CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Biochemical Sciences

&

RHEINISCHE FRIEDRICH-WILHELMS-UNIVERSITÄT BONN
FACULTY OF MATHEMATICS AND NATURAL SCIENCES

Department of Pharmaceutical Chemistry I

ROLE OF DISULPHIDE BONDS IN hA_{2A} SUBTYPE ADENOSINE RECEPTOR

Master's Thesis

Lukáš Zappe

Supervisors: Ing. Petra Matoušková, Ph.D.

PD Dr. Anke C. Schiedel

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ABSTRACT

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Biochemical Sciences

Candidate: Lukáš Zappe

Supervisor: Ing. Petra Matoušková Ph.D., PD Dr. Anke C. Schiedel

Title of diploma thesis: Role of disulphide bonds in hA_{2A} subtype adenosine receptor

The adenosine A_{2A} receptor belongs to the G protein coupled receptor family (GPCR). GPCRs are the targets of almost 40% of the drugs on the market. GPCRs are characterized by seven transmembrane helices, which are linked by three extracellular and three intracellular loops (ECL and ICL). The structure of the receptor has been revealed by crystallography, hence we know that ECL1 and ECL2 are connected by several disulphide bonds. The ECL2 is believed to be involved in ligand binding and recognition.

In order to understand the relevance of those disulphide bonds involved in this process, four adenosine A_{2A} receptor mutants were generated by one-site direct mutagenesis, in which the cysteine residues were replaced with serine residues (C146S, C159S, C166S and C146S-C159S). These receptor mutants were expressed in the mammalian cell line, CHO K1(Chinese Hamster Ovary) and the receptor expression was tested with ELISA (Enzyme-linked immunosorbent assay). The determination of ligand binding has been carried out by radioligand competition binding studies. Several adenosine receptor agonists (NECA, CGS-21680, PSB-826 and BAY60-6583) were tested against the radioligand [^{3}H]CGS-21680. Only the C146S receptor mutant was tested against [^{3}H]MSX-2. The function of the receptors was analyzed by cAMP accumulation assays using adenosine receptor agonists, including the endogenous agonist adenosine.

Data presented in this paper confirmed the important role of disulphide bonds for the agonist binding and recognition process. Some of the receptor mutants showed significant differences in ligand binding and function compared to the human A_{2A} adenosine receptor wildtype. In conclusion, disulphide bonds have a great importance in the ECL2.

ABSTRAKT

Univerzita Karlova v Praze

Farmaceutická Fakulta v Hradci Králové

Katedra Biochemicky věd

Kandidát: Lukáš Zappe

Vedoucí: Ing. Petra Matoušková Ph.D., PD Dr. Anke C. Schiedel

Název diplomové práce: Role disulfidických vazeb u hA_{2A} podtypu adenosinového receptoru

Adenosinový receptor podtyp A_{2A} je součástí rodiny receptorů spřažených s G proteinem (GPCR), která je nyní cílem téměř 40% všech léčivých látek. GPCRs se skládají ze sedmi transmembránových domén spojených třemi extracelulárními a třemi intracelulárními kličkami (ECL a ICL). Byla odkryta skutečná struktura receptoru, tedy víme, že ECL1 a ECL2 jsou spojeny disulfidickými vazbami. Z toho ECL2 je pravděpodobně zodpovědná za proces rozpoznání a navázání ligandu.

Abychom lépe pochopili, jak důležitá je úloha těchto disulfidických vazeb v tomto procesu, pomocí přímé jednobodové mutace byly vytvořeny čtyři adenosinové A_{2A} mutantní receptory, kde aminokyselina cystein byla nahrazena za serin (C146S, C159S, C166S a C146S-C159S). Ovlivnění receptorové exprese bylo zkoumáno pomocí metody ELISA (Enzyme-linked immunosorbent assay). Následný efekt na proces rozpoznání ligandu receptorem byl proveden pomocí kompetice mezi radioaktivně značeným ligandem [^{3}H]CGS-21680 (5 nM) a několika agonisty na adenosinových receptorech (NECA, CGS-21680, PSB-826, BAY60-6583). Pouze mutant C146S byl testován s [^{3}H]MSX-2 (1 nM). Funkčnost receptoru byla zjišťována pomocí cAMP akumulační studie, za použití agonistů popsaných výše a navíc byl zahrnut i endogenní agonista adenosin.

Zjištěné výsledky v této práci potvrzují důležitou roli disulfidických vazeb při procesu rozpoznání a navázání agonistů. Většina testovaných mutantních receptorů ukázala významný rozdíl v porovnání s lidským wild-type A_{2A} adenosinovým receptorem. Disulfidické vazby v ECL2 skutečně mají své opodstatnění.

1. INTRODUCTION

This thesis is a part of more extensive work which investigates the structure and function of the adenosine receptors. Furthermore the whole project determines possible involvement in ligand binding and recognition, aiming on the extracellular loops. Adenosine receptors belong to G protein coupled receptors (GPCRs), one of the largest groups of membrane proteins. They are coupled to G proteins, small complex of proteins, which work as trigger after the receptor activation (more details in *2.1.4*). They are characterized by the presence of seven transmembrane (TM) α-helical domains, therefore it is possible to call them 7TM receptors.[1, 2]

So far over 800 GPCRs have been discovered in the human body (**Fig. 1**) which cover approximately 4% of the human genome that codes for proteins. This large group of membrane proteins participates in cell communication and signalling. They are activated by a variety of very distinctive stimuli (light, ions, peptides, non-peptides, hormones, odorants, lipids etc.) and they subsequently influence many biological activities: vision, smell, taste, cardiovascular, neurological, endocrine and reproductive functions. Because of such an influence, many researchers investigated them very deeply during past few decades.[1, 2, 3]

2. THEORETICAL BACKGROUND

2.1. G protein coupled receptors

2.1.1. Division

The GPCRs can be divided in five major classes, according to the DNA sequence homology:

Rhodopsin-like (A)
Secretin (B)
Glutamate (C)
Adhesion
Frizzled/Taste2

Listed families are additionally sorted in subclasses. Adenosine receptors belong to Rhodopsin-like family (class A) so it will be described more detailed.[1, 2 ,3]
Class A is divided into subclasses α – γ and comprises 700 receptors that make it the largest class. Group α receptor binds monoamine neurotransmitters and acetylcholine, thus contains for example β-adrenergic receptors, dopamine receptors, histamine receptors, muscarinic acetylcholine receptors and also adenosine receptors. These receptors are widely involved in pathological processes, therefore very frequently used as pharmacological targets. Another very well known group is called γ, including opioid receptors, famous to be used as targets in the pain-release therapy and therefore belongs to the most comprehensively covered subclass so far.[4]

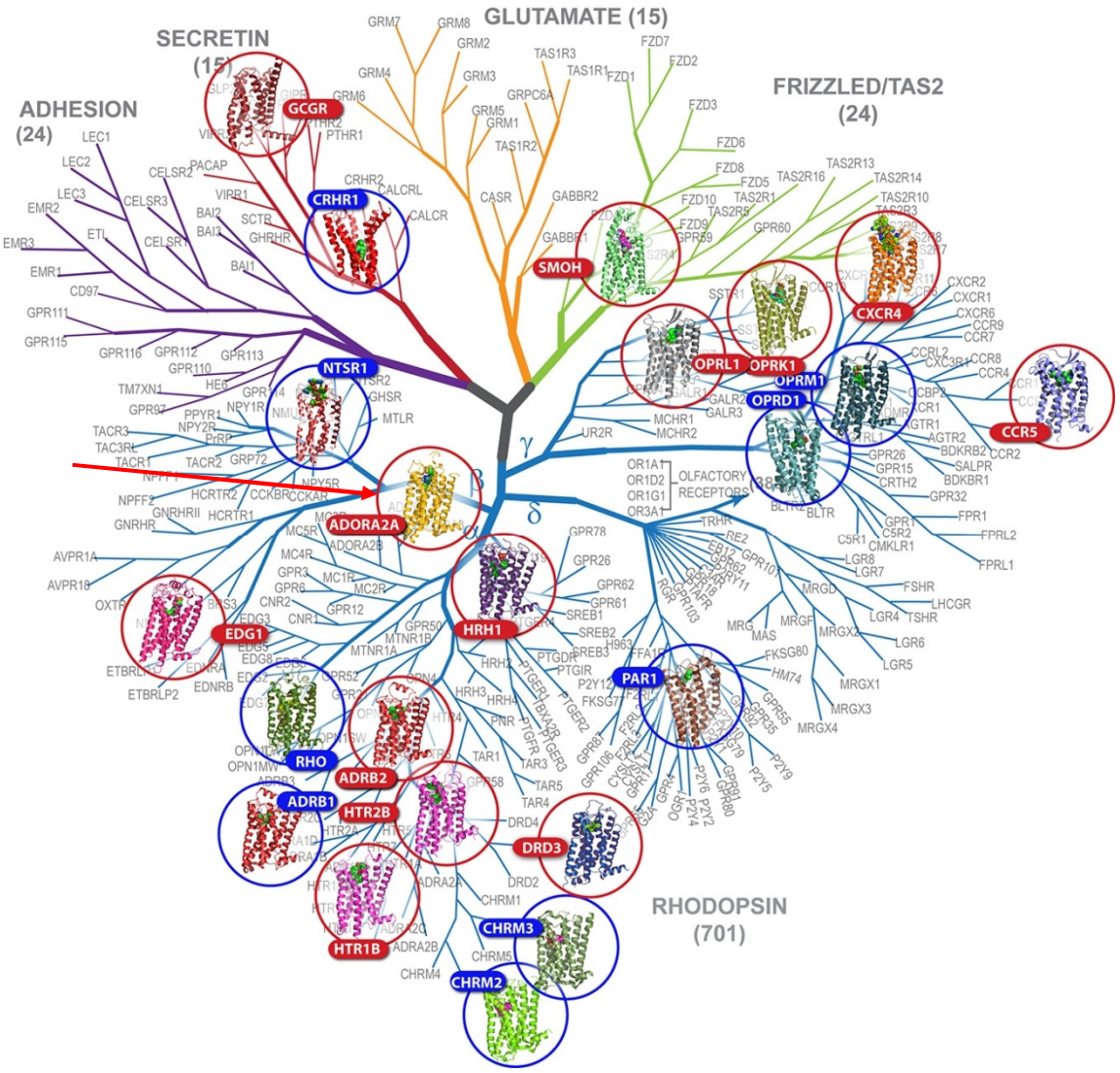

Fig. 1: Tree of GPCRs divided in five families according to DNA homology with number of proteins in each class (in brackets). Rhodopsin-like family (blue branch) is further divided into four subclasses (α – γ). Circled are those proteins with solved crystal structure (over 20). Red arrow points at the adenosine A_{2A} receptor, in particular. [5]

2.1.2. GPCR conformations

In order to better understand function and intercellular communication, I need to explain that the receptors occur in different states according to the conditions around or in the cell. There is a "*two-state model*" describing the receptor states: active (R*) and inactive (R) state, which would be in equilibrium, if any substance would not interfere. The basal activity of the receptor depends on this equilibrium, which is shifted by ligands. The inverse agonist shifts the receptor towards the inactive state, whereas the full agonist converts the receptor to the active form (R*). If a heterotrimeric G protein complex is present, this form might be further converted into a third state, called

"signalling state" (R^{*G}). Partial agonists have affinity for both states but induce only submaximal activation even at the saturation. The antagonist does not alter the basal activity, but block the access at the receptor to other ligands.[2, 4, 6]

Although the "*two-state model*" appears quite reasonable, the real process seems to be more complex and this model does not reflect the different ways of signalling (G protein, kinase, arrestin), called bias signalling, nor the receptor activation degree (*2.1.4*).[4]

2.1.3. Structure

Docking and virtual screening are now widely used for searching for new pharmacological molecules. This computer-based visualisation of possible receptor-molecule bonds is helpful, nevertheless the validation of the predicted structure by X-ray crystallography still remains the key part to understand the receptor functionality. Crystal structure is hence essential: until now over 20 different GPCRs crystal structures have been revealed (**Fig. 1**). The shortage of crystal structures is caused by several obstacles.

First we need a high amount of purified protein (tens of milligrams), which is no problem nowadays thanks to bacteria or infected cells production. The second issue is to guarantee the stability of purified protein in a detergent that is compatible with crystallography. GPCRs are usually stable in non-ionic detergents with long alkyl chains. But those chains create micelles and thus prevent the crystals to grow.[2,7] The third and probably the greatest obstacle is the conformational heterogeneity, which on one side is necessary for the proper receptor function, but on the other side it prevents the crystal growth.[2] This combination of fragility and flexibility is a major hurdle in obtaining good-quality crystals.[8] It has been demonstrated that using ligands bound to the receptor reduces decreased the heterogeneity by limiting TM movements and stabilises the proteins. The first observed GPCR in its inactive state was the rhodopsin receptor. It is quite stable compared to the other GPCRs and it was easy to extract in a high quantity from bovine retina. In 2008 the rhodopsin receptor was also the first one which provided us insight into the active state together with adenosine A_{2A} and beta-adrenergic β2 receptors.[2, 4]

The architecture of GPCRs has several common features (**Fig. 2**). Each receptor consists of a single polypeptide with an extracellular N-terminus and seven hydrophobic

transmembrane domains (TM1 – TM7) which create three extracellular loops (ECL1 – ECL3) and three intracellular loops (ICL1 – ICL3).[2] After the TM7 continues the amphipathic helical motif Helix-8, that is conserved among all class A GPCRs.[9] All is ended with an intracellular C-terminus.[2]

High diversity is caused by the lack of DNA homology they share. The highest similarity among all GPCRs occurs in the TM domains mainly in the regions connected with the cytoplasmic G proteins. On the other hand, they differ especially in: C-terminus, extracellular loops (ECL; differences in ECL2 among A class GPCRs presented in the **Fig. 3**) and N-terminus. Particularly the last one shows together with ECL the greatest diversity. The N-terminal chain varies not only in amino acid sequence but also in the length of the chain. The sequence is relatively short (10-50 amino acids) for monoamine and peptide receptors in contrast to glycoprotein hormone receptors and glutamate receptors (350 – 600 amino acids). The largest N-terminus domains are observed in the adhesion family.[2] The N-terminus might play the key role in the recognition of large ligand molecules.[10, 11]

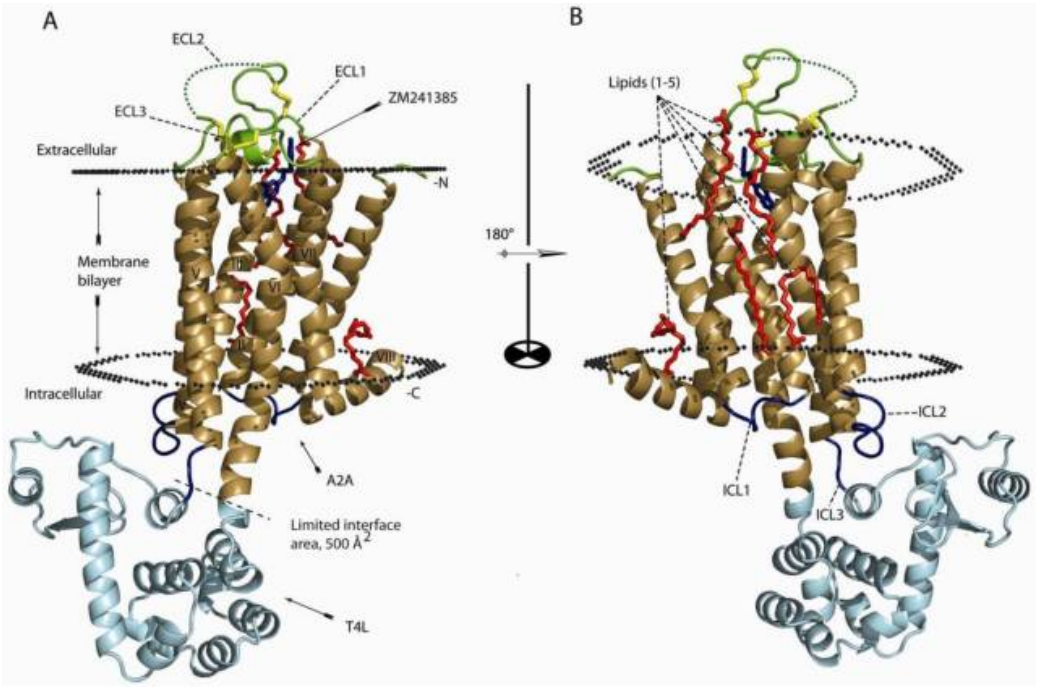

Fig. 2: Crystal structure of A_{2A}-T4L-ΔC. **A.** Overall topology of A_{2A}-T4L-ΔC. The transmembrane part of A_{2A}-ΔC structure is coloured in brown (helices I - VIII) and the T4L is in cyan. The structure is viewed perpendicular to the plasma membrane. ZM241385 is coloured light blue and the four lipid molecules bound to the receptor are coloured in red. The four disulphide bonds are yellow. The sulphate ions are omitted. The extracellular loops (ECL1-3) are coloured in green and the intracellular loops are coloured in blue. The membrane boundaries are adapted from the OPM database (http://opm.phar.umich.edu/) using β_2 adrenergic receptor-T4L (2RH1) as a model. **B.** Rotated 180° around the *x*-axis. The images were created with PyMOL.[12]

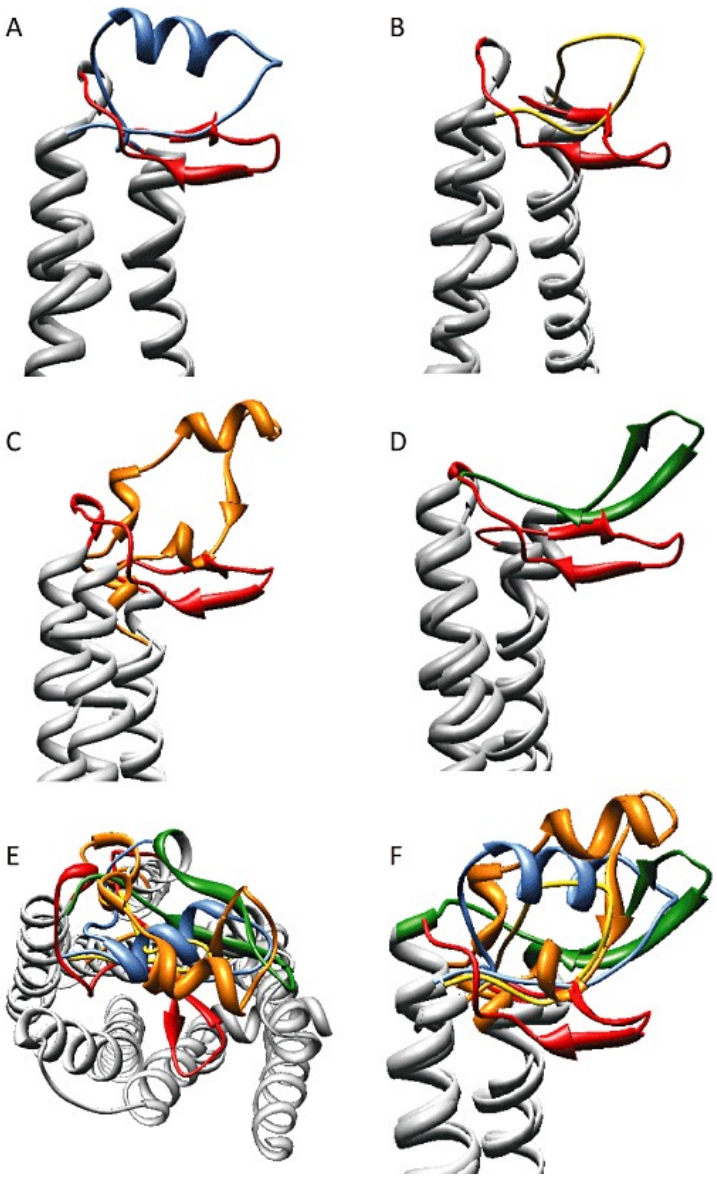

Fig. 3: ECL2 conformational diversity in Class A GPCRs. In Panels A–D, the ECL2 of the diffusible-ligand GPCR is compared with the ECL2 of rhodopsin (red; PDB accession 3CAP): A, β_2AR (blue; PDB accession 2RH1); B, D3R (yellow; PDB accession 3PBL); C, A_{2A}R (orange; PDB accession 2YDO); D, CXCR4 (green; PDB accession 3OE0). An overlay of ECL2 of all five GPCRs viewed from above (Panel E) or from within the plane of the membrane (Panel F). Only the TM bundle of rhodopsin is shown in Panels E and F for clarity.[13]

2.1.4. Function and signalling

After the binding of agonist or partial agonist, the receptor is activated. Guanidine diphosphate (GDP) is exchanged for guanidine triphosphate (GTP) bound to the Gα subunit of heterotrimeric G protein (consists of Gαβγ). The phosphorylation changes the conformation and activates the complex which usually causes dissociation of Gα-

subunit from the Gβγ subunit. It has been observed that the signalling pathway depends on Gα subunit. Since now 17 different Gα-subunits are known, occurring in the human body, grouped in four subfamilies (**Fig. 4**: **section a**):

- $G\alpha_s$ – stimulating the adenylate cyclase pathway
- $G\alpha_{i/o/t/z}$ – inhibiting the adenylate cyclase
- $G\alpha_q$ – activating the Phospholipase Cβ pathway
- $G\alpha_{12}$ – involved in RhoA signalling

There are two G protein complexes that influence adenylate cyclase. $G\alpha_s$ stimulates adenylate cyclase and supports the production of 3',5'-cyclic adenosine monophosphate (cAMP) converted from adenosine triphosphate (ATP). $G\alpha_i$ has the opposite effect and inhibits the cAMP production. cAMP serves as a second messenger in the cell and activates protein kinase A (PKA), that further regulates other cellular functions including the activation of L-type Ca^{2+} channel. After the receptor stimulation, the phosphodiesterase enzyme (PDE) down regulates the level of cAMP.

