Affinity-based profiling of the adenosine receptors

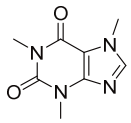
Beerkens, B.L.H.

Chapter 1

General Introduction

Caffeine and the Adenosine Receptors

Caffeine is a substance present in coffee, tea and chocolate, among other food and beverages, consumed widely across the world. Caffeine is well-known because of its effects upon consumption, resulting in a decreased feeling of tiredness for the consumer.[1] The molecular mechanisms behind the stimulatory effects have been elucidated about fifty years ago, when caffeine was found to block **adenosine-responsive receptors**.[2–4] In other words, caffeine prevents the molecule adenosine from binding to and stimulating 'its' receptor, thereby impairing the natural feeling of drowsiness caused by receptor activation. Up to today, four subtypes of adenosine receptor have been characterized: the **A_1**, **A_{2A}**, **A_{2B}** and **A_3 receptors** (coined A_1AR, A_{2A}AR, A_{2B}AR and A_3AR throughout this dissertation).[5] These subtypes vary in their molecular structure, induced effects upon activation and expression levels in certain cell- and tissue types.[5–7] All four subtypes share similar structural characteristics, as well as a mode of action that involves Guanine Nucleotide Binding Protein (G Protein) signaling, making them part of the so-called **G Protein-Coupled Receptor** (GPCR) family of proteins.[8]



Caffeine was first isolated as a pure substance in 1819 by the German Friedlieb Ferdinand Runge and termed 'Kaffeebase'. The molecule was later independently discovered by the French Pierre-Joseph Pelletier, who gave the substance the name 'caffeine'. Both names are derived from the word 'coffee'.

G Protein-Coupled Receptors (GPCRs)

Proteins within the family of GPCRs share several characteristics. Considering structure, GPCRs have an extracellular N-terminus, seven transmembrane helices and an intracellular C-terminus.[9] Upon activation, GPCRs change their conformation, resulting in the dissociation of the intracellularly bound G Protein.[10] In turn, the G Protein is able to induce various intracellular pathways, of which cyclic adenosine monophosphate (cAMP) generation is the most well-studied (Figure 1).[11] Next to G Proteins, GPCRs have been found to bind arrestin, a signaling protein that induces internalization of the receptor from the membrane into the cell.[12–15] These "classical" and fundamental GPCR pathways have been known for a couple of decades. Nowadays, however, it is becoming clear that there are more factors that influence the fate of GPCRs and their signaling pathways.[16–19]

One of the factors that influences the outcome GPCR signaling pathways is the ability of the GPCR to form **protein-protein interactions** (PPIs) with other proteins. Next to the well-known interactions with subtypes of G Protein and arrestin, PPIs between GPCRs and multiple other proteins have been discovered. Possible interactions partners are the same GPCR (homodimer formation), a different GPCR (heterodimer formation), other membrane proteins (e.g. adenylyl cyclase), or intracellular proteins (e.g. G protein-coupled receptor kinases (GRKs)).[16] A second factor of influence is the presence or absence of certain **post-translational modifications** (PTMs): covalent reversible modifications onto the protein that are not encoded within their DNA.[17,18] GPCR **signaling from other cellular compartments** has also been observed, e.g. from organelles and vesicles. Cellular localization of GPCRs is thus a third factor that influences GPCR signaling. All of these factors, PPIs, PTMs and cellular localization, are highly intertwined, thereby complicating the outcome of the induced signaling pathways.[16–19]

Knowledge of all different aspects of GPCR signaling is highly valuable when targeting a GPCR for medicinal purposes. In fact, GPCRs are one of the most popular drug targets, as roughly one third of all marketed drugs is targeting a specific GPCR, directly or indirectly.[23,24] The reason for their popularity is the important role of GPCR signaling in a wide range of physiological and pathological conditions. This is also true for all four of the adenosine receptors, that are involved in multiple pathophysiological conditions, ranging from immune regulation to cancer.[25]

