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Affinity-based profiling of the adenosine receptors

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Chapter 7

Conclusions and Future Prospects

Conclusions

On the development of covalent probes to target adenosine receptors

This thesis summarizes the development of various types of covalent probes to target the adenosine A₁, A_{2B} and A₃ receptors. As discussed in **chapter 2**, the use of covalent small molecules in GPCR labelling studies has several benefits, one being the opportunity to target *endogenous* receptors, another the possibility to use *denaturing* assay conditions. However, several aspects have to be taken into account upon developing a *valid* covalent probe for GPCR labelling studies:

1. Selectivity

First of all, covalent probes should bear sufficient selectivity towards their target GPCR, in order to prevent covalent binding to off-target proteins. Due to the low expression levels of GPCRs, off-target labelling can easily hamper the detection of target GPCRs (**chapters 4-6**). A selective pharmacophore should therefore be included in the design of covalent probes. Fortunately, over the past two decades, selective ligands have been developed for all four of the adenosine receptors. In **chapters 3** and **6** probe design was based on the A_{2B}AR antagonist PSB 1115.^[1] In **chapter 3** we observed a drop in selectivity upon substitution of the prime scaffold with a 'mere' propyl group, indicating that small changes to the molecular scaffold can have a big influence on the selectivity of the probe. In **chapter 4** probe design was based on the covalent A₁AR agonist LUF7746, in turn derived from the clinical candidate Capadenoson.^[2,3] In this study, we observed a loss in affinity for three out of four of the alkyne-substituted probes, indicating that location of the substitution also drastically effects the on-target affinity of the probe. In **chapter 5** probe design was based on the covalent A₃AR antagonist LUF7602.^[4] Contrary to the previous chapters, three different locations of the alkynyl substituent did not decrease affinity nor selectivity towards the A₃AR. Nevertheless, on- and off-target selectivity is greatly dependent on the selected molecular scaffold and thus should be investigated per case.

2. Reactivity

Secondly, a reactive warhead should be chosen that covalently binds the target nucleophilic amino acid residue, but not amino acid residues on unrelated proteins present in the sample. Thus, reactivity of the warhead also has a strong influence on overall selectivity of the probe. Besides that, the warhead should bear hydrolytic stability under standard biochemical assay conditions. **Chapter 2** lists the various types of warheads that have been implemented in covalent GPCR probes. In **chapter 3** we investigated three lysine-targeting warheads: fluorosulfonyl, fluorosulfonate and isothiocyanate groups, to covalently bind the A_{2B}AR. In this study the fluorosulfonyl group showed optimal reactivity, while the fluorosulfonate group was not reactive enough, presumably due to an unstable reaction product, and the isothiocyanate group was too reactive, as observed in the increased binding towards other adenosine receptors. The fluorosulfonyl group was therefore our electrophile of choice, also in **chapters 4** and **5**. In **chapter 6** we implemented the electrophilic *N*-acyl-*N*-alkyl sulfonamide (NASA) group as warhead in a ligand-directed probe. Although labelling of the A_{2B}AR was observed in SDS-PAGE experiments, the probe showed to be highly reactive under standard cell culture conditions. Changing the substituents of the NASA group, e.g. replacing the cyano moiety with a less electron withdrawing substituent, might decrease off-target reactivity and result in a more selective probe.^[5] Taken together, the fluorosulfonyl group shows a well-balanced reactivity, and new types of electrophiles should be thoroughly investigated prior and after their implementation in covalent probes.

3. Detectability

Lastly, binding of the covalent probe to the GPCR should lead to a measurable signal in biochemical assays. In **chapter 2** the various detection moieties are listed that have been used to covalently functionalize GPCRs. A distinction can be made between one-step (direct) and two-step (via click chemistry) labelling strategies.^[6–8] The affinity-based and ligand-directed probes from chapters **4**, **5** and **6** all contain an alkyne group that allows the substitution of an azide-conjugated detection moiety via the copper-catalyzed alkyne-azide cycloaddition (CuAAC).^[9,10] This two-step labelling strategy allows the choice between different detection moieties, while using the same stock of probe, in various biochemical assays. This prevents the need for the time-consuming synthesis of one-step probes individually functionalized with detection moieties, such as fluorescent ligands. A drawback of this strategy is the need for cytotoxic click reagents during additional incubation and washing steps, making the probes largely incompatible with live-cell experiments. Bioorthogonal strategies have been developed to overcome the use of cytotoxic reagents, using different click handles such as strained alkynes or tetrazines.^[11–13] However, alkyne groups are synthetically accessible, relatively stable and due to their small size more attractive to implement in pharmacological scaffolds than strained alkynes or tetrazines. Therefore we have limited ourselves to the use of alkyne groups as click handles within our experiments. Nevertheless, in **chapter 5** we managed to overcome the aforementioned drawbacks by performing the click reaction and subsequent purification prior to addition of the covalent probe to live cells. A similar strategy might be used in the future to functionalize other two-step probes targeting ARs prior to their use in live-cell experiments.