$G\alpha_q$ activates Phospholipase Cβ (PLCβ), that catalyzes the formation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 is then involved in the increasing Ca^{2+} concentration by opening the IP_3-gated calcium channels. DAG subsequently modulates protein kinase C (PKC).

The signal activation can be terminated in two ways (**Fig. 4 section b**). The first one is the dephosphorylation of GTP linked to Gα subunit performed by the subunit itself. The heterotrimeric complex is recreated and prepared for the next stimulation. In the presence of continuous activation, the second possibility of termination is exploited. The receptor cytoplasmic residues are phosphorylated by GPCR kinases (GRKs) and induce the affinity for arrestin. Arrestin is a regulatory protein that promotes extracellular signal-regulated kinases (ERK), which further influence the gene expression. It also promotes the receptor internalization through clathrin-coated pits.[1, 3, 14, 15]

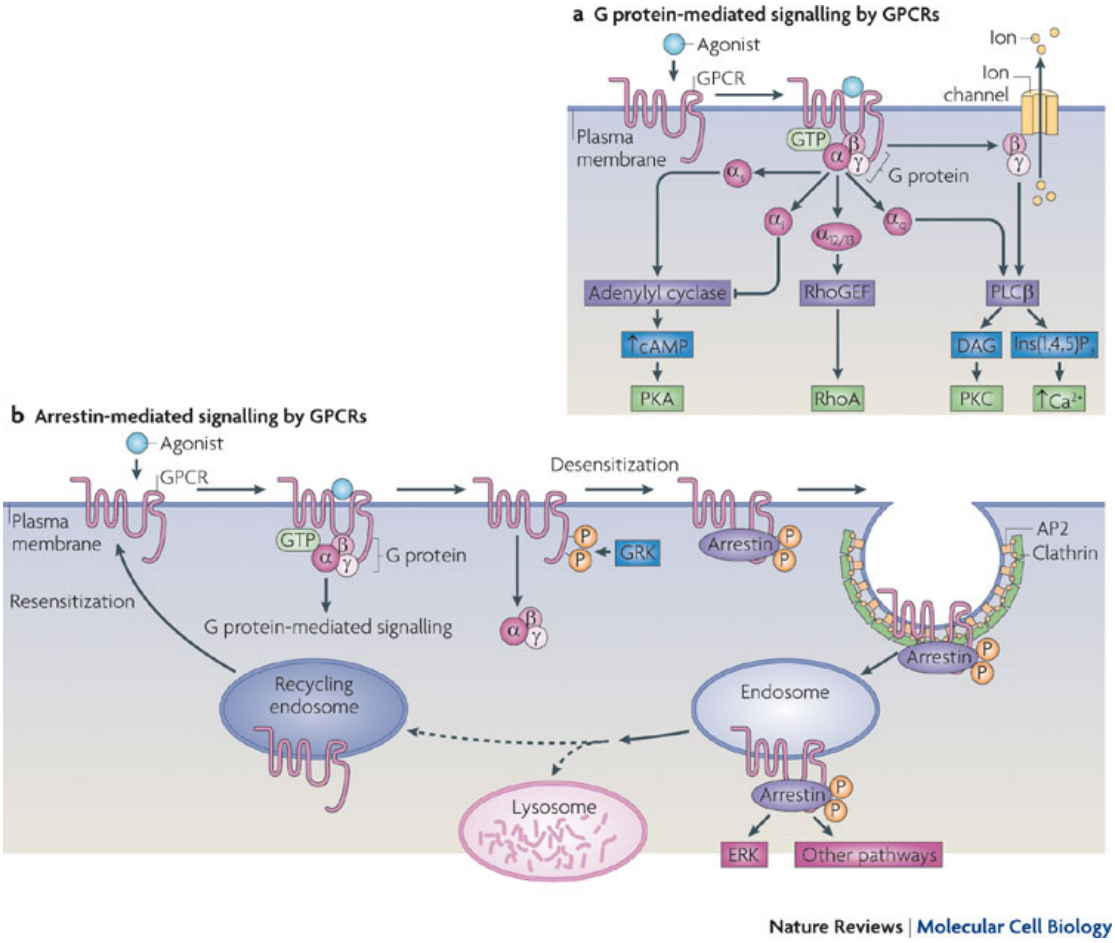

Fig. 4: GPCR signalling. Activation of the receptor by the agonist leads to the signalling cascade via different Gα subunits (section A). In the section B, phosphorylation of the receptor creates connection with arrestin which influences further faith of the receptor. Receptor is removed from cytoplasmic membrane via clathrin-coated pits and either recycled and sent back to membrane or destroyed in the lysosomes. Arrestin might promote extracellular signal-regulated kinases (ERK) that result in a change of gene expression.[15]

2.2. Purinergic receptors

2.2.1. Purinergic receptor classes

The discovery by Drury and Szent-Györgyi (1929) about possible role of adenosine as a ligand in the heart tissue [16] led to the rising interest of many researches. Since 1929, some chemically modified adenosine molecules were synthesized and the examination of the dose-response relationships suggested the presence of specific adenosine receptors.[17] In 1972, the effect of the ATP as a transmitter was observed and the concept of purinergic intercellular signalling was postulated.[18] Purinergic receptors (PR) were divided into two groups, according to their endogenous ligands. P1 group uses adenosine as an endogenous ligand and P2 group exploits ATP. Later, the P2 group was

split in P2Y (metabotropic) and P2X (ionotropic) subclasses and each group in several isoforms. This paper is focused on adenosine receptors so from now on I will describe only P1 group.[18, 19, 20]

2.2.2. Adenosine receptors

Currently, the P1 group is distinguished into the four subfamilies according to their pharmacological responses to inhibit or stimulate adenylate cyclase: A_1, A_{2A}, A_{2B}, A_3
The A_2 group is further divided in two isoforms by their affinity to adenosine (A_{2A}: high affinity, A_{2B} low affinity).[21]

A_1 and A_3 are coupled with $G_{i/o}$ family of G protein, thus inhibits the cAMP production, whereas A_{2A} and A_{2B} increase cAMP conversion via G_s. It has been observed that for $G_{i/o}$ coupling both C-terminus and ICL3 are important, whereas the influence of G_s coupling is promoted only by ICL3. The affinity for G_s is also modulated by G protein β-subunit, from which β_4 is the most potent one. Finally it has been revealed that all adenosine receptors are able to activate at least one subfamily of mitogen-activated protein kinases (MAPKs).[18, 20, 22]
Particularly, the A_{2A} receptor increases the activity of adenylate cyclase, thus supports the production of cAMP, which then stimulates the cAMP-dependent proteine kinase (PKA). The cAMP dependent PKA is then able to activate different pathways such as calcium channels, potassium channels, cAMP responsive element-binding (CREB), phospholipase C (PLC) or already mentioned MAPKs.[23]
The receptors were sorted according to the DNA sequence and ligand recognition. By now, all adenosine receptors were cloned from human, rat and mouse (**Fig. 5**). If we aim on the four human AR (adenosine receptor) subtypes, it has been observed that they share DNA sequence of 56%. The human A_{2A} receptor shares sequence identity of 49% with hA_1, 70% with hA_{2B} and 41% with hA_3.[24, 25] The 30 receptor residues considered as critical for ligand recognition show an average identity of 71%. In some parts of the receptor, the identity between hA_{2A} and hA_{2B} increases up to 90%. Most differences are typically found in the loop regions, especially in the extracellular loops (ECLs). Particularly ECL2 is involved in the ligand binding and recognition.[26] By sequence analysis has been determined that ECL1 and ECL2 presented in the A_{2A} and A_{2B} adenosine receptor subtype share 44% and 34% identity, additionally 56% and 46%

similarity, respectively. So ECL2 varies substantially even among subtypes. However focusing on the specific cysteine residues in ECL1-2 (C71, C77, C166 in the A_{2A} receptor and the corresponding residues C72, C78, C171 in the A_{2B} receptor) we can see a certain similarity. C166 in A_{2A}AR related to C171 in A_{2B}AR is even 100% similar.[27] Total amount of cysteine presented in the ECL2 of A_{2A}AR is three compared to four occurring in the A_{2B} adenosine receptor. Despite the high conservation of the regions responsible for the ligand recognition, the variety of ligands is still extensive.[25]

The adenosine receptors are asparagine-linked glycoproteins and only the hA_{2A} adenosine receptor has no access for palmitoylation.[20] Palmitoylation, together with phosphorylation, is involved in the receptor internalization and desensitization.[28] Furthermore adenosine A_{2A} receptor contains the longest C-terminus compared to the other adenosine receptors (e.g. 122 amino acids versus 36 amino acids in the A_1R).[29]

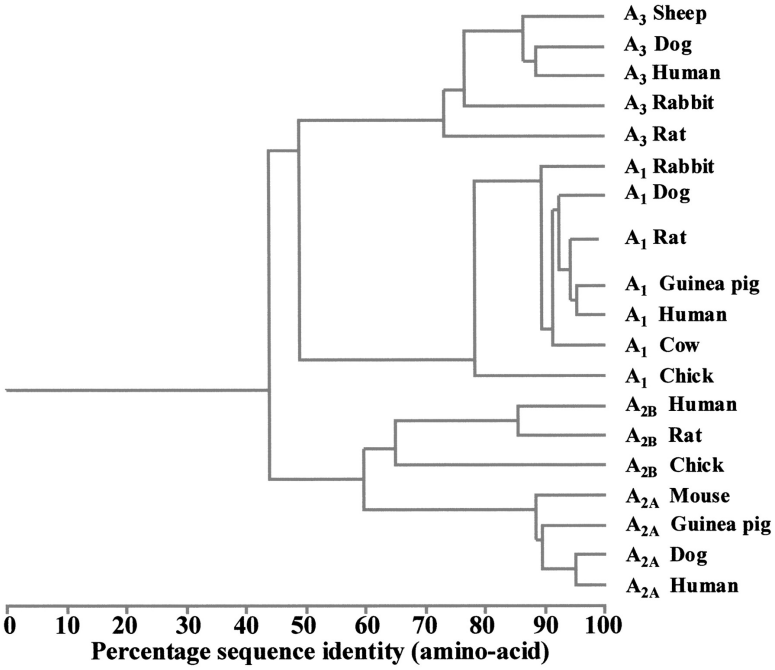

Fig. 5: Dendrogram showing the DNA similarity in adenosine receptors between different species. Figure is available at: http://pharmrev.aspetjournals.org/content/53/4/527/F1.expansion. Phylogenetic tree was automatically calculated by WHAT IF based on a neighbour-joining algorithm.

2.3. Function of human A_{2A} receptor

Adenosine biological affinity mediates effect on several cell types.[24] In the following lines there is a summary of the main possible pharmacological targets on the A_{2A}AR. Nevertheless only few of the drugs are used in the clinical practice. A complete summary of expression tissues, diseases and drug possible targets related to A_{2A} is shown in the **Fig. 6**.

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2.3.1. Cardiovascular system

The A_{2A}AR is expressed in the aorta and coronary arteries and it is involved in the vasodilatation, thus regulates the myocardial blood pressure. This fact leads to a better blood perfusion but may also cause hypotension. In the adenosine A_{2A} receptor knockout (KO) mice, slight hypertension has been observed.[24] In the 1960s and 1970s, A_{2A}AR agonists (such as CGS-21680) were tested as a potential medicament for hypertension. Unfortunately, the non-selectivity was proven on a dog model.

Activating the A_{2A}AR in platelets induced an antithrombotic effect, thus agonists might be used as an antiaggregation mediators. Nowadays there is an adenosine molecule (commercial name: Adenoscan from Astellas Pharma) that is used in imaging to evaluate coronary artery disease for patients who are not able to exercise properly.[30]

Together with A_1 and A_3, A_{2A}AR helps to protect cardiac tissue against ischemia and necrosis connected with infarct by activating the lymphocytes.[31]

2.3.2. Parkinson's disease

The A_{2A} receptors are widely expressed in the striatum where they are involved in the dopamine regulation.[24] It is believed that adenosine receptors are capable of creating dimers (two receptors connected together), either with itself (homodimers [32]) or like in this case with D_2 (dopamine) receptors (heterodimers).[21] It has been observed that stimulation of A_{2A}AR by an agonist (CGS-21680) decreases the affinity of D_2 agonist (L-(-)-N-[^{3}H]propylnorapomorphine) while the adenosine A_{2A} receptor antagonist (8-phenyltheophylline) potentiates the effect of D_2 agonist [33] and improves loco-motor activity or the receptor expression in striatum.[24] This might be the new possible treatment of Parkinson's disease. Parkinson's disease is caused by depletion of dopamine in substantia nigra in striatum. An A_{2A}AR antagonist can lead to a higher response on dopamine compare to the wild-type. Targeting heterodimers would eliminate some drug side-effects.[21] Using epidemiological studies it has been observed that there is an inverse relationship between caffeine (well known non-selective AR antagonist) consumption and Parkinson's disease. So it is not surprising that some of the

new promising antagonists entered in clinical trials.[30, 34] One example is KW-6002 called Istradefylline.[35]

2.3.3. Ischemie and neuroprotection

Adenosine subtype A_{2A} receptor is also connected with glutamate receptors in the brain tissue.[21] Experiments were performed using a highly selective A_{2A}AR antagonist, SCH58261 (K_i = 1.1 nM), on a lesioned model of rats which simulated Huntington's disease. This experiment resulted in the neuroprotective effect of the A_{2A} receptor antagonist by decreasing the releasing of quinolinic acid (QA). This has been proven only in a low dose range of SCH58261 (0.01 mg/kg). The inhibition of QA evoked by glutamate outflow appears to have a neuroprotective mechanism.[24, 36]

2.3.4. Interaction with ethanol

Ethanol inhibits adenosine re-uptake in the brain, therefore increases the concentration of adenosine, which leads to A_{2A}AR activation. The re-uptake inhibition process results in a higher amount of cAMP that leads to activation of PKA thereby CREB (cAMP response element-binding protein) activation. Alcohol and adenosine synergistically interacts with the activation of D_2Rs in the striatum/nucleus accumbens. Dopamine works in the nucleus accumbens as a reward neurotransmitter causing pleasant feeling. The A_{2A} knockout mice have been exposed to consumption of ethanol for 10 days and then researchers observed the withdrawal effect. The knockout mice better dealt with it than the wild-type. Mice to whom was administered the A_{2A} receptor antagonist ZM241385 (20 mg/kg) showed a significantly lower withdrawal effect compare to the wild-type. It is believed that, thank to this receptor cooperation, a voluntary alcohol consumption might be reduced. Therefore antagonizing the synergic system might be useful as an alcohol abuse treatment.[30, 37]

2.3.5. Inflammatory disorders

A_{2A} receptors are widely expressed on the cells of the immune system including macrophages, lymphocytes, monocytes and dendritic cells. Activating the A_{2A} receptors leads to the suppression of the immune system by increasing the cAMP concentration.

For example, the migration of neutrophils is influenced by interfering with the activity of surface protein on both neutrophils and endothelial cells. Surface proteins are responsible for migration into inflammatory site. Furthermore, intervention to the migration of the immune cells might prevent the inflammation.[23] The A_{2A} adenosine receptor activation inhibits the adherence of N-formyl-methionyl-leucyl-phenylalanine (fMLP) which normally supports the adherational process of neutrophils to the endothelial cells. In contrast it has been proven that A_1 receptor acts in the opposite way, thus activates proinflammatory pathways.[38] Another example includes T-lymphocytes, where adenosine through A_{2A} adenosine receptors inhibits T-cell activation, proliferation and reduces the production of the inflammatory cytokines. These cytokines normally attract other immune cells. Additionally adenosine supports production of the anti-inflammatory cytokines.[30] By using the A_{2A}AR agonist CGS-21680 it has been reached lower production of interleukin 2 (IL2) and tumor necrosis factor alpha (TNF-α) with nominal effect on interferon gamma (IFNγ) secretion. Reduction of IL2 was found only for A_{2A}AR and no other adenosine receptors were involved.[39]

The necessary presence of A_{2A} adenosine receptor for optimal effect of methotrexate has been observed. Methotrexate belongs to a DMARD (disease modifying antirheumatic drugs) and it is widely used to treat rheumatoid arthritis and the drug is considered as one of the most effective treatments.[40] The observation was proven when the A_{2A}AR knockout mice (administered with adenosine) did not show any decrease of the pro-inflammatory TNFα nor reduce the number of leukocytes compare to the wild-type. This also confirms the significant role of A_{2A}R in the inflammation process.[41]

