransmitters, chemokines or odorants[2]. Not surprisingly, GPCRs are currently the largest group of drug targets[3]. Membrane bound receptors consist of a single polypeptide, arranged in 7 transmembrane (7TM) helices that are oriented perpendicular to the membrane (Figure 1.1). Upon activation of the receptor by agonist binding, the G protein binds to the receptor and induces an intracellular signal via a second messenger, e.g. phospholipase A or C, or adenylyl cyclase. G proteins consist of an $\alpha$, $\beta$ and $\gamma$ subunit. Currently, 16 $\alpha$, 5 $\beta$ and 14 $\gamma$ isoforms are known, implicating a great variety in G proteins[4]. In the inactive state, the $G_\alpha$ subunit contains guanosine-5'-diphosphate (GDP) in its binding site. Upon activation of the receptor, GDP is exchanged for guanosine-5'-triphosphate (GTP), leading to activation or inhibition of a second messenger effector protein.

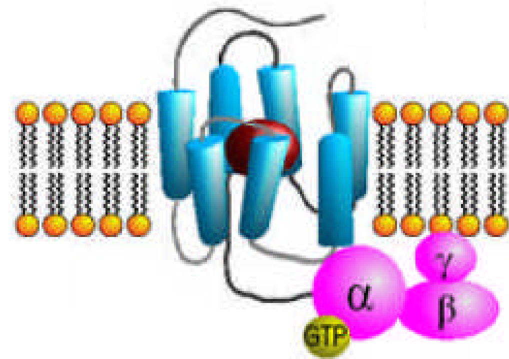


**Figure 1.1.** Structure GPCR

The GPCR superfamily can be divided into different sub-classes, based on similar structural features. Class A receptors or rhodopsin-like receptors form the largest sub-family, and share a series of conserved amino acid residues. Amongst others, adenosine receptors are members of this Class A receptors[5].

### *Adenosine receptors and their subtypes*

Adenosine receptors are named after their endogenous ligand, adenosine, which is widespread in the body with a basal concentration in the nM-µM range (Figure 1.2)[6]. Besides being a neuromodulator, adenosine is also acting as a local hormone. It has a very short half-life (seconds) and is produced instantaneously when and where it is needed[7]. Extracellularly, adenosine is formed by the breakdown of the abundantly present ATP by 5'-ectonucleotidase.

In 1929, Drury and Szent-Györgi were the first to describe the actions of adenosine on the heartbeat and arterial pressure[8]. Almost 50 years later Burnstock proposed two types of receptors, $P_1$ and $P_2$, which could be distinguished by their preference

for either adenosine or adenine nucleotides, respectively[9]. The $P_1$ receptor was subsequently divided into $A_1$ (or $R_i$) and $A_2$ (or $R_s$) adenosine receptors according to their ability to either inhibit or stimulate the cAMP production, respectively[10,11]. In 1983, it was found that the $A_2$ receptor showed high and low affinity binding sites in the brain[12]. Accordingly, the adenosine $A_2$ receptor was subdivided into $A_{2A}$ receptors with a high affinity binding site (striatum) and $A_{2B}$ receptors with a low affinity binding site (throughout the brain)[13]. The adenosine $A_1$, $A_{2A}$, and $A_{2B}$ receptors were discovered with help of ´classical´ ligand pharmacology. In contrast, the existence of the adenosine $A_3$ receptor subtype was discovered with help of molecular biology studies, and later confirmed with pharmacological experiments[14,15].

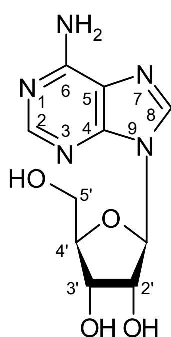


**Figure 1.2.** Chemical structure of adenosine

*Cloning*

As molecular biological techniques evolved in the 90's, all adenosine receptor subtypes were individually cloned and identified for a number of species. The adenosine $A_1$ receptor has been cloned from tissues from rat[16], cow[17], rabbit[18], mouse[19], guinea pig[20] and human[21,22]. Interestingly, a slight (5%) difference in sequence homology between bovine and human adenosine $A_1$ receptors accounts for considerable interspecies differences in ligand binding[23]. The adenosine $A_{2A}$ receptor has been cloned from rat[24,25], mouse[19], guinea pig[26], and human tissues[27]. The rat adenosine $A_{2B}$ receptor was cloned in 1992, and appeared to share only 50% sequence homology with the rat adenosine $A_1$ and $A_{2A}$ receptor[28] which explained its different pharmacological profile like a low affinity for the reference $A_{2A}$ receptor antagonist CGS21680. In addition, human[29], mouse[19] and chicken[30] adenosine $A_{2B}$ receptors have been cloned as well. Whereas the existence of adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptors was based on pharmacological experiments and later confirmed by cloning of the respective genes, the opposite was true for the adenosine $A_3$ receptor. After cloning of this receptor from rat testis[14], its pharmacology was determined[15]. Subsequently, the $A_3$ receptor was also cloned from sheep[31], rabbit[32] and human[33]. The sequence homology among adenosine $A_3$ receptors from different species is rather low e.g. the rat adenosine $A_3$ receptor shows only 74% sequence homology with the human receptor, which is also reflected in its pharmacology. For instance xanthine-based antagonists have a high affinity for human and sheep $A_3$ receptors but a much lower affinity for rat $A_3$ receptors[34].

