



Universiteit
Leiden
The Netherlands

Affinity-based profiling of the adenosine receptors

Beerkens, B.L.H.

Citation

Beerkens, B. L. H. (2023, November 9). *Affinity-based profiling of the adenosine receptors*. Retrieved from <https://hdl.handle.net/1887/3656497>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3656497>

Note: To cite this publication please use the final published version (if applicable).

Chapter 2

Covalent Functionalization of G Protein-Coupled Receptors by Small Molecular Probes

Bert L.H. Beerkens, Adriaan P. IJzerman and Daan van der Es.

Abstract

Roughly one third of all marketed drugs act via binding to one or multiple of the >800 human GPCRs, mostly through activation or inhibition via the orthosteric binding site. Next to that, novel strategies to alter GPCR functioning are being developed, including allosteric, biased and covalently binding ligands. Molecular probes play an important role to verify such drug molecules with new modes of action and provide information on all factors involved in GPCR-signaling. Various flavors of molecular probes have been developed, ranging from small molecules to antibodies, each bearing its own advantages and disadvantages. In this mini-review, a closer look is taken at small molecular probes that functionalize GPCRs in a covalent manner, e.g. through conjugation of reporter groups such as a fluorophore or biotin. Covalently bound reporter groups allow the investigation of GPCRs in an increasing amount of biochemical assay types, yielding new information on GPCR-signaling pathways. Here, a broad range of recently developed 'functionalized covalent probes' is summarized. Furthermore, the use of these probes in biochemical assays, as well as their applications in the field of GPCR research are discussed. Lastly, a view on possible future applications of these types of small molecular probes is provided.

1. Introduction

G protein-coupled receptors (GPCRs) are transmembrane proteins that share the same structural features: seven trans-membrane helices, three extra- and three intracellular loops, an extracellular N-terminus and an intracellular C-terminus. The human family of GPCRs comprises >800 members, divided over multiple subfamilies classified by their ligand binding partners.^[1] Upon binding to extracellular stimuli, GPCRs undergo conformational changes, in turn inducing a cascade of intracellular signaling events. GPCRs thus allow cells to respond to molecules in the extracellular environment. Signaling pathways that originate from GPCRs can have a major influence on the physiology of the cell, and many pathophysiological conditions have been linked to the activation or malfunctioning of GPCRs.^[2] Such findings have led to a surge in GPCR drug discovery at the end of the 20th century, resulting in a high amount (>500) of currently marketed drugs targeting GPCRs.^[3] Besides the development of 'classical' orthosteric ligands, current strategies to modulate GPCR functioning include new types of small molecules, examples being biased, allosteric, bitopic and covalent ligands.^[4] To take advantage of these novel modulation strategies, it is important to study these ligands and understand their molecular mechanisms of action.

Studying GPCRs however, has many challenges that share some common grounds. Firstly, most GPCRs have a relatively low basal expression level on native cells, as compared to e.g. cytosolic proteins, hindering purification and characterization.^[5] Secondly, most GPCRs are believed to contain multiple post-translational modifications (PTMs), such as extracellular glycan modifications, intracellular phosphorylated residues and an intracellular palmitoyl modification, all of which add another dimension of complexity to structure-based and functional studies.^[6,7] Thirdly, having seven transmembrane helices, GPCRs contain a large portion of hydrophobic amino acids that hamper solubilization and require additional surfactants in conventional assay buffers.^[5,8,9] Additionally, other factors, such as cellular localization, protein-protein interactions (PPIs) and proteolytic cleavage all influence the fate of a GPCR in its cellular context.^[7,10] Fortunately, a plethora of chemical and biological probes has been developed as tools to aid the molecular and pharmacological characterizations of GPCRs.^[11–14] Utilization of the right type of probe can help overcome and even study the challenges mentioned above.

Radioactive chemical probes have historically been a primary source for GPCR characterization.^[15] β -emitting radioligands are being used to precisely determine the binding affinity of putative ligands, while positron-emitting radioligands are being used to trace GPCR distribution *in vivo*. Radiolabeled chemical probes, however, require the use of radioactive material and specialized labs. Therefore, in addition to radioligands, fluorescent ligands have been and are being developed as chemical probes for GPCRs.^[13,14] Besides compound screening and visualizing GPCR distribution, fluorescent ligands aid in determining subcellular localization and cellular expression levels of GPCRs, using modern confocal microscopes and flow cytometry techniques, respectively. Next to that, GPCR-targeting antibodies are being developed as biological probes. The use of GPCR antibodies, however, is not without challenges.^[13] While some antibodies have shown successful applications,^[16,17] other antibodies suffer from low selectivity towards their target GPCR.^[17–19] One reason for the lack of selectivity is the low number of possible unique epitopes: the extracellular portion of a GPCR 'merely' consist of an N-terminus and three extracellular loops. Length of these extracellular domains, and thus the ability to be selectively targeted by an antibody, differs greatly per GPCR. Altogether, there is a broad overlap between the applications of radioligands, fluorescent ligands and antibodies.

The chemical and biological probes mentioned above mostly bind in a reversible fashion. A different strategy to study GPCRs is through covalent functionalization, in which the GPCR is

covalently functionalized with a detection group of interest, such as a fluorophore, biotin moiety or 'click' handle. A big advantage of covalent functionalization is the robustness of the bond between GPCR and detection group, allowing the use of washing steps, reductants, oxidants, surfactants and other chemicals in biochemical assays. This allows the investigation of GPCRs in an expanded set of experiment types, for instance SDS-PAGE and pull-down proteomics.

In this mini-review, we will discuss the recently reported small molecular probes that are able to covalently functionalize GPCRs. As the term 'covalent probes' is already used to describe covalent ligands, we will use the term 'functionalized covalent probes' or 'functionalized covalent ligands' throughout this mini-review. As such, we hope to put emphasis on both the reactive groups and the reporter groups. Although covalent GPCR functionalization might also be done through genetic or metabolic alterations,^[12,13] these strategies are not applicable to *native* GPCRs and are therefore beyond the scope of this review. Here, we list most if not all recently developed small molecular probes that covalently functionalize GPCRs. Additionally, we will briefly discuss their applications.

2. Types of Probes

In the next paragraphs, four different types of functionalized covalent probes are being discussed: affinity-based probes (AfBPs) (Figure 1A), ligand-directed probes (LD probes) (Figure 1B), glycan-targeting probes (Figure 1C) and metabolic probes (Figure 1D). Each of these probes label target GPCRs in their own specific manner.

2.1. Affinity-based probes

AfBPs are tool compounds that consist of three functional moieties: (1) a high affinity ligand that promotes selective binding to the protein target of interest, hence the term 'affinity-based'; (2) a reactive group ('warhead') that induces covalent binding to the protein target; (3) a reporter group that allows detection of the probe-bound protein in biochemical assays (Figure 1A). Although such probes have been synthesized for GPCRs for over three decades, the recent term 'affinity-based probes' is a derivation of the term 'activity-based probes', as first coined by Cravatt and coworkers.^[21] Activity-based probes are similar tool compounds but differ in reactivity, as their warheads target nucleophilic amino acid residues within the active site of enzymes. GPCRs on the other hand, do not have such an active site nucleophile that can be targeted. Therefore AfBPs for GPCRs require relatively more reactive warheads.

Two types of warheads can be distinguished: photoreactive groups and electrophilic groups. AfBPs with photoreactive groups, also named 'photo-affinity probes', covalently bind their target GPCR upon irradiation at specific wavelengths. Dependent on the type of photoreactive group, carbene or nitrene species are generated that will insert into neighboring hydrogen-heteroatom bonds.^[22] Due to this broad reactivity, most photo-affinity probes do not require a particularly reactive amino acid residue to be present in the binding pocket of the receptor. However, the broad reactivity might also cause an increased amount of off-target labeling. Electrophilic AfBPs on the other hand, covalently bind their target GPCR through attack of a proximate nucleophilic amino acid residue. Here, specific labeling of the target GPCR requires a balanced electrophile: reactive enough to be attacked by the weakly nucleophilic amino acid, but not randomly attacked by any amino acid residue in the proteome.^[23]

Considering the third functional moiety of AfBPs, the reporter group, a distinction can be made between 'one-step' and 'two-step' probes.^[24–26] In case of one-step AfBPs, the reporter group, e.g. a fluorophore or biotin moiety, is directly conjugated to the probe. Two-step AfBPs on the other hand, contain a bio-orthogonal group (click handle) that can be functionalized after covalent binding to the target GPCR. Multiple probes have been developed that contain either an alkyne or an azide group that can be functionalized using click chemistry. The advantage

of two-step AfBPs is the lack of bulky reporter groups that might strongly influence the affinity towards the target GPCR. Disadvantages are the introduction of an extra 'click' step in the assay protocol, as well as possible use of cytotoxic reagents such as copper. In the next paragraphs, the most recent advancements will be discussed considering the development of one- and two-step, photo-affinity and electrophilic AfBPs for GPCRs.