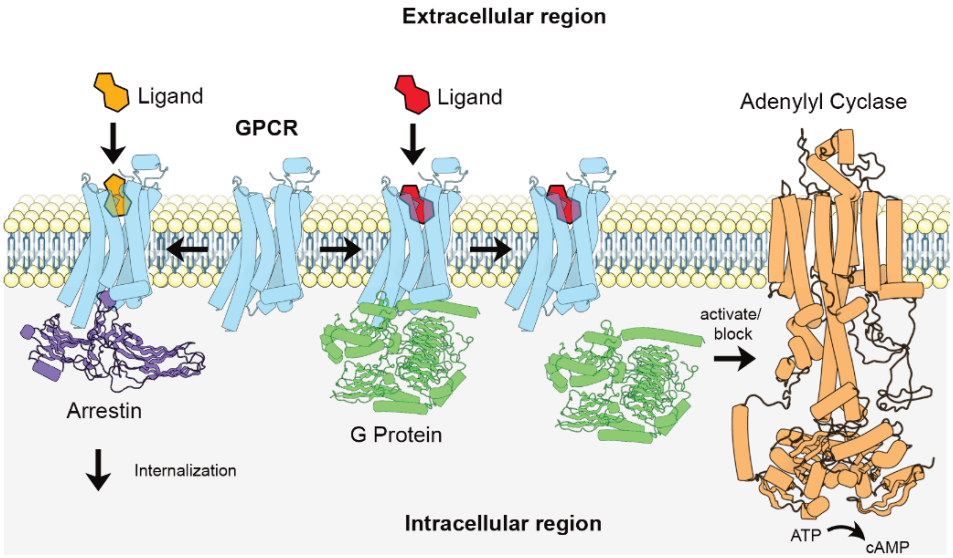


Figure 1. Activation of GPCRs results in intracellular signal transduction pathways, of which G protein-dependent stimulation or inhibition of adenylyl cyclase (right-hand side) and arrestin-mediated internalization (left-hand side) are the most well studied. Adenylyl cyclase in turn produces cAMP from adenosine triphosphate (ATP). This figure was partly created using Protein Imager,[20] using the structures of the A_{2A}AR (PDB: 7ARO), modified G_S Protein (PDB: 8HDO), β-Arrestin1 (PDB: 7SRS) and predicted structure of Adenylyl Cyclase type 6 (Alphafold: AF-043306-F1).[21,22]

Adenosine Receptors as Drug Target

Although the adenosine receptors are a target for caffeine, the receptors are named after their endogenous ligand: adenosine (Figure 2).[5] Adenosine is a signaling molecule that is formed, next to other biosynthetic pathways, through the breakdown of extracellular ATP.[6] ATP functions as energy carrier of the cell. Hence, a high extracellular concentration of adenosine indicates high levels of ATP consumption. Increased concentrations of adenosine have been found in various pathological conditions, for example during inflammation, hypoxia and in the tumor microenvironment.[26,27] Cells respond to this adenosine-rich environment via binding of adenosine to one or more of the four adenosine receptors (ARs), most often leading to an immunosuppressive response. Targeting the adenosine receptors is therefore an interesting strategy to modulate immune responses in a variety of pathologies, as further outlined below.

The **adenosine A_1 receptor** (A_1AR) is expressed in several brain regions (cortex, cerebellum, hippocampus), the heart (cardiomyocytes) and fat tissue (adipocytes), among other tissue types.[5] Activation of the receptor results in, for example, analgesic effects, reduction of ischemic injury or induction of lipolysis, however, this is highly dependent on cell- and tissue type.[30–32] Clinical trials of A_1AR-targeting drugs have mostly been focused on reducing and preventing heart damage, using either (partial) agonists or antagonists for the A_1AR.[30] More recent studies reveal a reduction of nociception upon treatment with an A_1AR allosteric modulator, implying novel therapeutic pathways for the treatment of pain.[32]

The **adenosine A_{2A} receptor** (A_{2A}AR) is expressed in multiple brain regions (a high A_{2A}AR receptor density is found in the basal ganglia), as well as on immune cells (granulocytes and lymphocytes, among other cells).[7] The stimulatory effects of caffeine are mostly caused by inhibition of A_1ARs and A_{2A}ARs in the brain.[33] Next to that, antagonism of A_{2A}ARs in brain regions dampens the effects of neurodegenerative diseases, such as Parkinson's and Alzheimer's, leading to FDA approval of the A_{2A}AR antagonist Istradefylline as therapy for Parkinson's disease.[34–36]

The **adenosine A_{2B} receptor** (A_{2B}AR) is expressed on smooth muscle cells, endothelial cells and immune cells (macrophages, dendritic cells and antigen-presenting cells, among other immune cells). Like the A_{2A}AR, activation of A_{2B}ARs leads to local immune suppression.[37] Pathways involving both A_{2A}ARs and A_{2B}ARs have been found beneficial for the proliferation of cancerous cells within the tumor micro-environment.[26,27,37] Therefore, multiple clinical trials are currently ongoing using antagonists to block A_{2A}ARs and A_{2B}ARs in certain types of cancer.[7]

