Non-ribose ligands for the human adenosine A1 receptor

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Summary

The effect of adenosine on heartbeat and arterial pressure was first described in 1929. Much later, almost 30 years ago, it became clear that certain seven transmembrane proteins were responsible for this effect of adenosine. These proteins belong to the large class of G protein-coupled receptors (GPCRs), a protein class which has proven itself to be very amenable as drug targets. Four GPCRs have been identified that recognise adenosine. All are expressed throughout the body and each contains its own specific characteristics concerning ligand binding and signal transduction. An introduction to the four adenosine receptors subtypes, their history and cloning, occurrence, functioning, trafficking and therapeutic potential is given in **Chapter 1.**

The process of desensitization and internalization of adenosine receptors in cell systems, tissues and *in vivo* studies is described in **Chapter 2.** An overview of the current literature concerning desensitization and internalization of the adenosine receptors is given and the regulation of the different subtypes upon agonist binding is discussed. For instance, the A_1R internalizes slowly, showing a typical half-life of several hours, while the other G_i-coupled adenosine receptor (A_3R) internalizes in a matter of minutes. In addition, molecular mechanisms involved in adenosine receptor desensitization are discussed.

In **Chapter 3**, human adenosine A_1 receptors fused to ^{351}Cys mutated $G_{i\alpha}$-subunits are used as tools to study inverse agonism. In addition, the enhancing effect of the allosteric modulator PD81,723 on agonist affinity is shown. It appeared that the physical linking of the $G_{i1\alpha}$ subunit to the adenosine A_1R in a 1:1 ratio, resulted in an eightfold higher affinity of the reference agonist CPA. However, the affinity of the antagonist DPCPX for the fusion protein was not affected, indicating that G protein-coupling does not affect antagonist affinity. Although the A_1R was precoupled to the $G_{i\alpha}$-subunit, thereby shifting the receptor equilibrium to the high affinity state, PD81,723 was still able to increase the affinity of CPA for the fusion proteins. Again, the affinity of DPCPX for the fusion proteins was hardly affected. Na^+-ions, acting as allosteric inhibitors, were unable to decrease agonist binding to the A_1-$G_{i1\alpha}$ fusion proteins, presumably because they exhibit their effect through uncoupling of the R-G complex. Exceptions were the ^{351}Pro and ^{351}Arg A_1-$G_{i\alpha}$ fusion proteins for which Na^+-ions did decrease the affinity of CPA. One may speculate that the side-chain characteristics of these amino acids (ring structure and positive charge respectively) may alter the conformation of the $G_{i\alpha}$ protein and with it the conformation of the fusion protein as a whole. [^{35}S]GTPγS binding experiments showed that all A_1-$G_{i1\alpha}$ fusion proteins tested had a higher basal receptor activity, thereby providing improved conditions to observe inverse agonism. It was also found that maximal receptor (de)activation depended on the amino acid at position 351 of the $G_{i1\alpha}$–

subunit. Especially the ^{351}Cys and ^{351}Ile mutants can be used as research tools to investigate inverse agonism, due to their increased readout window in [^{35}S]GTPγS binding experiments.

The influence of allosteric modulators on the internalization of the A_1R is detailed in **Chapter 4**. For that purpose, the adenosine A_1R was equipped with a C-terminal yellow fluorescent protein tag, resulting in an A_1YFP receptor. The adenosine A_1R appears to internalize very slowly, a matter of hours. Upon long-term exposure (16 h), CPA was able to internalize 25% and 40% of the receptors at a concentration of 400 nM or 4 μM, respectively. However, in the presence of PD81,723 a slight amount of internalization was already obtained at 40 nM of CPA, and at 400 nM CPA 59% of the receptors internalized, an increase of 34%. SCH-202676 on the other hand effectively prevented CPA-induced internalization of the receptor. Recent investigations, however, have shown that the action of SCH-202676 is irreversible and the result of a reaction with among others cysteine residues in a protein. These properties render SCH-202676 unsuitable as an allosteric inhibitor. Addition of either PD81,723 or SCH-202676 alone had no effect on internalization. PD81,723 was not able to accelerate the internalization process it was, however, able to reduce the threshold agonist concentration at which internalization takes place.

Recently, a series of selective agonists for human adenosine A_1 receptors with an unusual structure lacking the ribose moiety of traditional adenosine-like agonists were synthesized. The synthesis, affinity and activity of twelve of these non-ribose ligands, all 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles, are described in **Chapter 5**. Substitution at the phenyl ring mostly dictated affinity, whereas the substitution pattern at the sulfanyl position appeared to rather govern intrinsic activity. cAMP studies revealed that this series of compounds contains inverse agonists, partial agonists and very potent full agonists. It is quite remarkable to find such a great variety in potency in such a small series of compounds. From these series, the 3,4-methylenedioxyphenyl substituted compounds recognized two binding states/sites with a picomolar affinity for the high affinity site. These compounds were full agonists that largely superseded the prototypic agonist N^6-cyclopentyladenosine (CPA) in affinity and potency.

Chapter 6 evaluates the characteristics of LUF6037 a representative of these 3,4-methylenedioxyphenyl substituted compounds. LUF6037 recognized two binding states/sites on the human adenosine A_1 receptor with picomolar and nanomolar affinity, both much higher than the reference agonist CPA. Just like CPA, LUF6037 was able to inhibit the cAMP production, although again with a much lower EC_{50} value (picomolar range) than CPA (nanomolar range). In addition, thermodynamic radioligand binding experiments also classified LUF6037 as a full agonist. However, the allosteric enhancer PD81,723 did not influence the interaction of LUF6037 with

the receptor whereas the interaction of CPA with the receptor was affected. Furthermore, LUF6037 did not induce internalization whereas CPA was able to induce dose-dependent internalization of the adenosine A_1 receptor. Taking these results together, LUF6037 is a new, high affinity agonist that due the absence of internalization inducing properties can be preferred over classical agonists like CPA in drug treatment.

One of the other non-ribose agonists with high affinity for the A_1R, LUF5834, was chosen to be radioactively labelled. **Chapter 7** describes the evaluation of [^{3}H]LUF5834 as a new agonist radioligand. It appeared that [^{3}H]LUF5834 recognizes two high-affinity binding sites with only a small difference in K_d values, occupying all the receptor binding sites present on the cell membrane. This is a remarkable feature for an agonist radioligand, resembling the properties of the inverse agonist/antagonist [^{3}H]DPCPX. Displacement experiments revealed that LUF5834 is insensitive to PD81,723 or GTP. In addition, an 'extra' third site was revealed in displacement experiments with the inverse agonist DPCPX. This extra third site represents most probably a subdivision of the R conformation of the receptor, here named R^1 and R^2. Based on these results, an extended receptor equilibrium was proposed for the different receptor states.

Finally, in **Chapter 8,** general conclusions about the research described in this thesis are drawn. These are followed by some future perspectives and suggestions for research to be pursued, based on the interesting results obtained from this work.