Universiteit Leiden
The Netherlands

To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors
Yang, X.

Citation
Yang, X. (2019, December 4). *To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors*. Retrieved from https://hdl.handle.net/1887/81190

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/1887/81190

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

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/81190 holds various files of this Leiden University dissertation.

Author: Yang, X.
Title: To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors
Issue Date: 2019-12-04

\

Chapter 7

Conclusions and Future Perspectives

This thesis delves into the design and pharmacological profiling of covalent ligands for adenosine receptors (ARs). In this chapter, insights gained from case studies at three adenosine receptor subtypes are elaborated on, and remarks for further research on covalent probes for GPCRs are presented.

1. Conclusions

1.1. Guidelines for the design of covalent probes targeting ARs

Covalent probes, or affinity labels, are pharmacological tools that can be used to study the structural and functional properties of GPCRs [1]. As illustrated in **Chapter 2**, these ligands comprise three elements: a pharmacophore, a linker and a reactive group. The pharmacophore generates affinity and selectivity towards a specific adenosine receptor subtype, whereas a linker moiety connects the pharmacophore to the reactive moiety that forms a covalent bond with an amino acid residue near the binding pocket. An ideal reactive moiety, also referred to as warhead, shows a low nonspecific reactivity in solution or outside the binding pocket. Once accommodated in the binding pocket, it has the capacity to react with a neighboring nucleophilic amino acid residue to form a covalent interaction. In this regard, sulfonyl fluorides were chosen as warhead for all the covalent ligand design in this thesis (**Chapters 3-6)**. This privileged functionality holds a good combination of physiochemical properties (i.e., aqueous or thermodynamic stability) and electrophilic reactivity [2]. The targeted nucleophilic amino acid residues include serine, threonine, tyrosine, lysine, cysteine and histidine. Building on that, throughout this thesis I present examples that demonstrate the rational ligand design based on a well-defined structure-activity relationship (SAR) profile of the pharmacophore and extensive structural information of the binding site of the adenosine receptor.

First, a high-affinity pharmacophore is deemed to be an important starting point towards a successful design, as it increases the ligand concentration in the binding site, eventually triggering covalent interaction. Logically, it is essential that the introduction of a warhead to the original pharmacophore should not jeopardize the key properties of the ligand such as affinity and selectivity. As shown in Figure 1, the design of the covalent antagonist LUF7445 (**Chapter 3**) was based on a high-affinity non-xanthine antagonist, ZM241385, a molecular template co-crystalized with $hA_{2A}R$ (PDB: 4EIY) [3]. Analysis of the binding site in the crystal structure shows that the phenylethylamine chain in ZM241385 is directed toward the more solvent exposed extracellular region (EL2 and EL3), where this hydrophobic/hydrophylic interface offers us tremendous substituent flexibility. Based on

extensive SAR studies performed by our group [4, 5], we introduced the electrophilic fluorosulfonyl group at the phenylethylamine side chain to achieve a covalent interaction with amino acid K153^{ECL2}.

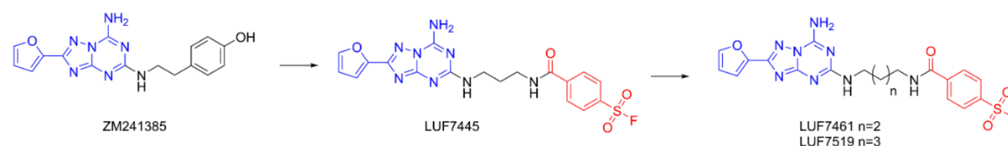

Figure 1. Chemical structures of covalent ligands for hA_{2A}R examined in Chapters 3 and 6. Here, the effect of the linker length between scaffold and warhead on affinity was further examined, yielding compound LUF7461 and, preferably, compound LUF7519 with an improved affinity.

A case based on an agonist scaffold is described in **Chapter 5**. The non-ribose agonists' scaffold was inspired by a former drug candidate, capadenoson [6, 7]. This dicyanopyridine scaffold with a benzo[1,3]dioxol-5-yl moiety at the C^4 position (LUF7174, Figure 2) showed good selectivity and efficacy at the hA_1R [8, 9]. This pharmacophore led to the discovery of a covalent partial agonist, LUF7746 (Figure 2), which maintained its high affinity and selectivity toward hA_1R.