*Occurrence of adenosine receptors*

The different adenosine receptors are distributed widely in varying levels throughout the body, thus explaining the plethora of effects of adenosine [35,36]. The adenosine $A_1$ receptor is highly expressed in the central nervous system (CNS) and adipocytes. The adenosine $A_{2A}$ receptor is found mostly in the striatum, spleen, thymus, leukocytes and blood platelets. The adenosine $A_{2B}$ receptor is expressed in low levels in the brain, but can be found in high levels in the colon and bladder. The adenosine $A_3$ receptor is hardly expressed in the CNS, however, and in the periphery the highest levels have been found in the lung and liver.

*Receptor Structure*

The cloning of all four adenosine receptor subtypes and alignment of the amino acid sequences, revealed that the adenosine receptors indeed belong to the GPCR superfamily, sharing the characteristic 7 transmembrane (TM) domain structure[37,38]. The human adenosine $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ receptors consist of 326, 412, 332 and 318 amino acids, respectively[21,27,31,32,39]. The TM domains consist of α helices containing 21 to 28 amino acids and are connected by three extracellular and three intracellular hydrophobic loops. The $A_{2A}$ receptor is the only subtype with an extraordinary long C-terminus of 122 amino acids, explaining its larger size compared to the other adenosine receptor subtypes[40]. The N-terminus of each protein is on the extracellular side, and the C-terminus is located on the cytoplasmic side of the membrane. The α helices are arranged in such a way that they form a core for ligand binding. Certain conserved amino acids contribute to ligand specificity within the binding pocket, e.g. Glu16 in TM1, Asp55 in TM2[41] and Thr277[42] and His278[17] in TM7 (numbering according to the human adenosine $A_1$ receptor).

*Fusion proteins*

Activation of G protein-coupled receptors results in an interaction of these receptors with their respective G proteins. Since it is known that upon activation the $G_\alpha$ subunit also interacts with intracellular domains including the C-terminus of receptors, constructs in which Gα subunits are physically linked to the C-termini of receptors have been prepared. One of the first fusion proteins, the $\beta_2$-adrenergic receptor fused to its $G_s\alpha$-subunit, was prepared by Bertin et al. in 1994. This $\beta_2$- $G_s\alpha$ fusion protein was shown to be functional in both ligand binding and in cAMP experiments[43]. Since then, many fusion proteins have been engineered between various receptors and their G proteins[44], e.g. the $\alpha_{2A}$-adrenergic with $G_{i1}\alpha$ or $G_0\alpha$ subunits[45-47], the serotonin 5-$HT_{1A}$ receptor with $G_{i1}\alpha$ or $G_0\alpha$ subunits[48-50], the $\delta$ opioid receptor with a $G_{i1}\alpha$ subunit[51] and the adenosine $A_1$ receptor with $G_{i1}\alpha$ or $G_0\alpha$ [52-54].

Milligan and coworkers constructed nine different fusion proteins between the human adenosine $A_1$ receptor and different $^{351}$Cys-mutated $G_{i1}$ α-subunits. They used these fusion proteins to investigate the ternary complex formation between agonist, receptor and G protein, and also demonstrated that there is no selectivity for any particular adenosine $A_1$ receptor – $G_{i1}\alpha/G_0\alpha$ combination, using different agonists[52,53]. Next to these G protein-receptor fusion proteins, fusion proteins between different receptors and fluorescent tags have been prepared to visualize the receptor. In 1997, Barak et al. were among the first to equip a GPCR with a fluorescent probe. They tagged the $\beta_2$-adrenergic receptor with a green fluorescent protein ($\beta_2$R-GFP), providing a tool to study intracellular trafficking and surface mobility of a functionally intact $\beta_2$-adrenergic receptor[55].

Concerning adenosine receptors, the human adenosine $A_{2A}$ receptor was recently C-terminally tagged with the green fluorescent protein (GFP) by Niebauer et al. This provided an easy and useful tool to investigate the expression levels over time of the adenosine $A_{2A}$R expressed in yeast[56].