Lastly, the **adenosine A_3 receptor** (A_3AR), is expressed on immune cells (granulocytes), various cancerous cell lines and in testes and lungs, among other tissue types.[5] Activation of the A_3AR can lead to various immunomodulatory effects, depending on cell and tissue type, and ranges from the release of immune mediators to chemotaxis.[38–40] Altering immune signaling through the activation or inhibition of A_3ARs is currently being investigated as treatment of rheumatoid arthritis, COVID-19 and psoriasis.[7]

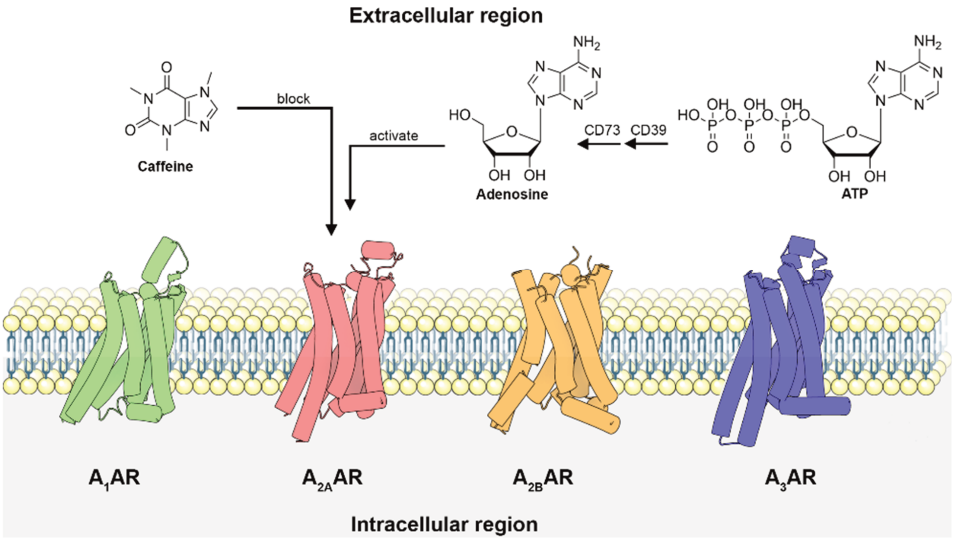


Figure 2. The four adenosine receptors are activated by their endogenous ligand adenosine and blocked by caffeine, although with differences in binding affinity between receptors, experiments and species.[7,28,29] ATP is a source of extracellular adenosine and is dephosphorylated by the membrane enzymes CD39 and CD73. This figure was partly created with Protein Imager,[20] using the structures of the A_1AR (PDB: 7LD4), A_{2A}AR (PDB: 7ARO), A_{2B}AR (PDB: 8HDO) and predicted structure of the A_3AR (Alphafold: AF-P0DMS8-F1).[21,22]

Challenges in Studying Adenosine Receptors

As evident from the examples above, the adenosine receptors are widely expressed throughout the human body. Consequently, activation of the adenosine receptors can cause a variety of downstream effects, dependent on cell type and cellular environment. Using the adenosine receptors to modulate immune responses in heart diseases, neurodegenerative diseases, cancers and immune disorders are interesting new strategies for drug discovery programs. Nevertheless, caution must be taken upon targeting the ARs, as the multitasking role of the receptors might cause unwanted side effects.[41] It is therefore of great importance to investigate the ARs and decipher all aspects of AR signaling, prior to the introduction of new drug candidates.

Studying the ARs, however, has many challenges. Being part of the GPCR family of proteins, ARs have various structural features that make them difficult to detect in standard biochemical assays. First of all, the seven hydrophobic transmembrane domains cause **poor solubility** of the receptors, thus requiring adjusted buffers and assay conditions.[9,42,43] This initially hampered the progress in crystallization studies of GPCRs but has become less of an issue with the rise of cryo-EM techniques.[43] Nevertheless, other biochemical assay types, for example chemical proteomics, still suffer from the poor solubility of GPCRs under standard assay conditions.[44]

Next to that, **low endogenous expression levels** of ARs (not to be confused with the wide expression on various cell types) hinder detection of ARs on endogenous cells, while low levels are still of physiological importance.[6,42,44] Most studies towards AR detection have therefore been carried out on AR-overexpressing cell lines, purified membrane fractions or purified receptors.

