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Non-ribose ligands for the human adenosine A1 receptor

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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, hormones¹, neurotransmitters, chemokines or odorants². Not surprisingly, GPCRs are currently the largest group of drug targets³. 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 α , β and γ subunit. Currently, 16 α , 5 β and 14 γ isoforms are known, implicating a great variety in G proteins⁴. In the inactive state, the G_α 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.

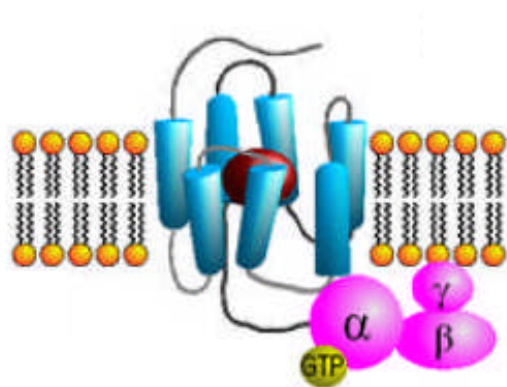


Figure 1.1. Structure GPCR

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 receptors⁵.

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- μ M 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 needed⁷. 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 pressure⁸. 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, respectively⁹. 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 brain¹². 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}.

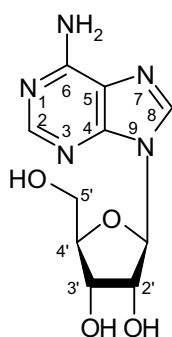


Figure 1.2. Chemical structure of adenosine

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 rat¹⁶, cow¹⁷, rabbit¹⁸, mouse¹⁹, guinea pig²⁰ 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 binding²³. The adenosine A_{2A} receptor has been cloned from rat^{24,25}, mouse¹⁹, guinea pig²⁶, and human tissues²⁷. 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} receptor²⁸ which explained its different pharmacological profile like a low affinity for the reference A_{2A} receptor antagonist CGS21680. In addition, human²⁹, mouse¹⁹ and chicken³⁰ 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 testis¹⁴, its pharmacology was determined¹⁵. Subsequently, the A_3 receptor was also cloned from sheep³¹, rabbit³² and human³³. 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 receptors³⁴.

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 A₁ 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 A₃ 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 A₁, A_{2A}, A_{2B}, and A₃ 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 subtypes⁴⁰. 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 TM2⁴¹ and Thr277⁴² and His278¹⁷ in TM7 (numbering according to the human adenosine A₁ 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 α subunit also interacts with intracellular domains including the C-terminus of receptors, constructs in which G α subunits are physically linked to the C-termini of receptors have been prepared. One of the first fusion proteins, the β_2 -adrenergic receptor fused to its G_s α -subunit, was prepared by Bertin et al. in 1994. This β_2 -G_s α fusion protein was shown to be functional in both ligand binding and in cAMP experiments⁴³. Since then, many fusion proteins have been engineered between various receptors and their G proteins⁴⁴, e.g. the α_{2A} -adrenergic with G_{i1} α or G₀ α subunits⁴⁵⁻⁴⁷, the serotonin 5-HT_{1A} receptor with G_{i1} α or G₀ α subunits⁴⁸⁻⁵⁰, the δ opioid receptor with a G_{i1} α subunit⁵¹ and the adenosine A₁ receptor with G_{i1} α or G₀ α ⁵²⁻⁵⁴.

Milligan and coworkers constructed nine different fusion proteins between the human adenosine A₁ receptor and different ³⁵¹Cys-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 A₁ receptor – G_{i1} α/G₀α 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 β₂-adrenergic receptor with a green fluorescent protein (β₂R-GFP), providing a tool to study intracellular trafficking and surface mobility of a functionally intact β₂-adrenergic receptor⁵⁵.

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 yeast⁵⁶.

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 there 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 A₁ receptors are not (readily) phosphorylated and internalize slowly which takes several hours. At the other extreme, adenosine A₃ 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 etc⁵⁹.

Agonists for the adenosine A₁ receptor: traditional and non-ribose agonists.