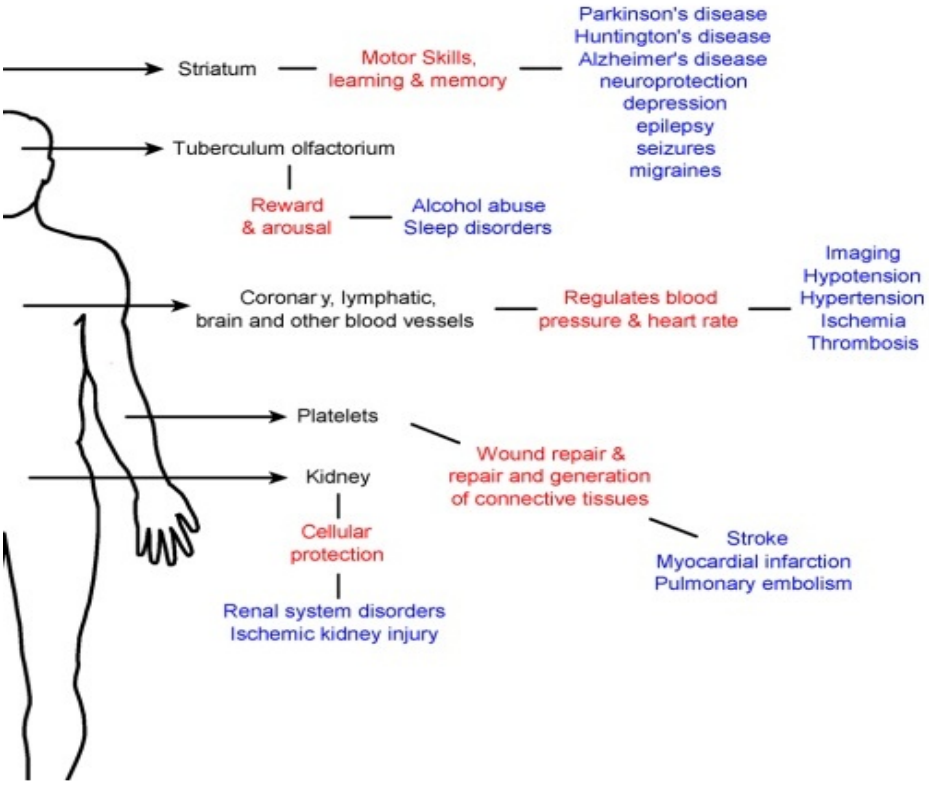
The A_{2A}AR agonist ATL-146e (formerly DWH-146e) prevents inflammatory cell adhesion. Mice skin was cyclically pressurized and subsequently occurring of a necrotic tissue was observed. Mice administrated with ALT-146e showed 65% reduction of the tissue necrosis, 31% less inhibition of average skin blood flow compare to vehicle group.[42] Inflammation is an important part of the healing process so a research group in New York University Medical Centre investigated how A_{2A}AR might be involved. They found out that A_{2A} adenosine receptor indeed participates in the wound healing process. Agonist CGS-21680 was applied into the wound of injured mice (healthy mice and also mice with the simulation of diabetes mellitus). Closing of the wound in the healthy mice was significantly faster compare to the control and the mice with simulated diabetes mellitus healed faster or equally as untreated mice.[43] The A_{2A} adenosine receptor agonist

MRE-0094 (thanks for its healing effect) was proposed as a treatment for neuropathic diabetic foot ulcers. It entered I. Phase of a clinical trials but it was terminated due to the lack of data.[30, 44]

Treatment of mice with the A_{2A} agonist ALT202 reduced the migration of neutrophils into a lung tissue by 50% compared to mice without any treatment. These findings were confirmed by A_{2A}AR knockout mice when no effect has been observed.[30, 45]

2.3.6. Learning and memory

Prefrontal cortex plays an important role in a learning, rewarding (nucleus accumbens) and memory retrieving (hippocampus) process.[21] Acquisition, the first part of memorising process, was improved when mice were administrated with the A_{2A}AR agonist CV-1808 (1 and 2 mg/kg, i.p.).[46] On the other hand, memory retrieving connected with olfactory system is possibly stimulated by adenosine antagonists. Experiments conducted by putting rats into the different cage with two compartments (one with familiar odours and the second one with strange odours). The time spent in the each compartment was measured. Also social memory was tested by putting the juvenile rat in the adult's rat cage together for five minutes. After this time juvenile rat was removed and placed in a different cage for half an hour. After this period of time they were put together again. During five minutes sniffing, nosing, grooming etc. was measured. Results suggested that the A_{2A}AR antagonist (ZM241385 or non-selective antagonist caffeine) improves aged-relative cognitive dysfunction.[47]



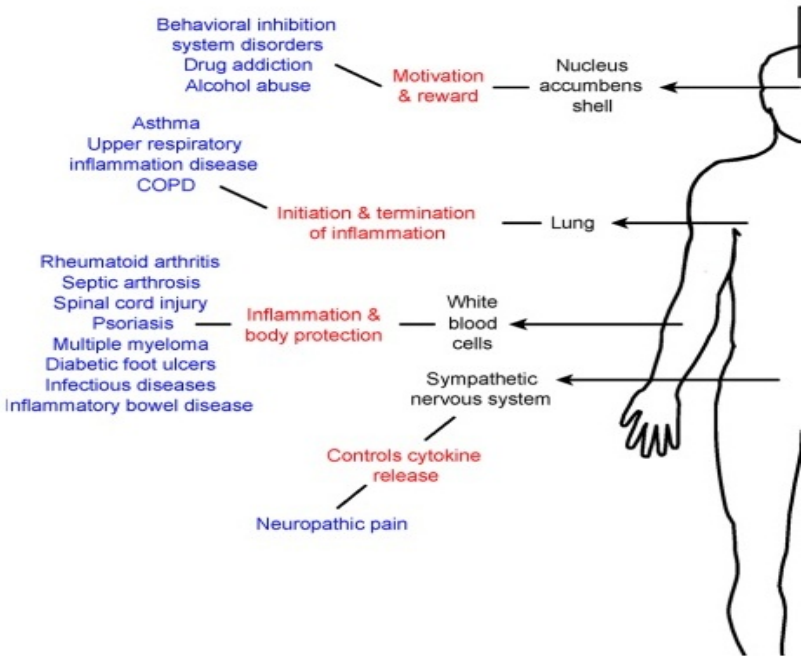


Fig. 6: Adenosine A_{2A} receptor location (black), cell or tissue function (red), and possible therapeutic applications (blue).[24]

2.4. Ligands of adenosine receptors

Adenosine (**Fig. 7**, **1**) is the endogenously occurring agonist for all four P1 subtype receptors (A_1, A_{2A}, A_{2B}, A_3), however the potency varies from each subtype. Under the basal condition in the body the extracellular concentration is held at 30 – 300 nM.[48]

Since adenosine is decomposed quickly by numerous enzymes, attempts to modify the structure have been conducted. It was observed that the best modifications are on N_6 or N_2 on adenosine structure. Otherwise purine structure can be preserved by modifying the 5' position on the ribose: NECA (*5'-N-ethylcarboxamidoadenosine*) is an example of this modification. It is a non-selective agonist with 20-fold increased potency than adenosine.[24] (**Fig. 7**, **2)**

CGS-21680 (see **Fig. 7**, **3**) with the chemical name: *4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid* [49] is highly selective A_{2A} adenosine receptor agonist, which can be used to distinguish A_{2A} from A_{2B}.[24]

The last used ligand was BAY60-6583 (seen in the **Fig. 7**, **4)** with the chemical name: *[2-({6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-yl}sulfanyl)acetamide]* According to the observation, BAY60-6583 seems to be a partial agonist at the adenosine A_{2B} receptors, moreover in high concentration of adenosine it may act as an antagonist.[50]

PSB-826 has been synthesized by *Prof. Dr. C. E. Müller chemical group at the Pharmaceutical Institute at the University of Bonn (Germany)*. It has been observed that PSB-826 acts as a selective A_{2A}AR agonist (**Fig. 7**, **5)**.

MSX-2 (**Fig. 7**, **6**) is a newly synthesized potent and selective adenosine A_{2A} receptor antagonist. Chemical name: *(3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine)*

The last mentioned compound was not used to determined the experiments. However it is mentioned several times in the thesis, so it more than appropriate to show the structure. ZM241385 (**Fig. 7**, 7) is a non-xanthine A_{2A} selective adenosine receptor antagonist, with chemical name:*(4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin- 5-yl amino]ethyl) phenol)*.

Affinities of some ligands are listed in the **Table 1**.

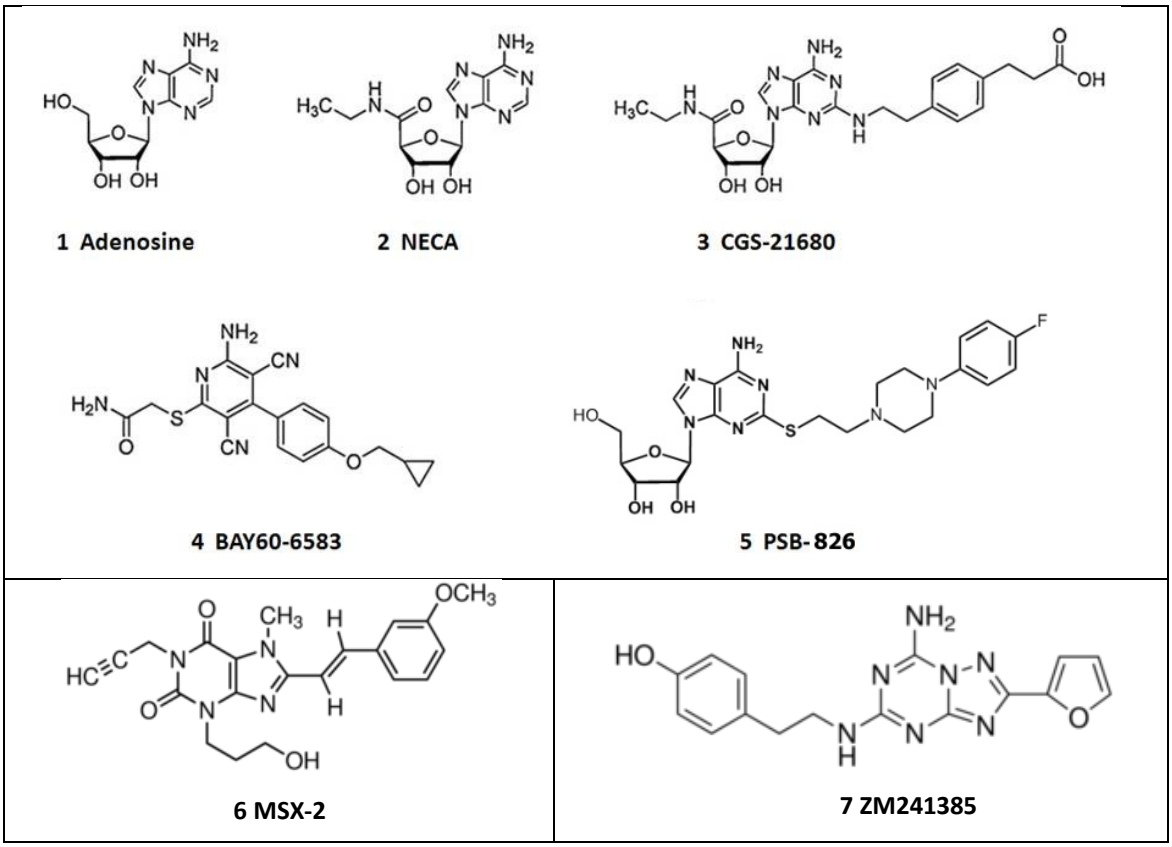


Fig. 7:Chemical structures of four adenosine A_{2A} receptor agonists (adenosine, NECA, CGS-21680, PSB-826) and one adenosine A_{2B} receptor partial agonist, BAY60-6583 that have been used for determination of the binding and ligand recognition.

Table 1: Adenosine is presented by EC_{50} values in nM. [48] K_i values of NECA on the adenosine receptors: A_1, A_{2A}, A_{2B}, A_3 were converted from pK_i (formula: $pK_i = -\log K_i$).[51] K_i values of CGS-21680.[24] Affinity of the selective A_{2B}AR agonist BAY60-6583 was obtained using radioligand binding studies versus [^{3}H]PSB-603. K_i value was calculated based on the K_D obtained from homologous radioligand binding.[26] And K_i values of ZM241385 were converted from pK_i (formula: $pK_i = -\log K_i$).[52] All listed values are K_i in nM otherwise is noted.

Ligand	A_1	A_{2A}	A_{2B}	A_3
Adenosine	100	309	15,000	288
NECA	6-5000	20	140	4-32
CGS-21680	290	27	>10 000	67
BAY60-6583	n.d.	n.d.	114	n.d.
ZM241385	251-794	0.8-1.6	6.3-158	741

3. AIM OF THE THESIS

The thesis aims on the role of the disulphide bonds formed between ECL1 and ECL2 (extracellular loop). In the Master's Thesis following questions has been examined:

- What is the impact on the human A_{2A} adenosine receptor agonists if the disulphide bonds are disrupted?
- How do disulphide bonds influence the expression and trafficking of the receptor within the cell?
- Is the affinity of A_{2A}AR agonists changed?
- How important are the disulphide bonds for ligand recognition?
- What is the difference in efficacy of the A_{2A} adenosine receptor agonists when some of the disulphide bonds in ECL2 are disrupted?

4. MATERIALS AND METHODS

4.1. Cell culture

4.1.1. General conditions

Chinese Hamster Ovary (CHO) K1 cells were cultured at 37°C 5% CO_2. Cells were kept in flasks of different volumes (25 cm^2, 75 cm^2, 175 cm^2, Cell Star). Cells were detached using 1-2 ml of 0.01% trypsin / 0.6mM EDTA solution for 2 min in the incubator under the same conditions during all the experiments.

4.1.2. CHO K1 untransfected cells

Cells were incubated in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, Gibco-Life Technologies) medium enriched with 10% of FCS (Fetal Calf Serum, Sigma) and 1% Penicillin/Streptomycin.

4.1.3. CHO K1 transfected cells

For transfected CHO K1 cells, 0.2% Geniticin (G_{418}) has been additionally added to keep the selection process between transfected and untransfected. Low passage cells (<15) were used for all experiments.

4.1.4. Counting cells

For all experiments as precise amount of cells as possible was required, hence cells were counted before seeding into the well. Counting was performed with Neubauer chamber which is a thick crystal slide with the size of a glass slide (30 x 70 mm and 4 mm thickness) with a grid on it.[53] 15µl of the cell suspension was dropped on the Neubauer chamber and counted under optical microscope. Consequently needed amount of pure medium and cell suspension was calculated according desired cell concentration.

4.1.5. Reagents for cell culture

PBS solution

Listed compounds are dissolved in deionised water, autoclaved and stored at RT with pH adjusted to 7.2.

NaCl..... 150 mM

KCl ..... 2.5 mM

Na_2HPO_4 x $2H_2O$..... 7.5 mM

KH_2PO_4..... 1.5 mM

Trypsin solution

PBS..... 500 ml

EDTA (0.6 mM, pH = 7.6) ..... 0.6 ml

Trypsin (0.01%, steril.)..... 10 ml

4.2. Retroviral transfection

To guarantee stable expression of desired protein, coding sequence should be inserted into cell genome.[54] For this purpose genetically modified retroviruses (only genes responsible for viral RNA encapsulation, reverse transcription and integration were kept) carried the coding information.

Packaging cells (GP+envAM12, derived from mouse fibroblast) which was the experiment conducted with, were already transfected with two plasmids. One coding *env* gene responsible for viral envelope and the second one consist of *gag* and *pol* genes of murine leukemia virus (MuLV) that presents the group-specific antigen on capsids and the viral polymerase. In order to kept only cells with these three genes medium was enriched with hygromycin B, hypoxanthine, xanthine and mycophenolic acid. The packaging cell were then transfected with third plasmid carrying gene for packaging signal sequence (Ψ) necessary for proper RNA insertion into capsids, along with that also our gene of interest occurred (gene coding hA_{2A}). Both of the genes were bordered with LTRs (long terminal repetitions) where viral promoters and transcriptional enhancers were located. To improve our chances for retroviral transfection, vector encoding the glycoprotein G of the vesicular stomatitis virus (VSV-G) was added. This glycoprotein enable the virus enter the cell not only via specific receptor but also

through phospholipids unspecifically. This process is called pseudotyping and it allows viruses to infect cells from different species.[55]

Packaging cells GP^+envAM12 were cultivated in the medium, consisted of DMEM medium with 10% FCS, 1% PS, 0.2 mg/ml Hygromycin B, 250 µg/ml Xanthin, 15 µg/ml Hypoxanthin, 25 µg/ml and Mycophenolic acid, until they were 70% confluent then 1.5 x 10^6 cells were seeded in a 25 cm^2 flask in 5 ml seeding medium (without Xanthin, Hypoxanthin and Mycophenolic acid) and incubated 24 hours at 37°C with 5% CO_2. On the second day, a lipofectamine transfection of two plasmids (pcDNA3.1-VSV-G and pQCXIN, retroviral vectors containing the gene of interest) was performed as described in the section above.

On the third day, to increase the virus production, the culture medium was exchanged with 3 ml of fresh DMEM medium supplemented with 30 µl of sodiumbutyrate. Incubation continued at 32°C for 48 hours after this procedure. On the fourth day, 3 x 10^5 of target cells (CHO-K1 cell line) were placed in a 25 cm^2 flask with seeding medium and incubated 24 hours. On the fifth day, virus particles were harvested from the packaging cells. 3 ml of the virus supernatant were taken from the small flask containing the packaging cells and sterile filtered using a 0.2 µm filter. The CHO medium was replaced with 3 ml of virus-containing supernatant supplemented with 6 µl of polybrene solution (4 mg/ml in water, sterile and filtered) that enhanced the virus transduction. Target cells were incubated together with virus particles for 2.5 hours at 37°C. Then the supernatant was replaced by normal CHO medium. Two days later, CHO medium was changed with selective DMEM/F12 medium (supplemented with 0.8 mg/ml G_{418} antibiotic) to distinguish successfully transfected and untransfected cells. Every second day the medium was changed until the selection process ended and only the stable cells survived.

4.3. ELISA (Enzyme-Linked ImmunoSorbent Assay)

The expression of the receptors on the cell surface was verified by ELISA. CHO K1 cells stably transfected with wt and mutant receptors linked to HA tag (hemagglutinin) were generated. As positive control (set as 100%), CHO K1 expressing the wt hA_{2A} adenosine receptor, was used and as negative control (set as 0%) untransfected CHO K1 cells were used. 100,000 cells/well were seeded in 24well plate and incubated for 24h. (All solutions and cells were kept on ice during the procedure, otherwise is noted). The

medium was removed and cells were washed with PBS (10 min) and then 1% BSA sol. blocked the unspecific binding sites (5 min). The first antibody (Ab), mouse anti-HA (300µl, 60 min, RT), was added. Cells were washed with PBS (3x10 min) after incubation time and fixed with Methanol/Aceton (v/v 1:1) at -20°C for 15 min. Then cells were washed with PBS (10 min) and blocked unspecific binding sites inside the cell again with 1% BSA (10 min). The 2. Ab, goat anti-mouse linked with HRP (horseradish peroxidase) (300µl, 60 min, RT) was used to recognize the 1. Ab. Then washed with PBS (4x10 min) and 300µl of ABTS chromophor reagent was added for 50 min at RT. HRP causes ABTS reagent oxidation and subsequent colour change of the solution, that is measured at 405nm (total volume of 170µl in 96well plate with flat bottom).

4.3.1. Antibodies

Antibody was added into DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, Gibco-Life Technologies) where 1% of BSA was already dissolved. 1st Ab. (Covance) diluted 1:1000 and 2nd Ab. (Jackson Immunoresearch) diluted 1:2500.

4.4. Membrane preparation for radioligand binding assay

4.4.1. Cell culture

When CHO K1 cells expressing wt or mutant hA_{2A} adenosine receptor reached 80-90% confluency, they were split1:20 in 150mm dishes. After 3 days of incubation (under the same conditions as mention before) valproic acid was added in the final concentration 0.5 mM to increase the receptor expression and cells were kept in the incubator over night.[56]

4.4.2. Membrane preparation

The 4th day dishes were washed with PBS buffer and frozen (-20°C) over night. Then dishes has been gradually thawed out, cells were melted and scraped off using ice-cold 5 mM TRIS-HCl / 2 mM EDTA buffer (pH = 7.4) and collected into a beaker on ice to prevent protein degradation. Then the cell suspension was homogenized by ULTRA-TURAX® (6° rate level, 2x15 sec) and centrifuged 10min at 1000 x g (4°C).

Supernatant was kept and centrifuged 60min at 48,000 x g (4°C). The pellet was resuspended in appropriate amount (1ml / 10 dishes) of 50 mM TRIS-HCl (pH = 7.4). Aliquots of protein suspension were stored at -80°C.

4.4.3. Buffers and solutions

EDTA / TRIS-HCl buffer

EDTA ..... 2 mM

TRIS-HCl..... 5 mM

All the compounds were weighted and dissolved in appropriate amount of Millipore water. pH was adjusted to 7.4.