Lastly, the factors that influence GPCR signaling, as discussed above, also increase the **complexity of AR signaling**. These include PPIs, PTMs and (sub)cellular localization. All the ARs interact with other proteins, contain multiple PTM sites and partake in internalization pathways after agonist-induced activation.[45] The A_{2A}AR has been the most extensively studied, resulting in the discovery of PPIs with multiple other proteins, such as members of the subfamilies of cannabinoid and dopamine receptors.[46–48] Next to the A_2AR, homo- and/or heteromeric PPIs have been observed for the A_1AR, A_{2B}AR and A_3AR, although the physiological relevance of these PPIs has yet to be understood.[49,50]

Fortunately, various chemical and biochemical tools are being developed to aid the detection of ARs, as well as AR-induced signaling pathways. These include genetic alterations of the receptor, e.g. incorporation of FRET- and BRET-based proteins or tags, the development of antibodies, and the development of chemical probe molecules.[51,52] Of these tools, genetic incorporation of fluorescent sensors is not possible when looking at endogenous AR expression in native systems. Next to that, GPCR antibodies are often hindered by their low selectivity.[53,54] Therefore, this thesis focuses on the development and use of selective chemical probes to target and study the adenosine receptors.

Targeting the Adenosine Receptors with Chemical Probes

Over the past decades, various types of chemical probes have been developed to target the adenosine receptors.[51] Practically, these chemical probes can be divided into two categories: reversible and covalent probes. Reversible chemical probes bind to ARs in a similar manner as adenosine and caffeine: through intermolecular forces in the binding pocket of the receptor. Reversible probes can leave the receptor binding pocket after binding, thereby generating an equilibrium between bound and unbound receptor (Figure 3A). Reversible probes for adenosine receptors include ligands that have been functionalized with radioactive isotopes (radioligands) or fluorophores (fluorescent ligands).[51,55,56]

Covalent probes on the other hand, contain an electrophilic or photoreactive group that reacts with an amino acid residue near the probe binding pocket, inducing a covalent bond between probe and receptor (Figure 3B).[57] Covalent probes have an 'infinite' residence time and therefore show a time-dependent increase in receptor occupancy. Ligands functionalized with an electrophilic or photoreactive group (covalent ligands) have been used as tool to permanently block the adenosine receptors. A summary of most of the previously (before 2020) developed radioligands, fluorescent ligands and covalent ligands can be found in a recent review paper.[51]

Building upon this, covalent ligands have been functionalized with reporter groups, such as radioactive isotopes, fluorophores and biotin moieties. Contrary to the reversible radioligands and fluorescent ligands, these reporter groups are attached to the receptor in a covalent manner, allowing detection of ARs in assay types that require thorough washing steps or the use of denaturing conditions. Different strategies to covalently functionalize GPCRs with small molecular probes are reviewed in chapter 2. In case of the ARs, two different types of functionalized covalent probes have been developed: affinity-based probes and ligand-directed probes.