In the following paragraphs, a closer look is provided on the detectability of GPCRs, in particular the adenosine receptors, in the various biochemical assay types that have been performed as part of this thesis.

A. SDS-PAGE

Throughout **chapters 4**, **5** and **6** SDS-PAGE experiments have been performed to detect labelled A₁AR, A₃AR and A_{2B}AR. SDS-PAGE has been successfully used in the past as a technique to detect the presence of adenosine receptors, especially the A₁AR and A_{2A}AR, in various tissue types, by making use of radiolabeled photo-affinity probes as tool compounds.^[14–22] In our experiments, detection of the respective AR was most clearly visible upon performing the experiment in overexpressing cell lines, prior to membrane fractionation, click reaction and protein separation. Interestingly, endogenous N-glycosylation of the receptors hampered their initial detection, as the receptor bands appeared as ‘smear’ over a range of molecular weights.^[23–26] In each instance, incubation with PNGase resulted in clear bands at roughly the expected molecular weights. Similar enzymatic incubation steps might also be used in the future to study other post-translational modifications (PTMs). Performing the experiments in membrane fractions, however, resulted in an increase in off-target binding for all of the probes and even prevented detection of the A_{2B}AR with LUF8019 (**chapter 6**). As speculated in **chapters 4** and **5**, we presume the increase in off-target labelling to be caused by a combination of the low expression levels of the respective receptor, the electrophilic character of probes, the high density of other proteins as compared to live cell assays and the presence of intracellular membrane proteins in our membrane fractions. Detection of adenosine receptors in non-overexpressing cell lines showed to be even more cumbersome. Attempts have been done using LUF7909 (A₁AR) in adipocytes (**chapter 4**), but also using LUF7487 (A_{2A}AR) and LUF7960 (A₃AR) in various cancerous cell lines with detectable mRNA expression levels of the respective receptor (data not included). In the latter two experiments, no labelling of the respective AR was observed, while labelling of off-targets was evident. Thus, it seems that the low expression levels of the ARs hinder proper detection in SDS-PAGE experiments, partly due to the inherent reactivity of the electrophilic groups. Taken together,

the herein developed covalent probes should be used in SDS-PAGE experiments to verify binding of ligands, probes and other tool molecules to purified, overexpressed or highly expressed ARs, but not as a technique to detect expression levels of endogenous receptors on cells and tissues.

B. Fluorescent Microscopy

In **chapters 4** and **5** fluorescent microscopy has been used as a technique to detect probe labelled ARs. A considerable amount of fluorescent ligands for ARs has already been developed in the past,^[27] yet it still is a lively topic of research.^[28–33] These studies deviate from our work by developing one-step and reversible probes, thereby requiring extensive synthetic steps and limiting the number of applications. The herein reported experiments on the A₁AR and A₃AR have been *proof of concept* studies to showcase selective labelling of the respective receptors in microscopy experiments. A drawback of the used experimental setup is the need for a fixation step, prior to incubation with click reagents. However, this hurdle might be overcome by performing the click reaction *a priori* to the first cellular incubation steps, as has been done in case of the flow cytometry experiments in **chapter 5**. Although selective labelling of the A₁ARs and A₃ARs was observed on overexpressing CHO cells, future work should unveil whether selective labelling of the ARs can also be detected in native/primary cells and tissues. Interestingly, selective labelling of the A_{2A}AR on a breast cancer cell line has recently been performed using a ligand-directed probe, showing the future potential of covalently binding probes.^[34] Next to that, labelling by the partial agonist probe LUF7909 (**chapter 4**) yielded information on internalization of the A₁AR. Taken together, the use of affinity-based probes in fluorescent microscopy experiments is thus a valid strategy to study (sub)cellular receptor localization in overexpressing cell lines and potentially receptor expression in non-overexpressing cells.