TRIS-HCl buffer

TRIS-HCl..... 50 mM

Dissolved in Millipore water and adjusted pH = 7.4

Valproic acid solution

Valproic acid..... 500 mM

Valproic acid was dissolved in Millipore water and then filtrated through 0.45µm filter under the sterile conditions and kept at -20°C.

4.4.4. Bradford assay for calculation the protein concentration

Bradford assay is widely used to determine protein concentration. Coomassie Brillant Blue G-250 (**Fig. 8**) is brown in its neutral form. Basic (Arginin) and aromatic amino acids are mainly responsible for the charge change in the dye molecule, which turns the compound into anionic blue form and is measured at 595nm (highest peak of the anionic form) by spectrophotometer.[57]

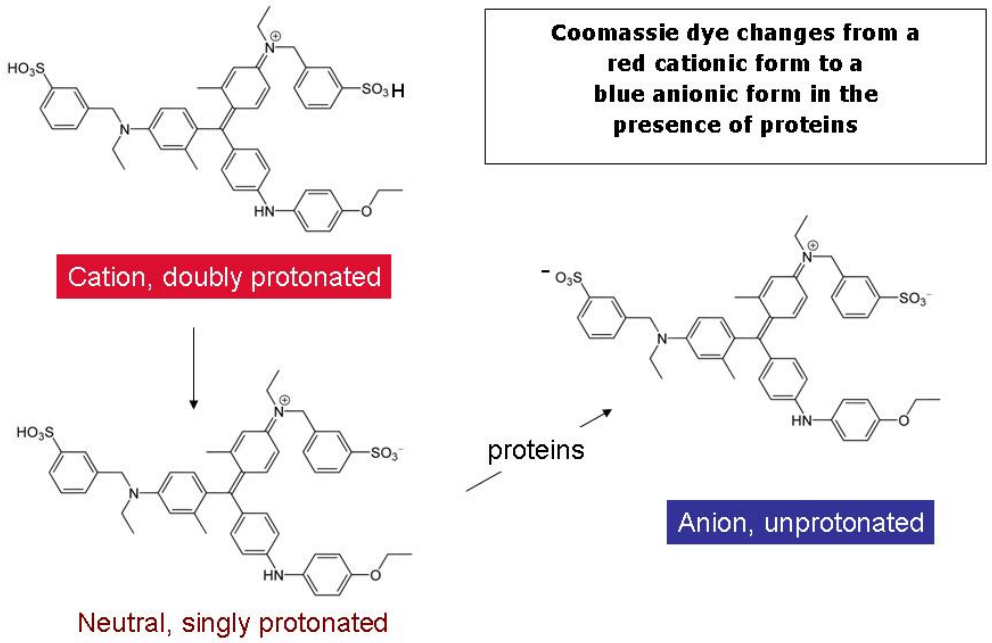


Fig. 8: Charge changes in Coomassie dye molecule in the presence of proteins.[58]

10µl of protein suspension was incubated into a 96well plate (flat bottom) with 190µl of *Quick Start™ Bradford 1x Dye Reagent* (BioRad), diluted with Millipore water in ratio 1:5, until blue colour occurred (5 min) and absorbance was measured. Simultaneously, dilutions of BSA (Bovine Serum Albumin, 1 – 0.1mg/ml) have been prepared as standards. Measurements were performed in triplicates.

4.4.5. Bradford reagent

Bradford reagent (*Quick Start™ Bradford 1x Dye Reagent*, BioRad) is diluted 1:5 in Millipore water. The solution is filtered by 0.45µm filter to remove undissolved particles and stored at 4°C.

4.4.6. BSA standards

Different concentrations of BSA were prepared by dissolving 10 mg of BSA in 10 ml of 50 mM TRIS-HCl buffer (pH = 7.4) (**Table 2**). By using dilutions in Tris-buffer, different BSA concentrations were tested.

Table 2:Table of different BSA (bovine serum albumin) concentration used to determine concentration-absorbance curve.

Concentration [mg/ml]	1.0	0.7	0.6	0.5	0.4	0.3	0.2	0.1

4.4.7. Calculation of protein concentration

Absorbance of the mixture Bradford reagent and protein suspension was measured at 595 nm. Since the Lambert-Beer law defines the absorbance range between 0 and 1, dilutions of the protein suspensions were prepared. Based on the BSA calibration function (**Fig. 9**), obtained absorbance values were used to calculate the protein concentration of each sample.

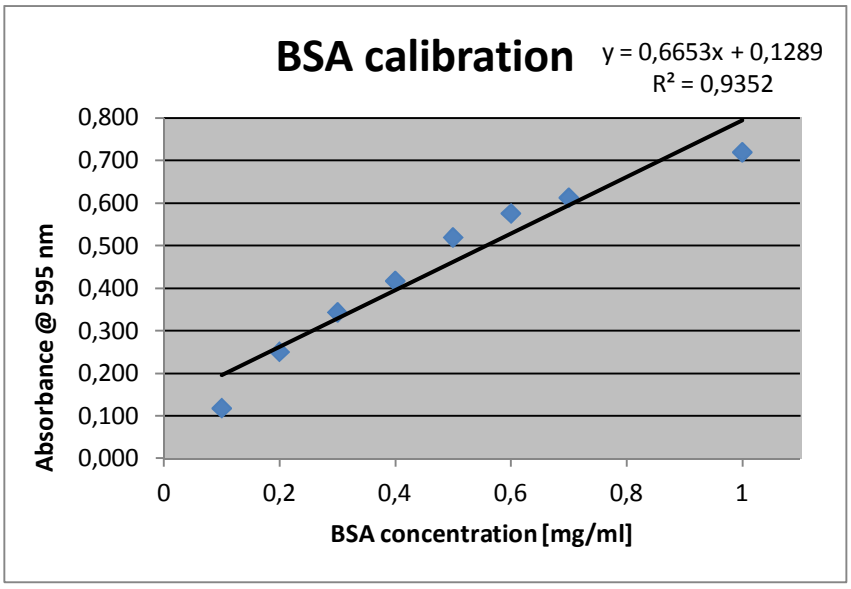


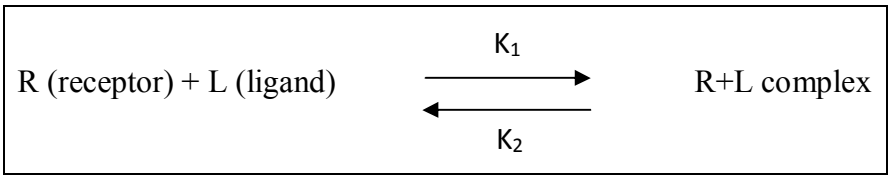
Fig. 9: Absorbnce-protein concentration curve of BSA standards used to calculate protein concentration in suspension gained from membrane preparation. *X*- axis represents variety of BSA concentration and on *y-axis* is the absorbance at 595nm.

4.5. Radioligand competitive assay

4.5.1. Basics of radioligand binding studies

Binding studies are used to determine the affinity of a certain compound (ligand) to a receptor. Affinity is the ability of a molecule to create bonds with other molecules. Higher is the affinity, stronger are the bonds.[59] The affinity is displayed by K_D value which is the concentration of ligand needed to occupy 50% of the receptor binding sites. It's a state where association (K_1) and dissociation (K_2) constants are in equilibrium (**Equation 1**). A different parameter might be calculated using radioligand binding studies: B_{max} value. It represents binding density, thus the concentration of tested receptor in the total protein suspension.[60]

Equation 1:Standard calibration curve determined by Bradford assay with BSA concentrations from 0.1 – 1 mg/ml.



4.5.2. Competitive binding versus [^{3}H]CGS-21680

Experiments with [^{3}H]CGS-21680, a selective A_{2A} agonist, were performed in total volume of 400µl. Different concentrations of tested compounds, were prepared in DMSO and added into vials (final volume 10µl). As nonspecific binding, 50 µM of NECA (with mutant C146S–C159S higher concentration needed: 100 – 150 µM) was set as 0% for the normalized data. And as total binding, which was considered 100%, pure DMSO was used. Then 90µl of 50 mM TRIS-HCl buffer (pH = 7.4) was added together with 100µl 40 mM $MgCl_2$. The radioligand was diluted at 5 nM in 50 mM TRIS-HCl (final volume 100µl). At last 100µl of protein, diluted in the same TRIS-HCl buffer, were added after 20min of preincubation with Adenosine deaminase (ADA, 2U/mg of protein) at RT. ADA decomposes naturally occurring adenosine, thus prevent the competition at the receptor with tested substances. After 60 min of incubation at RT,

the solution was harvested through GF/B (glass microfiber) filter using a Brandel Harvester. Before harvesting, filter was soaked with Millipore water and then washed 3 times with cold (4°C) 50 mM TRIS-HCl buffer (pH = 7.4). After harvesting, the filter was washed 3 times with the same TRIS-HCl buffer to remove unbound radioligand from the filter. Then the filter was inserted into scintillation vials with 2.5 ml of scintillation cocktail (LUMAsafe). The vials were counted in LSC-counter after 9h of incubation. The experiments were performed in duplicates and all the curves in triplicates (n = 3) otherwise is noted.

4.5.3. Competitive binding versus [^{3}H]MSX-2

Competitive binding with A_{2A} antagonist [^{3}H] MSX-2 were performed in a total volume of 400µl. An unspecific binding 50 µM of NECA was configured as 0%. And as total binding which was considered as 100%, 10µl of pure DMSO was used. Then 190µl of 50 mM TRIS-HCl buffer (pH = 7.4) was added. 100µl of radioligand in the very same buffer with concentration 1 nM was added and at last added 100µl of protein dissolved in the same TRIS-HCl buffer (preincubated with ADA (2U/mg of protein) at RT for 20 min. Adenosine deaminase (ADA) catalyze the deamination of adenosine. After 30 min of incubation time at RT vials were harvested using Brandel Harvester through GF/B (glass microfiber) filter. Filter was incubated in 0.3% polyethylenamin solution at 4°C for 50 min before use. Then washed 3 times with cold 50 mM TRIS-HCl buffer (pH = 7.4) before harvesting - prevent degradation process. After harvesting filter was again washed 3 times with 1-3 ml of 50 mM TRIS-HCl buffer (pH = 7.4) to washed away unbind radioligand molecules. Transferred into scintillation vials with 2.5 ml of scintillation cocktail (LUMAsafe) and after 8h of incubation time counted in LSC-counter. All the experiments were performed in duplicates and all the curves in triplicates (n = 3) otherwise is noted.

4.5.4. Buffers and solutions

$MgCl_2$ solution

$MgCl_2$ (anhydric) ..... 40 mM

Dissolved in Millipore water.

4.6. cAMP accumulation assay

4.6.1. Basics of cAMP accumulation assay

Cyclic adenosine monophosphat (cAMP) occurs naturally in cells as a secondary messenger. It is converted from ATP (adenosine triphosphate) by an enzyme called adenylate cyclase, which is mostly activated by $G\alpha_s$ subunit. Then it releases different cascade of action, targeting mainly PKA (protein kinase A), the guanine-nucleotide-exchange factor (GEF) EPAC and cyclic-nucleotide-gated ion channels. cAMP is then destroyed by enzyme nucleotide phosphodiesterase (PDE) (**Fig. 10**).[61] For more detailed information see *2.1.4*.

In the cAMP accumulation assays different concentrations of A_{2A} and A_{2B} agonists have been tested. As positive control (set as 100% for the normalisation) 100 µM forskolin was used, known as a direct activator of adenylate cyclase. [62]

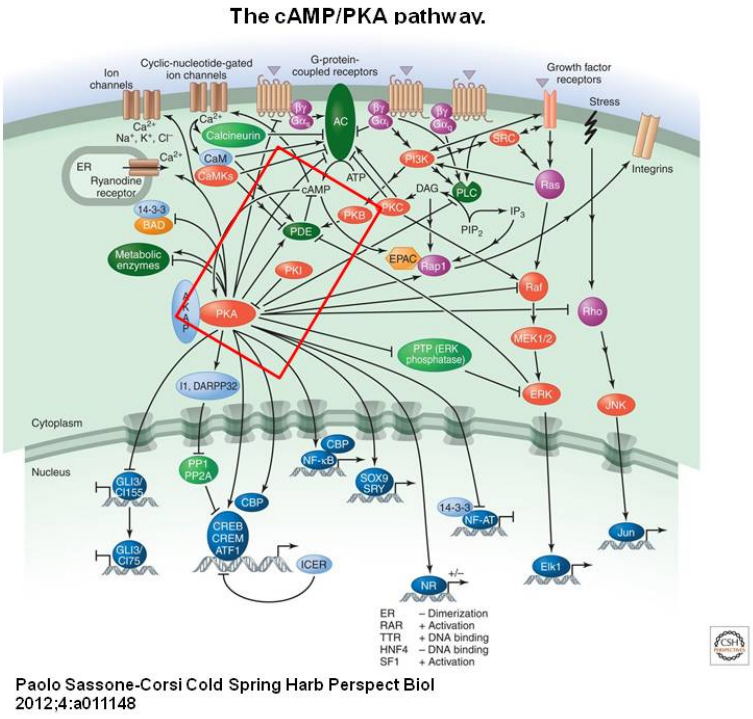


Fig. 10: The cAMP/PKA pathway. The cAMP is involved in different pathways in the cell. From creation by adenylate cyclase (AC) to interference in the cell cascade, influencing protein kinases (PKA), cyclic-nucleotide-gated

ion channels and the guanine-nucleotide-exchange factor (GEF) EPAC up to destruction by nucleotide phosphodiesterase (PDE).[61]

4.6.2. cAMP accumulation assay with human A_{2A}

Cells were incubated in 24well plate for 24h (150 000 cells/well). After 24h, cells were washed with 300µl of HBSS (37°C) and then incubated for 2h with 300µl HBSS (37°C) + ADA (1U/ml) solution – to prevent the activation of the receptors by endogenously occurring adenosine. Only cells tested with adenosine as an agonist were incubated with 300µl HBSS (37°C) without ADA. After the incubation time, 100µl of phosphodiesterase inhibitor Ro20-1724 (Hoffman La Roche, Grenzach, Germany, final concentration: 40 µM) was added and left on portable thermostat (37°C, 200 rpm) for 15 min. This inhibitor stopped decomposing of created cAMP after ligand stimulation. Then different dilutions of compounds (in 5% DMSO-HBSS) were added in total volume of 100µl and incubated 15 min. As negative control, the same amount of 5% DMSO-HBSS was used. And as positive control, forskolin (final concentration: 100 µM), known for direct activation of adenylate cyclase [62], was used. Then the supernatant was removed and 500µl of 90°C lysis buffer was added. The cell lysate was frozen at -20°C. The frozen cell lysate was then defrosted on ice. 50µl of the lysate was transferred to vials. Simultaneously background (consisting of radioligand and lysis buffer), total binding (contains lysis buffer instead of cell lysate) (see in **Table 3**) and cAMP standards were prepared (calculations in 4.6.4).

Table 3: Content of cAMP accumulation assay vials.

	Tested compound	Background	Total binding
Cell lysate	50 µl		
Lysis Buffer		90 µl	50 µl
Radioligand	30 µl	30 µl	30 µl
Binding protein	40 µl		40 µl
Total volume	120 µl	120 µl	120 µl

Then 30µl of [^{3}H] cAMP solution (final concentration: 3 nM) was added to each vial. As a last, 40µl of cAMP binding protein (cAMP dependent protein kinase extracted from a bovine in a concentration of 80 µg/vial) was added. The vials were incubated 2h on ice, to prevent cAMP degradation. Then the solutions were harvested using a 48er

Brandel Harvester through GF/B (glass fibres) filter which was previously soaked with Millipore water. The filter was then washed 3 times with 50 mM TRIS-HCl buffer (pH = 7.4). The filters were transferred into scintillation vials and 2.5 ml of scintillation cocktail was added. After 9h the radioactivity inside the vials was counted using a LSC-counter.

4.6.3. Buffers and solutions

HBSS buffer

NaCl..... 13 mM

HEPES..... 20 mM

Glucose..... 5.5 mM

$MgSO_4$..... 1.0 mM

$CaCl_2$..... 1.25 mM

$NaHCO_3$..... 4.2 mM

KCl..... 5.4 mM

KH_2PO_4..... 0.44 mM

Na_2HPO_4..... 0.34 mM

All components were dissolved in autoclaved Millipore water. Then adjust pH = 7.4

LYSIS buffer

EDTA..... 4 mM

Triton..... 0.01 %

Dissolved in autoclaved Millipore water. Then adjust pH = 7.4

4.6.4. Calculations

Dilution row of cAMP standards:

Table 4: Table of variety concentrations of cAMP standards.

Concentration [pmol]	40	20	10	5	2.5

The mean of background in cpm was subtracted from cpm values of standards plotted in **Table 4**. Call it SB (standard minus background). Then background was subtracted from the mean of total binding in cpm, name it TB (total binding minus background).

Then TB value was divided by SB and T/S was obtained (**Equation 2**). This step was repeated with all five SB values and reached numbers were plotted in the graph **Fig. 11**, in order to obtain equation to calculate the tested samples.

Equation 2: Calculation of the T/S.

(Total binding – Background) / (Standard – Background) = T/S

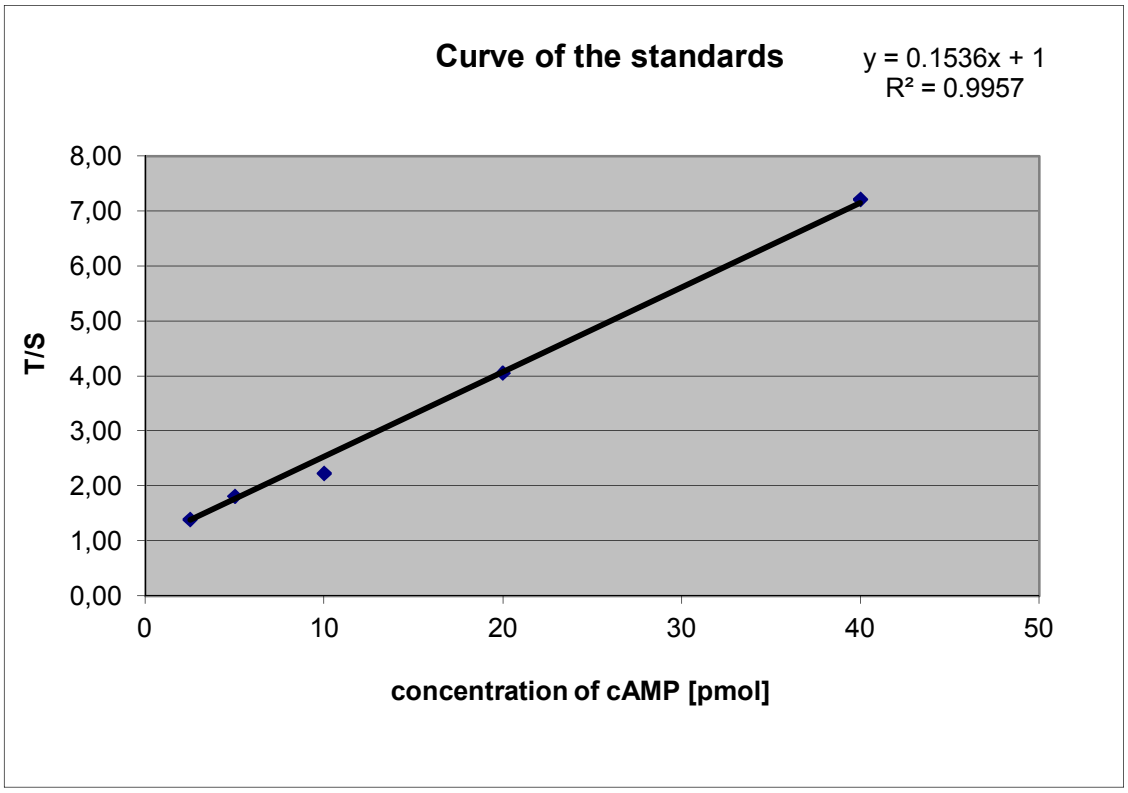


Fig. 11: cAMP standard curve used to calculate the concentration of cAMP (pmol) in samples. For every day of the experiments new curve was made. On the *x*-axis is the concentration of cAMP in pmol and on *y*-axis is the ratio between the results obtained from total binding and the standards (obtained from **Equation 2**). Each point represents one concentration of cAMP standards.