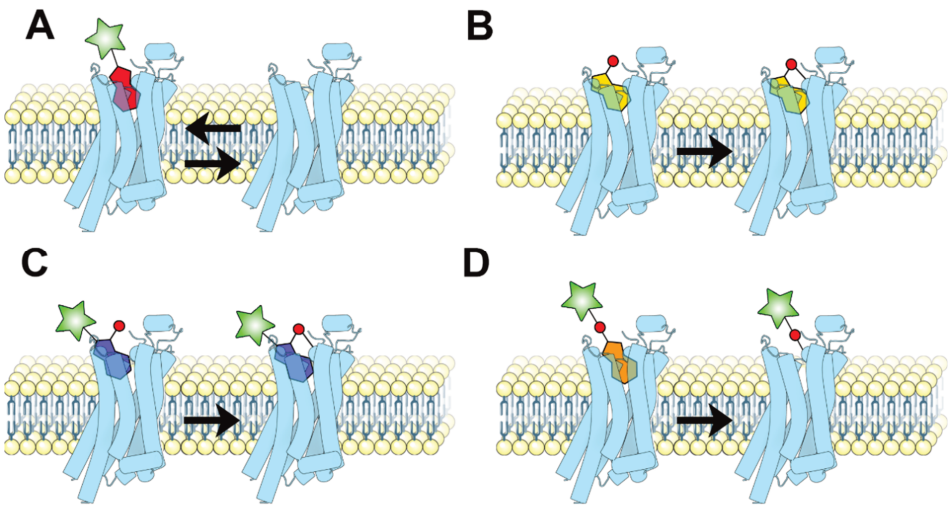


Figure 3. Schematic overview of the types of probes that have been developed for the adenosine receptors. (A) Reversible probes; (B) Covalent ligands; (C) Affinity-based probes; (D) Ligand-directed probes. This figure was partly created with Protein Imager,[20] using the structure of the A_{2A}AR (PDB: 7ARO).

Affinity-based probes are covalent ligands functionalized with reporter groups and consist of three parts: a high affinity ligand to induce selectivity; an electrophilic or photoreactive group ('warhead') that ensures covalent bond formation between probe and receptor; and a reporter group that allows detection of the probe-bound receptor in biochemical assays (Figure 3C). Early examples of affinity-based probes for ARs contain a radioisotope that is directly conjugated to the molecular scaffold (one-step probes),[58–60] while more recent examples of affinity-based probes use click chemistry to 'click' detection moieties onto the probe-bound receptors *in situ* (two-step probes).[61–64]

Ligand-directed probes consist of the same three parts as affinity-based probes: a high affinity ligand to induce selectivity; an electrophilic group that reacts with a nearby amino acid residue; and a reporter group that allows detection of all probe-bound receptors. However, ligand-directed probes use a different type of electrophile: upon binding covalently to the receptor, the high affinity-ligand acts as leaving group (Figure 3D). This means that the binding pocket of the receptor is 'free' to bind other ligands, which allows studies towards receptor activation upon binding to various (partial) agonists. Both one-step and two-step ligand-directed probes have been developed for the ARs.[65,66]

Aim and Outline of This Thesis

The adenosine receptors are interesting protein targets from a drug discovery perspective. However, targeting specific AR pathways is hampered by the wide expression of ARs and their multitude of functionalities. Besides that, the inherent properties of being GPCRs, such as poor solubility, low expression levels, PPIs, PTMs and subcellar localization, all add extra layers of complexity to AR behavior. Therefore, in this thesis, we aim to develop new chemical probes that allow the detection of ARs in a broad range of assay types, in order to both overcome and study the abovementioned complexities. These chemical probes include covalent ligands, affinity-based probes and ligand-directed probes.

Chapter 2 provides an overview of all the small molecular probes that have been developed to covalently functionalize the whole family of GPCRs. Various types of probes are discussed, as well as their potential applications in GPCR research. **Chapter 3** describes the development of a covalent ligand for the adenosine A_{2B} receptor. A set of potential covalent ligands for the A_{2B}AR was synthesized and the effect of location and type of electrophile ('warhead') is evaluated in this chapter. In **Chapter 4** the development of an affinity-based probe for the adenosine A_1 receptor is reported. The synthesis of the probe is described, as well as the evaluation of the probe in radioligand binding assays. Furthermore, utilization of the affinity-based probe in SDS-PAGE, pull-down proteomics and microscopy experiments is described. **Chapter 5** reports the development of an affinity-based probe for the adenosine A_3 receptor. Likewise, synthesis and pharmacological evaluation of the affinity-based probe are reported. This chapter also shows the application of the affinity-based probe in SDS-PAGE, microscopy and flow cytometry experiments to detect both overexpressed and endogenous A_3AR. **Chapter 6** builds onto the work of chapter 3 and describes the development of a ligand-directed probe based on the aforementioned A_{2B}AR covalent ligand. Reactivity, selectivity and functionality of the ligand-directed probe are evaluated in this chapter. Finally, **Chapter 7** gives an overview of the developed probes and their use in various types of biochemical assays. In conclusion, future applications of the herein presented probe types are this discussed in this chapter.

References

[1] B. B. Fredholm, K. Bättig, J. Holmén, A. Nehlig, E. E. Zvartau, *Pharmacol Rev* **1999**, *51*, 83–133.

[2] T. de Gubareff, W. Jr. Sleator, *J Pharmacol Exp Ther* **1965**, *148*, 202–214.

[3] H. Shimizu, J. W. Daly, C. R. Creveling, *J Neurochem* **1969**, *16*, 1609–1619.