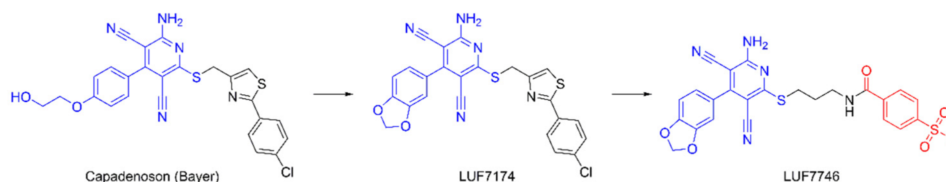

Figure 2. Chemical structures of covalent partial agonists examined in Chapter 5. The lead compound, capadenoson, a selective hA_1R partial agonist inspired the design of covalent partial agonist, LUF7746.

Of note, in the case with limited structural insights of the receptor binding pocket, altering the linker length is not the only way in which key properties of ligands (affinities) can be affected. **Chapter 4** presents the case of covalent antagonist design for the hA_3R, in which both the linker type and orientation of the warhead appeared relevant. The position of the warhead and linker type and length were given equal weight to the design strategy. It was found that the 4-position of the sulfonyl fluoride on the warhead's phenyl group was favored for high affinity. More importantly, the replacement of the ester group with the more metabolically stable amide linker resulted in irreversible probes with a slightly increased affinity. This led to the discovery of LUF7602 (Figure 3) as a best-in-class ligand among the series.

Lastly, in some cases the incorporation of a linker unit can be likewise important to maintain the properties of the pharmacophore and orient the ligand into a suitable direction for covalent interaction. As illustrated in **Chapter 6**, an extension of linker length based on LUF7445 (Figure 1) slightly increased the apparent affinity. This concurs with available literature reporting an electrophilic probe for the cannabinoid CB_1 receptor, also demonstrating that a significantly improved affinity resulted from a longer linker [10]. This might be the result of more steric freedom allowing the warhead to reach the adjacent nucleophilic amino acid residue in the receptor binding site more efficiently.

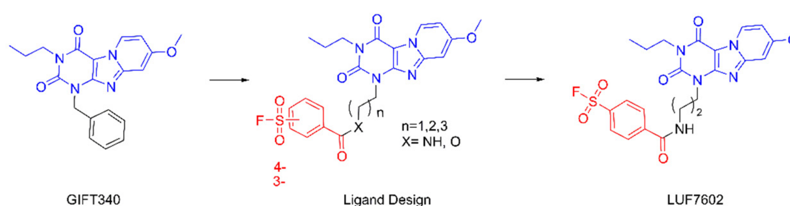

Figure 3. Chemical structures examined in Chapter 4. The antagonist scaffold, 1H,3H-pyrido[2,1-*f*]purine-2,4-dione (blue color), was employed to study the effect of the linker length, type and position of warheads on affinity. It led to the discovery of LUF7602 as a best-in-class covalent antagonist in this study.

In summary, cases presented in this thesis provide insight and clues towards a universal strategy for the design of covalent ligands. The efficiency of the ligation reaction is ultimately dependent on an optimal combination of 1) the affinity of the pharmacophore for the target receptor, 2) the warhead's proximity to an appropriate nucleophilic amino acid residue and 3) the warhead's reactivity.

1.2. Research workflow to characterize the covalent ligands

The examples summarized in thesis (**Chapter 3-6**) demonstrate a research workflow (Figure 4) to profile the pharmacological activities of the covalent ligand-receptor interaction. As summarized in section 7.1.1, the design of covalent ligands needs to be balanced between high-affinity pharmacophore, linker unit and reactive functionality. Moreover, a control ligand is required to maintain a similar binding mode but in a nonreactive reversible manner, where it is crucial to maintain a structural similarity with the corresponding covalent ligand. In **Chapter 3** for instance, both a long residence time compound LUF6632 and a short-residence time prototypical ligand ZM241385 are employed as control ligands to perform the pharmacological evaluations. Although, these control compounds were structurally very similar to the covalent ligand, even closer analogues were possible. Hence, in the cases presented in **Chapters 4** and **5** non-reactive electron-withdrawing methylsulfonyl derivatives

were designed as control ligands, resulting in significant, if not the best possible, structural similarities with covalent ligand. Subsequently, both covalent ligand and control ligand are subjected to streamlined biological evaluation, including time-dependent affinity determination, binding kinetics investigation, wash-out assays, mutagenesis study and functional characterization (Figure 4).