The cpm values from tested compound were subtracted with the mean of background (CB). Subtraction of total binding and background (TB) was divided by CB. This number was plotted instead of y in **Equation 3** obtained from **Fig. 11**.

Equation 3: Equation to calculate cAMP concentration in pmol using equation from the **Fig. 11**

$$x = (y - 1)/0.1536$$

(Total binding – Background) / (Tested sample – Background) = y

Concentration of cAMP in pmol was obtained from **Equation 3** as x. Same amount of the cells in each well can't be guaranteed every measurement. So in order to compare the data from different days 100μM of forskolin as 100% and 5% DMSO solution in HBSS as 0% was configured.

5. RESULTS

The aim of the project is to investigate disulphide bonds in the human A_{2A} adenosine receptor, where they link ECL1 and ECL2. Furthermore their importance in the flexibility, ligand recognition and binding has been examined. According to the A_{2A} receptor crystal structure, three disulphide bonds are formed between C77-C166, C71-C159 and C74-C146 (**Fig. 12**). The experiments were conducted using CHO K1 cells stably transfected with the human A_{2A} adenosine receptor and mutated variations of this receptor. By site-directed mutagenesis, cysteine residues were replaced with a serine, thus three single mutants (C166S, C146S, C159S) and one double mutant (C146S-C159) were generated. The following information has been inserted in the plasmid: the genetic information for the receptor, coding sequence for hemagglutinin at the N-terminal of the receptor and antibiotic resistance. After the retroviral transfection 0.8 mg/ml of G_{418} antibiotic was added to distinguish successfully transfected cells.

These mutants were tested with radioligand binding studies, focused on affinity and cAMP accumulation assay to investigate the influence on the potency and efficacy of adenosine receptor agonist. ELISA (Enzyme-linked immunosorbent assay) was carried out to investigate the receptor expression.

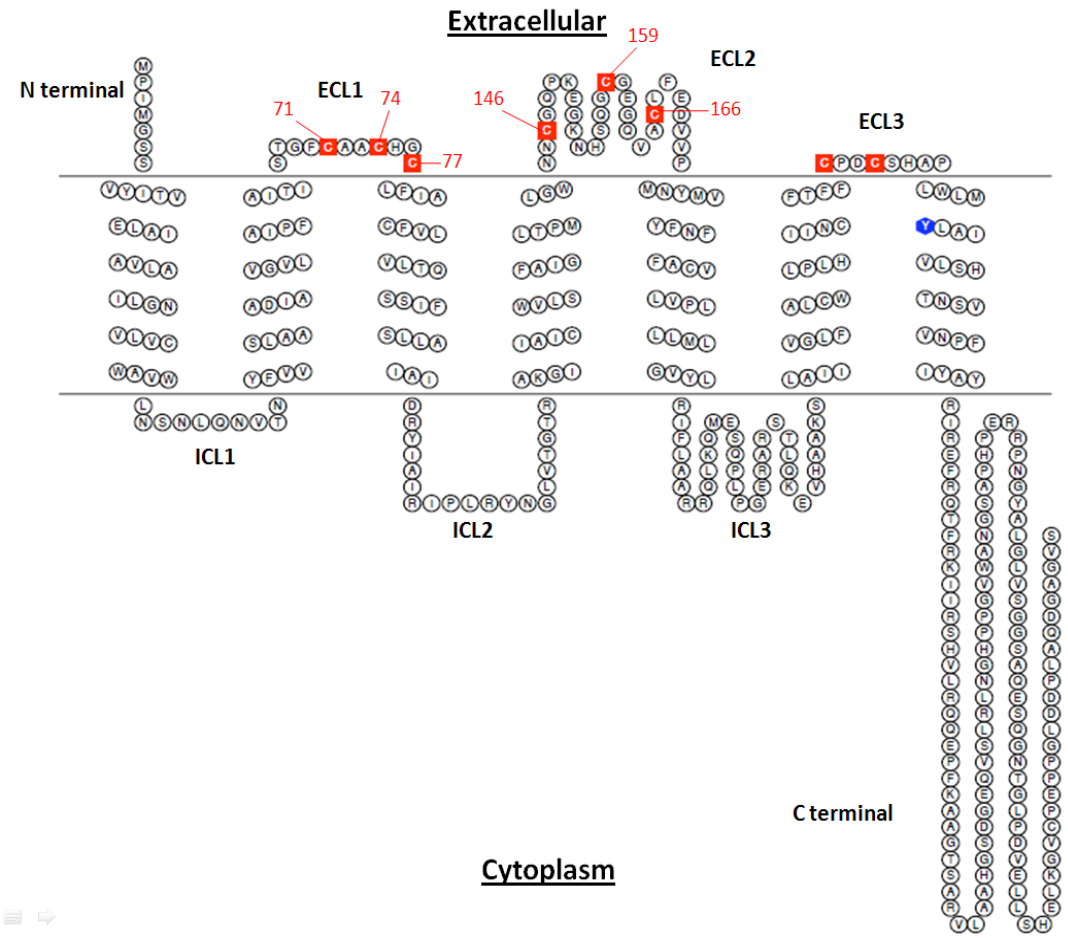


Fig. 12: Topology model of the hA_{2A} receptor. By replacing cysteine in ECL2 with serine (marked red) four mutants were generated. The amino acids residue Y271 (marked blue) was used to be mutated to asparagines amino acid in order to simulate the hA_{2B} binding site in the different project. 2D plot is generated with TOPO2 online software.

5.1. ELISA

In order to prevent the reduction of receptor expression, only cell lines with low passages (below 15) were used. Experiment conditions are described in the chapter **4.3**. In the **Fig. 13** the double mutant (C146S – C159S – 87.4%) was expressed nearly as the wt hA_{2A} adenosine receptor. On the other hand, the expression of two single mutant receptors (C146S and C166S) was lower if compared to the wt $hA_{2A}AR$ expression (C146S – 24.8% and C166S – 40.1%). The C159S mutant receptor was higher expressed (153%) than the wt hA_{2A} adenosine receptor, according to the ELISA methodology.

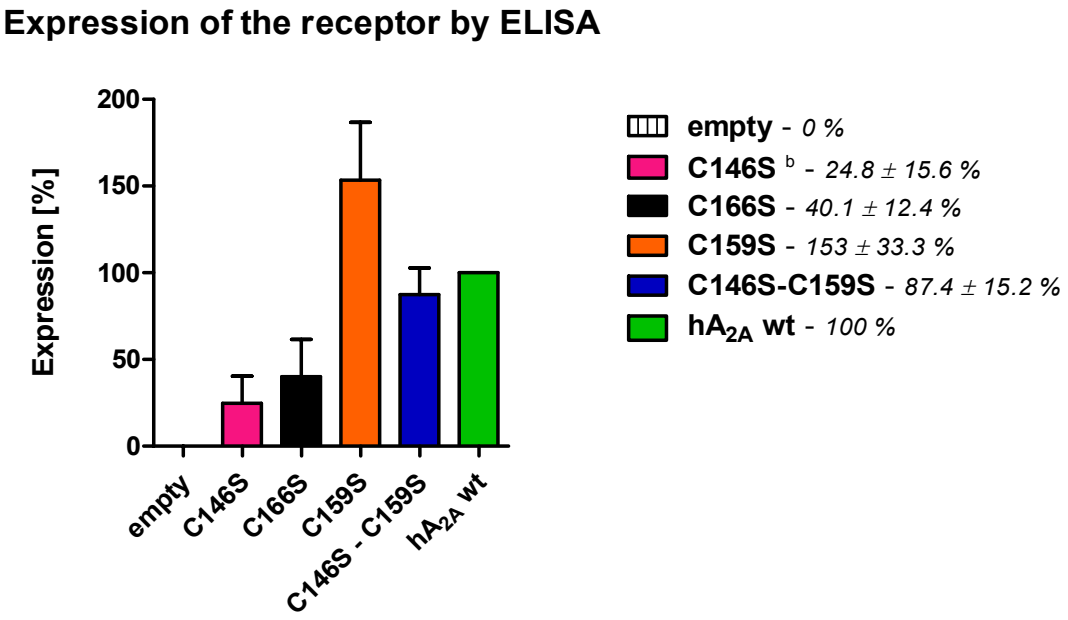


Fig. 13: The expression of the mutant receptors on the cell surface of stably transfected CHO K1 cells compared to the wt $hA_{2A}AR$ and untransfected cells. Untransfected cells were set as 0% and the wt $hA_{2A}AR$ was considered as 100%. Independent experiments were performed in triplicates, otherwise noted (C146S mutant is n = 2).

5.2. Radioligand competitive binding

5.2.1. Homologous competitive binding

Homologous competitive binding is conducted with the same two ligands, from which one of them is radioactively labelled (CGS-21680 versus [^{3}H]CGS-21680). Values obtained from the experiment are B_{max} and K_D and the two-tailed test is conducted to prove the statistical significance. B_{max} represents the incidence of the receptor in the cell, not only on the membrane surface, as ELISA detects. K_D is called dissociation constant and it displays the affinity of a compound to the receptor.[60]

We consider the expression of the wt hA_{2A} adenosine receptor as 100% in order to compare the homologous binding data with the ELISA data. The double mutant C146S–C159S receptor has an expression level of 73% of the wt hA_{2A}AR, which quite corresponds with the expression data obtained by ELISA (87.4%). The mutant C166S receptor is expressed at the 48% of the wt A_{2A}AR level. This data is in agreement with the one obtained by ELISA (40% of the wt hA_{2A} receptor). The only contradictory data we found is the C159S mutant receptor expression: the results obtained by ELISA showed an over expression (153%) of the mutant receptor while in homologous competitive binding the expression level is 49% of the wt hA_{2A}AR.

The mutant C146S receptor has not been obtained since we used different radioligand for the competitive binding. Window range between the total binding (TB) and the nonspecific binding (NB) was too narrow, so experiments were conducted with A_{2A}AR antagonist [^{3}H]MSX-2 (see the TB/NB comparison in the **Fig. 15**).

From the **Table 5** we can observe that the K_D values of two of the mutant receptors (C166S, C146S-C159S) are similar to the wt hA_{2A}AR receptor and only the mutant C159S receptor shows 2.8-fold higher affinity.

Table 5: Expression levels and affinities of the wt $hA_{2A}AR$ compared to the mutant A_{2A} adenosine receptors. B_{max} represents the density of the receptor obtained from membrane preparations in fmol/mg of protein, using stably transfected CHO K1 cells. The K_D value corresponds to the dissociation constant (nM). The ELISA results are expressed in percentage and they are compared to the B_{max} values. The two-tailed t-test was performed to determine the statistical significance between the values of the independent experiments. Values are means ± SEM from at least three independent experiments performed in duplicates, unless otherwise noted.

wt hA_{2A} or mutant	B_{max} ± SEM [fmol/mg of protein]	K_D ± SEM [nM]	ELISA Mean ± SEM [%]
wt hA_{2A}	478 ± 57	127 ± 2	100
C146S	n.d.	n.d.	24.8 ± 15.6 [b]
C146S-C159S	352 ± 70 [c, ns] (p = 0.0632)	124 ± 9 [c, ns] (p = 0.6434)	87.4 ± 15.2
C159S	236 ± 21 [a, **] (p = 0.0013)	46.2 ± 5.6 [a, ***]	153 ± 33
C166S	230 ± 33 [a, **] (p = 0.0018)	110 ± 3 [c, ns] (p = 0.1367)	40.1 ± 12.4

[a] n = 4

[b] n = 2

[c] n = 5

n.d. *not determined*

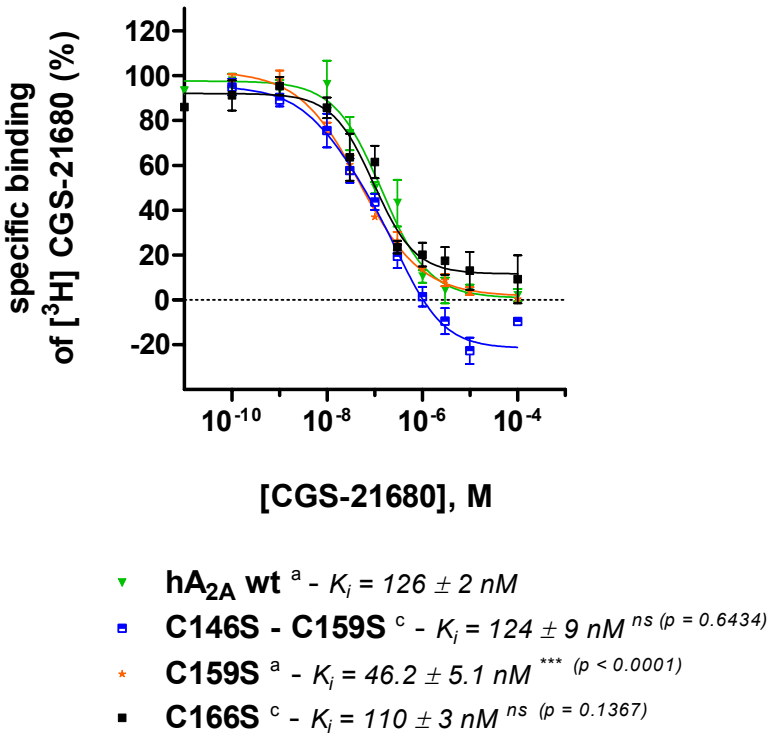
* $p < 0.05$ *(determined using the two-tailed t-test)*.

** $p < 0.01$

*** $p < 0.001$

ns *Not significantly different from wildtype $hA_{2A}AR$*

The **Fig. 14** shows that the cysteine mutant receptors have similar affinity for CGS-21680, a high selective $A_{2A}AR$ agonist, if compared to the wt $hA_{2A}AR$. Only the C159S mutant receptor moderately presents an increased affinity (2.8-fold) than the wt A_{2A} adenosine receptor. The binding curve for the double mutant C146S-C159S receptor reaches an "under zero" level due to the lost in affinity for the compound used as non-specific binding, NECA. For future experiments, increasing the NECA concentration will set the range of the curve between 0 and 100%.



[a] n = 4
[b] n = 2
[c] n = 5

Fig. 14: Homologous competition binding studies at the wt $hA_{2A}AR$ and hA_{2A} cysteine mutant receptors versus [^{3}H] CGS-21680 (5 nM). On the *y* axis is percentage of the binding, pure DMSO is set as 100% and nonspecific binding (NECA 50 µM) as 0%. The three independent experiments were performed in duplicates, unless otherwise noted.

5.2.2. Radioligand binding versus [^{3}H]CGS-21680

Radioligand competitive binding was used to determine the affinity of different compounds to the cysteine mutant receptors (**Fig. 17**). K_i is called inhibition constant and is calculated using K_D obtained in homologous binding studies. K_D is used if we conduct the homologous binding, thus two same compounds versus each other and K_i is used when are the compounds different. See the calculation of K_i in the **Equation 4**.

Equation 4: Prusoff equation where L is the concentration of radioligand used, Kd is the affinity of the radioligand and IC_{50} is the affinity of tested substance.

$$Ki = \frac{IC50}{1 + \frac{L}{Kd}}$$

An Overview of the K_i values sorted according to the cell line and ligands is listed in **Table 11**.

The first tested compound was PSB-826 (**Fig. 17**, **section A)**, a selective $hA_{2A}AR$ agonist. The mutant C159S receptor showed higher affinity for this compound (29.2 nM) compared to the wt $hA_{2A}AR$ (115 nM). The double mutant (C146S-C159S) and the C166S mutant receptor had moderately lower affinity (278 nM and 197 nM, respectively) compared to the wt hA_{2A} adenosine receptor (115 nM).

BAY60-6583 (**Fig. 17**, **section B**), a selective $A_{2B}AR$ partial agonist. None of the mutant receptors showed affinity for this compound.

The last tested compound was NECA, a non-selective adenosine receptor agonist (

Fig. 17, **section C**). The double mutant (C146S–C159S) showed a significant loss of affinity (27-fold) compared to the wt hA_{2A} receptor. The mutant C166S receptor has a K_i value similar to the wt $hA_{2A}AR$ (K_i = 110 nM compared to wt $hA_{2A}AR$ K_i = 92.9 nM) On the other hand, the mutant C159S adenosine receptor showed 5-fold higher affinity at NECA than the wt $hA_{2A}AR$. The binding curve of the C159S mutant fits a biphasic curve, therefore both sigmoidal and two-site competition K_i values were calculated (low K_i = 4.17 nM and high K_i = 257 nM)

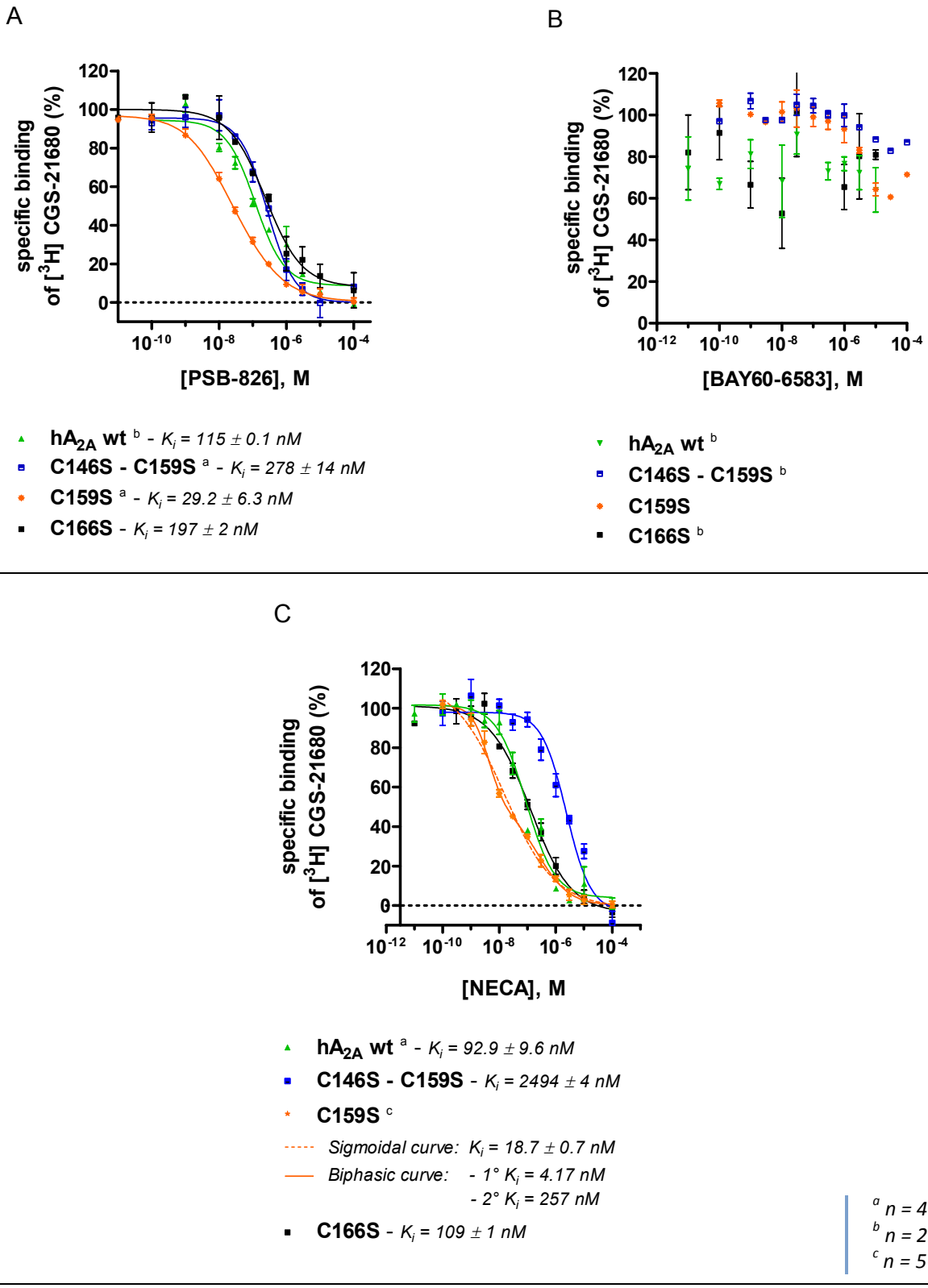


Fig. 17: Radioligand competitive binding studies at the wt $hA_{2A}AR$ and hA_{2A} cysteine mutant receptors using a selective $A_{2A}AR$ agonist PSB-826 (A), $A_{2B}AR$ agonist BAY60-6583 (B) and non-selective AR agonist NECA (C) versus [^{3}H]CGS-21680 (5 nM). On the *y* axis is percentage of the binding, pure DMSO is set as 100% and unspecific binding (NECA 50 μM) as 0%. The independent experiments were performed in duplicates, unless otherwise noted.