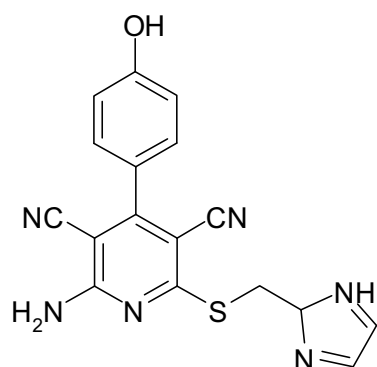


Figure 1.3. Structure of LUF5834, one of the non-adenosine compounds

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 A₁ 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 A₁ 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 subtypes⁶⁰⁻⁶². One of those new, non-adenosine compounds, LUF5834 (Figure 1.3), was characterized as a full agonist for the human adenosine A₁ 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 affinity⁶³. Another non-adenosine compound, LUF5831, appeared to be a partial agonist with an affinity of 18 ± 1 nM for the adenosine A₁ receptor⁶⁴. 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 CPA⁶⁴. 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 eighties⁶⁵. 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

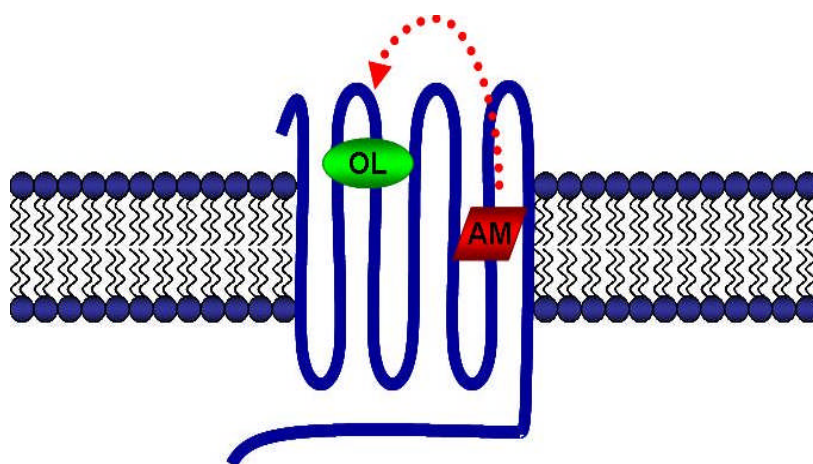


Figure 1.4. Schematic representation of the mechanism of allosteric modulation. OL = orthosteric ligand, AM = allosteric modulator.

potential as analgesics and neuroprotective agents⁶⁷. 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 receptor⁶⁹. 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_3AR but not at A_1 - and $A_{2A}AR$ s. Allosteric enhancers for the adenosine A_3 receptors may be useful against ischemic conditions⁷⁰. At this moment, allosteric modulators for adenosine receptors receive increasing interest and as described above may have therapeutic advantages over orthosteric ligands⁷¹.

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 A₁ 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 intestine⁷²⁻⁷⁴. In view of this broad distribution pattern, many therapeutic applications of adenosine A₁ receptor compounds have been proposed. For example, adenosine A₁ agonists or the high release of adenosine during cerebral ischaemia have been shown to reduce neuronal damage in cerebral ischaemia by inhibiting glutamate release⁷⁵. These findings provide hope for the treatment of neurological disorders observed in Huntington's disease and Alzheimer⁷⁶. Next to the neuroprotective effects of adenosine A₁ 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 performance⁷⁷⁻⁷⁹. 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 A₁ receptors in analgesia in the periphery and at spinal sites. The expression of the adenosine A₁ receptor on atrial tissue suggests a role for this receptor in cardiovascular diseases. Activation of adenosine A₁ receptors was demonstrated to play a protective role upon myocardial ischaemia and subsequent reperfusion⁸⁰. Patients with congestive heart failure often suffer from fluid retention caused by elevated adenosine levels in the kidneys. Administration of A₁ antagonists prevents renal failure and increases the urine flow in these patients⁸¹.

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 A₁ receptors was given. In earlier work we have demonstrated our ability to design selective adenosine A₁ 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 A₁ receptors and G_{iα}-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 A₁ receptor concerning allosteric modulation and internalization.

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