Figure 4. Research workflow to design, select and validate covalent ligands for GPCRs

In detail, the first hint of a covalent interaction by the probe is represented by a leftward shift of the concentration-dependent radioligand displacement curve at different incubation times. A longer incubation time renders the covalent ligand to be more potent in displacing the radioligand from the receptor, resulting in an increased apparent affinity. However, it is far from conclusive to verify a covalent interaction from an affinity shift alone, as pseudo-irreversible interactions can also occur caused by slow dissociation rates. This is actually shown in **Chapter 3**, where a long residence time compound, LUF6632, also achieves a better affinity with a longer incubation time. To this end, the binding kinetic profiles are explored as a second hint for covalence, with specific focus on a ligand's dissociation rate constant (k_{off}) and residence time (RT = 1/k_{off}; Figure 4). The covalent ligands presented in **Chapters 3-5** cause an initial 'overshoot' of the competition association curve, followed by a linear decline over time indicating that no equilibrium was reached. The data analysis of these cases yield a negligible dissociation rate, and a concomitant, almost infinite residence time. In addition, the inadequacy of the Motulsky-Mahan equations to fit this data is further evidence for the non-equilibrium features of the irreversible interaction with the receptor [11]. Thirdly, a "washout" experiment to ascertain the irreversible ligand-receptor interaction are often performed (Figure 4). Here, the washing treatment failed to regenerate the binding capacity of the radioligand used, which demonstrated wash-resistance of the covalent ligand at the receptor.

A final (fourth) confirmation, is obtained by locating the target amino acid of the warhead responsible for the covalent interaction of the ligand in the receptor binding pocket (Figure 4). Structural knowledge of the receptor binding pocket greatly aids in docking the covalent compound, and thus pinpointing towards a potential anchor point. Finding the anchor point for covalent probes of GPCR families is always challenging. Mutagenesis of nucleophilic residues near the ligand's binding pocket appears to be one of the most useful tools to identify where the ligands bind covalently. Under the circumstance that receptor mutation does not alter the receptor binding site and functionality, a regained recovery of radioligand binding capacity in "wash-out" assays demonstrates the involvement of covalent bonding with the selected amino acid residue. In the studies of LUF7445 (**Chapter 3)** and LUF7746 **(Chapter 5)**, the mutation led to the identification of the primary anchor point engaged in the covalent interaction. Still, this recovery failed to regenerate a 100% radioligand binding capacity, suggesting that other unidentified residues may play a similar role. In this context, an incomplete receptor activation recovery was observed in the functional [^{35}S] GTPγS assay for covalent partial agonist (LFU7746, **Chapter 5**). Of note, site-directed mutagenesis studies for hA_3R (**Chapter 4**) showed that removal of the nucleophilic group of the targeted amino acid residue resulted in the complete loss of covalent binding, validating that Y265$^{7.36}$ is the only anchor point of reactive covalent ligand LUF7602. The results presented in this thesis therefore agree with previous investigations to some degree, i.e. a high-affinity pharmacophore is a crucial starting point for a successful design of covalent ligands. Nevertheless, in future research, more extensive knowledge of receptor structure may improve the feasibility to locate the covalent interaction anchoring point. Overall, mutagenesis of nucleophilic residues near the ligand binding pocket is useful to study the mode and site of interaction, but may also drive the covalent ligand to react with secondary nucleophilic amino acid residues.