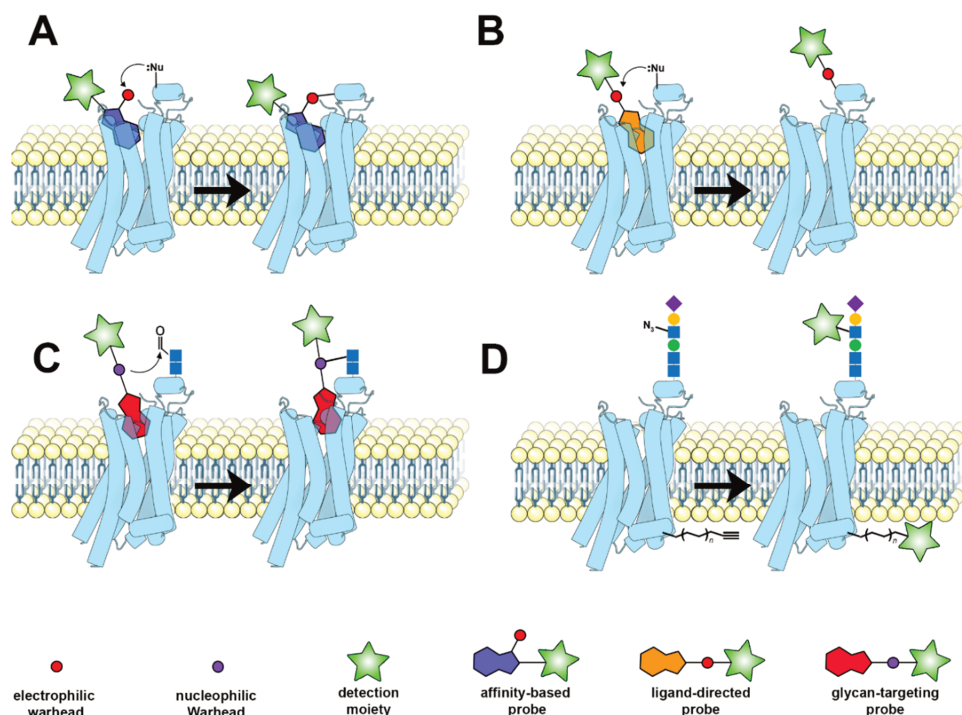


Figure 1. Schematic overview of the various functionalized covalent probes described in this review. (A) Affinity-based probes (AfBPs). After reversible binding of an AfBP to the target GPCR, a nearby nucleophilic residue attacks the electrophilic warhead, resulting in a covalently bound AfBP; (B) Ligand-directed probes (LD probes). Upon reversible binding of an LD probe to the target GPCR, a nearby nucleophilic residue attacks the electrophilic warhead, resulting in intramolecular bond cleavage and subsequent donation of the reporter group to the GPCR; (C) Glycan-targeting probes. First, aldehydes are generated through oxidation of the extracellular glycan chain of the GPCR. Next, the glycan-targeting probe binds reversibly to the GPCR and the nucleophilic warhead attacks the generated aldehyde, resulting in covalently bound glycan-targeting probe; (D) Metabolically incorporated probes. First, fatty acids or sugar molecules derivatized with click groups are added to the cell culture medium. These molecules are then post-translationally incorporated in the GPCR structure, allowing functionalization of the GPCR via click chemistry. This figure was partly generated with Protein Imager,^[20] using the structure of the adenosine A_{2A} receptor (PDB: 7ARO).

2.1.1. One-step photo-affinity probes

The introduction of photoreactive groups in the molecular structure of GPCR ligands has a long history, as photo-affinity ligands have been widely used to decipher the location of binding pockets in GPCRs.^[27] In some cases, these photo-affinity ligands have been equipped with reporter groups, such as a fluorophore, biotin, or a radioisotope, to allow detection of the probe-bound residues by SDS-PAGE and MS-techniques. Technological advances in the fields of

microscopy and MS-based proteomics have led to new approaches to use photo-affinity ligands to study GPCRs, resulting in the recent development of novel one-step photo-affinity probes (Figure 2).^[28–34] While initially most photo-affinity probes were equipped with benzophenone and aryl azide groups, most modern probes contain a diazirine as photoreactive group. The reasons for this are the relatively small size, favorable absorption range and improved synthetic accessibility.^[22] On the other hand, Hayashi *et al.* implemented multiple photoreactive groups into the scaffold of a known Dopamine D₂ Receptor (D₂R) ligand, including diazirines, and found the 2-aryl 5-carboxytetrazole-containing probe (**4**) to bind less off-targets in proteomic studies.^[31] Thus, the right balance needs to be found between size and reactivity of the photoreactive group.

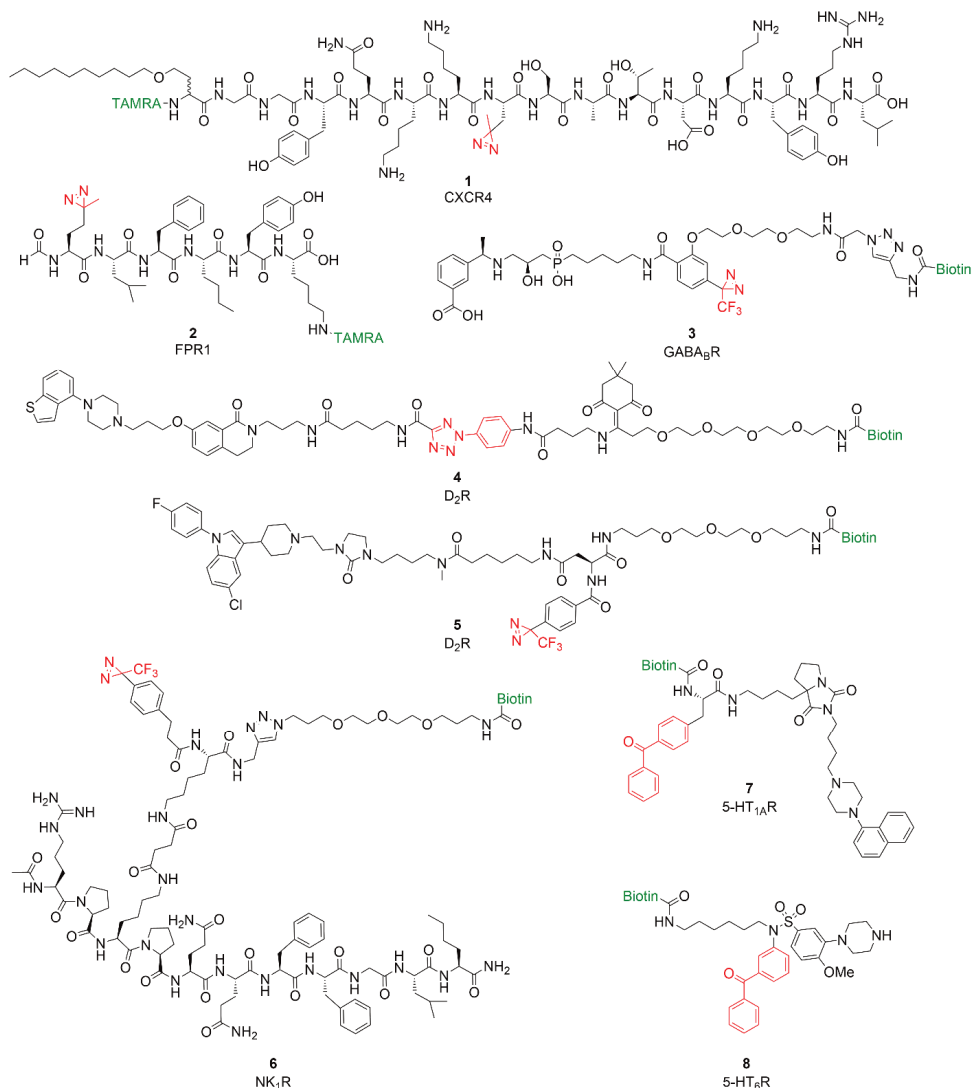


Figure 2. Molecular structures of one-step photo-affinity probes and their target GPCRs. Show in red are the electrophilic groups and in green the reporter groups.