C. Flow Cytometry

In **chapter 5** flow cytometry has been used as technique to detect probe labelled A₃ARs. Instead of fixing cells, as has been done throughout the microscopy assays, covalent probe LUF7960 was clicked to a Cy5 fluorophore prior to the addition to live cells. The pre-click step yielded a probe, LUF7960-Cy5, that had a ~20-fold lower affinity towards the A₃AR (data not included), but allowed us to circumvent an additional incubation step with cytotoxic click reagents. Gratifyingly, the pre-clicked probe showed A₃AR-selective labelling in A₃AR-overexpressing CHO cells, as well as native eosinophils. Fluorescent ligands for the A_{2A}AR, A_{2B}AR and A₃AR have been used in flow cytometry experiments before, however mostly for competition binding experiments.^[30,33,35–37] To the best of our knowledge, utilization of chemical probes for the detection of AR expression on native cells has thus far been reported only once.^[37] In our experiments, commercial A₃AR antibodies did not show selective labelling of the A₃AR, presumably due to off-target activity.^[38] As opposed to antibodies, chemical probes compete with agonists and antagonists for a ligand binding pocket on their respective receptor target. Competition experiments can therefore be used as control to verify selective labeling of the receptor, while such control experiments are not possible for the often poorly selective antibodies.^[38–40] Altogether, covalent probes are interesting tool molecules for the detection of AR expression on native cells and tissues in flow cytometry experiments and show more promise than AR-targeting antibodies. The combination of covalent probes and flow cytometry is therefore an interesting strategy to map expression of the ARs in pathological and physiological conditions.

D. Chemical Proteomics

In **chapter 4** biotin-click and subsequent proteomic pull-down experiments have been performed to detect the A₁AR using LC-MS/MS. Gratifyingly, almost 50% of the A₁AR peptide sequence was detected, an amount that is remarkably high for a GPCR. Detection of non-purified GPCRs by LC-MS/MS techniques is often hampered by the low expression levels of GPCRs, even in overexpressing cell lines. The use of covalent probes to pull-down target GPCRs from a complex protein mixture, thereby strongly reducing background noise, is therefore a path forward for detection in LC-MS/MS experiments. Next to that, we found the type of digestion enzyme (e.g. trypsin vs. chymotrypsin) to play an important role in the successful detection of the resulting peptides. Remarkably, pull-down experiments on GPCRs are often carried out using trypsin as digestion enzyme,^[41–47] while the seven transmembrane helices contain little to zero sites that are susceptible to trypsin cleavage. In an ideal situation, multiple digestion enzymes are screened to find the optimal conditions for peptide formation. Next to the experiments on the A₁AR, we also performed pull-down experiments with LUF7960 (**chapter 5**) as a tool for the detection of the A₃AR on overexpressing CHO cells. Unfortunately, we were not able to reliably detect the receptor. There are multiple possible reasons for this, such as the low expression of A₃AR as compared to the A₁AR on CHO cells, the poor solubility of the receptor due to its relatively small extracellular regions, or poor compatibility with the digestion enzyme chymotrypsin. It is therefore still questionable whether the current assay setup allows successful pull-down experiments on cells endogenously expressing ARs. However, we think that optimizing the aforementioned problems of solubility and digestion, as well as further reducing the background noise, will lead to an improved detection of the ARs. Ultimately, the detection of ARs in pull-down experiments will yield information that is difficult to obtain in SDS-PAGE, flow cytometry and fluorescent microscopy experiments, such as the presence of PTMs and interactions with other (non-defined) proteins. Taken together, using covalent probes in pull-down proteomics has the promise to be an important strategy for future studies on the ARs.

Altogether, the herein developed covalent probes are promising tool molecules to detect and study the ARs in a multitude of biochemical assays. Each of the abovementioned biochemical assays bears its own challenges, ranging from off-target labelling to lack of sensitivity. The work described in this thesis addresses these issues, bringing the application of covalent AR probes one step further towards general usage. Having this ‘toolbox’ of covalent probes allows investigations towards many interesting aspects of AR signaling, that differ from the applications of reversible probes, antibodies and genetic techniques.