Although covalence can be confirmed by the experiments listed above, the irreversible activation/inhibition can be further evaluated on receptor function (Figure 4). For example, the covalent antagonistic nature of LUF7445 (**Chapter 3)** was confirmed in a cAMP functional assay, as it irreversibly blocked $hA_{2A}R$-mediated cAMP accumulation by agonist NECA. For G_i-coupled receptors, a membrane functional assay using [^{35}S]GTPγS was employed. An insurmountable antagonism caused by the covalent interaction was demonstrated for LUF7602 (**Chapter 4**), while a persistent activation by LUF7746 (**Chapter 5**) was validated by its resistance to be blocked by an inverse agonist. Importantly, ligands of

interest in **Chapter 5** were further evaluated for their covalent partial agonistic behavior in the label-free xCELLigence assay detecting changes in cell morphology. Compared to conventional *in vitro* functional assays, these assays provide new opportunities, as they determine integrated receptor-mediated responses under near-physiological conditions at the cellular level that are recorded in real time without the need for any labels. Consistent with an irreversible mode of receptor activation in a membrane-based [^{35}S]GTPγS assay, the obtained results validated the irreversible activation induced by LUF7746, also by its resistance to inhibition by antagonist DPCPX.

1.3. Applications of covalent ligands

The application of covalent ligands summarized in this thesis mainly focuses on the investigation of the topography of the GPCR-ligand binding pockets, as well as on receptor-signaling. However, in **Chapter 6**, we developed a covalent antagonist LUF7445 into an affinity-based probe LUF7487, which was used for two-step labeling of adenosine receptors. The previously synthesized covalently binding ligand LUF7445 (described in **Chapter 3**) was diversified to yield a few novel derivatives with different linker lengths. The most potent ligand was further equipped with a terminal alkyne allowing conjugation to azide-tailed fluorescent dyes, yielding probe LUF7487. Once bound, probe LUF7487 was concentration-dependently reacted with a fluorescent Cy3 moiety onto purified hA_{2A}Rs via a bio-orthogonal copper catalyzed azide-alkyne click-ligation reaction. We further demonstrated that this affinity-based covalent labeling of the purified $hA_{2A}R$ by probe LUF7487 was inhibited by $hA_{2A}R$ selective antagonists. Lastly, we showed successful labeling of the receptor in cell membranes overexpressing $hA_{2A}R$ making probe LUF7487 a promising affinity-based probe (AfBP) that will be useful in identifying and profiling the presence of the $hA_{2A}R$ in complex biological samples.

In summary, by applying this research workflow (Figure 4), including rational design of covalent ligands and non-reactive control compounds, covalent interaction characterization and anchoring position capture, we shed light on the molecular mechanism of covalent modulation of the adenosine receptors. The obtained insights are valuable for the design of covalent antagonists (**Chapter 3** and **4**) and agonists (**Chapter 5**) for the orthosteric ligand binding site, for which novel assays were set up to study receptor pharmacology (**Chapters 3, 4 and 5**). The results obtained with novel tool compounds (**Chapters 6**) depict native receptor binding, and bridge the fields of chemical biology and molecular pharmacology to better investigate receptor-ligand interactions. Together, these insights are valuable in future

discovery projects for drugs targeting the adenosine receptor, and this research methodology may serve as an example to study the structural details on ligand recognition for other GPCR subtypes.

2. Future perspectives

2.1. The "druggability" of adenosine receptors

Adenosine receptors have been recognized as therapeutic targets for various diseases, such as cerebral and cardiac ischemic diseases [12], sleep disorders [12], immune and inflammatory disorders [13] and cancer [14]. Therefore, numerous attempts to develop potent and selective ligands have been made in recent decades [12, 15]. In the end, the first approved AR ligand is a selective $A_{2A}R$ agonist, regadenoson (Lexiscan®), for medical use related to myocardial perfusion imaging [16]. Another example is a xanthine-derived $A_{2A}R$ antagonist, istradefylline, (NOURIAST®), for the treatment of Parkinson's disease in Japan [17]. Many other clinical trials targeting ARs are currently in progress with a focus on indications including Parkinson's disease, chronic heart failure and inflammatory and autoimmune disorders [15, 18]. More recently, with the discovery of the adenosine-mediated immunosuppressive mechanism in cancer therapy [14], several A_{2A} antagonists, initially developed for the CNS system, have entered clinical trials as immune-oncology agents alone and in combination with anti-PD1 or anti-PDL1 therapies [19]. Since oxygen deprivation in the tumor microenvironment causes an augmented extracellular adenosine level [14], it is essential that high-affinity adenosine antagonists are able to maintain their potencies in competition with the local adenosine levels, especially for small molecule antagonists with a fairly short *in vivo* half-life. In this regard, a covalently binding antagonist such as LUF7445 (described in **Chapter 3**), potentially prolonging the duration of action, may be a better proposition under these conditions. Moreover, as discussed in **Chapter 1**, the complexity of adenosine-mediated signaling and potential side effects compromises the development of pharmacological agents for specific applications. In such cases, the current fundamental research efforts on receptor structure and function still provide novel insights to facilitate the development of clinical candidates for adenosine receptors. More importantly, novel covalent AR ligands, including those discussed in this thesis, should be developed to investigate the topography of the GPCR-ligand binding pocket, as well as receptor-signaling and further pharmacological research. It is anticipated that such future molecules will possess enhanced properties and may therefore emerge as future drugs targeting ARs.