One-step photo-affinity probes functionalized with a fluorophore for confocal imaging have been developed for the C-X-C chemokine receptor type 4 (CXCR4)^[28] and the Formyl Peptide Receptor 1 (FPR1).^[29] Both probes **1** and **2** were able to visualize agonist-induced internalization upon binding their target GPCR. One-step photo-affinity probes functionalized with biotin for proteomic pull-down (receptor capture) experiments have been developed for the GABA_B receptor (GABA_BR),^[30] D₂R,^[31,32] Neurokinin 1 receptor (NK₁R),^[33] 5-HT_{1A} receptor (5-HT_{1A}R) and 5-HT₆ receptor (5-HT₆R).^[34] Of these one-step photo-affinity probes, **3** (GABA_BR) has been used to elucidate PPIs within the GABA_BR signaling complex, while **5** (D₂R), **7** (5-HT_{1A}R) and **8** (5-HT₆R) have been used to profile the protein interactome of their respective molecular scaffolds.

2.1.2. Two-step photo-affinity probes

Most of the reported two-step photo-affinity probes were developed using a strategy that combines the photoreactive group and the click handle onto the same 'R' group, substituted at a location on the pharmacological scaffold that does not impair binding to the target GPCR (Figure 3). However, having this option is strongly dependent on both the molecular scaffold and GPCR binding pocket. The diazirine and benzophenone groups are the most popular warheads among the two-step photo-affinity probes, but phenyl azide and acetophenone groups have also been reported.^[34–41] Two-step photo-affinity probes do not only avoid the use of bulky reporter groups, as mentioned above, but also allow functionalization of the probe-bound GPCR by any detection moiety of interest. This is reflected in the wide variety of assays that have been performed, i.e. two-step photo-affinity probes **9–12** have been used in SDS-PAGE experiments to detect the 5-HT_{1A}R,^[34] 5-HT₆R,^[34] metabotropic glutamate receptor 2 (mGlu₂R)^[35] and metabotropic glutamate receptor 5 (mGlu₅R)^[36]; **13** in flow cytometry experiments to investigate cannabinoid receptor type 2 (CB₂R)^[37] expression on lymphocytes; **14–16** in target identification experiments to identify GPR39,^[38] GPR75^[39] and GPRC5A^[40] as their respective protein targets; and **17** in pull-down experiments to map possible PPIs of the D₂R.^[41]

2.1.3. Two-step electrophilic affinity-based probes

As general interest in covalent ligands is currently emerging, more and more electrophilic covalent ligands are being developed to target GPCRs. Covalent ligands for GPCRs and their wide variety of warheads are extensively reviewed elsewhere.^[42] To the best of our knowledge no one-step electrophilic AfBP for GPCRs has recently been developed. In case of the two-step electrophilic AfBPs, the fluorosulfonyl group is the most popular warhead, reacting through an S_N2 mechanism with a lysine or tyrosine residue on the target GPCR.^[43–46] Next to that, thiocyanate and acrylamide groups have been implemented in AfBPs to target cysteine residues, reacting through reductive disulfide formation or a Michael addition, respectively (Figure 4).^[47] In general, the strategy for the development of electrophilic AfBPs has been substitution of a click handle, with or without extra linker, onto the scaffold of an existing covalent ligand. Two-step probes provide the advantage of being used in a wide variety of assay types due to 'click' functionalization *in situ*. This has resulted in the use of electrophilic AfBPs in a broad range of assay types, such as SDS-PAGE, confocal microscopy, flow cytometry and proteomic pull-down experiments, as has been showcased for the adenosine A₁, A_{2A} and A₃ receptors (A₁AR, A_{2A}AR and A₃AR)^[43–46] and the chemokine receptor subtype 2 (CCR2).^[47] Of these electrophilic AfBPs, **18**, **20** and **22** have been used to study *N*-glycosylation of the A₁AR, A₃AR and CCR2, respectively; **18** to observe internalization of the A₁AR; and **20** to detect endogenously expressed A₃AR on human primary cells.

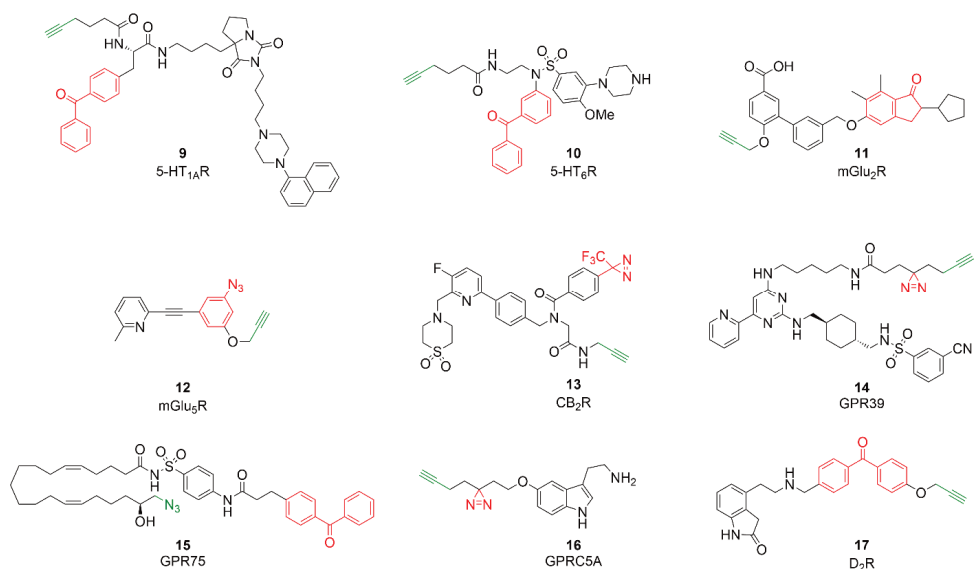


Figure 3. Molecular structures of two-step photo-affinity probes and their target GPCRs. Shown in red are the photoreactive groups, shown in green are the click handles for functionalization.

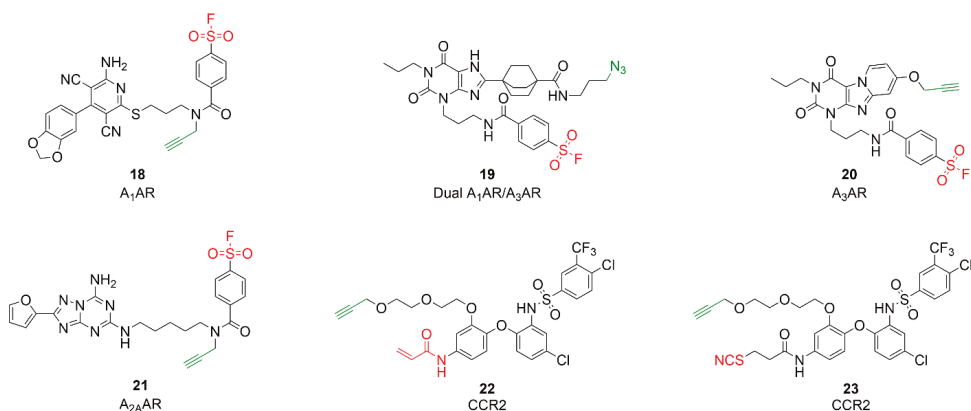


Figure 4. Molecular structures of electrophilic affinity-based probes and their target GPCRs. Shown in red are the electrophiles, shown in green are the click handles for functionalization.