*Desensitization and Internalization*

Desensitization is generally defined as the phenomenon in which previous or continued exposure of receptor to agonist results in a diminished functional response of the receptor upon prolonged agonist treatment. Signal duration, intensity or quality is attenuated upon receptor desensitization. This does not necessarily mean that the receptor will disappear from the plasma membrane, it can remain their in the inactive state. Desensitization of a signal can occur at different levels of the signal transduction cascade, thereby terminating cellular responses. The process of desensitization follows a series of sequential steps. For most receptors, desensitization is initiated by the phosphorylation of serine and threonine residues in the third intracellular loop and C-terminus of the receptor. Subsequently, the phosphorylated receptors recruit the protein β-arrestin, which sterically inhibits G protein coupling and is also able to target the receptor to clathrin coated pits for internalization. During internalization, the receptor moves away from the plasma membrane and is targeted to endosomes or lysosomes, depending on which β-arrestin is attracted. Upon internalization, receptors can either be rapidly recycled to the plasma membrane, targeted to larger endosomes and slowly recycled, or degraded in lysosomes[57,58].

The adenosine receptor subtypes display different rates of desensitization. For example, adenosine $A_1$ receptors are not (readily) phosphorylated and internalize slowly which takes several hours. At the other extreme, adenosine $A_3$ receptors which are also $G_i$-coupled, require only a few minutes to internalize. The $A_{2A}$ and $A_{2B}$ receptors, which are both $G_s$-coupled, show a fast downregulation with kinetics

usually less than 1h for short-term desensitization. Apparently, receptor trafficking is regulated via different pathways, which are specific for receptor type, cell type, metabolic state of the cell, cell-specific factors etc[59].

*Agonists for the adenosine $A_1$ receptor: traditional and non-ribose agonists.*

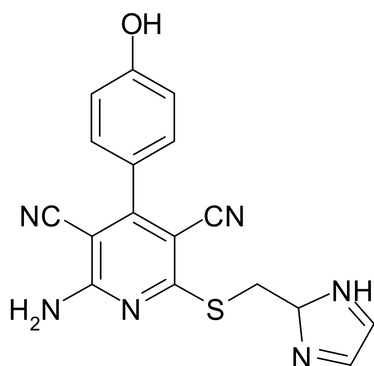


**Figure 1.3.** Structure of LUF5834, one of the non-adenosine compounds

As mentioned before, adenosine is the endogenous ligand for all adenosine receptor subtypes. Adenosine is composed of a purine group linked to a ribose moiety, see Figure 1.2. Examples of potent and selective adenosine $A_1$ agonists are: *N*$^6$-cyclopentyladenosine (CPA), 2-chloro-*N*$^6$-cyclopentyladenosine (CCPA), *N*$^6$-cyclohexyladenosine (CHA), (*R*)-*N*$^6$-(2-Phenylisopropyl)adenosine (*R*-PIA) and *N*$^6$-cyclopentyl-2-(3-phenylaminocarbonyltriazene-1-yl)adenosine (TCPA). All these prototypic $A_1$ receptor agonists are based on the endogenous ligand adenosine, and the ribose group of adenosine has long been considered essential for agonistic activity on adenosine receptors. However, Rosentreter *et al.,* (2003, 2004) recently described a new class of adenosine receptor ligands, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitriles, displaying significant affinity and efficacy towards different adenosine receptor subtypes[60-62]. One of those new, non-adenosine compounds, LUF5834 (Figure 1.3), was characterized as a full agonist for the human adenosine $A_1$ receptor with a very high affinity comparable to the $K_{i,\ high}$-value of CPA (2.2 nM), and as a partial agonist for the $A_{2B}$ receptor with high affinity[63]*.* Another non-adenosine compound, LUF5831, appeared to be a partial agonist with an affinity of $18 \pm 1$ nM for the adenosine $A_1$ receptor[64]. From this study it also appeared that allosteric modulators such as PD81,723 and GTP seem to have no or much less effect on the binding of LUF5831 than they have on the binding of the traditional adenosine analogue CPA[64]. We have further investigated the properties of promising non-ribose agonists which have revealed some remarkable novel features.

*Allosteric Modulation*

The ligand binding site of the endogenous receptor ligands is also referred to as the orthosteric binding site. Next to such an orthosteric modulation more recently allosteric modulation of receptors was discovered. The term ‘allosteric modulation’ with respect to receptor binding, was first mentioned in literature in the early eighties[65]. The word ‘allosteric’ is derived from Greek and composed of two words: ‘allo’ and ‘stere’ which literally mean ‘other’ and ‘site’ or ‘shape’. Allosteric modulation