2.2. Covalent inhibitors for drug targets - what are the future opportunities?

In this thesis I have presented a design strategy for covalent probes that entails the identification of a reversibly binding lead compound, which is further optimized and used as a scaffold to incorporate a warhead for the targeted receptor. Notwithstanding the successful cases studied in this thesis, there are concerns that the addition of warheads and/or linkers might alter the key properties of the synthesized ligands. Meanwhile, the full dependency on the topology information of the binding pocket sometimes limits the successful application of this strategy to new targets. As in the hA_3R study in **Chapter 4**, one solution is to build a homology model based on other target, in this case $hA_{2A}R$ subtype, with similar binding pockets. Knowledge of targets with similar binding pockets and anchoring locations is a valuable resource for designing selective compounds, as overlaying structures can often highlight where modifications can be made to gain selectivity. Still, this strategy can only be applicable if a suitable reversible lead exists.

Alternatively, covalent fragment screening appears to be a potential solution to cases where no available reversible ligands/inhibitors for the targets are known [20, 21]. It starts with a low molecular weight or drug-like fragment with an electrophilic reactive center, which is then developed into a fragment library. Specifically, when the electrophilic fragments bind proximal to the nucleophilic residue on the target protein, they will be covalently trapped on the target protein surface and detected using X-ray crystallography, mass spectrometry and digestion studies to identify the residue targeted [22]. The robust discovery of covalent ligands relies on the size of fragment libraries covering a broad range of reactivity. Nevertheless, potential issues that arise from this approach reveal that such simple hits will be dominated by strong reactivity of the fragments instead of specific recognition (as is the case for a pharmacophore), consequently increasing the risk of non-specific labeling, off-target toxicity, and promiscuous activity [21].

In addition, the availability of proper bioassays to measure covalent binding is essential. The examples illustrated in this thesis focus on the time-dependent radioligand binding assay and site-directed mutagenesis studies aided by computational docking studies. Another technique, mass spectrometry (MS)-based proteomics, emerged as a powerful structural biological tool to characterize protein confirmation providing further structural insight [23, 24]. Depending on the availability of high-resolution structures, MS-based structural strategies can provide valuable, previously inaccessible information on protein conformational changes and dynamics, protein flexibility, and ligand-protein binding [25, 26].

The power of combining mass spectrometry-based proteomics and site-directed covalent labeling in the elucidation of GPCR ligand binding sites has shown great impact on the understanding of the structural features involved in ligand binding [27-29]. As summarized in **Chapter 2,** so far there is only one case that uses a photoaffinity probe to investigate the precise nucleophilic anchor point in the $hA_{2A}R$ binding pocket by mass spectrometry [30]. In this regard, the development of diverse experimental paradigms opens promising avenues for covalent ligands to obtain focused insights in structure-enabled GPCR ligand design. Ultimately, this may help in increasing the number and the quality of drug candidates targeting adenosine receptors as well as other GPCRs in the near future.

2.3. Covalent probes for GPCRs - where should we go?

Covalent ligands for GPCRs have shown to be valuable tool compounds to facilitate GPCR structure and function determination [31]. In the field of the adenosine A_3 receptor, an experimental crystal or cryo-EM structure would be a valuable addition to the currently available structures in the adenosine receptor family. The covalent ligands described in **Chapter 4** could be valuable tools for the elucidation of the inactive state of the human adenosine A_3 receptor structure.