Future Prospects

Detection of endogenous adenosine receptor expression

The first application of covalent AR probes that one might think of is the detection of AR expression levels in pathophysiological conditions, e.g. using cell lines, tissues or disease models. Examples would be the investigation of A₁AR expression in fat tissue under various lipolytic conditions,^[48] the investigation of A_{2A}AR and A_{2B}AR expression in tumor models,^[49] and the investigation of A₃AR expression during inflammatory conditions.^[50] The first steps towards covalent endogenous AR detection have been taken in **chapters 4** and **5**, as well as recent work on the A_{2A}AR.^[34] As mentioned in the conclusions, flow cytometry as a technique has, in our view, shown the highest potential to study endogenous expression levels of the ARs. Flow cytometry has already been used to verify binding of covalent probes to other GPCRs.^[44,51–55] For example, experiments on the μ opioid receptor (MOR) showed that the fluorescence intensity after probe labeling was similar to the fluorescent intensity upon antibody staining.^[55] Next to that, experiments on the Cannabinoid Receptor 2 (CB₂R) revealed good correlations between the observed fluorescence intensity and mRNA levels in various leukocytes.^[44] Covalent AR probes therefore have the potential to be used complementarily to mRNA techniques, as tool molecules to quantify relative receptor expression between various cell lines. Ideally, both non- and overexpressing cell lines are included in flow cytometry experiments to determine a binding window for thorough quantifications. In the future, such experiments might be performed to determine the total receptor expression level per cell type, as an alternative to the expensive radioligand binding experiments currently used for these purposes.

A glimpse of other possible future applications of covalent GPCR probes has already been given in the outlook of **chapter 2**. These applications will be further elaborated in the paragraphs below.

Investigation of (sub)cellular receptor localization

Upon agonist-induced activation and G protein dissociation, the intracellular regions of GPCRs are prone to phosphorylation and subsequent arrestin binding, in turn leading to internalization of the GPCR via endosomal vesicles. According to current understanding, the fate of internalized GPCRs is dependent on multiple factors, such as PTMs, protein-protein interactions (PPIs) and protein-lipid interactions.^[56,57] The most well-studied consequences of internalization are proteolytic degradation of the GPCR in lysosomes, recycling of the GPCR to the plasma membrane and intracellular signaling via arrestin. All of the former lead to a reduced response (desensitization) towards extracellular ligand binding. Over the past decade, a great amount of work has been carried out to unravel signaling activity of GPCRs upon internalization into cellular vesicles, exemplified by recent crystallographic work on the adrenergic receptors.^[58,59] In case of the adenosine receptors, various extents of desensitization have been observed between cell- and tissue types.^[57,60] However, receptor presence and/or activity via intracellular vesicles is still uncertain. Covalent functionalization of adenosine receptors with fluorescent groups may give answers to these questions, as covalent probes allow fluorescently labelled receptors to be traced in confocal microscopy experiments. Unfortunately agonist-induced internalization cannot be studied by an antagonist AfBP (LUF7960; **chapter 5**), though agonist AfBPs (LUF7909; **chapter 4**) and ligand-directed probes (LUF8019; **chapter 6**) can be suitable tools for this purpose.

Taking the A₁AR as an example, internalization of the receptor has been studied by genetically engineering a yellow fluorescent protein (YFP),^[61] or a HiBiT tag onto the receptor,^[62] or by using anti-A₁AR antibodies.^[63–66] This has yielded valuable insights into receptor half-life time, endocytosis and PPIs of the A₁AR, yet requires the use of engineered receptors or ‘large’, often poorly selective, antibodies. Functionalization of the A₁AR by a small alkyne group for click chemistry is therefore an interesting opportunity to study internalization of native A₁ARs. Taking the A₃AR as another example, localization and clustering has been detected at the leading edge and on specific microdomains of membranes from activated neutrophils.^[67,68] Most interestingly, a covalent ligand-directed probe has recently been used in imaging flow cytometry experiments to measure CB₂R localization on cells.^[54] Studying location-dependent signaling of the A₃AR via imaging flow cytometry might therefore be an interesting case study for the use of the herein developed covalent probes.