is thus achieved by adding compounds which act on a site of the receptor distinct from the orthosteric binding site, thereby inducing a conformational change of the receptor (Figure 1.4). Allosteric modulators for the adenosine receptors have been described in literature. Amiloride analogues and sodium ions were demonstrated to be common allosteric modulators for at least three subtypes of the adenosine receptors, $A_1$, $A_{2A}$, and $A_3$[66]. Next to these non-selective allosteric modulators also subtype selective modulators have been identified. The most well-known selective allosteric modulator is probably PD81,723, a benzoylthiophene derivative. It was demonstrated that several benzoylthiophene derivatives were selective enhancers of agonist binding to the adenosine $A_1$ receptors, with little or no effect on other adenosine receptor subtypes. Allosteric enhancers at the adenosine $A_1$ receptor have received attention as anti-arrhythmic and anti-lipolytic agents. In addition, they may also have therapeutic potential as analgesics and neuroprotective agents[67]. Allosteric modulation of $A_{2A}$R has so far only been described for the already mentioned sodium ions and amiloride analogues. In 2004, Van den Nieuwendijk et al, described a class of small organic molecules, 2,3,5-substituted [1,2,4]thiadiazoles, that seemed to allosterically modulate the $A_{2A}$R[68]. However, investigation of the mechanism of action of these compounds revealed that they acted as sulfhydryl modifiers rather than as allosteric modulators of the receptor[69]. Recently, two classes of $A_3$ AR allosteric modulators were characterized: 3-(2-pyridinyl)isoquinolines (e.g. VUF5455) and 1H-imidazo-[4,5-c]quinolin-4-amines (e.g. DU124183), which selectively decrease the agonist dissociation rate at the human $A_3$AR but not at $A_1$- and $A_{2A}$ARs. Allosteric enhancers for the adenosine $A_3$ receptors may be useful against ischemic conditions[70]. At this moment, allosteric modulators for adenosine receptors receive increasing interest and as described above may have therapeutic advantages over orthosteric ligands[71].

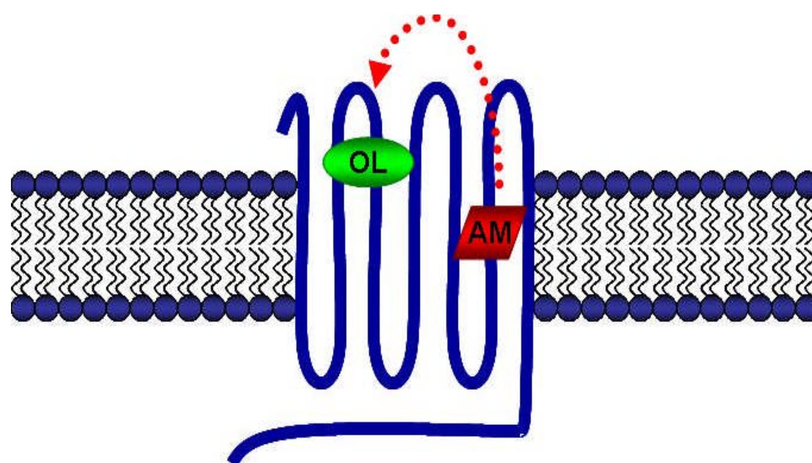


**Figure 1.4.** Schematic representation of the mechanism of allosteric modulation. OL = orthostheric ligand, AM = allosteric modulator.

*Therapeutic potential of adenosine $A_1$ receptor regulation*

As mentioned, the adenosine $A_1$ receptor is abundantly expressed in the brain with the highest levels in the hippocampus and the cerebral cortex. In addition, the

adenosine $A_1$ receptor is widely distributed and expressed at varying levels throughout the body, e.g. vas deferens, testis, white adipose tissue, stomach, spleen, adrenal gland, heart, aorta, liver, eye and bladder. Lower levels were found in the lung, kidney and small intestine[72-74]. In view of this broad distribution pattern, many therapeutic applications of adenosine $A_1$ receptor compounds have been proposed. For example, adenosine $A_1$ agonists or the high release of adenosine during cerebral ischaemia have been shown to reduce neuronal damage in cerebral ischaemia by inhibiting glutamate release[75]. These findings provide hope for the treatment of neurological disorders observed in Huntington's disease and Alzheimer[76]. Next to the neuroprotective effects of adenosine $A_1$ receptor activation in the central nervous system, also sedation, decreased locomotor activity and anticonvulsant effects have been observed. Compounds that are able to block the receptor from receiving its endogenous ligand in the CNS (antagonists) may be beneficial to counteract the sedation and negative locomotor effects of adenosine. Antagonists have also been found to enhance cognition, leading to an improvement in memory performance[77-79]. They may therefore be potentially useful in the treatment of neurological disorders such as Alzheimer's disease. The depth and levels of sleep are also dependent on the amount of adenosine present in the brain. Furthermore, adenosine plays an important role via adenosine $A_1$ receptors in analgesia in the periphery and at spinal sites. The expression of the adenosine $A_1$ receptor on atrial tissue suggests a role for this receptor in cardiovascular diseases. Activation of adenosine $A_1$ receptors was demonstrated to play a protective role upon myocardial ischaemia and subsequent reperfusion[80]. Patients with congestive heart failure often suffer from fluid retention caused by elevated adenosine levels in the kidneys. Administration of $A_1$ antagonists prevents renal failure and increases the urine flow in these patients[81].