The advent of covalent probes for chemical biology has been assisted by the development of click chemistry methods [32-34]. As demonstrated in **Chapter 6**, these covalent probes equipped with a ligation handle are paired with tags (e.g., biotin and/or a fluorophore) after they covalently bound to the receptors. This strategy underlays chemical biology or proteomics studies, to gain deeper insights into receptor localization and target engagement. The uncoupling of the ligand binding from the reporter tag labeling steps by click chemistry allows for tracking tissue and organ distribution of covalent probes *in vivo* [35]. In future research on adenosine receptors, different tags may be introduced; for instance, a biotin-tag would allow for streptavidin-mediated receptor enrichment for in-depth profiling using the Multi-dimensional Protein Identification Technology (MudPIT) analysis [36]. Similarly, the approach developed in this study may be applied to other GPCRs in similar physiological and pathological conditions.

2.4. Covalent drugs for GPCRs - worth a try?

In the history of drug discovery, covalent drugs have returned to the stage of the drug market in recent years, despite the past reluctance to pursue a covalent mechanism of interaction due to potential off-target toxicities. In approximately one-third of all enzyme targets for which there is an FDA-approved inhibitor, there is an example of an approved covalent drug [37].

The number of literature citations concerning covalent drugs appears to be rapidly increasing [37-41]. The continued and renewed interest in covalent drugs came from several recognized benefits, such as increased target potency, prolonged duration of action and the decreased therapy-induced drug resistance in clinical studies of cancer or infection. There is currently only one covalent drug targeting GPCR families, chopidogrel [42, 43]. It blocks the $P2Y_{12}$ receptor to inhibit platelet aggregation, occurring in e.g., thrombosis. However, clopidrogel can come with unwanted (on target) side-effects, such as extensive bleeding and thrombotic thrombocytopenic purpura.

In general, the bar to introduce a covalent drug for GPCRs is higher than is the case for kinases, a target class which has so far been well recognized and represented by several recently approved covalent drugs (ibrutinib, afatinib, and osimertinib) [44, 45]. Part of the reason is owing to the absence of an active reaction center in receptors and thus in the deficiency of mechanism-based inhibitors. Several considerations for rational design summarized in the conclusion paragraph may increase the rate of success. In addition, designing a compound targeting a poorly conserved residue may improve the selectivity of a compound. For instance, cysteine residues are in low abundance in proteins and possess a high nucleophilicity, appealing to be targeted with a low-reactivity warhead, Michael acceptors being key examples [46, 47]. This warhead reactivity is certainly an important consideration, demonstrating a balance between target engagement and idiosyncratic adverse drug reactions. To this end, warheads with low reactivity are generally preferred as a “safer” choice for a future drug candidate. Overall, the successful development of covalent kinase inhibitors as safe and efficacious cancer therapies will support the efforts towards other targets, such as GPCRs. If the selectivity and thus the safety of covalent molecules can be guaranteed these molecules provide valuable opportunities for future drug therapy.

Final notes

This thesis is focused on rational design and pharmacological profiling paradigms of covalent probes for adenosine receptors. The results obtained in this thesis contribute to an improved understanding of the molecular aspects of receptor structure and function. We provide evidence that covalent modulation of GPCRs adds indispensable information on structural insights. Besides, we set up a work flow of *in vitro* pharmacological assays as a robust tool for measuring and quantifying covalent modulation. Finally, we developed affinity-based probes, which allow monitoring of GPCR expression in cell fragments.

Hopefully, all findings from this thesis add to a further molecular understanding of covalent ligand-receptor interactions, and contribute to the design of better covalent ligands with an appropriate profile, multiple tool compounds for future target validation, and ultimately suitable evaluation schemes for a better translation towards effective and safe drugs.