2.1.4. Broad-spectrum affinity-based probes

Besides targeting one specific GPCR, a (sub)family of GPCRs might also be targeted by a 'broad-spectrum' AfBP. Broad-spectrum activity-based probes have already found their widespread use in proteomic studies towards various families of enzymes, such as hydrolases, proteases and kinases. In case of GPCRs, the 'high affinity' moiety of the probe should be a molecular scaffold that binds to multiple GPCRs. Steroids are a good example, as these molecules have shown to allosterically bind GPCRs in crystallization studies. In fact, both cholesterol and bile acid have been transformed in broad spectrum photo-affinity probes **24** and **25** for proteomics studies.^[48,49] Next to that, the $\Delta^8/9$ -Tetrahydrocannabinol (THC)-based

probes **26** and **27** have been developed for the investigation of all THC-binding proteins besides their target cannabinoid receptors (Figure 5).^[50,51] However, the number of GPCRs detected by these broad-spectrum probes is still smaller than expected, presumably due to low expression levels of GPCRs as compared to other proteins binders. For example, C-X-C motif chemokine receptor 4 (CXCR4) is one of the GPCRs detected by utilization of probe **24**, though presumably picked up due to its relatively high expression levels in the cell line used.^[52] Improvements to the enrichment of GPCRs, e.g. during protein solubilization steps,^[53] is therefore necessary for the future detection of GPCRs with broad-spectrum AfBPs.

2.2. Ligand-directed probes

LD probes are very similar to AfBPs as they also consist of three functional moieties, (1) a high affinity-ligand that induces selectivity, (2) an electrophilic group that reacts with a nucleophilic amino acid residue, and (3) a reporter group for detection in chemical biological assays. The main difference between AfBPs and LD probes is the electrophilic group. Upon reacting with a nucleophilic amino acid residue, the electrophilic group of an LD probe induces bond cleavage between the high affinity ligand and the reporter group (Figure 1B), allowing the high affinity-ligand to leave the binding pocket after donation of the reporter group to the protein. LD probes are therefore interesting new tools to label native GPCRs, without occupying the GPCR ligand binding pockets.

The idea of LD probes has been developed by Hamachi and coworkers, where multiple electrophilic groups have been investigated for their use in LD chemistry, examples being the tosyl, dibromo benzoate, acyl imidazole and *N*-acyl, *N*-alkyl sulfonamide groups.^[54–57] An important note is that the right balance should be found between selectivity and reactivity of the electrophile, to prevent off-target labeling of the LD probe. The same team also provided evidence for the first LD probe capable of tagging a GPCR.^[55,58] Over the past decade, multiple research groups have followed, resulting in a recent increase in LD probes as tools to study GPCRs. Also in case of LD probes, one-step and two-step probes have been developed. These will be discussed in the paragraphs below.

2.2.1. One-step ligand-directed probes

Early examples of one-step LD probes use the acyl imidazole group as electrophile,^[55,59] while more recent one-step LD probes have also implemented 2-nitrophenyl esters, 2-fluorophenyl esters and *N*-acyl, *N*-alkyl sulfonamides as electrophilic groups (Figure 6).^[60–62] An interesting strategy is the use of the *O*-nitrobenzoxadiazole moiety as both the electrophile and the fluorophore in LD probes **35** and **36**.^[62,63] Upon nucleophilic attack by a proximal lysine residue, the moiety becomes fluorescent, resulting in a measurable 'turn-on' signal upon receptor binding. Besides the nitrobenzoxadiazole group, one-step LD probes have been conjugated to biotin and fluorophore moieties and used in SDS-PAGE, flow cytometry and confocal microscopy experiments. One-step LD probes have been developed for the bradykinin B₂ receptor (B₂R),^[64] A_{2A}AR,^[60,61] metabotropic glutamate receptor 1 (mGlu₁R),^[65] μ opioid receptor (MOR),^[59] dopamine D1 receptor (D₁R),^[66] CB₂R,^[62] and smoothened receptor (SMOR).^[63] Of these one-step LD probes, **30** has been used to selectively target endogenous A_{2A}ARs on breast cancer cells; and one-step LD Probes **31** and **32** have been used to selectively label endogenously expressed mGlu₁R and MOR, respectively, in brain slices derived from rodents.^[59,65]

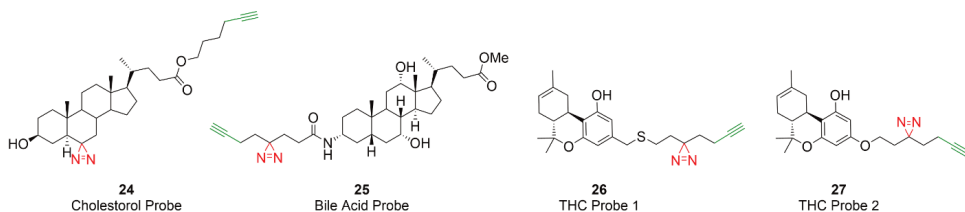


Figure 5. Molecular structures of broad-spectrum affinity-based probes and the names of their parent molecules. Shown in red are the photoreactive groups, shown in green are the click handles for functionalization.

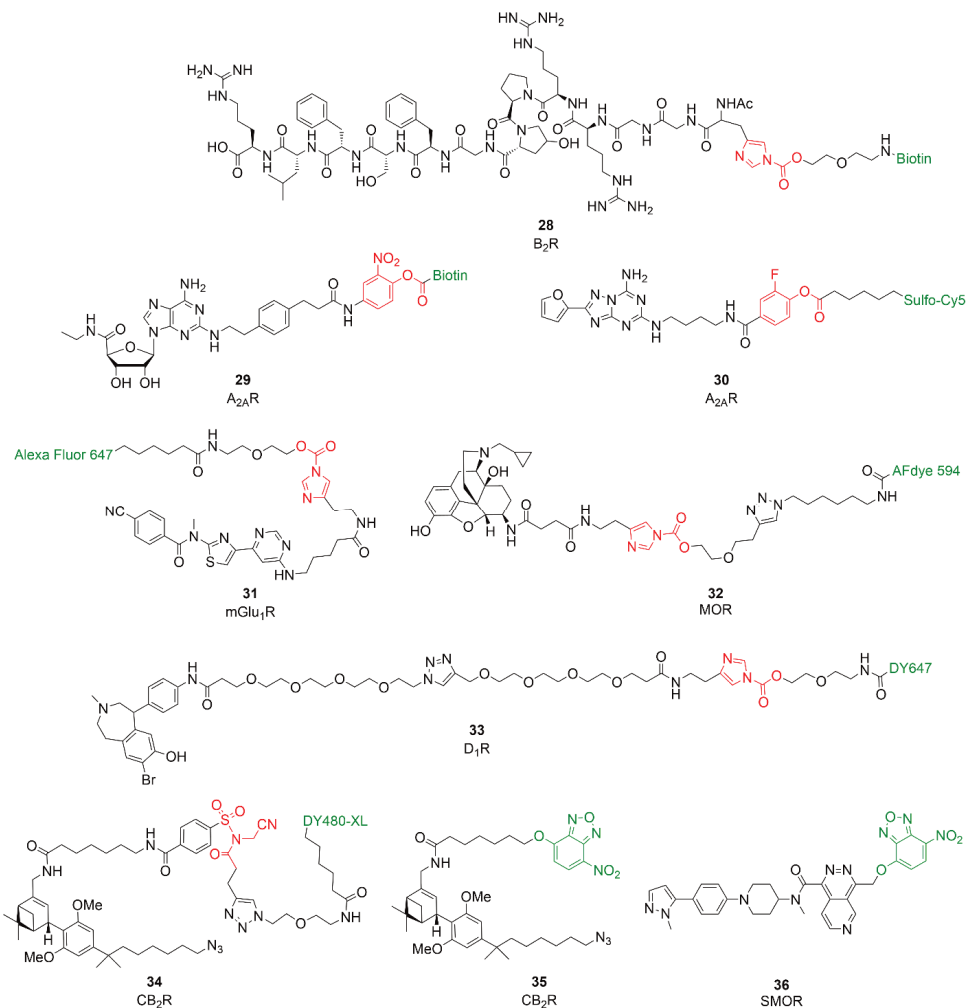


Figure 6. Molecular structures of one-step ligand-directed probes and their GPCR targets. Shown in red are the electrophilic groups and in green the reporter groups.