Table 11: Affinities of A_{2A} adenosine receptor agonists and an A_{2B}AR partial agonist (BAY60-6583) for the wt hA_{2A} receptor and the cysteine mutant receptors are presented by the K_i values. They are mean ± SEM (nM) and are obtained from radioligand competitive binding studies vs [^{3}H]CGS-21680 (5nM). Three independent experiments were performed in duplicates, otherwise noted.

wt or mutant	Ki ± SEM [nM]			
	CGS-21680	NECA	PSB-826	BAY60-6583
hA_{2A} wt	126 ± 2 [a]	92.9 ± 9.6 [a]	115 ± 1 [b]	>10 000
C146S	n.d.	n.d.	n.d.	>10 000
C146S-C159S	124 ± 9 [c, ns] (p = 0.7467)	2494 ± 4 ***	278 ± 14 [a, ***] (p = 0.0002)	>10 000
C159S	46.2 ± 5.1 [a, ***]	18.7 ± 0.7 [c, ***]	29.2 ± 6.3 [a, ***]	>10 000
C166S	110 ± 3 [c, ***] (p = 0.0001)	109 ± 1 [ns] (p = 0.0556)	197 ± 2 ***	>10 000

[a] n = 4

[b] n = 2

[c] n = 5

* p < 0.05 *(determined using the two-tailed t-test).*

** p < 0.01

*** p < 0.001

ns *Not significantly different from wildtype $hA_{2A}AR$*

5.2.3. Radioligand binding studies versus [^{3}H]MSX-2

The competition binding studies with the mutant C146S receptor were performed with a different radioligand because [^{3}H]CGS-21680 has shown a limited window range between non-specific and total binding, which might be considered as a noise. Therefore an A_{2A}AR antagonist, [^{3}H]MSX-2, was used because the window resulted more adequate than the one obtained with [^{3}H]CGS-21680 (**Fig. 15**). In order to compare the binding data, the wt hA_{2A}AR was tested versus [^{3}H]MSX-2, as well.

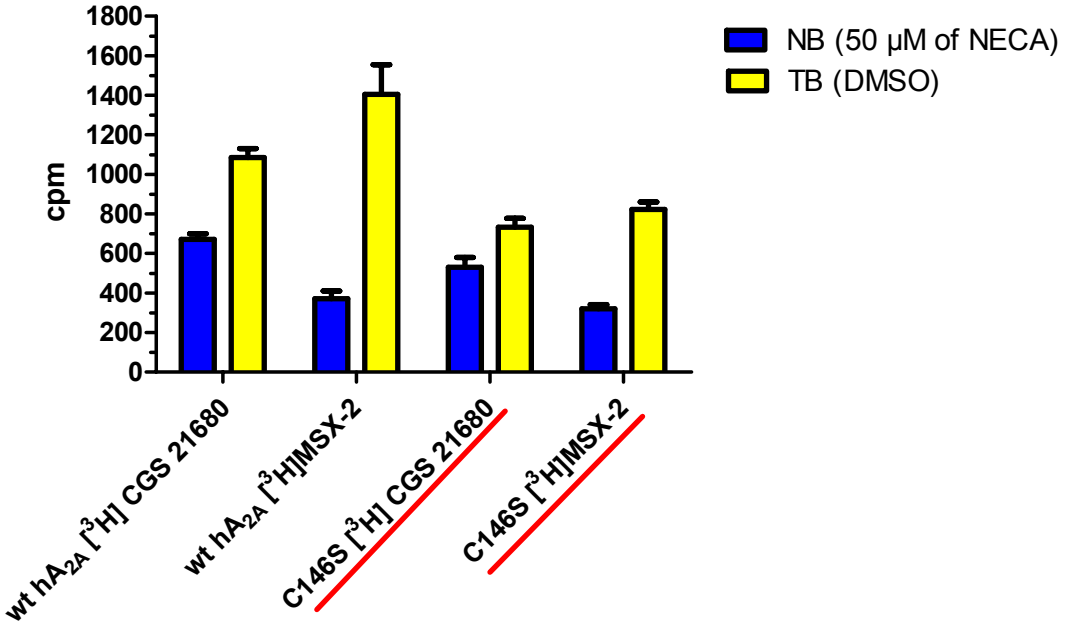
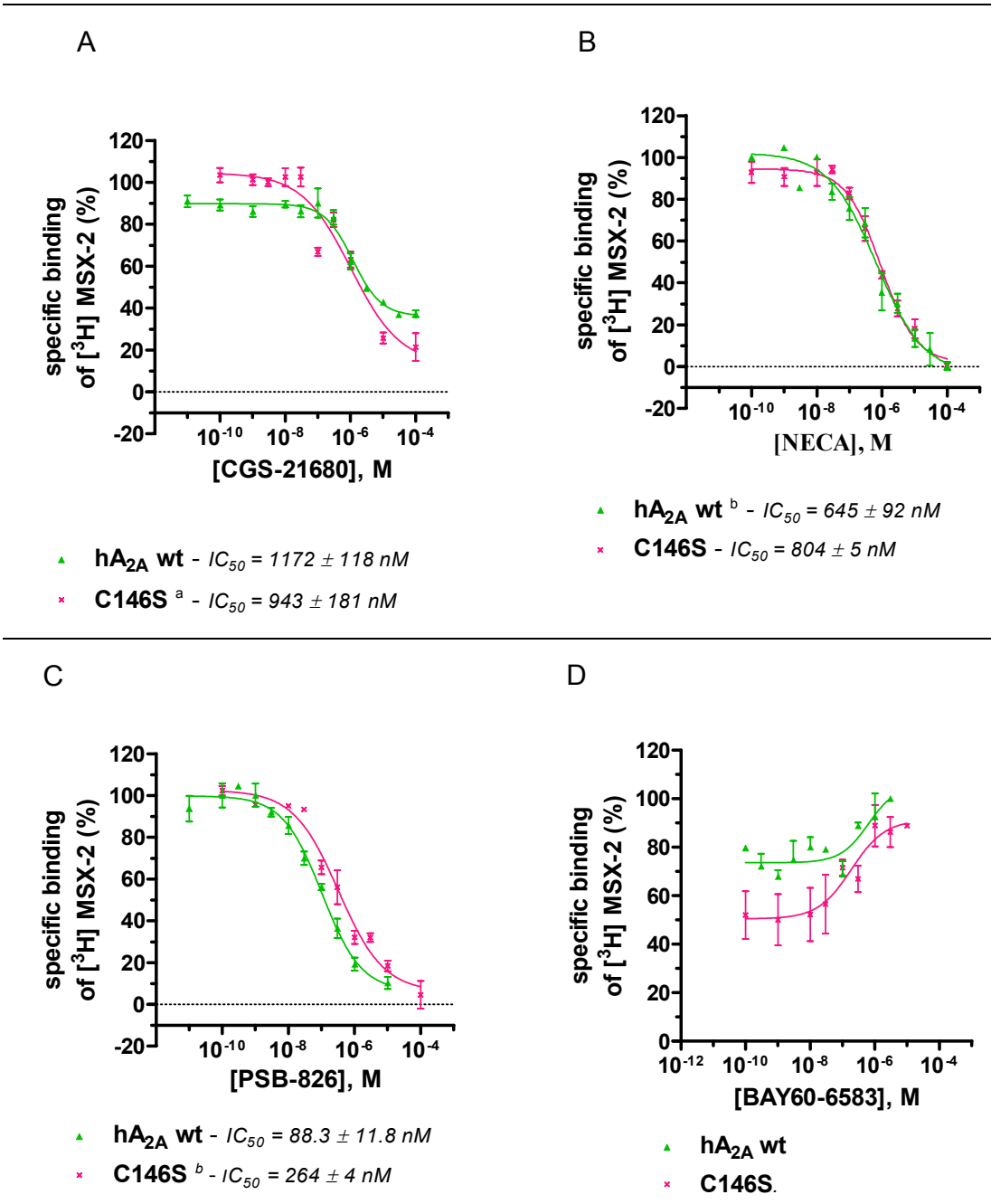


Fig. 15: The cpm (counts per minute; common unit to measure the radioactivity) window between non-specific binding (NB), marked in blue (NECA 50 µM) and total binding (TB), marked in yellow (pure DMSO) of competition binding studies. On the *x*-axis are coupled NB and TB for each cell line tested versus different radioligands. On the *y*-axis the cpm are plotted. The window range of the mutant C146S receptor tested against the two different radioligands is underlined with red.

The affinity is presented by IC_{50} value, the concentration needed for inhibition the receptor by 50%. The C146S mutant receptor was tested against the same compounds as the other mutants. The mutant C146S receptor slightly gained the affinity against CGS-21680 (**Fig. 16, A**) in comparison with the wt hA_{2A} receptor (943 nM compared to 1171 nM of the wt hA_{2A}AR). NECA (**Fig. 16, B**) did not show any remarkable difference in affinity compared to the wt hA_{2A}AR (804 nM compared to 645 nM for the wt hA_{2A}AR).

On the other hand, the only significant difference was observed at PSB-826 (**Fig. 16, C**). The affinity of PSB-826 for the C146S mutant receptor (264 nM) was 3-fold lower than the wt hA_{2A} receptor (88.3 nM).Finally, the hA_{2B} partial agonist BAY60-6583 showed no binding at both the wt hA_{2A}AR and the C146S mutant receptor (**Fig. 16, D**).

An Overview of the IC_{50} values is presented in the **Table 6**.



[a] n = 4
[b] n = 2
[c] n = 5

Fig. 16: Radioligand competitive binding studies at the wt $hA_{2A}AR$ and hA_{2A} cysteine mutant receptors using a selective $A_{2A}AR$ agonists CGS-21680 (A) and PSB-826 (C), non-selective AR agonist NECA (B) and $A_{2B}AR$ agonist BAY60-6583 (D) versus [^{3}H]MSX-2 (1 nM). On the y-axis is percentage of the binding, pure DMSO is set as 100% and non-specific binding (NECA 50 µM) as 0%. On x-axis is the concentration of the tested ligand. The three independent experiments were performed in duplicates, unless otherwise noted.

Table 6: Affinities of $hA_{2A}AR$ agonists and a hA_{2B} partial agonist (BAY60-6583) for the wt hA_{2A} receptor and the cysteine mutant receptors are presented by the IC_{50} values. Displayed values are mean ± SEM (nM) obtained from radioligand competitive binding studies versus [^{3}H]MSX-2 at 1 nM final concentration. Three independent experiments were performed in duplicates, otherwise noted.

wt or mutant	**IC_{50} ± SEM [nM]**			
	CGS-21680	**NECA**	**PSB-826**	**BAY60-6583**
wt hA_{2A}	1171 ± 118	645 ± 92	88.3 ± 11.8	>10 000
C146S	943 ± 181 [a, ns] (p = 0.1846)	804 ± 5 [ns] (p = 0.0697)	264 ± 4 [b, ***] (p = 0.0005)	>10 000

[a] n = 4

[b] n = 2

* $p < 0.05$ *(determined using the two-tailed t-test).*

** $p < 0.01$

*** $p < 0.0001$

ns *Not significantly different from wildtype $hA_{2A}AR$*

5.3. cAMP accumulation assay

In order to investigate the function of the presented agonists at the wt $hA_{2A}AR$ in comparison with the cysteine mutant receptors, the cAMP accumulation assay was performed stimulating the receptors with the four hA_{2A} adenosine receptor agonists (adenosine, NECA, CGS-21680, PSB-826) and a hA_{2B} partial agonist (BAY60-6583). EC_{50} represents the concentration needed to induce 50% of the maximum effect of an agonist, also called potency. The efficacy refers to the maximal cAMP production.
Graphs are divided into two groups:

- The first group (**Fig. 17**, left column labelled with **nr. 1**) represents raw data without normalization. On the y-axis the pmol of agonist-induced cAMP is plotted.

- The second group (**Fig. 17**, right column labelled with **nr. 2**) shows cAMP production data after normalization. Data are normalized with 100 µM forskolin,

set as 100%. Forskolin induces cAMP by direct activation of adenylate cyclase, so the cAMP production is only dependent by the cell number.[62]

Adenosine, the endogenous AR (adenosine receptor) agonist, showed lower potency at all tested mutant receptors. The most remarkable difference in comparison to the wt hA_{2A} adenosine receptor was noticed at the C146S–C159S mutant receptor: the potency was 247-fold lower than the wt $hA_{2A}AR$ (46935 nM compared to 190 nM, respectively), however the efficacy improved. The efficacy rose up nearly to 130% compared with 50% of the wt $A_{2A}AR$. The potency of adenosine on the mutant receptors C159S and C166S (3687 nM and 5111 nM) more approached to the values obtained from the A_{2B} adenosine receptor (7435 nM). Only the C146S mutant receptor did not show any response after the ligand stimulation. (**Fig. 17**, **A**)

CGS-21680 (**Fig. 17**, **B**), a selective $hA_{2A}AR$ agonist, induced activation of the wt hA_{2B}, at high concentrations (9385 nM). The EC_{50} value at the wt hA_{2A} adenosine receptor is 6.6 nM and both the C159S and C146S–C159S mutant receptors slightly gain potency (3.67 nM and 6.87 nM, respectively). The efficacy for these two mutant receptors was similar to the one of the wt $hA_{2A}AR$. The potency of the C146S mutant receptor (0.84 nM) significantly increased compared to wt hA_{2A} adenosine receptor but the efficacy of this mutant receptor was extremely decreased (20% in comparison with 55% of the wt $hA_{2A}AR$).

NECA, (**Fig. 17**, **C**) a non-selective AR agonist, is more potent at the wt hA_{2A} receptor than at the wt hA_{2B} receptor (10.3 nM and 171 nM, respectively). The potency of the double mutant C146S-C159S receptor significantly decreased of 450-fold (4636 nM) in comparison to the wt $hA_{2A}AR$. However the efficacy of the double mutant is 2-fold higher than the one of the wt $hA_{2A}AR$ (60%). The C166S mutant receptor showed a 5-fold reduced potency (53.8 nM) if compared to the wt $hA_{2A}AR$. The rest of the mutant receptors (C146S and C159S) showed higher potency than the wt $hA_{2A}AR$: 1.30 nM at C146S and 2.12 nM at C159S. The C146S mutant receptor showed a reduced efficacy in comparison to the wt hA_{2A} receptor, similar to the one induced by CGS-21680.

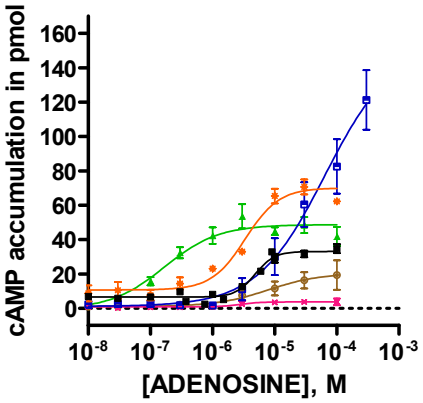
PSB-826, (**Fig. 17**, **D**) newly synthesised hA_{2A} agonist, confirmed its subtype-selectivity since it did not stimulate cAMP production at the wt hA_{2B}. Along with the wt

hA_{2B} receptor also the mutant receptor C146S did not show any activation. Increased potency was determined at the mutant receptors C159S (3.67 nM) and C166S (53.9 nM) compared with hA_{2A}AR (66.7. nM), however the efficacy was lower for both mutant receptors C166S and C159S (50%) in contrast to wt hA_{2A} receptor efficacy (80%). The double mutant receptor C146S-C159S showed moderately decreased potency in comparison to the wt hA_{2A} receptor (97.5 nM compared to 66.7 nM) and a similar efficacy.

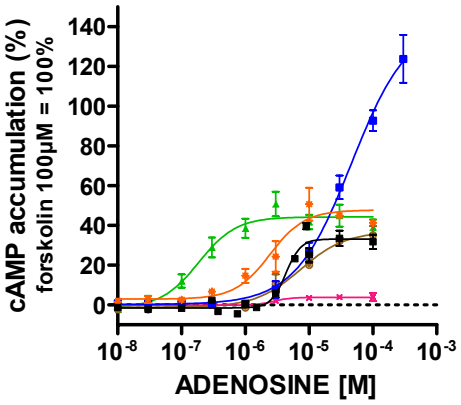
The last tested compound was the hA_{2B} partial agonist BAY60-6583 (**Fig. 17**, **E**). This compound did not activate the hA_{2A} tested receptors (wt nor cysteine mutants). However the mutant receptor C159S showed a slight increase at concentrations 3-10 μM (EC_{50}: 6118 nM).

A summary of the EC_{50} values obtained by cAMP accumulation assays is listed in the **Table 7**.