Lastly, a large number of GPCRs has also been found to signal from the membranes of intracellular organelles, such as the mitochondria, nucleus, endoplasmic reticulum (ER) and Golgi apparatus.^[69] This has thus far not been observed for the four ARs. However, covalent probes may contribute to the discovery of novel intracellular signaling ‘hotspots’ through functionalization of the receptor with fluorescent groups and detection by confocal microscopy. One requirement for such assays would be proper membrane permeability of the probe, which therefore should be investigated *a priori*.

Characterization of post-translational modifications

All GPCRs are predicted to contain one or more PTMs of which the functions range from altering signaling pathways to intracellular trafficking from and to the plasma membrane.^[70] Phosphorylation is the most well-studied PTM and, as mentioned above, plays a role in the internalization and desensitization of GPCRs. Phosphorylation of GPCRs takes place on the intracellular domain and is mediated by the protein family of GPCR Kinases (GRKs). More recently, specific phosphorylation patterns (‘barcodes’) have been found to induce specific downstream signaling pathways that vary from other phosphorylation barcodes.^[71,72] Thus, the amount and pattern of phosphorylation are of physiological importance. In case of the ARs, for example the A₁AR, phosphorylation has been studied by using radioactive phosphorous isotopes in SDS-PAGE experiments.^[73,74] More recent experiments on other GPCRs avoid the use of radioactive isotopes and have moved to antibodies in SDS-PAGE experiments and purified GPCRs in mass spectrometry experiments.^[71,72,75] The herein reported covalent probes may offer a more sophisticated approach to study phosphorylation of ARs. First of all, covalent probes can be used in SDS-PAGE experiments *in tandem* with phospho-specific antibodies to investigate presence of phosphorylated residues. This, however, will not yield information about the phosphorylation barcode itself. Instead, using covalent probes to pull-down the respective AR for phosphoproteomics experiments can yield quantitative information on the exact peptides and/or residues that have been phosphorylated,^[76] avoiding the need for extensive purification procedures.

Besides phosphorylation, all GPCRs are predicted to contain one or more glycosylated asparagine residue(s) (*N*-glycosylation) in the extracellular region, necessary for trafficking of the GPCR from the ER and Golgi to the plasma membrane.^[70,77] Next to that, *N*-glycosylation might have an effect on ligand binding and (biased) signaling.^[70] Glycosylation of serine or threonine residues (*O*-glycosylation) has also been observed for a number of GPCRs. Most interestingly, it has been proposed that *O*-glycosylation, together with tyrosine sulfonylation, may dictate a barcode that regulates signaling of GPCRs, case of study being the Chemokine Receptor 5 (CCR5).^[78] Contrary to the chemokine receptors, the ARs contain short intra- and extracellular regions and are therefore not predicted to be *O*-glycosylated. However, as observed in **chapters 4-6**, *N*-glycosylation of ARs is evident and can be studied with the herein

developed covalent probes. Utilization of the covalent probes in SDS-PAGE and pull-down proteomics experiments may yield information on the presence and location of the *N*-glycosylated residues, e.g. by combining with site-directed mutagenesis experiments or sophisticated proteomics analyses. An interesting development is the field of glycoproteomics, in which the glycan chains on specific proteins are analyzed on their sequence and structure by LC-MS/MS techniques.^[79] In the future, such experiments might be combined with pull-down steps to enrich probe-bound GPCRs from native samples. However, detection problems that arise from the low expression levels of GPCRs first have to be overcome.

Further than that, other PTMs have been observed on GPCRs, examples being ubiquitination, palmitoylation, SUMOylation, S-nitrosylation and methylation.^[70,77] While palmitoylation of ARs has been observed in early studies,^[80,81] it is still questionable whether any other PTMs are present on the ARs. Covalent probes may help to elucidate the presence/absence of PTMs in a manner as mentioned before: through labelling in SDS-PAGE experiments, e.g. in combination with antibodies or labels specific towards the target PTM, or through pull-down experiments with subsequent LC-MS/MS analysis.

Lastly, endogenous GPCRs can be prone to proteolytic cleavage. As example, cleavage of the *N*-terminus of the β 1-Adrenergic receptor resulted in a population of cleaved and non-cleaved receptors, that both regulated their own signaling pathways *in vivo*.^[82] In our studies, we also observed two populations of A_{2A}AR upon labelling in SDS-PAGE experiments (data not included), although this is most likely due to the presence of proteases in the used membrane fractions. Nevertheless, covalent probes may be used to decipher the possible physiological impact of proteolytic cleavage.