2.2.2. Two-step ligand-directed probes

Incorporation of a click handle increases the versatility of the LD probe, however also demands the implementation of an additional 'click' step during labeling assays. The latter might hamper the use of LD probes in cellular environments. The first two-step LD probes for GPCRs were based on a catalytic mode of action: a high-affinity ligand was conjugated to a catalytic group, e.g. a dimethyl aminopyridine (DMAP) or pyridinium oxide (PyrOx) moiety, that catalyzes the reaction of the reporter molecule with a nearby nucleophilic amino acid residue (Figure 7).^[58,67] This strategy has been used to substitute fluorophores onto the B₂R, among other protein targets,^[58] and allowed receptor labeling in SDS-PAGE and confocal microscopy experiments. These reactions however, are not specific between two functional groups and might therefore result in unwanted off-target labeling. Instead, a click handle has been implemented in the recently developed two-step LD probe **38** for the adenosine A_{2B} receptor (A_{2B}AR) (chapter 6). This LD probe incorporated an *N*-acyl, *N*-alkyl sulfonamide group as electrophile for donation of a clickable terminal alkyne group to the A_{2B}AR, as observed in SDS-PAGE experiments.

2.3. Glycan-targeting probes

Glycosylation is a PTM expected to be present on all cell surface GPCRs.^[6] A strategy has been developed that makes use of glycosylation to covalently label all cell surface proteins, including GPCRs.^[68,69] First, the oligosaccharides within the glycan chain are mildly oxidized to generate aldehyde groups that function as electrophiles. Next, a trifunctional probe is added, again containing three functional moieties, (1) a high affinity ligand; (2) a nucleophilic group; and (3) a reporter group for detection. The trifunctional probe binds to the target GPCR and subsequently forms a covalent bond with an aldehyde of a proximal glycan chain (Figure 1C), allowing detection of the GPCR in biochemical assays. The first glycan-targeting probe (coined 'TRICEPS') implemented trifluoroacetylated hydrazine as nucleophile, while later probes implemented aminooxy groups ('ASB' probe) and acetone protected hydrazine groups ('HATRIC' probe) (Figure 8).^[68–71] Noteworthy, all the reported glycan-targeting probes need to be 'pre-coupled' to a GPCR ligand prior to their utilization in biochemical assays. Pre-coupling is carried out via the electrophilic *N*-hydroxy succinimide ester or the nucleophilic thiol group. Biotin has been the reporter moiety of choice in all these examples. Of these glycan-targeting probes, **39** has been used in target identification experiments, leading to the detection and identification of the Apelin Receptor (APLNR)^[68] and Latrophilin 2 receptor (LPHN2R)^[72] via pull-down proteomics.

2.4. Metabolic incorporation of two-step probes

Huge efforts have been performed in the labs of Lin and Sakmar on the incorporation of unnatural amino acids into the peptide sequence of GPCRs.^[12,73] These include amino acids that contain photoreactive or clickable groups. Although these probes are beyond the scope of this review, there are two interesting strategies that metabolically incorporate probes without altering the genetic code of the GPCR (Figure 1D). First, clickable oligosaccharide **42** has been incorporated in the glycan chains of the Histamine H₃ Receptor (H₃R) (Figure 9). This has led to the detection of the H₃R in FRET-based assays.^[74] Secondly, clickable variants of palmitic acid **43** and **44** have been metabolically incorporated as S-palmitoyl groups. This allowed identification of the palmitoylation sites at the α_1 adrenergic receptor (α_1 R),^[75] β_1 and β_2 adrenergic receptors (β_1 R and β_2 R),^[76,77] MOR^[78] and D₂R^[79] through SDS-PAGE and western blot experiments.

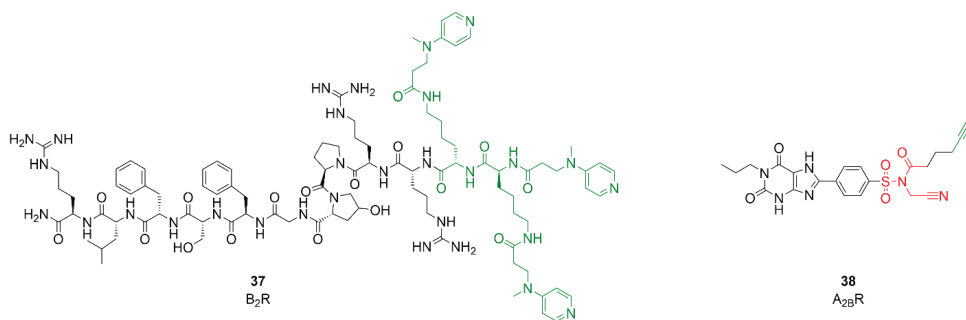


Figure 7. Molecular structures of two-step ligand directed probes and their GPCR targets. Shown in green are the chemical groups that allow functionalization of the receptor, shown in red are the electrophilic groups.

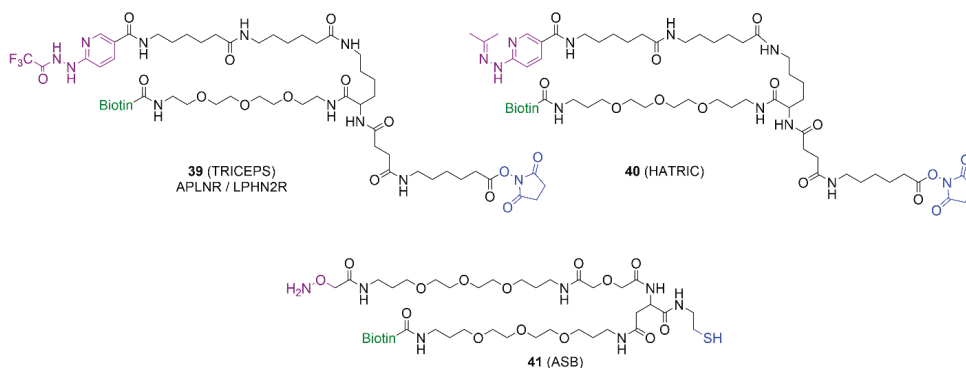


Figure 8. Molecular structures of glycan-targeting probes and their GPCR targets. Shown in purple are the nucleophiles responsible for glycan binding, shown in green are the detection moieties and shown in blue are the chemical groups that allow ligand incorporation.

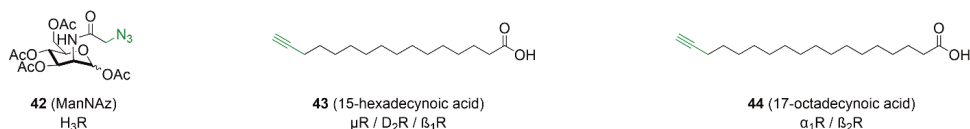


Figure 9. Molecular structures of two-step probes for metabolic incorporation and their target GPCRs. Shown in green are the click handles for functionalization.

3. Concluding Remarks

Over the past decade a modest set of roughly 40 small molecular probes has been developed for the covalent functionalization of GPCRs (Table 1). These include affinity-based probes, ligand-directed probes, glycan-targeting probes and metabolic probes, using either a one- or two-step labeling strategy. Depending on the envisioned assay setup, a specific type of probe might be preferable, e.g. one-step probes might be suitable for live-cell imaging experiments, while ligand-directed probes might be used to track receptors upon ligand binding.

Thus far, most of the reported functionalized covalent probes have been used to detect the presence of the receptor using overexpressing cell lines, although in some studies receptor expression was assessed in human blood cells,^[37,45] mouse adipocytes,^[43] or brain slices from rats and mice.^[59,65] Similar studies on GPCR expression have also been carried out using reversible ligands, either fluorescent or radiolabeled, and are therefore not a specific application of covalent probes. However, functionalized covalent probes can be used to track GPCR localization inside the cell without possible loss of reversibly bound detection moieties. This is exemplified in literature by the detection of agonist-induced internalization.^[28,29,43,64]

Next to that, covalent functionalization of GPCRs has also revealed the presence of several PTMs, of which *N*-glycosylation has been the most evident.^[29,37,43,45,58,64] Glycan-targeting and clickable sugar moieties are based on the idea of receptor glycosylation.^[68–71,74] Nevertheless, many questions remain regarding location and sequence of *N*- and *O*-glycosylation, as well as their regulatory effects on receptor functioning.^[6,7,80] *S*-palmitoylation as a PTM has been studied with two-step metabolic fatty acid probes to investigate agonist-induced internalization,^[76] receptor stability and trafficking for their respective GPCRs.^[77,79]

Furthermore, covalent probes have shown to be elegant tools for the target identification of bioactive molecules using pull-down proteomics.^[40,48–50,72] Yet, such proteomic strategies have not always yielded a detectable signal of the target GPCR.^[39,41,81] Careful examination of multiple variables, e.g. expression level, solubilization and digestion methods, is therefore of great importance in target identification studies.^[8,32,43,53,70] Further complicating, GPCRs also form signaling complexes, interacting with both membrane and cytosolic proteins. Several of the aforementioned probes have already identified potential interaction partners of GPCRs.^[30,32,34,39,41] However, in such experiments, careful analysis is necessary to distinguish between off-target proteins and true PPI partners.