*The Scope and Content*

In the previous paragraphs, an introduction into the history, occurrence, functioning, trafficking and therapeutic potential of adenosine receptors and in particular the adenosine $A_1$ receptors was given. In earlier work we have demonstrated our ability to design selective adenosine $A_1$ receptor ligands with high affinity. Moreover, we extended this ligand repertoire to include allosteric modulators and non-ribose agonistic ligands. In this thesis we have expanded our knowledge space by investigating the interaction of these compounds with the adenosine receptors in more detail. To study in addition the role of these compounds in novel concepts such as constitutive activity and trafficking we have applied recombinant DNA techniques, among others to construct fusion proteins that can serve as tracers. In chapter 2, the state of affairs is established with a review of the current literature concerning the desensitization and internalization of adenosine receptors. Results from *in vitro, ex*

*vivo* and *in vivo* studies are summarized, followed by the molecular mechanisms involved in adenosine receptor desensitization and internalization. The role of accessory proteins and the influence of receptor mutations are also taken into account.

In chapters 3 and 4, the use of fusion proteins between the human adenosine $A_1$ receptors and $G_{i\alpha}$-subunits or a yellow fluorescent protein as tools to investigate inverse agonism or receptor trafficking is described, respectively. Furthermore, we analysed if these constructs were still subject to allosteric modulation[54,82]. In Chapter 5, 6 and 7, the structure-activity relationships of differently substituted 2-amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles are reported. These series of compounds display a wide variety of intrinsic efficacies, ranging from inverse agonists, to partial agonists and very potent full agonists. I present the first evidence that the properties of these non-adenosine agonists are very different from the traditional agonists for the adenosine $A_1$ receptor concerning allosteric modulation and internalization.

**References**


1. Bartfai T, Benovic JL, Bockaert J, Bond RA, Bouvier M, Christopoulos A, Civelli O, Devi LA, George SR, Inui A, Kobilka B, Leurs R, Neubig R, Pin JP, Quirion R, Roques BP, Sakmar TP, Siefert R, Stenkamp RE, Strange PG (2004) *Nature Rev. Drug Discov.,* 3:574-626.
2. Premont RT, Gainetdinov RR. (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu. Rev. Physiol.,* 69:511-534.
3. Patny A, Desai PV, Avery MA. (2006) Homology modeling of G-protein-coupled receptors and implications in drug design. *Curr. Med. Chem.,* 13:1667-1691.
4. Milligan G, Kostenis E. (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol.,* 147 Suppl 1:S46-55.
5. www.gpcr.org
6. Levene PA, Tipson RS. (1931) The ring structure of adenosine. *Science*, 74:521.
7. Moser GH, Schrader J, Deussen A. (1989) Turnover of adenosine in plasma of human and dog blood. *Am. J. Physiol.,* 256:C799-806.
8. Drury AN, Szent-Györgyi A. (1929) The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol.,* 68:213-237.
9. Burnstock G. (1978) A basis for distinguishing two types of purinergic receptor. *Cell membrane receptors for drugs and hormones,* 107-118.
10. Van Calker D, Müller M, Hamprecht B. (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem.,* 33:999-1005.
11. Londos C, Cooper DM, Wolff J. (1980) Subclasses of external adenosine receptors. *Proc Natl Acad Sci U S A*., 77:2551-2554.
12. Daly JW, Butts-Lamb P, Padgett W. (1983) Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell Mol Neurobiol.,* 3:69-80.
13. Bruns RF, Lu GH, Pugsley TA. (1986) Characterization of the $A_2$ adenosine receptor labeled by [$^3$H]NECA in rat striatal membranes. *Mol Pharmacol.,* 29:331-346.
14. Meyerhof W, Muller-Brechlin R, Richter D. (1991) Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.,* 284:155-160.
15. Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O. (1992) Molecular cloning and characterization of an adenosine receptor: the $A_3$ adenosine receptor. *Proc Natl Acad Sci U S A.,* 89:7432-7436.
16. Mahan LC, McVittie LD, Smyk-Randall EM, Nakata H, Monsma FJ Jr, Gerfen CR, Sibley DR. (1991) Cloning and expression of an $A_1$ adenosine receptor from rat brain. *Mol Pharmacol.,* 40:1-7.