References

1. Jorg M., and Scammells P.J. *ChemMedChem.* **2016**. 11(14): 1488-1498.
2. Narayanan A., and Jones L.H. *Chem Sci.* **2015**. 6(5): 2650-2659.
3. Jaakola V.P., Griffith M.T., Hanson M.A., Cherezov V., Chien E.Y., Lane J.R., IJzerman A.P., and Stevens R.C. *Science.* **2008**. 322(5905): 1211-1217.
4. Guo D., Xia L.Z., van Veldhoven J.P.D., Hazeu M., Mocking T., Brussee J., IJzerman A.P., and Heitman L.H. *Chemmedchem.* **2014**. 9(4): 752-761.
5. de Zwart M., Vollinga R.C., Beukers M.W., Sleegers D.F., von Frijtag Drabbe Künzel J.K., de Groote M., and IJzerman A.P. *Drug Dev Res.* **1999**. 48(3): 95-103.
6. Sherman W., Day T., Jacobson M.P., Friesner R.A., and Farid R. *J Med Chem.* **2006**. 49(2): 534-553.
7. Rosentreter U., Henning R., Bauser M., Krämer T., Vaupel A., Hübsch W., Dembowsky K., Salcher-Schraufstätter O., Stasch J.-P., Krahn T., and Perzborn E. *WO2001/025210, .* **2001**: April 12.
8. Chang L.C.W., Kunzel J.K.V.F., Mulder-Krieger T., Spanjersberg R.F., Roerink S.F., van den Hout G., Beukers M.W., Brussee J., and IJzerman A.P. *J Med Chem.* **2005**. 48(6): 2045-2053.
9. Louvel J., Guo D., Soethoudt M., Mocking T.A.M., Lenselink E.B., Mulder-Krieger T., Heitman L.H., and IJzerman A.P. *Eur J Med Chem.* **2015**. 101: 681-691.
10. Picone R.P., Fournier D.J., and Makriyannis A. *J Pept Res.* **2002**. 60(6): 348-356.
11. Motulsky H.J., and Mahan L.C. *Mol Pharmacol.* **1984**. 25(1): 1-9.
12. Jacobson K.A., and Gao Z.G. *Nat Rev Drug Discov.* **2006**. 5(3): 247-264.
13. Hasko G., Linden J., Cronstein B., and Pacher P. *Nat Rev Drug Discov.* **2008**. 7(9): 759-770.
14. Vijayan D., Young A., Teng M.W.L., and Smyth M.J. *Nat Rev Drug Discov.* **2017**. 17(12): 765-765.
15. Muller C.E., and Jacobson K.A. *Biochim Biophys Acta.* **2011**. 1808(5): 1290-1308.
16. Ghimire G., Hage F.G., Heo J., and Iskandrian A.E. *J Nucl Cardiol.* **2013**. 20(2): 284-288.
17. Dungo R., and Deeks E.D. *Drugs.* **2013**. 73(8): 875-882.
18. Chen J.F., Eltzschig H.K., and Fredholm B.B. *Nat Rev Drug Discov.* **2013**. 12(4): 265-286.
19. Congreve M., Brown G.A., Borodovsky A., and Lamb M.L. *Expert Opin Drug Discov.* **2018**. 13(11): 997-1003.
20. Lonsdale R., and Ward R.A. *Chem Soc Rev.* **2018**. 47(11): 3816-3830.
21. Kathman S.G., and Statsyuk A.V. *Medchemcomm.* **2016**. 7(4): 576-585.
22. Tailor A., Waddington J.C., Meng X.L., and Park B.K. *Chem Res Toxicol.* **2016**. 29(12): 1912-1935.
23. Xiao K., Chung J., and Wall A. *J Recept Signal Transduct Res.* **2015**. 35(3): 213-229.
24. Shukla A.K., Westfield G.H., Xiao K., Reis R.I., Huang L.Y., Tripathi-Shukla P., Qian J., Li S., Blanc A., Oleskie A.N., Dosey A.M., Su M., Liang C.R., Gu L.L., Shan J.M., Chen X., Hanna R., Choi M., Yao X.J., Klink B.U., Kahsai A.W., Sidhu S.S., Koide S., Penczek P.A., Kossiakoff

7

A.A., Woods V.L., Jr., Kobilka B.K., Skiniotis G., and Lefkowitz R.J. *Nature.* **2014**. 512(7513): 218-222.