1A

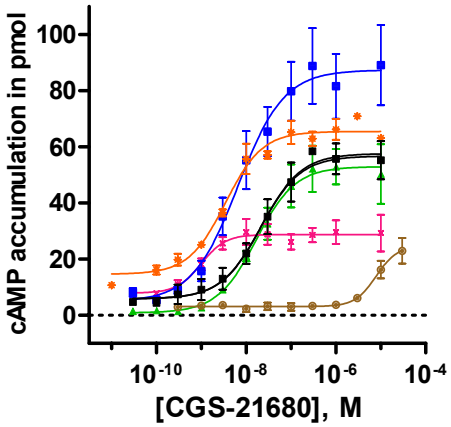


2A

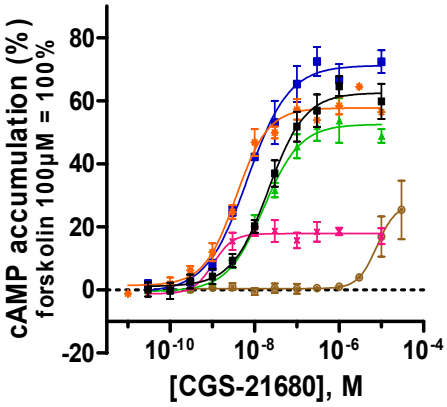


- **hA_{2A} wt** - *EC_{50} = 190 ± 45 nM*
- **hA_{2B} wt** [b] - *EC_{50} = 7435 ± 43 nM*
- **C146S** - *n.d.*
- **C146S-C159S** [a] - *EC_{50} = 46935 ± 16889 nM*
- **C159S** [a] - *EC_{50} = 3687 ± 880 nM*
- **C166S** [a] - *EC_{50} = 5111 ± 57 nM*

1B



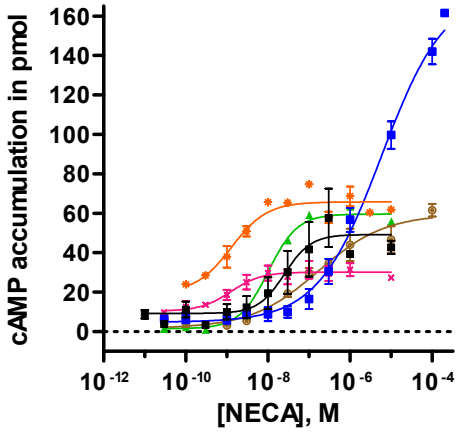
2B



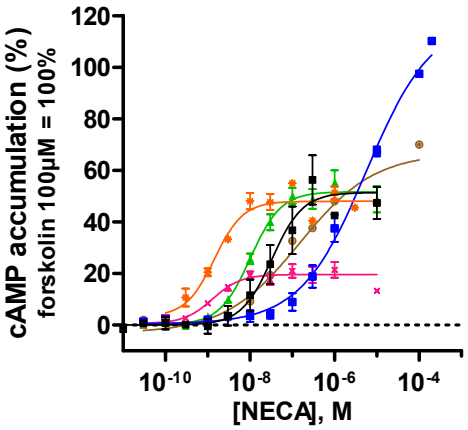
- **hA_{2A} wt** [a] - *EC_{50} = 16.6 ± 1.3 nM*
- **hA_{2B} wt** - *EC_{50} = 9385 ± 2011 nM*
- **C146S** - *EC_{50} = 0.84 ± 0.01 nM*
- **C146S-C159S** - *EC_{50} = 6.87 ± 0.63 nM*
- **C159S** - *EC_{50} = 3.67 ± 0.09 nM*
- **C166S** [a] - *EC_{50} = 21.5 ± 1.5 nM*

[a] *n = 4*
[b] *n = 2*
[c] *n = 5*

1C

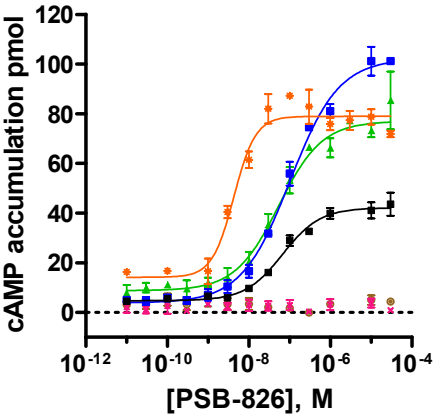


2C

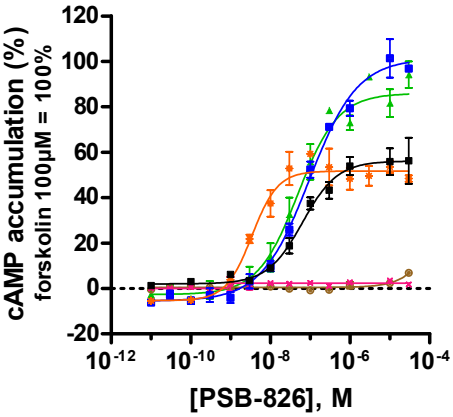


- **hA$_{2A}$ wt** - *EC$_{50}$ = 10.3 ± 0.1 nM*
- **hA$_{2B}$ wt** [a] - *EC$_{50}$ = 171 ± 12.7 nM*
- **C146S** - *EC$_{50}$ = 1.30 ± 0.13 nM*
- **C146S-C159S** - *EC$_{50}$ = 4635 ± 340 nM*
- **C159S** [a] - *EC$_{50}$ = 2.12 ± 0.53 nM*
- **C166S** [a] - *EC$_{50}$ = 53.8 ± 6.0 nM*

1D

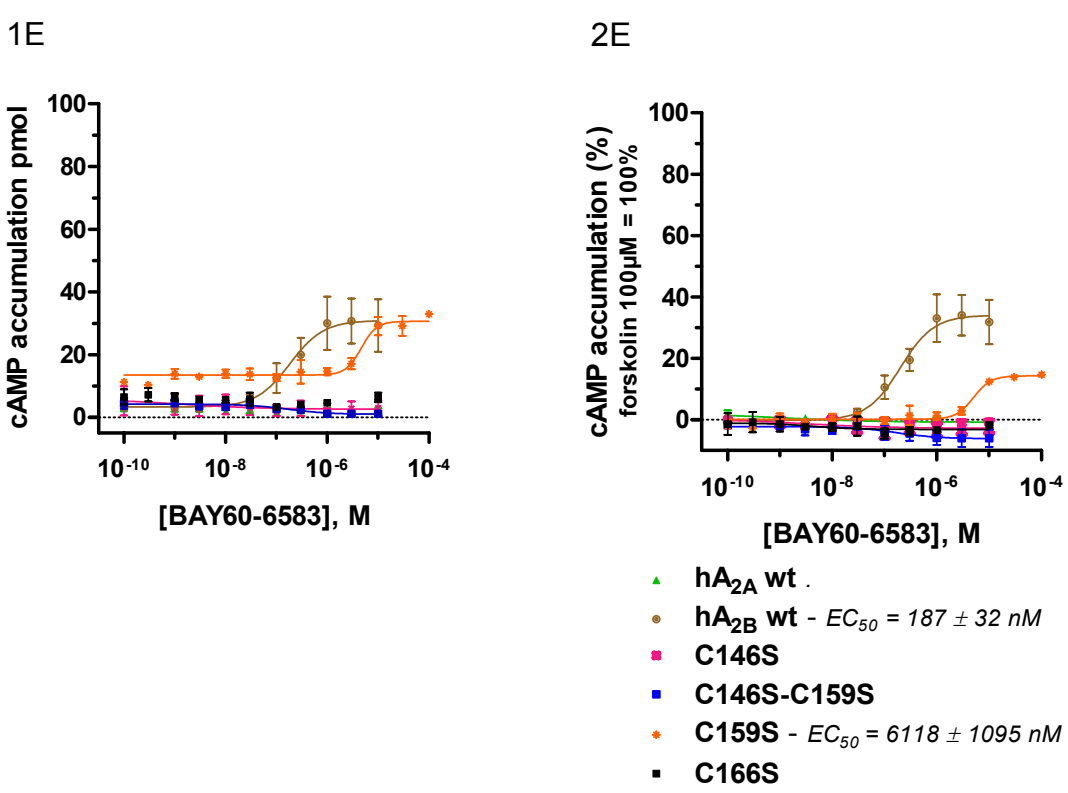


2D



- **hA$_{2A}$ wt** - *EC$_{50}$ = 66.7 ± 20.0 nM*
- **hA$_{2B}$ wt** - *EC$_{50}$ = n.d.*
- **C146S** - *EC$_{50}$ = n.d.*
- **C146S-C159S** - *EC$_{50}$ = 97.5 ± 13.3 nM*
- **C159S** - *EC$_{50}$ = 3.67 ± 0.04 nM*
- **C166S** [b] - *EC$_{50}$ = 53.9 ± 2.9 nM*

[a] *n = 4*
[b] *n = 2*
[c] *n = 5*



[a] *n = 4*
[b] *n = 2*
[c] *n = 5*

Fig. 17: Agonist-induced cAMP accumulation in CHO K1 cells stably transfected with the following adenosine receptors: wt hA_{2A}, hA_{2B} wt and four cysteine hA_{2A} mutant receptors. On *y*-axis is either cAMP accumulation in pmol (left column, 1) or cAMP accumulation in percentage (%) after normalization (right column, 2; 100 µM of forskolin was set as 100%). Data represent mean curves ± SEM from three independent experiments performed in duplicates, otherwise noted.

Table 7: The EC_{50} values obtained by agonist-induced cAMP accumulation assays with stably transfected CHO K1 cells expressing the wt and the cysteine hA_{2A} mutant receptors. EC_{50} values are mean ± SEM in nM after normalization on 100µM forskolin. Data are mean ± SEM in three independent experiments performed in duplicates, unless otherwise noted.

wt or mutant	**EC_{50} ± SEM [nM]**				
	CGS-21680	**NECA**	**PSB-826**	**BAY60-6583**	**ADENOSINE**
wt hA_{2A}	16.6 ± 1.3	10.3 ± 0.1	66.7 ± 21.0	>10 000	190 ± 45
wt hA_{2B}	9385 ± 2011	171 ± 13 [a]	>30 000	187 ± 32	7435 ± 43 [b]
C146S	0.84 ± 0.01 ***	1.30 ± 0.13 ***	>30 000	>10 000	>100 000
C146S-C159S	6.87 ± 0.63 *** (p = 0.0006)	4636 ± 340 ***	97.5 ± 13.3 ns (p = 0.1545)	>10 000	46935 ± 16889 a, ** (p = 0.0098)
C159S	3.76 ± 0.09 *** (p = 0.0001)	2.12 ± 0.53 a, ***	3.67 ± 0.04 * (p = 0.0132)	6118 ± 1095	3687 ± 880 a, ** (p = 0.0021)
C166S	21.5 ± 1.5 a, * (p = 0.0125)	53.8 ± 6.0 a, * (p = 0.0346)	53.9 ± 2.9 b, ns (p = 0.5536)	>10 000	5111 ± 57 a, ***

[a] n = 4

[b] n = 2

[c] n = 5

* p < 0.05 *(determined using the two-tailed t-test).*

** p < 0.01

*** p < 0.001

ns *Not significantly different from wildtype $hA_{2A}AR$*

6. DISCUSSION

The extracellular loops (ECL1-3), are not only important as linkers and hold the functionality of the transmembrane domains, but also play an important role in the ligand binding and recognition. The diversity between ECLs among GPCRs subfamilies is striking [8] and their essentiality has been demonstrated on the bovine rhodopsine receptor.[63] The adenosine A_{2A} receptor contains eight cysteine residues in the ECLs creating four disulphide bonds. Three of them link ECL1 and ECL2: Cys71 - Cys159 and Cys74 - Cys146 are unique to the A_{2A} adenosine receptor while the third Cys77 - Cys166 is considered as a conserved bond among many class A GPCRs. The fourth disulphide bond is formed in the ECL3: Cys259 - Cys262 (topology model in the **Fig. 12** and 3D crystal structure model in the **Fig. 2**). Mutations of Cys262 did not demonstrate any effect on radioligand binding.[12] All disulphide bonds contribute to rigidifying the receptor structure.[8] Since disulphide bonds are important and since homology alignments suggested that there is a conserved disulphide bond among many class A GPCRs (this bond corresponds to Cys77-Cys166 in the adenosine A_{2A} receptor),[64] we decided to study the importance of each disulphide bond occurring between ECL1 and ECL2 by generating four mutant adenosine A_{2A} receptors (C146S, C159S, C166S and C146S-C159S) where cysteine residues were replaced with serine. This amino acid is considered as a sterically and electronically similar to cysteine, but looses the ability to participate in disulphide bond formation, nevertheless serine is still able to form H-bonds.[27]

6.1. Receptor expression and trafficking

Replacing a single amino acid in the receptor might have fatal consequences on the receptor conformation which is closely connected with the receptor trafficking.[65] Comparison between the results obtained from B_{max} and ELISA might help to clarify the importance of disulphide bonds during the trafficking process. ELISA is focused on the presence of the receptor on the cell surface in contrast to B_{max} which represents the total amount of the receptor occurring in the cell. We are able to determine the influence of the disulphide bonds on the receptor trafficking by comparing these two values. Transportation process starts in ER (endoplasmic reticulum) where the receptor is synthesized and disulphide bonds are formed by thiol-disulphide oxidoreductases

family.[66] The protein subsequently continues through Golgi apparatus (posttranslational modifications) and eventually ends in the cell membrane. If the conformation is not properly formed, the protein is decomposed to the single amino acids.[67] It has been observed that lack of disulphide bridges leads to the higher possibility of degradation [27], that might be a consequence of the glycosylation process, which is naturally prevented by the disulphide bonds.[68] That explains the decreased expression of the mutant A_{2A} receptors (C146S, C166S, C146S-C159S), seen in the **Table 5**. However the double mutant receptor C146S-C159S showed similar expression as a wt A_{2A}AR, the both single mutant receptors (C146S and C166S) were expressed in a lower amount. The mutant receptor C146S seems to be the most important in the trafficking since disrupting this disulphide bond resulted in 25% of the wt A_{2A}AR expression. However disrupting both Cys146 and Cys159 led only to the small decline of the receptor expression, thus the Cys166 might have taken over the function of the Cys146. The only overly expressed mutant receptor C159S (153% of the wt A_{2A}AR expression) did not correlate with the B_{max} value (50% of the adenosine wt A_{2A} receptor), which might be caused by extremely efficient trafficking or an error occurred during the ELISA measurements. Except this fluctuation, all data are related with the results which Naranjo and co-workers obtained.[69] Nevertheless the whole quality control process remains unclear, while replacing two cysteine residues (C146S-C159S) leads to similar or even higher expression, whereas the receptors with only single mutation (C146S, C159S, C166S) are more deposited in the ER. Further investigation might improve our understanding of the molecular factors responsible for the distribution of GPCRs in the cell.[69]

6.2. The ligand binding and recognition

Extracellular loop two (ECL2) is the most diverse region between the class A of GPCRs and it plays an important role in ligand binding and recognition. The flexibility of the ECL2 predicts the conformational state of the receptor, because the ECL2 behaves as a lid or a "gatekeeper" to the binding pocket. It can either enable binding the ligand and subsequently close the "gate" after the binding or prevent the binding at all.[8] This hypothesis has been confirmed by creating a new disulphide bond in M_2 muscarinic receptor, thus restrict the flexibility of ECL2.[70] Whereas I disrupted the disulphide bonds and support the flexibility of ECL2, with the biggest intervention at the double

mutant receptor C146S-C159S. Disrupting two disulphide bonds thus, interference into the rigidity so large, however negatively influenced the affinity of small molecules such as NECA or possibly adenosine, since it has been shown by crystal structures that they share the same binding pocket.[71] The analogous molecular structure belongs to the most probably explanations, the structure is seen in **Fig. 7**. Testing NECA, the double mutant receptor C146S-C159S lowered its affinity by nearly 27-fold compared to wt A_{2A} adenosine receptor. However this reduced affinity was shown only at NECA, considerably smaller molecule than other tested substances. The presence of a long hydrophobic chain substituted on N_2 in the purine molecule may contribute to higher affinity for CGS-21680 and PSB-826 and possibly stabilise them in the receptor. The supposed conserved disulphide bond Cys166-Cys77 turned out not to be essential for ligand recognition, moreover all the A_{2A}AR agonists tested at C166S showed similar or slightly lower affinity than the wt A_{2A} adenosine receptor. Replacing Cys159 by serine contributed to increasing affinity for all tested agonists. The mutant receptor C159S might be an example of how increasing flexibility is involved in the simplification of the binding process, nevertheless it remains unclear the exact disulphide bond formation after mutating the receptors. While disrupting only one cysteine that forms disulphide bond increases the affinity, whereas replacing both cysteine (C71A-C159A) led to the suppression of affinity at the A_{2A}AR agonist CGS-21680.[69] Since the mutant receptor C146S did not show sufficiently high window range between NB and TB (**Fig. 15**) while [^{3}H]CGS-21680 was tested, the experiment was proceeded with the antagonist [^{3}H]MSX-2. Both agonist and antagonist differ on the molecular level (see in the **Fig. 7**), thus the binding pocket for both is diverse. The ribose part of the agonist extends deeper into the binding pocket and creates polar interactions with residues in the helical transmembrane region 7 (TM7) (Ser277 and His278). On the contrary selective A_{2A} adenosine receptor antagonist ZM241385 does not interact with any of these residues. Furthermore it is suggested that ZM241385 sterically prevents the conformational change in TM5, which would consequently result in the activation of G protein. Also has been proven that antagonists are capable of binding to inactive state of the receptor, which occurs in the cell in numerously higher amount.[71] This might be an explanation why the curves in the **Fig. 16** did not reach the zero. Altering the Cys146 with serine eventually ended in decreasing affinity for all tested compounds. Additionally all the mutant receptors (C146S, C159S, C166S, C146S-C159S) were not binding to the selective A_{2B}AR partial agonist, which proved that cysteine residues have probably no

role in the subfamily recognition. Investigating the affinity of CGS-21680 Naranjo et al. came to the similar conclusion with the saturation radioligand binding experiments and my competitive radioligand studies make the data only more valid.[69]

Determination of the ECL2 functionality was conducted by cAMP accumulation assay (**Fig. 17**). It has been examined, the ligand concentration that is needed to stimulate 50% of the receptors and the amount of cAMP produced.

The mutant receptor C146S showed activity only after stimulation with NECA and CGS-21680. Both molecules have the same substituent at the 5' position of the ribose (ethylcarboxamide). Additionally PSB-826 and adenosine also share the same structure at the 5' position of the ribose (hydroxyl), which on contrary resulted in no response at all (see the chemical structures in **Fig. 7**). The ethylcarboxamide structure possibly provides a structural support in the binding pocket during the absence of Cys146. However during testing NECA and CGS-21680 on C146S results reached the lowest cAMP concentration of all tested receptors. That might be induced by the minor receptor expression on the cell surface or the inefficient trigger mechanism.

The double mutant receptor C146S-C159S confirmed the difficulty to bind small molecules such as NECA or adenosine, when potency of NECA was decreased by 460-fold and adenosine by 247-fold compared to wt A_{2A}AR. On the other hand, molecules with large hydrophobic tail substituted on N_2 (CGS-21680 or PSB-826) resulted in comparable potency as the wt A_{2A} adenosine receptor.

Only the C159S mutant receptor revealed higher potency of all the chemically modified tested A_{2A} adenosine agonists, which also correlate with increased affinity to this receptor.

Cys166 was considered that participates on forming the essential disulphide bond, which was eventually disproven. First by replacing Cys166 with alanine and additionally to prevent the formation of the disulphide bond at all, by replacing both Cys166 and Cys77. None of which led to loss of the receptor activity.[69] I achieved the same conclusion with my results, moreover the C166S mutant receptor preserved highest similarity to the wt A_{2A} adenosine receptor in comparison with other mutant receptors (adenosine excluded).

Adenosine as an endogenous agonist demonstrated decreased potency in the range from 19-fold to 247-fold compared to the wt $A_{2A}AR$, in all the tested mutant receptors. In addition to that, the data approached the potency obtained from the A_{2B} adenosine receptor. This only corroborated the importance of the cysteine residues placed in the ECL2 for physiological function.

7. CONCLUSION

It has been confirmed the importance of the disulphide bonds on the ligand binding and recognition but also the impact on trafficking. Additionally has been partially revealed how chemical structure might be involved in this process. Nevertheless further investigation in trafficking might be needed, in order to better understand the whole quality control process that could be consequently applicable also on other GPCRs. I also suggest generating more variations of the mutant adenosine A_{2A} receptors. More heterogeneous combinations would, with the most probability, reveal which disulphide bonds are necessarily involved in diverse processes of the receptor functionality and which are not. This eventually could lead to more precise ligand synthesis with increased subtype selectivity and thus eliminate the side effects. The further determination would include creating more double mutant receptors to ensure that disulphide bond has been truly disrupted and examine the impact on the ligand binding and functionality. Since we know that double mutant receptors showed higher expression than single mutant receptors, disulphide bonds might decrease the trafficking process.[69]

8. ABREVIATIONS

$A_{2A}AR$	Adenosine subtype A_{2A} receptor
Ab	Antibody
AC	Adenylate cyclase
ADA	Adenosine deaminase
ALT-146e	$A_{2A}AR$ agonist
ALT202	$A_{2A}AR$ agonist
AR	Adenosine receptor
ATP	Adenosine triphosphate
B_{max}	Density of the receptor
BSA	Bovine serum albumin
cAMP	3',5'-cyclic adenosine monophosphate
CGS-21680	Adenosine receptor A_{2A} agonist
CHO	Chinese hamster ovaries
cpm	Count per minute (measuring the radioactivity)
CREB	cAMP responsive element-binding
CV-1808	A_{2A} adenosine receptor agonist
D_2Rs	Dopamine subtype 2 receptor
DAG	Diacylglycerol
DMARD	Disease modifying antirheumatic drugs
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal effective concentration
ECL2	Extracellular loop 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum Golgi intermediate complex
ERK	Extracellular signal-regulated kinases
FCS	Fetal Calf Serum
GPCRs	G protein coupled receptors

GRKs	GPCR kinases
GTP	Guanosine triphosphate
$hA_{2A}AR$	Human A_{2A} adenosine receptor
HBSS	Hank's Balanced Salt Solution
HRP	Horseradish Peroxidase
IC_{50}	Half maximal inhibitory concentration
IFNγ	Interferon gamma
IL2	Interleukin 2
IP_3	Inositol-1,4,5-trisphosphate
K_D	Dissociation constant
K_i	Inhibition constant
MAPKs	Mitogen-activated protein kinases
MRE-0094	A_{2A} adenosine receptor agonist
NB	Nonspecific binding
PDE	Phosphodiesterase enzyme
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PR	Purinergic receptors
RT	Room temperature
SEM	Standard error of the mean
SRP	Signal recognition particle
TB	Total binding
TGN	Trans-Golgi network
TM	Transmembrane
TNF-α	Tumor necrosis factor alpha
ZM241385	A_{2A} adenosine receptor antagonist

9. REFERENCES

(1) Rosenbaum, D. M.; Rasmussen, S. G. F.; Kobilka, B. K. The Structure and Function of G-Protein-Coupled Receptors. *Nature* **2009**, *459* (7245), 356–363.

