Corpora non agunt nisi fixata : ligand receptor binding kinetics in G protein-coupled receptors

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Chapter 7

Conclusions and Future Perspectives

About this chapter

In this thesis, the binding kinetics of ligands to several human GPCR, i.e. cannabinoid receptor 1 (hCB_1), adenosine A_1 and A_3 (hA_1 and hA_3) receptors, were studied to provide a better and multi-faceted understanding of drug-target interactions. In this concluding chapter, new insights are elaborated on, and suggestions for future investigations in this research area are outlined.

Conclusions from this thesis

Binding kinetics (i.e. knowledge of association and dissociation rate constants of ligands binding to and unbinding from their target) are gaining importance in the early phases of drug discovery. Therefore, throughout all the chapters of this thesis, we have characterized the binding kinetics of several series of compounds on different GPCR (i.e. hCB_1, hA_1 and hA_3). Firstly, binding kinetics of 1,2-diarylimidazol-4-carboxamide derivatives and 1-(4,5-diarylthiophene-2-carbonyl)-4-phenylpiperidine-4-carboxamide derivatives as hCB_1 receptor antagonists have been investigated in **Chapters 2** and **3**, respectively. Secondly, for the hA_3 receptor, both antagonists (i.e. pyrido[2,1-f]purine-2,4-dione derivatives) and agonists (i.e. ribofurano and (N)-methanocarba derivatives) have been subjected to kinetic characterization (**Chapters 4** and **5**). Lastly, the binding kinetics of a few reference agonists and antagonists for the hA_1 receptor that were obtained through a novel methodology have been compared with results stemming from traditional filtration assays (**Chapter 6**).

First of all the spectrum of their kinetics, especially the residence times (RTs), varied significantly, although experimental conditions (e.g., temperature) were different. We found that the shortest RT antagonist at hCB_1 receptor was rimonabant (14 min at 30 ^{o}C, from **Chapter 2** and **3**), the drug that was withdrawn from the market. The longest RT antagonist is a 1-(4,5-diarylthiophene-2-carbonyl)-4-phenylpiperidine-4-carboxamide derivative (**Chapter 3**) with a RT of 2222 min (>36h at 30 ^{o}C). Another reported example of a ligand with such a rare long-lasting RT is tiotropium, a M_3 muscarinic receptor antagonist.[1] In the case of the hA_3 receptor antagonists (**Chapter 4**), divergent RTs from 2.2 min to 391 min at 10 ^{o}C were obtained; under similar experimental conditions the hA_3 receptor agonists (**Chapter 5**) presented a greater range of RTs, from 10 min to 1961 min (>30h). In addition, the RTs variation (from 0.68 min to 167 min) of hA_1 receptor reference ligands was also significant (**Chapter 6**), which were obtained at 28 ^{o}C. We speculate that variation in ligand dissociation rate constants may be due to the following three aspects: 1) the nature of the receptors (i.e. lipid hCB_1R vs hA_1 and hA_3R); 2) different GPCR conformations (inactive state vs active, G protein-bound state); 3)

the ligand's class-related binding mode (two series of hCB_1R antagonists or two series of hA_3R agonists).

Interestingly, in the current thesis all the determined association rate constants are significantly below the so-called diffusion limit (i.e. 10^7 $M^{-1}s^{-1}$)[2]. This observation indicates that target engagement for ligands is more hampered than imposed by the diffusion limit alone. Specifically, the association rate constants of hA_1 receptor ligands varied substantially (by 100-fold), which was similar to a previous report.[3] In contrast, for hCB_1 receptor and hA_3 receptor ligands, the differences in association rate constants were less pronounced, approximately 5- to 30-fold, respectively. Interestingly, we found that hA_3 receptor agonists' association rate constants, albeit not that divergent, correlated significantly with their affinities. Such correlation between affinities and on-rates has been reported in the case of other GPCR[4] and ion channels.[5, 6] It is therefore necessary to examine both association and dissociation rate constants of a ligand for better ligand optimization.