25. Qin S.S., Meng M.M., Yang D.H., Bai W.W., Lu Y., Peng Y., Song G.J., Wu Y.R., Zhou Q.T., Zhao S.W., Huang X.P., McCorvy J.D., Cai X.Q., Dai A.T., Roth B.L., Hanson M.A., Liu Z.J., Wang M.W., Stevens R.C., and Shui W.Q. *Chem Sci.* **2018**. 9(12): 3192-3199.
26. Massink A., Holzheimer M., Holscher A., Louvel J., Guo D., Spijksma G., Hankemeier T., and IJzerman A.P. *Purinergic Signal.* **2015**. 11(4): 581-594.
27. Mallipeddi S., Kreimer S., Zvonok N., Vemuri K., Karger B.L., Ivanov A.R., and Makriyannis A. *J Proteome Res.* **2017**. 16(7): 2419-2428.
28. Zvonok N., Xi W., Williams J., Janero D.R., Krishnan S.C., and Makriyannis A. *J Proteome Res.* **2010**. 9(4): 1746-1753.
29. Szymanski D.W., Papanastasiou M., Melchior K., Zvonok N., Mercier R.W., Janero D.R., Thakur G.A., Cha S.W., Wu B., Karger B., and Makriyannis A. *J Proteome Res.* **2011**. 10(10): 4789-4798.
30. Muranaka H., Momose T., Handa C., and Ozawa T. *ACS Med Chem Lett.* **2017**. 8(6): 660-665.
31. Weichert D., and Gmeiner P. *Acs Chem Biol.* **2015**. 10(6): 1376-1386.
32. Gregory K.J., Velagaleti R., Thal D.M., Brady R.M., Christopoulos A., Conn P.J., and Lapinsky D.J. *ACS Chem Biol.* **2016**. 11(7): 1870-1879.
33. Soethoudt M., Stolze S.C., Westphal M.V., van Stralen L., Martella A., van Rooden E.J., Guba W., Varga Z.V., Deng H., van Kasteren S.I., Grether U., IJzerman A.P., Pacher P., Carreira E.M., Overkleeft H.S., Ioan-Facsinay A., Heitman L.H., and van der Stelt M. *J Am Chem Soc.* **2018**. 140(19): 6067-6075.
34. Yang X., Michiels T.J.M., de Jong C., Soethoudt M., Dekker N., Gordon E., van der Stelt M., Heitman L.H., van der Es D., and IJzerman A.P. *J Med Chem.* **2018**. 61(17): 7892-7901.
35. Speers A.E., and Cravatt B.F. *Chem Biol.* **2004**. 11(4): 535-546.
36. Speers A.E., and Cravatt B.F. *Curr Protoc Chem Biol.* **2009**. 1: 29-41.
37. Singh J., Petter R.C., Baillie T.A., and Whitty A. *Nat Rev Drug Discov.* **2011**. 10(4): 307-317.
38. Kalgutkar A.S., and Dalvie D.K. *Expert Opin Drug Discov.* **2012**. 7(7): 561-581.
39. Bauer R.A. *Drug Discov Today.* **2015**. 20(9): 1061-1073.
40. Adeniyi A.A., Muthusamy R., and Soliman M.E.S. *Expert Opin Drug Discov.* **2016**. 11(1): 79-90.
41. De Cesco S., Kurian J., Dufresne C., Mittermaier A.K., and Moitessier N. *Eur J Med Chem.* **2017**. 138: 96-114.
42. Savi P., Pereillo J.M., Uzabiaga M.F., Combalbert J., Picard C., Maffrand J.P., Pascal M., and Herbert J.M. *Thromb Haemost.* **2000**. 84(5): 891-896.
43. Ding Z., Kim S., Dorsam R.T., Jin J., and Kunapuli S.P. *Blood.* **2003**. 101(10): 3908-3914.
44. Baillie T.A. *Angew Chem Int Ed.* **2016**. 55(43): 13408-13421.
45. Liu Q., Sabnis Y., Zhao Z., Zhang T., Buhrlage S.J., Jones L.H., and Gray N.S. *Chem Biol.* **2013**. 20(2): 146-159.
46. Abranyi-Balogh P., Petri L., Imre T., Szijj P., Scarpino A., Hrast M., Mitrovic A., Fonovic U.P., Nemeth K., Barreteau H., Roper D.I., Horvati K., Ferenczy G.G., Kos J., Ilas J., Gobec S., and Keseru G.M. *Eur J Med Chem.* **2018**. 160: 94-107.
47. Shannon D.A., and Weerapana E. *Curr Opin Chem Biol.* **2015**. 24: 18-26