4. Outlook

For future studies, the toolbox with functionalized covalent probes will aid thorough investigations of GPCRs. Example studies in the near future can be: (i) covalently tagging GPCRs with fluorophores to track GPCRs intracellularly, investigating possible signaling activity on intracellular organelles.^[10] (ii) Covalently tagging GPCRs with biotin to perform pull-down proteomics and subsequent MS experiments, identifying PTMs through sophisticated MS analysis software.^[6,82] (iii) Covalently tagging GPCRs with fluorophores or biotin to detect protein interaction partners, using cross-linking chemistry (pull-down proteomics) or counterstaining (fluorescent microscopy) methods.^[83,84]

Altogether, there are many possibilities to make smart use of functionalized covalent probes. However, it must be noted that these probes do not replace reversible probes, genetic techniques or metabolic techniques to functionalize receptors. Instead, these techniques are complementary towards one another, all yielding their own subset of information. In the future, this combined toolbox filled with reversible, covalent, genetic and metabolic probes will therefore be of great use in answering fundamental questions regarding GPCRs.

Table 1. List of functionalized covalent probes for GPCRs, sorted by GPCR name.

Target GPCR	Probe	Name	Type of probe	Electrophile	Reporter	Detection Methods	Ref
5-HT _{1A} R	7	5	one-step photo-affinity	benzophenone	biotin	mass spectrometry	[34]
5-HT _{1A} R	9	7	two-step photo-affinity	benzophenone	alkyne	SDS-PAGE	[34]
5-HT ₆ R	8	16	one-step photo-affinity	benzophenone	biotin	mass spectrometry	[34]
5-HT ₆ R	10	18	two-step photo-affinity	benzophenone	alkyne	SDS-PAGE	[34]
A ₁ AR	18	LUF7909	two-step electrophilic	fluorosulfonyl	alkyne	SDS-PAGE, microscopy, mass spectrometry	[43]
A ₁ AR, A ₃ AR	19	UODC9	two-step electrophilic	fluorosulfonyl	alkyne	SDS-PAGE	[44]
A _{2A} AR	30	1	one-step ligand-directed	2-fluorophenyl ester	Cy5	SDS-PAGE, TR-FRET, microscopy	[61]
A _{2A} AR	29	3c	one-step ligand-directed	2-nitrophenyl ester	biotin	-	[60]
A _{2A} AR	21	LUF7487	two-step electrophilic	fluorosulfonyl	alkyne	SDS-PAGE	[46]
A _{2B} AR	38	LUF8019	two-step ligand-directed	<i>N</i> -acyl, <i>N</i> -alkyl sulfonamide	alkyne	SDS-PAGE	-
A ₃ AR	20	LUF7960	two-step electrophilic	fluorosulfonyl	alkyne	SDS-PAGE, flow cytometry, microscopy	[45]
B ₂ R	28	3	one-step ligand-directed	Acyl imidazole	biotin	SDS-PAGE, microscopy	[64]
B ₂ R	37	9	two-step ligand-directed	-	tri-DMAP	SDS-PAGE, microscopy	[58]
CB ₂ R	34	15	one-step ligand-directed	<i>N</i> -acyl, <i>N</i> -alkyl sulfonamide	DY-480XL	Imaging flow cytometry, microscopy	[62]
CB ₂ R	35	2b	one-step ligand-directed	<i>O</i> -nitro benzoxa diazole	<i>O</i> -nitro benzoxa diazole	TR-FRET	[62]
CB ₂ R	13	LEI121	two-step photo-affinity	diazirine	alkyne	SDS-PAGE, flow cytometry, mass spectrometry	[37]
CCR2	22	LUF7834	two-step electrophilic	acrylamide	alkyne	SDS-PAGE, mass spectrometry	[47]

Chapter 2

CCR2	23	LUF7835	two-step electrophilic	thiocyanate	Alkyne	SDS-PAGE, mass spectrometry	[47]
CXCR4	1	ATI-2766	one-step photo-affinity	diazirine	TAMRA	SDS-PAGE, microscopy	[28]
D ₁ R	33	5h	one-step ligand-directed	acyl imidazole	DY647	TR-FRET, microscopy	[66]
D ₂ R	17	5	two-step photo-affinity	benzophenone	alkyne	SDS-PAGE, flow cytometry, microscopy, mass spectrometry	[41]
D ₂ R	5	CPT-00031	one-step photo-affinity	diazirine	biotin	SDS-PAGE, mass spectrometry	[32]
D ₂ R	4	Probe 7	one-step photo-affinity	2-aryl, 5-carboxy tetrazole	biotin	mass spectrometry	[31]
FPR1	2	Probe-TAMRA	one-step photo-affinity	diazirine	TAMRA	SDS-PAGE, flow cytometry, microscopy	[29]
GABA _B	3	CGP 64213 ^B	one-step photo-affinity	diazirine	biotin	SDS-PAGE	[30]
GPR39	14	1A	two-step photo-affinity	diazirine	alkyne	mass spectrometry	[38]
GPR75	15	20-ApheDa	two-step photo-affinity	benzophenone	azide	SDS-PAGE, mass spectrometry	[39]
GPRC5A	16	x-alk-TA	two-step photo-affinity	diazirine	alkyne	SDS-PAGE, microscopy, mass spectrometry	[40]
mGlu ₁ R	31	CmGlu1 M	one-step ligand-directed	acyl imidazole	Alexa Fluor 647	SDS-PAGE, microscopy	[65]
mGlu ₂ R	11	(±)- 14	two-step photo-affinity	acetophenone	alkyne	SDS-PAGE, microscopy	[35]
mGlu ₅ R	12	8	two-step photo-affinity	phenyl azide	alkyne	SDS-PAGE	[36]
MOR	32	NAI-A594	one-step ligand-directed	acyl imidazole	Alexa 594	SDS-PAGE, flow cytometry, microscopy	[59]
NK ₁ R	6	Ac-Nle-SP-probe	one-step photo-affinity	diazirine	biotin	SDS-PAGE, microscopy, mass spectrometry	[33]
SMOR	36	9	one-step ligand-directed	O-nitro benzoxa diazole	O-nitro benzoxa diazole	SDS-PAGE	[63]

[a] Nucleophilic group that reacts with oxidized glycan chains.