(2) Kobilka, B. K. G Protein Coupled Receptor Structure and Activation. *Biochim. Biophys. Acta* **2007**, *1768* (4), 794–807.

(3) Zhang, R.; Xie, X. Tools for GPCR Drug Discovery. *Acta Pharmacol. Sin.* **2012**, *33* (3), 372–384.

(4) Katritch, V.; Cherezov, V.; Stevens, R. C. A. O.-10. 1146/annurev-pharmto.-032112-135923. Structure-Function of the G Protein–Coupled Receptor Superfamily. *Annual Review of Pharmacology and Toxicology*, 531–556.

(5) Liu, W.; Wacker, D.; Gati, C.; Han, G. W.; James, D.; Wang, D.; Nelson, G.; Weierstall, U.; Katritch, V.; Barty, A.; Zatsepin, N. a; Li, D.; Messerschmidt, M.; Boutet, S.; Williams, G. J.; Koglin, J. E.; Seibert, M. M.; Wang, C.; Shah, S. T. a; Basu, S.; Fromme, R.; Kupitz, C.; Rendek, K. N.; Grotjohann, I.; Fromme, P.; Kirian, R. a; Beyerlein, K. R.; White, T. a; Chapman, H. N.; Caffrey, M.; Spence, J. C. H.; Stevens, R. C.; Cherezov, V. Serial Femtosecond Crystallography of G Protein-Coupled Receptors. *Science* **2013**, *342* (6165), 1521–1524.

(6) Kobilka, B. K.; Deupi, X. Conformational Complexity of G-Protein-Coupled Receptors. *Trends Pharmacol. Sci.* **2007**, *28* (8), 397–406.

(7) Ostermeier, C.; Michel, H. Crystallization of Membrane Proteins. *Curr. Opin. Struct. Biol.* **1997**, *7* (5), 697–701.

(8) Peeters, M. C.; Van Westen, G. J. P.; Li, Q.; Ijzerman, a. P. Importance of the Extracellular Loops in G Protein-Coupled Receptors for Ligand Recognition and Receptor Activation. *Trends Pharmacol. Sci.* **2011**, *32* (1), 35–42.

(9) Sensoy, O.; Weinstein, H. A Mechanistic Role of Helix 8 in GPCRs: Computational Modeling of the Dopamine D2 Receptor Interaction with the GIPC1–PDZ-Domain. *Biochim. Biophys. Acta - Biomembr.* **2015**, *1848* (4), 976–983.

(10) Fan, Q. R.; Hendrickson, W. A. Structure of Human Follicle-Stimulating Hormone in Complex with Its Receptor. *Nature* **2005**, *433* (7023), 269–277.

(11) Fillion, D.; Lemieux, G.; Basambombo, L. L.; Lavigne, P.; Guillemette, G.; Leduc, R.; Escher, E. The Amino-Terminus of Angiotensin II Contacts Several Ectodomains of the Angiotensin II Receptor AT1. *J. Med. Chem.* **2010**, *53* (5), 2063–2075.

(12) Jaakola, V.-P.; Griffith, M. T.; Hanson, M. A.; Cherezov, V.; Chien, E. Y. T.; Lane, J. R.; Ijzerman, A. P.; Stevens, R. C. The 2.6 Angstrom Crystal Structure of a Human A2A Adenosine Receptor Bound to an Antagonist. *Science* **2008**, *322* (5905), 1211–1217.

(13) Wheatley, M.; Wootten, D.; Conner, M. T.; Simms, J.; Kendrick, R.; Logan, R. T.; Poyner, D. R.; Barwell, J. Lifting the Lid on GPCRs: The Role of Extracellular Loops. *Br. J. Pharmacol.* **2012**, *165* (6), 1688–1703.

(14) Brock; G., T. G Proteins and Their Coupled Receptors | Article Library || Cayman Chemical | Supplier https://www.caymanchem.com/app/template/Article.vm/article/2188 (accessed Apr 7, 2015).

(15) Ritter, S. L.; Hall, R. A. Fine-Tuning of GPCR Activity by Receptor-Interacting Proteins. *Nat. Rev. Mol. Cell Biol.* **2009**, *10* (12), 819–830.

(16) Drury, A. N.; Szent-Györgyi, A. The Physiological Activity of Adenine Compounds with Especial Reference to Their Action upon the Mammalian Heart. *J. Physiol.* **1929**, *68* (3), 213–237.

(17) Cobbin, L. B.; Einstein, R.; Maguire, M. H. Studies on the Coronary Dilator Actions of Some Adenosine Analogues. *Br. J. Pharmacol.* **1974**, *50* (1), 25–33.

(18) Abbracchio, M. P.; Burnstock, G.; Verkhratsky, A.; Zimmermann, H. Purinergic Signalling in the Nervous System: An Overview. *Trends Neurosci.* **2009**, *32* (1), 19–29.

(19) Burnstock, G.; Wood, J. N. Purinergic Receptors: Their Role in Nociception and Primary Afferent Neurotransmission. *Curr. Opin. Neurobiol.* **1996**, *6* (4), 526–532.

(20) Fredholm, B. B.; IJzerman, A. P.; Jacobson, K. a; Klotz, K. N.; Linden, J.; Ap, I. J. International Union of Pharmacology. XXV. Nomenclature and Classification of Adenosine Receptors. *Pharmacol. Rev.* **2001**, *53* (4), 527–552.

(21) Chen, J.; Lee, C.; Chern, Y. *Adenosine Receptor Neurobiology : Overview*, 1st ed.; Elsevier Inc., 2014; Vol. 119.

(22) Klotz, K. N. Adenosine Receptors and Their Ligands. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **2000**, *362* (4-5), 382–391.

(23) Lapa, R.; José, S.; Júnior, M.; Cerutti, M. L.; Roberto, A.; Santos, S. Pharmacology of Adenosine Receptors and Their Signaling Role in Immunity and Inflammation. **1970**.

(24) De Lera Ruiz, M.; Lim, Y.; Zheng, J. Adenosine A2A Receptor as a Drug Discovery Target. *J. Med. Chem.* **2014**, *57* (9), 3623–3650.

(25) Choudhary, M. *Frontiers in Drug Design and Discovery*; Bentham Science Publishers, 2010.

(26) Seibt, B. F.; Schiedel, A. C.; Thimm, D.; Hinz, S.; Sherbiny, F. F.; Müller, C. E. The Second Extracellular Loop of GPCRs Determines Subtype-Selectivity and Controls Efficacy as Evidenced by Loop Exchange Study at A2 Adenosine Receptors. *Biochem. Pharmacol.* **2013**, *85* (9), 1317–1329.

(27) Schiedel, A. C.; Hinz, S.; Thimm, D.; Sherbiny, F.; Borrmann, T.; Maass, A.; Müller, C. E. The Four Cysteine Residues in the Second Extracellular Loop of the Human Adenosine A2B Receptor: Role in Ligand Binding and Receptor Function. *Biochem. Pharmacol.* **2011**, *82* (4), 389–399.

(28) Klaasse, E. C.; IJzerman, A. P.; de Grip, W. J.; Beukers, M. W. Internalization and Desensitization of Adenosine Receptors. *Purinergic Signalling*, 2008, *4*, 21–37.

(29) Klinger, M.; Freissmuth, M.; Nanoff, C. Adenosine Receptors: G Protein-Mediated Signalling and the Role of Accessory Proteins. *Cell. Signal.* **2002**, *14* (2), 99–108.

(30) Jacobson, K. a.; Gao, Z.-G. Adenosine Receptors as Therapeutic Targets. *Nat. Rev. Drug Discov.* **2012**, *5* (3), 247–264.

(31) Yang, Z.; Day, Y.-J.; Toufektsian, M.-C.; Ramos, S. I.; Marshall, M.; Wang, X.-Q.; French, B. A.; Linden, J. Infarct-Sparing Effect of A2A-Adenosine Receptor Activation Is Due Primarily to Its Action on Lymphocytes. *Circulation* **2005**, *111* (17), 2190–2197.

(32) Canals, M.; Burgueño, J.; Marcellino, D.; Cabello, N.; Canela, E. I.; Mallol, J.; Agnati, L.; Ferré, S.; Bouvier, M.; Fuxe, K.; Ciruela, F.; Lluis, C.; Franco, R. Homodimerization of Adenosine A2A Receptors: Qualitative and Quantitative Assessment by Fluorescence and Bioluminescence Energy Transfer. *J. Neurochem.* **2004**, *88* (3), 726–734.

(33) Ferre, S.; von Euler, G.; Johansson, B.; Fredholm, B. B.; Fuxe, K. Stimulation of High-Affinity Adenosine A2 Receptors Decreases the Affinity of Dopamine D2 Receptors in Rat Striatal Membranes. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88* (16), 7238–7241.

(34) Kanda, T.; Jackson, M. J.; Smith, L. A.; Pearce, R. K. B.; Nakamura, J.; Kase, H.; Kuwana, Y.; Jenner, P. Adenosine A(2A) Antagonist: A Novel Antiparkinsonian Agent That Does Not Provoke Dyskinesia in Parkinsonian Monkeys. *Ann. Neurol.* **1998**, *43* (4), 507–513.

(35) KW-6002 (istradefylline) FDA Approval Status - Drugs.com http://www.drugs.com/history/kw-6002.html (accessed Apr 25, 2015).

(36) Popoli, P.; Pintor, A.; Domenici, M. R.; Frank, C.; Tebano, M. T.; Pèzzola, A.; Scarchilli, L.; Quarta, D.; Reggio, R.; Malchiodi-Albedi, F.; Falchi, M.; Massotti,

M. Blockade of Striatal Adenosine A2A Receptor Reduces, through a Presynaptic Mechanism, Quinolinic Acid-Induced Excitotoxicity: Possible Relevance to Neuroprotective Interventions in Neurodegenerative Diseases of the Striatum. *J. Neurosci.* **2002**, *22* (5), 1967–1975.

(37) El Yacoubi, M.; Ledent, C.; Parmentier, M.; Daoust, M.; Costentin, J.; Vaugeois, J. M. Absence of the Adenosine A2A Receptor or Its Chronic Blockade Decrease Ethanol Withdrawal-Induced Seizures in Mice. *Neuropharmacology* **2001**, *40* (3), 424–432.

(38) Cronstein, B. N.; Levin, R. I.; Philips, M.; Hirschhorn, R.; Abramson, S. B.; Weissmann, G. Neutrophil Adherence to Endothelium Is Enhanced via Adenosine A1 Receptors and Inhibited via Adenosine A2 Receptors. *J. Immunol. (Baltimore, Md. 1950)* **1992**, *148* (7), 2201–2206.

(39) Erdmann, A. A.; Gao, Z. G.; Jung, U.; Foley, J.; Borenstein, T.; Jacobson, K. A.; Fowler, D. H. Activation of Th1 and Tc1 Cell Adenosine A2A Receptors Directly Inhibits IL-2 Secretion in Vitro and IL-2-Driven Expansion in Vivo. *Blood* **2005**, *105* (12), 4707–4714.

(40) Methotrexát v léčbě revmatoidní artritidy – Přehledy, názory, diskuse – Články – Remedia – farmakoterapeutický časopis http://www.remedia.cz/Clanky/Prehledy-nazory-diskuse/Methotrexat-v-lecbe-revmatoidni-artritidy/6-F-IP.magarticle.aspx (accessed Apr 10, 2015).

(41) Montesinos, M. C.; Desai, A.; Delano, D.; Chen, J. F.; Fink, J. S.; Jacobson, M. A.; Cronstein, B. N. Adenosine A2A or A3 Receptors Are Required for Inhibition of Inflammation by Methotrexate and Its Analog MX-68. *Arthritis Rheum.* **2003**, *48* (1), 240–247.

(42) Peirce, S. M.; Skalak, T. C.; Rieger, J. M.; Macdonald, T. L.; Linden, J. Selective A(2A) Adenosine Receptor Activation Reduces Skin Pressure Ulcer Formation and Inflammation. *Am. J. Physiol. Heart Circ. Physiol.* **2001**, *281* (1), H67–H74.

(43) Montesinos, M. C.; Gadangi, P.; Longaker, M.; Sung, J.; Levine, J.; Nilsen, D.; Reibman, J.; Li, M.; Jiang, C. K.; Hirschhorn, R.; Recht, P. A.; Ostad, E.; Levin, R. I.; Cronstein, B. N. Wound Healing Is Accelerated by Agonists of Adenosine A2 (G Alpha S-Linked) Receptors. *J. Exp. Med.* **1997**, *186* (9), 1615–1620.

(44) Safety and Efficacy Study of MRE0094 to Treat Large, Single or Multiple, Chronic, Neuropathic, Diabetic Foot Ulcers - Full Text View - ClinicalTrials.gov https://www.clinicaltrials.gov/ct2/show/NCT00318214) (accessed Apr 25, 2015).

(45) Reutershan, J.; Cagnina, R. E.; Chang, D.; Linden, J.; Ley, K. Therapeutic Anti-Inflammatory Effects of Myeloid Cell Adenosine Receptor A2a Stimulation in Lipopolysaccharide-Induced Lung Injury. *J. Immunol.* **2007**, *179* (2), 1254–1263.

(46) Kim, D. H.; Ryu, J. H. Activation of Adenosine A2A Receptor Impairs Memory Acquisition but Not Consolidation or Retrieval Phases. *Biomol. Ther.* **2008**, *16* (4), 320–327.

(47) Prediger, R. D. S.; Batista, L. C.; Takahashi, R. N. Caffeine Reverses Age-Related Deficits in Olfactory Discrimination and Social Recognition Memory in Rats: Involvement of Adenosine A1 and A2A Receptors. *Neurobiol. Aging* **2005**, *26* (6), 957–964.

(48) Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, C. C. (2013). adenosine | Ligand page | IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?tab=biology&ligandId=2844 (accessed Apr 29, 2015).

(49) CGS 21680 hydrochloride Supplier | CAS 124431-80-7 | Tocris Bioscience | CGS21680 http://www.tocris.com/dispprod.php?ItemId=2216 (accessed Apr 14, 2015).

(50) Hinz, S.; Lacher, S. K.; Seibt, B. F.; Müller, C. E. BAY60-6583 Acts as a Partial Agonist at Adenosine A2B Receptors. *J. Pharmacol. Exp. Ther.* **2014**, *349* (3), 427–436.

(51) Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, C. C. (2013). Adenosine receptors | G protein-coupled receptors | IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=3 (accessed Apr 29, 2015).

(52) Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, C. C. (2013). Adenosine receptors | G protein-coupled receptors | IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=3 (accessed Apr 29, 2015).

(53) Bastidas, O. *Cell Counting with Neubauer Chamber (Basic Hemocytometer Usage)*; 2014.

(54) Grigoryev, Y. Principles and Mechanisms of Mammalian Cell Transfection.

(55) Spiegel, M.; Bitzer, M.; Schenk, A.; Rossmann, H.; Neubert, W. J.; Seidler, U.; Gregor, M.; Lauer, U. Pseudotype Formation of Moloney Murine Leukemia Virus with Sendai Virus Glycoprotein F. *J. Virol.* **1998**, *72* (6), 5296–5302.

(56) Backliwal, G.; Hildinger, M.; Kuettel, I.; Delegrange, F.; Hacker, D. L.; Wurm, F. M. Valproic Acid: A Viable Alternative to Sodium Butyrate for Enhancing Protein Expression in Mammalian Cell Cultures. *Biotechnol. Bioeng.* **2008**, *101* (1), 182–189.

(57) BioRad. Quick Start™ Bradford Protein Assay.

(58) coomassie_forms.jpg (JPEG obrázek, 960 × 720 bodů) - Měřítko (46%) http://lightboxkit.com/index_files/Images/BradfordAssay/coomassie_forms.jpg (accessed Apr 8, 2015).

(59) Ligand (biochemistry) - Wikipedia, the free encyclopedia http://en.wikipedia.org/wiki/Ligand_%28biochemistry%29 (accessed Apr 8, 2015).

(60) Deupree, J. D.; Bylund, D. B. Introduction Receptor Binding Studies Are Used to Determine the Affinity of Various Drugs for a Receptor as Well as the Binding Site Density (B. *Tocris Rev. No. 18,*.

(61) Sassone-Corsi, P. The Cyclic AMP Pathway. *Cold Spring Harb. Perspect. Biol.* **2012**, *4* (12), a011148–a011148.

(62) Takeda, J.; Adachi, K.; Halprin, K. M.; Itami, S.; Levine, V.; Woodyard, C. Forskolin Activates Adenylate Cyclase Activity and Inhibits Mitosis in in Vitro in Pig Epidermis. *J. Invest. Dermatol.* **1983**, *81* (3), 236–240.

(63) Karnik, S. S.; Khorana, H. G. Assembly of Functional Rhodopsin Requires a Disulfide Bond between Cysteine Residues 110 and 187. *J. Biol. Chem.* **1990**, *265* (29), 17520–17524.

(64) Palczewski, K. Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science (80-.).* **2000**, *289* (5480), 739–745.

(65) Scholl, D. J.; Wells, J. N. Serine and Alanine Mutagenesis of the Nine Native Cysteine Residues of the Human A1 Adenosine Receptor. *Biochem. Pharmacol.* **2000**, *60* (11), 1647–1654.

(66) Ellgaard, L.; Helenius, A. Quality Control in the Endoplasmic Reticulum. *Nat. Rev. Mol. Cell Biol.* **2003**, *4* (3), 181–191.

(67) Vitale, a; Denecke, J. The Endoplasmic Reticulum-Gateway of the Secretory Pathway. *Plant Cell* **1999**, *11* (4), 615–628.

(68) McGinnes, L. W.; Morrison, T. G. Disulfide Bond Formation Is a Determinant of Glycosylation Site Usage in the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus. *J. Virol.* **1997**, *71* (4), 3083–3089.

(69) Naranjo, A. N.; Chevalier, A.; Cousins, G. D.; Ayettey, E.; McCusker, E. C.; Wenk, C.; Robinson, A. S. Conserved Disulfide Bond Is Not Essential for the Adenosine A2A Receptor: Extracellular Cysteines Influence Receptor Distribution within the Cell and Ligand-Binding Recognition. *Biochim. Biophys. Acta - Biomembr.* **2015**, *1848* (2), 603–614.

(70) Avlani, V. a.; Gregory, K. J.; Morton, C. J.; Parker, M. W.; Sexton, P. M.; Christopoulos, A. Critical Role for the Second Extracellular Loop in the Binding of Both Orthosteric and Allosteric G Protein-Coupled Receptor Ligands. *J. Biol. Chem.* **2007**, *282* (35), 25677–25686.

(71) Lebon, G.; Warne, T.; Edwards, P. C.; Bennett, K.; Langmead, C. J.; Leslie, A. G. W.; Tate, C. G. Agonist-Bound Adenosine A2A Receptor Structures Reveal Common Features of GPCR Activation. *Nature* **2011**, *474* (7352), 521–525.