17. Olah ME, Ren H, Ostrowski J, Jacobson KA, Stiles GL. (1992) Cloning, expression, and characterization of the unique bovine $A_1$ adenosine receptor. Studies on the ligand binding site by site-directed mutagenesis. *J Biol Chem.,* 267:10764-10770.
18. Bhattacharya S, Dewitt DL, Burnatowska-Hledin M, Smith WL, Spielman WS. (1993) Cloning of an adenosine $A_1$ receptor-encoding gene from rabbit. *Gene.,* 128:285-288.
19. Marquardt DL, Walker LL, Heinemann S. (1994) Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J Immunol.,* 152:4508-4515.
20. Meng F, Xie GX, Chalmers D, Morgan C, Watson SJ Jr, Akil H. (1994) Cloning and characterization of a pharmacologically distinct $A_1$ adenosine receptor from guinea pig brain. *Brain Res Mol Brain Res.,* 26:143-155.
21. Townsend-Nicholson A, Shine J. (1992) Molecular cloning and characterisation of a human brain $A_1$ adenosine receptor cDNA. *Brain Res Mol Brain Res.,* 16:365-70.
22. Ren H, Stiles GL. (1994) Characterization of the human $A_1$ adenosine receptor gene. Evidence for alternative splicing. *J Biol Chem.,* 269:3104-3110.
23. Tucker AL, Linden J. (1993) Cloned receptors and cardiovascular responses to adenosine. *Cardiovasc Res.,* 27:62-67.
24. Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM, Reppert SM. (1992) Molecular cloning of the rat $A_2$ adenosine receptor: selective co-expression with $D_2$ dopamine receptors in rat striatum. *Brain Res Mol Brain Res.,* 14:186-195.
25. Chern Y, King K, Lai HL, Lai HT. (1992) Molecular cloning of a novel adenosine receptor gene from rat brain. *Biochem Biophys Res Commun.,* 185:304-309.
26. Meng F, Xie GX, Chalmers D, Morgan C, Watson SJ Jr, Akil H. (1994) Cloning and expression of the $A_{2A}$ adenosine receptor from guinea pig brain. *Neurochem Res.,* 19:613-621.
27. Furlong TJ, Pierce KD, Selbie LA, Shine J. (1992) Molecular characterization of a human brain adenosine $A_2$ receptor. *Brain Res Mol Brain Res.,* 15:62-66.
28. Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD, Reppert SM. (1992) Molecular cloning and expression of the cDNA for a novel $A_2$-adenosine receptor subtype. *Mol Endocrinol.,* 6:384-393.
29. Pierce KD, Furlong TJ, Selbie LA, Shine J. (1992) Molecular cloning and expression of an adenosine $A_{2B}$ receptor from human brain. *Biochem Biophys Res Commun.,* 187:86-93.
30. Worpenberg S, Burk O, Klempnauer KH. (1997) The chicken adenosine receptor $2_B$ gene is regulated by v-myb. *Oncogene.,* 15:213-221.
31. Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, Rivkees SA, Fink JS, Reppert SM. (1993) Molecular cloning and functional expression of a sheep $A_3$ adenosine receptor with widespread tissue distribution. *Mol Pharmacol.,* 44:524-532.
32. Salvatore CA, Jacobson MA, Taylor HE, Linden J, Johnson RG. (1993) Molecular cloning and characterization of the human $A_3$ adenosine receptor. *Proc Natl Acad Sci U S A.,* 90:10365-10369.
33. Hill RJ, Oleynek JJ, Hoth CF, Kiron MA, Weng W, Wester RT, Tracey WR, Knight DR, Buchholz RA, Kennedy SP. (1997) Cloning, expression and pharmacological characterization of rabbit adenosine $A_1$ and $A_3$ receptors. *J Pharmacol Exp Ther.,* 280:122-128.
34. Linden J. (1994) Cloned adenosine $A_3$ receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol Sci.,* 15:298-306.
35. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev.,* 53:527-552.
36. Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. (2000) Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch Pharmacol.,* 362:364-374.
37. Palmer TM, Stiles GL. (1995) Adenosine receptors. *Neuropharmacology.,* 34:683-694.
38. Olah ME, Stiles GL. (1995) Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu Rev Pharmacol Toxicol.,* 35:581-606.
39. Libert F, Van Sande J, Lefort A, Czernilofsky A, Dumont JE, Vassart G, Ensinger HA, Mendla KD. (1992) Cloning and functional characterization of a human $A_1$ adenosine receptor. *Biochem Biophys Res Commun.,* 187:919-926.
40. Klinger M, Freissmuth M, Nanoff C. (2002) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signal.,* 14:99-108.
41. Barbhaiya H, McClain R, IJzerman A, Rivkees SA. (1996) Site-directed mutagenesis of the human $A_1$ adenosine receptor: influences of acidic and hydroxy residues in the first four transmembrane domains on ligand binding. *Mol Pharmacol.,* 50:1635-1642.
42. Townsend-Nicholson A, Schofield PR. (1994) A threonine residue in the seventh transmembrane domain of the human $A_1$ adenosine receptor mediates specific agonist binding. *J Biol Chem.,* 269:2373-2376.