Unravelment of protein-protein interactions

The most well-known protein interaction partners of GPCRs are the G protein subunits and arrestin variants. Classically, GPCRs have been found to signal via either G protein or arrestin. However, recent findings hint towards the formation of 'megacomplexes' in endosomal vesicles: signaling units that consists of a GPCR bound to both G protein and arrestin.^[83] More interestingly, signaling 'nanodomains' of the glucagon-like peptide 1 receptor (GLP1R), as well as β 2-adrenergic receptor (β 2AR) have been observed on the plasma membranes of model cell lines.^[84] Within these so-called receptor-associated independent cAMP nanodomains ('RAINS'), agonist-induced cAMP formation was only observed within a few nanometers of the respective GPCR. Taken together, this indicates the presence of GPCR-signaling compartments in and near the plasma membrane, as well as on intracellular vesicles.^[84] In fact, modern electron microscopy experiments reveal cells to be highly packed with vesicles, organelles and proteins.^[85,86] It is therefore highly likely that GPCRs, even prior to agonist-binding, reside in close proximity to the proteins that are involved in their signal transduction pathways.

The ARs have been found to interact with multiple types of G proteins,^[87] β -arrestins, GRKs and clathrin, among other proteins.^[57] Next to that, interactions between ARs and the same (homodimers) or other (heterodimers) GPCRs have been observed,^[88] mostly through co-immunoprecipitation or FRET-based assays. Interestingly, the C-terminus tail of the A_{2A}AR is significantly longer than the C-terminus of other ARs and has been found to interact with various proteins such as actinin,^[89] calmodulin,^[90,91] and Cathepsin D.^[92] Multiple factors, e.g. state of the receptor, cell type, point in time, and molecular composition of the 'nanodomain', presumably influence protein binding towards the A_{2A}AR, since it is unlikely that all of the reported proteins simultaneously occupy the receptor.^[93] It would therefore be very interesting, also from a physiological perspective, to be able to map all the PPIs of ARs at specific points in space and time. Covalent probes may be the right tool molecules for this purpose.

First, dual labelling of the respective AR and its PPI might be studied in SDS-PAGE experiments, similar to co-immunoprecipitation studies. However, this requires predetermined knowledge about the nature of the PPI, as well as verified probes to label each protein interaction partner. More promising would be the detection of PPIs in a high throughput fashion, e.g. in chemical proteomics experiments. As an example, peroxidase-catalyzed proximity labelling has shown to be a promising strategy for the MS-based detection of GPCR PPIs, likely requiring the use of genetically altered receptors.^[94] Interestingly, after a pull-down of the A₁AR (**chapter 4**) we observed a significant enrichment of the G protein beta-1 subunit, a well-known PPI of the A₁AR. Similarly, presumable PPIs of the dopamine D₂ receptor have recently been mapped using a photo-affinity probe.^[47] In both cases however, enrichment of the protein interaction partners has been dependent on the reversible nature of the PPI. Such binding is easily disrupted during one of the many denaturing steps in a pull-down assay protocol and therefore not always reliable. More interestingly, pull-down strategies with covalent probes might be combined with cross-linking proteomics experiments.^[95] Within these experiments, a cross-linking agent is added to cross-link proteins that are within a determined range of one another. Subsequent pull-down experiments with a covalent probe will then pull-down the respective AR, as well as all of its cross-linked proteins. This in turn leads to the detection of a whole ‘interactome’ of signaling proteins, dependent on the ‘space and time’ of the addition of the crosslinking agent. Altogether, this might lead to the detection of physiologically important PPIs involving the ARs.

Final notes

This thesis describes the development, verification and application of various types of covalent probes to target the adenosine receptors. These include a covalent ligand for the A_{2B}AR, affinity-based probes for the A₁AR and A₃AR and a ligand-directed probe for the A_{2B}AR. The applicability of the covalent probes has been investigated in SDS-PAGE, confocal microscopy, flow cytometry and chemical proteomics experiments, using either membrane fractions, model cell lines or native cells. Altogether, we hope that this thesis offers valuable information on the usage and limitations of covalent probes in various types of biochemical assays. Finally, we hope that the herein developed probes lead to new insights regarding adenosine receptor signaling, ultimately leading to more rational targeting of the adenosine receptors within drug discovery programs.

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