References

- [1] S. M. Foord, T. I. Bonner, R. R. Neubig, E. M. Rossier, J. P. Pin, A. P. Davenport, M. Spedding, A. J. Harmar, *Pharmacol Rev* **2005**, *57*, 279–288.
- [2] T. Schöneberg, I. Liebscher, *Pharmacol Rev* **2021**, *73*, 89–119.
- [3] 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.
- [4] K. A. Jacobson, *Biochem Pharmacol* **2015**, *98*, 541–555.
- [5] D. M. Rosenbaum, S. G. F. Rasmussen, B. K. Kobilka, *Nature* **2009**, *459*, 356–363.
- [6] A. Patwardhan, N. Cheng, J. Trejo, *Pharmacol Rev* **2021**, *73*, 120–151.
- [7] C. K. Goth, U. E. Petäjä-Repo, M. M. Rosenkilde, *ACS Pharmacol Transl Sci* **2020**, *3*, 237–245.
- [8] A. O. Helbig, A. J. R. Heck, M. Slijper, *J Proteomics* **2010**, *73*, 868–878.
- [9] S. Elschenbroich, Y. Kim, J. A. Medin, T. Kislinger, *Expert Rev Proteomics* **2010**, *7*, 141–154.
- [10] M. Ali, M. Nezhady, J. C. Rivera, S. Chemtob, *iScience* **2020**, *23*, 101643.
- [11] M. M. Shchepinova, A. C. Hanyaloglu, G. S. Frost, E. W. Tate, *Curr Opin Chem Biol* **2020**, *56*, 98–110.
- [12] T. Huber, T. P. Sakmar, *Chem Biol* **2014**, *21*, 1224–1237.
- [13] 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.
- [14] Y. Wu, B. Zhang, H. Xu, M. He, X. Deng, L. Zhang, Q. Dang, J. Fan, Y. Guan, X. Peng, W. Sun, *Coord Chem Rev* **2023**, *480*, 215040.
- [15] C. A. Flanagan, *Methods Cell Biol* **2016**, *132*, 191–215.
- [16] C. J. Hutchings, M. Koglin, W. C. Olson, F. H. Marshall, *Nat Rev Drug Discov* **2017**, *16*, 787–810.
- [17] 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.
- [18] H. Cernecka, P. Ochodnický, W. H. Lamers, M. C. Michel, *Naunyn-Schmiedeberg's Arch Pharmacol* **2012**, *385*, 875–882.
- [19] Y. Marchalant, P. W. Brownjohn, A. Bonnet, T. Kleffmann, J. C. Ashton, *Journal of Histochemistry and Cytochemistry* **2014**, *62*, 395–404.
- [20] G. Tomasello, I. Armenia, G. Molla, *Bioinformatics* **2020**, *36*, 2909–2911.
- [21] Y. Liu, M. P. Patricelli, B. F. Cravatt, *PNAS* **1999**, *96*, 14694–14699.
- [22] P. P. Geurink, L. M. Prely, G. A. Van Der Marel, R. Bischoff, H. S. Overkleeft, *Top Curr Chem* **2012**, *324*, 85–113.
- [23] M. Gehringer, S. A. Laufer, *J Med Chem* **2019**, *62*, 5673–5724.
- [24] H. Ovaa, P. F. Van Swieten, B. M. Kessler, M. A. Leeuwenburgh, E. Fiebigier, A. M. C. H. Van den Nieuwendijk, P. J. Galaray, G. A. Van der Marel, H. L. Ploegh, H. S. Overkleeft, *Angew Chem Int Ed* **2003**, *42*, 3626–3629.
- [25] A. E. Speers, G. C. Adam, B. F. Cravatt, *J Am Chem Soc* **2003**, *125*, 4686–4687.
- [26] J. A. Prescher, C. R. Bertozzi, *Nat Chem Biol* **2005**, *1*, 13–21.
- [27] A. Grunbeck, T. P. Sakmar, *Biochemistry* **2013**, *52*, 8625–8632.
- [28] J. M. Janz, Y. Ren, R. Looby, M. A. Kazmi, P. Sachdev, A. Grunbeck, L. Haggis, D. Chinnappen, A. Y. Lin, C. Seibert, T. McMurry, K. E. Carlson, T. W. Muir, S. Hunt, T. P. Sakmar, *J Am Chem Soc* **2011**, *133*, 15878–15881.
- [29] D. H. Field, J. S. White, S. L. Warriner, M. H. Wright, *RSC Chem Biol* **2023**, *4*, 216–222.
- [30] X. Lin, X. Li, M. Jiang, L. Chen, C. Xu, W. Zhang, H. Zhao, B. Sun, X. Xu, F. Nan, J. Liu, *Biochemical Journal* **2012**, *443*, 627–634.
- [31] R. Miyajima, K. Sakai, Y. Otani, T. Wadatsu, Y. Sakata, Y. Nishikawa, M. Tanaka, Y. Yamashita, M. Hayashi, K. Kondo, T. Hayashi, *ACS Chem Biol* **2020**, *15*, 2364–2373.
- [32] C. Blex, S. Michaelis, A. K. Schrey, J. Furkert, J. Eichhorst, K. Bartho, F. Gyapon Quast, A. Marais, M. Hakelberg, U. Gruber, S. Niquet, O. Popp, F. Kroll, M. Sefkow, R. Schüle, M. Dreger, H. Köster, *ChemBioChem* **2017**, *18*, 1639–1649.
- [33] F. M. Müskens, R. J. Ward, D. Herkt, H. van de Langemheen, A. B. Tobin, R. M. J. Liskamp, G. Milligan, *Mol Pharmacol* **2019**, *95*, 196–209.
- [34] A. M. Gamo, J. A. González-Vera, A. Rueda-Zubiaurre, D. Alonso, H. Vázquez-Villa, L. Martín-Couce, Ó. Palomares, J. A. López, M. Martín-Fontecha, B. Benhamú, M. L. López-Rodríguez, S. Ortega-Gutiérrez, *Chemistry - A European Journal* **2016**, *22*, 1313–1321.
- [35] S. D. Hellyer, S. Aggarwal, A. N. Y. Chen, K. Leach, D. J. Lapinsky, K. J. Gregory, *ACS Chem Neurosci* **2020**, *11*, 1597–1609.
- [36] K. J. Gregory, R. Velagaleti, D. M. Thal, R. M. Brady, A. Christopoulos, P. J. Conn, D. J. Lapinsky, *ACS Chem Biol* **2016**, *11*, 1870–1879.
- [37] M. Soethoudt, S. C. Stolze, M. V. Westphal, L. van Stralen, A. Martella, E. J. van Rooden, W. Guba, Z. V. Varga, H. Deng, S. I. van Kasteren, U. Grether, A. P. IJzerman, P. Pacher, E. M. Carreira, H. S. Overkleeft, A. Ioan-Facsinay, L. H. Heitman, M. van der Stelt, *J Am Chem Soc* **2018**, *140*, 6067–6075.
- [38] J. R. Thomas, S. M. Brittain, J. Lipps, L. Llamas, R. K. Jain, M. Schirle, in *Proteomics for Drug Discovery: Methods and Protocols* (Eds.: I. M. Lazar, M. Kontoyianni, A. C. Lazar), Springer New York, New York, NY, **2017**, pp. 1–18.
- [39] V. Garcia, A. Gilani, B. Shkolnik, V. Pandey, F. F. Zhang, R. Dakarapu, S. K. Gandham, N. R. Reddy, J. P. Graves, A. Gruzdev, D. C. Zeldin, J. H. Capdevila, J. R. Falck, M. L. Schwartzman, *Circ Res* **2017**, *120*, 1776–1788.
- [40] X. Zhao, K. R. Stein, V. Chen, M. E. Griffin, H. C. Hang, *bioRxiv* **2021**, 2021.12.16.472979.
- [41] S. T. Kim, E. J. Doukmak, R. G. Flax, D. J. Gray, V. N. Zirimu, E. De Jong, R. C. Steinhardt, *ACS Chem Neurosci* **2022**, *13*, 3008–3022.
- [42] D. Weichert, P. Gmeiner, *ACS Chem Biol* **2015**, *10*, 1376–1386.
- [43] B. L. H. Beekens, C. 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.
- [44] P. N. H. Trinh, D. J. W. Chong, K. Leach, S. J. Hill, J. D. A. Tyndall, L. T. May, A. J. Vemall, K. J. Gregory, *J Med Chem* **2021**, *64*, 8161–8178.
- [45] B. L. H. Beekens, 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.