43. Bertin B, Freissmuth M, Jockers R, Strosberg AD, Marullo S. (1994) Cellular signaling by an agonist-activated receptor/$G_s$ $\alpha$ fusion protein. *Proc Natl Acad Sci U S A.,* 91:8827-8831.
44. Milligan, G. (2000) Insights into ligand pharmacology using receptor-G-protein fusion proteins. *Trends Pharmacol Sci.,* 21:24-28.
45. Rauly I, Ailhaud M, Wurch T, Pauwels PJ. (2000) Alpha$_{2A}$-adrenoceptor: $G_{\alpha i1}$ protein-mediated pertussis toxin-resistant attenuation of $G_s$ coupling to the cyclic AMP pathway. *Biochem Pharmacol.,* 59:1531-1538.
46. Pauwels PJ, Tardif S, Colpaert FC, Wurch T. (2001) Modulation of ligand responses by coupling of $\alpha_{2A}$-adrenoceptors to diverse $G_\alpha$-proteins. *Biochem Pharmacol.,* 61:1079-1092.
47. Jackson VN, Bahia DS, Milligan G. (1999) Modulation of relative intrinsic activity of agonists at the *Alpha*-2A adrenoceptor by mutation of residue 351 of G protein $G_{i1\alpha}$. *Mol Pharmacol.,* 55:195-201.
48. Dupuis DS, Tardif S, Wurch T, Colpaert FC, Pauwels PJ. (1999) Modulation of 5-$HT_{1A}$ receptor signalling by point-mutation of cysteine[351] in the rat $G_{\alpha 0}$ protein. *Neuropharmacology.*, 38:1035-1041.
49. Welsby PJ, Kellett E, Wilkinson G, Milligan G. (2002) Enhanced detection of receptor constitutive activity in the presence of regulators of G protein signaling: applications to the detection and analysis of inverse agonists and low-efficacy partial agonists. *Mol Pharmacol.,* 61:1211-1221.
50. Welsby PJ, Carr IC, Wilkinson G, Milligan G. (2002) Regulation of the avidity of ternary complexes containing the human 5-$HT_{1A}$ receptor by mutation of a receptor contact site on the interacting G protein α subunit. *Br J Pharmacol.,* 137:345-352.
51. Moon HE, Bahia DS, Cavalli A, Hoffmann M, Milligan G. (2001) Control of the efficiency of agonist-induced information transfer and stability of the ternary complex containing the δ opioid receptor and the α subunit of $G_{i1}$ by mutation of a receptor/G protein contact interface. *Neuropharmacology.,* 41:321-330.
52. Waldhoer M, Wise A, Milligan G, Freissmuth M, Nanoff C. (1999) Kinetics of ternary complex formation with fusion proteins composed of the $A_1$-adenosine receptor and G protein α-subunits. *J Biol Chem.,* 274:30571-30579.
53. Wise A, Sheehan M, Rees S, Lee M, Milligan G. (1999) Comparative analysis of the efficacy of $A_1$ adenosine receptor activation of $G_{i/o\alpha}$ G proteins following coexpression of receptor and G protein and expression of $A_1$ adenosine receptor-$G_{i/o\alpha}$ fusion proteins. *Biochemistry.,* 38:2272-2278.
54. Klaasse E, de Ligt RA, Roerink SF, Lorenzen A, Milligan G, Leurs R, IJzerman AP. (2004) Allosteric modulation and constitutive activity of fusion proteins between the adenosine $A_1$ receptor and different [351]Cys-mutated $G_i$ alpha-subunits. *Eur J Pharmacol.,* 499:91-98.
55. Barak LS, Ferguson SS, Zhang J, Martenson C, Meyer T, Caron MG. (1997) Internal trafficking and surface mobility of a functionally intact $\beta_2$-adrenergic receptor-green fluorescent protein conjugate. *Mol Pharmacol.,* 51:177-184.
56. Niebauer RT, Wedekind A, Robinson AS. (2004) Decreases in yeast expression yields of the human adenosine $A_{2A}$ receptor are a result of translational or post-translational events. *Protein Expr Purif.,* 37:134-143.
57. Pierce KL, Premont RT and Lefkowitz RJ. (2002) SEVEN-TRANSMEMBRANE RECEPTORS. *Nature Reviews Molecular Cell Biology,* 3:639-650.
58. Ferguson SSG. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev,* 53:1-24.
59. Chini B, Parenti M. (2004) G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *Journal of Molecular Endocrinology,* 32:325-338.
60. Rosentreter U, Kraemer T, Shimada M, Huebsch W, Diedrichs N, Krahn T, Henninger K, Stasch J-P. (2003) Substituted 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines and their use as adenosine receptor-selective ligands. WO patent 2003/03008384.
61. Rosentreter U, Kramer T, Shimada M, Hubsch W, Diedrichs N, Krahn T, Henninger K, Stasch J-P. (2004) Substituted 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines and their use as adenosine receptor-selective ligands. US patent 2004/0176417.
62. Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Roerink SF, van den Hout G, Beukers MW, Brussee J, IJzerman AP. (2005) A series of ligands displaying a remarkable agonistic-antagonistic profile at the adenosine $A_1$ receptor. *J Med Chem.,* 48:2045-2053.
63. Beukers MW, Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Brussee J, IJzerman AP. (2004) New, non-adenosine, high-potency agonists for the human adenosine $A_{2B}$ receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem.,* 47:3707-3709.
64. Heitman LH, Mulder-Krieger T, Spanjersberg RF, von Frijtag Drabbe Künzel JK, Dalpiaz A and IJzerman AP. (2006) Allosteric modulation, thermodynamics and binding to wild-type and mutant