[4] A. Sattin, T. W. Hall, *Mol Pharmacol* **1970**, *6*, 13–23.

[5] B. B. Fredholm, A. P. IJzerman, K. A. Jacobson, K.-N. Klotz, J. Linden, *Pharmacol Rev* **2001**, *53*, 527–552.

[6] B. B. Fredholm, A. P. IJzerman, K. A. Jacobson, J. Linden, C. E. Müller, *Pharmacol Rev* **2011**, *63*, 1–34.

[7] A. P. IJzerman, K. A. Jacobson, C. E. Müller, B. N. Cronstein, R. A. Cunha, *Pharmacol Rev* **2022**, *74*, 340–372.

[8] S. M. Foord, T. I. Bonner, R. R. Neubig, E. M. Rosser, J. P. Pin, A. P. Davenport, M. Spedding, A. J. Harmar, *Pharmacol Rev* **2005**, *57*, 279–288.

[9] D. M. Rosenbaum, S. G. F. Rasmussen, B. K. Kobilka, *Nature* **2009**, *459*, 356–363.

[10] M. M. Papasergi-Scott, G. Pérez-Hernández, H. Batebi, Y. Gao, G. Eskici, A. B. Seven, O. Panova, D. Hilger, M. Casiraghi, F. He, L. Maul, P. Gmeiner, B. K. Kobilka, P. W. Hildebrand, G. Skiniotis, *bioRxiv* **2023**, 2023.03.20.533387.

[11] N. Wettschureck, S. Offermanns, *Physiol Rev* **2005**, *85*, 1159–1204.

[12] H. Kühn, U. Wilden, *Journal of Receptors and Signal Transduction* **1987**, *7*, 283–298.

[13] J. L. Benovic, H. Kchnt, I. Weyandt, J. Codinat, M. G. Caron, R. J. Lefkowitz, *Proceedings of the National Academy of Sciences* **1987**, *84*, 8879–8882.

[14] M. J. Lohse, J. L. Benovic, J. Codina, M. G. Caron, R. J. Lefkowitz, *Science (1979)* **1990**, *248*, 1547–1550.

[15] S. K. Shenoy, R. J. Lefkowitz, *Trends Pharmacol Sci* **2011**, *32*, 521–533.

[16] D. Wootten, A. Christopoulos, M. Marti-Solano, M. M. Babu, P. M. Sexton, *Nat Rev Mol Cell Biol* **2018**, *19*, 638–653.

[17] C. K. Goth, U. E. Petäjä-Repo, M. M. Rosenkilde, *ACS Pharmacol Transl Sci* **2020**, *3*, 237–245.

[18] A. Patwardhan, N. Cheng, J. Trejo, *Pharmacol Rev* **2021**, *73*, 120–151.

[19] M. Ali, M. Nezhady, J. C. Rivera, S. Chemtob, *iScience* **2020**, *23*, 101643.

[20] G. Tomasello, I. Armenia, G. Molla, *Bioinformatics* **2020**, *36*, 2909–2911.

[21] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S. A. A. Kohl, A. J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, *Nature* **2021**, *596*, 583–589.

[22] M. Varadi, S. Anyango, M. Deshpande, S. Nair, C. Natassia, G. Yordanova, D. Yuan, O. Stroe, G. Wood, A. Laydon, A. Žídek, T. Green, K. Tunyasuvunakool, S. Petersen, J. Jumper, E. Clancy, R. Green, A. Vora, M. Lutfi, M. Figurnov, A. Cowie, N. Hobbs, P. Kohli, G. Kleywegt, E. Birney, D. Hassabis, S. Velankar, *Nucleic Acids Res* **2022**, *50*, D439–D444.

[23] A. S. Hauser, M. M. Attwood, M. Rask-Andersen, H. B. Schiöth, D. E. Gloriam, *Nat Rev Drug Discov* **2017**, *16*, 829–842.

[24] D. Yang, Q. Zhou, V. Labroska, S. Qin, S. Darbalaei, Y. Wu, E. Yuliantie, L. Xie, H. Tao, J. Cheng, Q. Liu, S. Zhao, W. Shui, Y. Jiang, M. W. Wang, *Signal Transduct Target Ther* **2021**, *6*, 7.

[25] P. A. Borea, S. Gessi, S. Merighi, F. Vincenzi, K. Varani, *Physiol Rev* **2018**, *98*, 1591–1625.