Multiple binding kinetics assays have been performed in the present thesis. Especially, the radioligand competition association assay based on the Motulsky-Mahan model served as a working horse throughout all the research topics.[7] Indeed, it turned out to be a good approach for the quantitative measurement of unlabeled ("cold") ligands' binding kinetics. However, this assay's low-throughput and the (safety) restrictions imposed by labeled ("hot") ligands hamper its application in the kinetic profiling and concomitant structure kinetics relationships (SKR) studies. In the initial "kinetics screening" phase we adopted a simplified in-house validated competition association assay;[3] by sampling only two assay (time) points per ligand. With that a kinetic rate index (KRI) value was determined as a semi-quantitative descriptor for a ligand's dissociation rate. This relative high-throughput assay could be generally applied for kinetics studies at diverse drug targets. This screening assay does have limitations, namely 1) it provides kinetics information on the dissociation rate only, and, 2) the resolution of KRI values is not high enough for detailed SKRs, unlike full kinetic parameters.[8] In **Chapter 6** of this thesis, we developed and validated a kinetic scintillation proximity

assay (SPA), a homogenous technology with a "mix and measure" format, allowing monitoring of radioligand binding over time in a single well, from which association and dissociation rate constants of unlabeled ligands were reliably and quickly determined. Since this assay technology brings the benefit of greater efficiency in kinetic radioligand binding experiments, it can be of general use for kinetics studies at other drug targets as well.[4] One drawback of this technology is that the temperature of the microplate counter cannot be controlled (i.e. in our lab it was fixed at 28 ± 1 °C), preventing some targets from being kinetically investigated currently. In contrast, with a typical filtration assay, kinetic radioligand binding experiments can be performed at virtually any temperature, which allows for assay temperature optimization according to the characteristics of the radioligand—target interaction. Thus the SPA technology would greatly benefit from a counting device with temperature setting.

Last but not least, this thesis provides substantial evidence that studying SKR is of great importance in the early phases of drug discovery. We have provided quite a few examples of ligands within the same chemical scaffold with very similar affinities that present very divergent binding kinetics. Thus detailed SKR analysis is able to offer added value to the traditional SAR. This can be exemplified by one of the hCB_1 receptor antagonists, 14d (**Chapter 2**). On the basis of its high, picomolar affinity alone 14d seems a potent lead, but its close-to-unity KRI value indicates this may not be the case. Another example from **Chapter 4**: hA_3 receptor antagonist 9 with a pKi = 8.5 would be considered a better ligand than antagonist 11 with a pKi = 8.2, according to traditional SAR. However, their RTs are 28 min and 278 min, respectively, making antagonist 11 kinetically favorable. Thus, ignorance of the RT (by solely focusing on affinity) may lead to discontinuation of a compound that would likely perform better *in vivo*. Importantly the hA_3 receptor agonist MRS5698 has been confirmed an interesting candidate lead compound for neuropathic pain, which might (in retrospect) be due to its optimal binding kinetics as shown in Chapter 5 (RT = 1961 min). Hence, the strategic combination of SAR and SKR analyses results in a better understanding of a ligand-receptor interaction, which comprises not only the equilibrium state but also metastable intermediates and/or transition states

during the course of ligand association and dissociation. This strategy may be generally applied for efficient drug design and provide improved drug candidates.

Future perspectives

Opportunities for binding kinetics on hCB_1 receptor.

The challenge in the development of hCB_1R antagonists/inverse agonists as antiobesity drugs is their poor safety profile. Our strategy has been described in **Chapters 2** and **3**: a potent hCB_1R antagonist should not only be periphery-selective but also have a slow dissociation profile from the receptor. There might be other strategies as well. One approach is developing an allosteric modulator, aiming to "fine-tune" the pharmacological activity of the endogenous orthosteric agonists.[9] In this case, a negative allosteric modulator (NAM) would be aimed for,[10] potentially having certain advantages over orthosteric ligands, like an increased target selectivity and the ability to maintain the spatial and temporal signaling profile of the endogenous ligands.[11-13] Existing (positive) allosteric modulators such as ORG27569 tend to act as non-competitive antagonists in functional assays. The reason why is not entirely clear, but may be due to increased receptor desensitization induced by the compound,[11] also indicating that there is no clear cut between allosteric and orthosteric efficacy, as observed in previous research.[14, 15] Thus, development of a true NAM and a subsequent NAMs-hCB_1 binding kinetics study may provide additional ligand-receptor information, yielding a better understanding of a NAM's mechanism of action and, possibly, NAMs as drug candidates.