(T277A) adenosine $A_1$ receptors of LUF5831, a novel non-adenosine like agonist. *Br J Pharmacol.,* 147:533-541.
65. Goldenberg K. (1980) Molecular interactions in biomedicine: modulation of regulatory behavior by cross reactivity: relevance to analysis of receptor function. *Med Hypotheses.,* 6:13-19.
66. Soudijn W, van Wijngaarden I, IJzerman AP. (2002) Allosteric modulation of G protein-coupled receptors. *Curr Opin Drug Discov Devel.,* 5:749-755.
67. Baraldi PG, Iaconinoto MA, Moorman AR, Carrion MD, Cara CL, Preti D, López OC, Fruttarolo F, Tabrizi MA, Romagnoli R. (2007) Allosteric enhancers for $A_1$ adenosine receptor. *Mini Rev Med Chem.,* 7:559-569.
68. van den Nieuwendijk AM, Pietra D, Heitman L, Göblyös A, IJzerman AP. (2004) Synthesis and biological evaluation of 2,3,5-substituted [1,2,4]thiadiazoles as allosteric modulators of adenosine receptors. *J Med Chem.,* 47:663-672.
69. Göblyös A, de Vries H, Brussee J, IJzerman AP. (2005) Synthesis and biological evaluation of a new series of 2,3,5-substituted [1,2,4]-thiadiazoles as modulators of adenosine $A_1$ receptors and their molecular mechanism of action. *J Med Chem.,* 48:1145-1151.
70. Gao ZG, Kim SK, IJzerman AP, Jacobson KA. (2005) Allosteric modulation of the adenosine family of receptors. *Mini Rev Med Chem.,* 5:545-553.
71. Akkari R, Burbiel JC, Hockemeyer J, Muller CE. (2006) Recent progress in the development of adenosine receptor ligands as antiinflammatory drugs. *Curr Top Med Chem.,* 6:1375-1399.
72. Palmer TM, Stiles GL (1995) Adenosine receptors. *Neuropharmacology*, 34: 683-694.
73. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev.,* 53:527-552.
74. Yaar R, Jones MR, Chen JF, Ravid K (2005) Animal models for the study of adenosine receptor function. *J Cell Physiol.,* 202:9-20
75. Pearson T, Damian K, Lynas RE, Frenguelli BG (2006) Sustained elevation of extracellular adenosine and activation of A1 receptors underlie the post-ischaemic inhibition of neuronal function in rat hippocampus in vitro. *J Neurochem.,* 97:1357-1368.
76. Blum D, Hourez R, Galas MC, Popoli P, Schiffmann SN. (2003) Adenosine receptors and Huntington's disease: implications for pathogenesis and therapeutics. *Lancet Neurol.,* 2:366-374.
77. Dunwiddie TV, Masino SA. (2001) The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci.,* 24:31-55.
78. Ribeiro JA, Sebastiao AM, de Mendonca A. (2002) Adenosine receptors in the nervous system: pathophysiological implications. *Prog Neurobiol.,* 68:377-392.
79. Cunha RA. (2001) Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int.,* 38:107-125.
80. Hayes ES. (2003) Adenosine receptors and cardiovascular disease: the adenosine-1 receptor $A_1$ and $A_1$ selective ligands. *Cardiovasc Toxicol.,* 3:71-88.
81. Modlinger PS, Welch WJ. (2003) Adenosine $A_1$ receptor antagonists and the kidney. *Curr Opin Nephrol Hypertens.,* 5:497-502.
82. Klaasse EC, van den Hout G, Roerink SF, de Grip WJ, IJzerman AP, Beukers MW. (2005) Allosteric modulators affect the internalization of human adenosine A1 receptors. *Eur J Pharmacol.,* 522:1-8.