[26] C. Cekic, J. Linden, *Nat Rev Immunol* **2016**, *16*, 177–192.

[27] B. Allard, D. Allard, L. Buisseret, J. Stagg, *Nat Rev Clin Oncol* **2020**, *17*, 611–629.

[28] P. J. M. Van Galen, A. H. Van Bergen, C. Gallo-Rodriguez, N. Melman, M. E. Olah, A. P. IJzerman, G. L. Stiles, K. A. Jacobson, *Mol Pharmacol* **1994**, *45*, 1101–1111.

[29] K. A. Jacobson, A. P. IJzerman, J. Linden, *Drug Dev Res* **1999**, *47*, 45–53.

[30] D. Meibom, B. Albrecht-Küpper, N. Diedrichs, W. Hübsch, R. Kast, T. Krämer, U. Krenz, H. G. Lerchen, J. Mittendorf, P. G. Nell, F. Süssmeier, A. Vakalopoulos, K. Zimmermann, *ChemMedChem* **2017**, *12*, 728–737.

[31] Y. Yun, J. Chen, R. Liu, W. Chen, C. Liu, R. Wang, Z. Hou, Z. Yu, Y. Sun, A. P. IJzerman, L. H. Heitman, X. Yin, D. Guo, *Biochem Pharmacol* **2019**, *164*, 45–52.

[32] C. J. Draper-Joyce, R. Bhola, J. Wang, A. Bhattarai, A. T. N. Nguyen, I. Cowie-Kent, K. O'Sullivan, L. Y. Chia, H. Venugopal, C. Valant, D. M. Thal, D. Wootten, N. Panel, J. Carlsson, M. J. Christie, P. J. White, P. Scammells, L. T. May, P. M. Sexton, R. Danev, Y. Miao, A. Glukhova, W. L. Imlach, A. Christopoulos, *Nature* **2021**, *597*, 571–576.

[33] G. Faudone, S. Arifi, D. Merk, *J Med Chem* **2021**, *64*, 7156–7178.

[34] M. A. Schwarzschild, L. Agnati, K. Fuxe, J. F. Chen, M. Morelli, *Trends Neurosci* **2006**, *29*, 647–654.

[35] J. F. Chen, R. A. Cunha, *Purinergic Signal* **2020**, *16*, 167–174.

[36] S. V. Da Silva, M. G. Haberl, P. Zhang, P. Bethge, C. Lemos, N. Gonçalves, A. Gorlewicz, M. Malezieux, F. Q. Gonçalves, N. Grosjean, C. Blanchet, A. Frick, U. V. Nägerl, R. A. Cunha, C. Mulle, *Nat Commun* **2016**, *7*, 11915.

[37] Z. G. Gao, K. A. Jacobson, *Int J Mol Sci* **2019**, *20*, 5139.

[38] Y. Chen, R. Corriden, Y. Inoue, L. Yip, N. Hashiguchi, A. Zinkernagel, V. Nizet, P. A. Insel, W. G. Junger, *Science (1979)* **2006**, *314*, 1792–1795.

[39] R. Corriden, T. Self, K. Akong-Moore, V. Nizet, B. Kellam, S. J. Briddon, S. J. Hill, *EMBO Rep* **2013**, *14*, 726–732.

[40] K. A. Jacobson, S. Merighi, K. Varani, P. A. Borea, S. Baraldi, M. Aghazadeh Tabrizi, R. Romagnoli, P. G. Baraldi, A. Ciancetta, D. K. Tosh, Z.-G. Gao, S. Gessi, *Med Res Rev* **2018**, *38*, 1031–1072.

[41] J.-F. Chen, H. K. Eltzschig, B. B. Fredholm, *Nat Rev Drug Discov* **2013**, *12*, 265–286.

[42] A. O. Helbig, A. J. R. Heck, M. Slijper, *J Proteomics* **2010**, *73*, 868–878.

[43] S. J. Piper, R. M. Johnson, D. Wootten, P. M. Sexton, *Chem Rev* **2022**, *122*, 13989–14017.

[44] O. Vit, J. Petrak, *J Proteomics* **2017**, *153*, 8–20.

[45] E. C. Klaasse, A. P. IJzerman, W. J. de Grip, M. W. Beukers, *Purinergic Signal* **2008**, *4*, 21–37.

[46] P. Carriba, G. Navarro, F. Ciruela, S. Ferré, V. Casadó, L. Agnati, A. Cortés, J. Mallol, K. Fuxe, E. I. Canela, C. Lluís, R. Franco, *Nat Methods* **2008**, *5*, 727–733.