- [46] 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.
- [47] L. S. den Hollander, S. Dekkers, B. L. H. Beerkens, J. P. D. van Veldhoven, N. V. O. Ortiz-Zacharias, C. van der Horst, I. Sieders, B. de Valk, J. Wang, A. P. IJzerman, D. van der Es, L. H. Heitman, *manuscript in preparation* n.d.
- [48] J. J. Hulce, A. B. Cognetta, M. J. Niphakis, S. E. Tully, B. F. Cravatt, *Nat Methods* **2013**, *10*, 259–264.
- [49] S. Zhuang, Q. Li, L. Cai, C. Wang, X. Lei, *ACS Cent Sci* **2017**, *3*, 501–509.
- [50] M. Soethoudt, G. Alachouzos, E. J. Van Rooden, M. D. Moya-Garzon, R. J. B. H. N. Van Den Berg, L. H. Heitman, M. Van Der Stelt, *Cannabis Cannabinoid Res* **2018**, *3*, 136–151.
- [51] Y.-J. Lim, G. Tang, Z. Ye, C.-J. Zhang, J. Wu, S. Q. Yao, *Chemistry – A European Journal* **2023**, e202300531.
- [52] C. Park, J. W. Lee, K. Kim, D. S. Seen, J. Y. Jeong, W. K. Huh, *Sci Rep* **2023**, *13*, 1894.
- [53] J. S. Behnke, L. H. Urner, *Anal Bioanal Chem* **2023**, DOI 10.1007/s00126-023-04584-z.
- [54] T. Tsukiji, M. Miyagawa, Y. Takaoka, T. Tamura, I. Hamachi, *Nat Chem Biol* **2009**, *5*, 341–343.
- [55] S. H. Fujishima, R. Yasui, T. Miki, A. Ojida, I. Hamachi, *J Am Chem Soc* **2012**, *134*, 3961–3964.
- [56] Y. Takaoka, Y. Nishikawa, Y. Hashimoto, K. Sasaki, I. Hamachi, *Chem Sci* **2015**, *6*, 3217–3224.
- [57] T. Tamura, T. Ueda, T. Goto, T. Tsukidate, Y. Shapira, Y. Nishikawa, A. Fujisawa, I. Hamachi, *Nat Commun* **2018**, *9*, 1870.
- [58] H. Wang, Y. Koshi, D. Minato, H. Nonaka, S. Kiyonaka, Y. Mori, S. Tsukiji, I. Hamachi, *J Am Chem Soc* **2011**, *133*, 12220–12228.
- [59] S. Arttamangkul, A. Plazek, E. J. Platt, H. Jin, T. F. Murray, W. T. Birdsong, K. C. Rice, D. L. Farrens, J. T. Williams, *Elife* **2019**, *8*, e49319.
- [60] S. M. Moss, P. S. Jayasekara, S. Paoletta, Z. G. Gao, K. A. Jacobson, *ACS Med Chem Lett* **2014**, *5*, 1043–1048.
- [61] L. A. Stoddart, N. D. Kindon, O. Otun, C. R. Harwood, F. Patera, D. B. Vepintsev, J. Woolard, S. J. Briddon, H. A. Franks, S. J. Hill, B. Kellam, *Commun Biol* **2020**, *3*, 722.
- [62] M. Kosar, D. A. Sykes, A. E. G. Viray, J. Rosa, M. Vitale, R. C. Sarott, R. L. Ganzoni, D. Onion, J. M. Tobias, P. Leippe, C. Ullmer, E. A. Zirwes, W. Guba, U. Grether, J. A. Frank, D. B. Vepintsev, E. M. Carreira, *ChemRxiv* **2022**, DOI 10.26434/chemrxiv-2022-z58fd.
- [63] D. Xue, L. Ye, J. Zheng, Y. Wu, X. Zhang, Y. Xu, T. Li, R. C. Stevens, F. Xu, M. Zhuang, S. Zhao, F. Zhao, H. Tao, *Org Biomol Chem* **2019**, *17*, 6136–6142.
- [64] T. Miki, S. H. Fujishima, K. Komatsu, K. Kuwata, S. Kiyonaka, I. Hamachi, *Chem Biol* **2014**, *21*, 1013–1022.
- [65] H. Nonaka, S. Sakamoto, K. Shiraiwa, M. Ishikawa, T. Tamura, K. Okuno, S. Kiyonaka, E. A. Susaki, C. Shimizu, H. R. Ueda, W. Kakegawa, I. Arai, I. Hamachi, *bioRxiv* **2023**, 2023.01.16.524180.
- [66] X. Gómez-Santacana, M. Boutonnet, C. Martínez-Juvés, J. L. Catena, E. Moutin, T. Roux, E. Trinquet, L. Lamarque, J. Perroy, L. Prézeau, J. M. Zwier, J.-P. Pin, A. Llebaria, *ChemRxiv* **2022**, DOI 10.26434/chemrxiv-2022-mqqtz-v2.
- [67] T. Tamura, Z. Song, K. Amaike, S. Lee, S. Yin, S. Kiyonaka, I. Hamachi, *J Am Chem Soc* **2017**, *139*, 14181–14191.
- [68] A. P. Frei, O. Y. Jeon, S. Kilcher, H. Moest, L. M. Henning, C. Jost, A. Plückthun, J. Mercer, R. Aebersold, E. M. Carreira, B. Wollscheid, *Nat Biotechnol* **2012**, *30*, 997–1001.
- [69] A. P. Frei, H. Moest, K. Novy, B. Wollscheid, *Nat Protoc* **2013**, *8*, 1321–1336.
- [70] T. L. Tremblay, J. J. Hill, *Sci Rep* **2017**, *7*, 46574.
- [71] N. Sobotzki, M. A. Schafroth, A. Rudnicka, A. Koetemann, F. Marty, S. Goetze, Y. Yamauchi, E. M. Carreira, B. Wollscheid, *Nat Commun* **2018**, *9*, 1519.
- [72] G. N. Yin, D. K. Kim, J. I. Kang, Y. Im, D. S. Lee, A. reum Han, J. Ock, M. J. Choi, M. H. Kwon, A. Limanjaya, S. B. Jung, J. Yang, K. W. Min, J. Yun, Y. Koh, J. E. Park, D. Hwang, J. K. Suh, J. K. Ryu, H. M. Kim, *Exp Mol Med* **2022**, *54*, 626–638.
- [73] S. K. Gangam, Q. Lin, in *Methods Enzymol*, Academic Press Inc., **2020**, pp. 95–111.
- [74] H. Stockmann, V. Todorovic, P. L. Richardson, V. Marin, V. Scott, C. Gerstein, M. Lake, L. Wang, R. Sadhukhan, A. Vasudevan, *J Am Chem Soc* **2017**, *139*, 16822–16829.
- [75] E. P. Marin, L. Jozsef, A. di Lorenzo, K. F. Held, A. K. Luciano, J. Melendez, L. M. Milstone, H. Velazquez, W. C. Sessa, *Arterioscler Thromb Vasc Biol* **2016**, *36*, 370–379.
- [76] D. M. Zuckerman, S. W. Hicks, G. Charron, H. C. Hang, C. E. Machamer, *Journal of Biological Chemistry* **2011**, *286*, 19014–19023.
- [77] N. Adachi, D. T. Hess, P. McLaughlin, J. S. Stamler, *Journal of Biological Chemistry* **2016**, *291*, 20232–20246.
- [78] B. Ebersole, J. Petko, R. Levenson, *Anal Biochem* **2014**, *451*, 25–27.
- [79] B. Ebersole, J. Petko, M. Woll, S. Murakami, K. Sokolina, V. Wong, I. Stagljar, B. Lüscher, R. Levenson, *PLoS One* **2015**, *10*, e0140661.
- [80] L. Verhallen, J. J. Lackman, R. Wendt, M. Gustavsson, Z. Yang, Y. Narimatsu, D. M. Sørensen, K. Mac Lafferty, M. Gouwy, P. E. Marques, G. M. Hjort, M. M. Rosenkilde, P. Proost, C. K. Goth, *Cellular and Molecular Life Sciences* **2023**, *80*, 55.
- [81] A. Garelli, F. Heredia, A. P. Casimiro, A. Macedo, C. Nunes, M. Garcez, A. R. M. Dias, Y. A. Volonte, T. Uhlmann, E. Caparros, T. Koyama, A. M. Gontijo, *Nat Commun* **2015**, *6*, 8732.
- [82] J. v. Olsen, M. Mann, *Molecular and Cellular Proteomics* **2013**, *12*, 3444–3452.
- [83] O. Klykov, B. Steigenberger, S. Pektaş, D. Fasci, A. J. R. Heck, R. A. Scheltema, *Nat Protoc* **2018**, *13*, 2964–2990.
- [84] A. R. B. Thomsen, B. Plouffe, T. J. Cahill, A. K. Shukla, J. T. Tarrasch, A. M. Dosey, A. W. Kahsai, R. T. Strachan, B. Pani, J. P. Mahoney, L. Huang, B. Breton, F. M. Heydenreich, R. K. Sunahara, G. Skiniotis, M. Bouvier, R. J. Lefkowitz, *Cell* **2016**, *166*, 907–919.