[47] G. Navarro, P. Carriba, J. Gandía, F. Ciruela, V. Casadó, A. Cortés, J. Mallol, E. I. Canela, C. Lluis, R. Franco, *ScientificWorldJournal* **2008**, *8*, 1088–1097.

[48] S. Ferré, F. Ciruela, *J Caffeine Adenosine Res* **2019**, *9*, 89–97.

[49] C. T. Lauren May, E. A. Vecchio, J.-A. Baltos, A. T. N Nguyen, A. Christopoulos, P. J. White, L. T. May, E. Vecchio, J. Baltos, *Br J Pharmacol* **2018**, *175*, 4036.

[50] R. Franco, A. Cordomí, C. Llinas del Torrent, A. Lillo, J. Serrano-Marín, G. Navarro, L. Pardo, *Cellular and Molecular Life Sciences* **2021**, *78*, 3957–3968.

[51] X. Yang, L. H. Heitman, A. P. IJzerman, D. van der Es, *Purinergic Signal* **2021**, *17*, 85–108.

[52] M. Soave, L. A. Stoddart, C. W. White, L. E. Kilpatrick, J. Goulding, S. J. Briddon, S. J. Hill, *FEBS Journal* **2021**, *288*, 2585–2601.

[53] M. Jo, S. T. Jung, *Exp Mol Med* **2016**, *48*, e207.

[54] L. Dahl, I. B. Kotliar, A. Bendes, T. Dodig-Crnković, S. Fromm, A. Elofsson, M. Uhlén, T. P. Sakmar, J. M. Schwenk, *bioRxiv* **2022**, 2022.11.24.517810.

[55] E. Kozma, P. Suresh Jayasekara, L. Squarcialupi, S. Paoletta, S. Moro, S. Federico, G. Spalluto, K. A. Jacobson, *Bioorg Med Chem Lett* **2013**, *23*, 26–36.

[56] S. Federico, L. Lassiani, G. Spalluto, *Pharmaceuticals* **2019**, *12*, 168.

[57] D. Weichert, P. Gmeiner, *ACS Chem Biol* **2015**, *10*, 1376–1386.

[58] A. Patel, R. H. Craig, S. M. Daluge, J. Linden, T. Burroughs, *Mol Pharmacol* **1988**, *33*, 585–591.

[59] W. W. Barrington, K. A. Jacobson, G. L. Stiles, *Mol Pharmacol* **1990**, *38*, 177–183.

[60] D. R. Luthin, K. S. Lee, D. Okonkwo, P. Zhang, J. Linden, *J Neurochem* **1995**, *65*, 2072–2079.

[61] X. Yang, T. J. M. Michiels, C. de Jong, M. Soethoudt, N. Dekker, E. Gordon, M. van der Stelt, L. H. Heitman, D. van der Es, A. P. IJzerman, *J Med Chem* **2018**, *61*, 7892–7901.

[62] P. N. H. Trinh, D. J. W. Chong, K. Leach, S. J. Hill, J. D. A. Tyndall, L. T. May, A. J. Vernall, K. J. Gregory, *J Med Chem* **2021**, *64*, 8161–8178.

[63] B. L. H. Beerkens, Ç. Koç, R. Liu, B. I. Florea, S. E. Le Dévédec, L. H. Heitman, A. P. IJzerman, D. van der Es, *ACS Chem Biol* **2022**, *17*, 3131–3139.

[64] B. L. H. Beerkens, I. M. Snijders, J. Snoeck, R. Liu, A. T. J. Tool, S. E. Le Dévédec, W. Jespers, T. W. Kuijpers, G. J. P. van Westen, L. H. Heitman, A. P. IJzerman, D. van der Es, *ChemRxiv* **2023**, DOI 10.26434/chemrxiv-2023-6g59z.

[65] S. M. Moss, P. S. Jayasekara, S. Paoletta, Z. G. Gao, K. A. Jacobson, *ACS Med Chem Lett* **2014**, *5*, 1043–1048.

[66] L. A. Stoddart, N. D. Kindon, O. Otun, C. R. Harwood, F. Patera, D. B. Veprintsev, J. Woolard, S. J. Briddon, H. A. Franks, S. J. Hill, B. Kellam, *Commun Biol* **2020**, *3*, 722.