Another direction is developing neutral hCB_1R antagonists to avoid some side effects because since the withdrawal of rimonabant the scientific community has been debating the compound's inverse agonism in this respect.[16, 17] Some of the compounds reduced peripheral nervous system (PNS) disorders. However, no improvement in reducing CNS side effects was observed, which might be due to the fact that their bioavailability was not restricted to the periphery.[18, 19] Another possible explanation might be that inverse agonism with respect to G protein-independent pathways (e.g., β-

arrestin recruitment) was neglected,[20] i.e. the neutral antagonists developed so far were not neutral with respect to all relevant signaling pathways. To date, the concept of biased signaling has gained increasing attention in the GPCR field,[21, 22] and the first evidence is mounting that binding kinetics can have a profound impact on this phenomenon.[23, 24]

Opportunities for binding kinetics on hA_3 receptor.

There is no doubt that the hA_3 receptor possesses tremendous therapeutic potential and clinical indications, due to its overexpression on cancer and inflammatory cells.[25-29] However, the hA_3 receptor has a peculiar and rapidly changing expression profile in cultured cells.[30] Moreover, certain hA_3 receptor ligands have been described as cytoprotective or cytotoxic merely depending on the concentration employed, highlighting the difficulties that arise when one aims to characterize hA_3 receptor compounds.[31,32] Such observed "dichotomy" in different therapeutic applications might be significantly influenced by both ligand RTs and desensitization/internalization-related receptor vulnerability (i.e. receptor-turnover): would such rapid receptor turnover off-set some agonist's long RT? For instance, IB-MECA (with a RT of 95 min at 10 °C, in **Chapter 5**) has been proven safe and well tolerated but its anti-inflammatory effect may be hampered by its relatively quick onset of internalization of about 35 min – 60 min at 37 °C by different cellular assays. [33, 34] For other longer RT antagonists and agonists as determined in the current thesis (e.g., MRS5698), further cellular investigation is required, since knowledge of binding kinetics and the effect this has on internalization/desensitization will help to determine which kinetic profile is needed for an hA_3 receptor ligand to display a beneficial therapeutic profile.

Another interesting, but complicating factor may be hA_3 receptor homodimerization. This has recently been well illustrated by fluorescently labelled ligand-receptor binding kinetics studies on intact cells.[35-37] A ligand's binding kinetics on the hA_3 receptor dimer is significantly different from its binding interaction with the receptor monomer; as a result, its concomitant functional effect could

be profoundly influenced. It would be of great interest to test our hA_3 receptor ligands in such a system and provide binding kinetics profiles for receptor dimers as well.

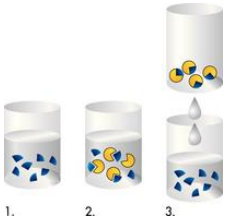
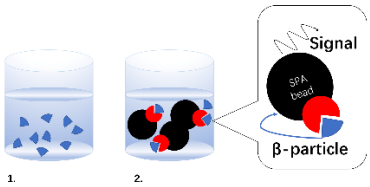
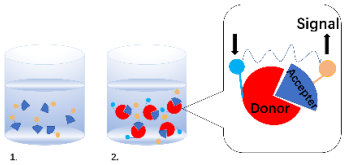
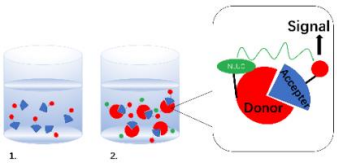
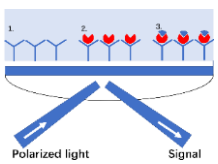
General opportunities in binding kinetics for GPCR and beyond.

- Assays and technologies for binding kinetics.

A comprehensive overview of biochemical and biophysical assays and technologies for assessing ligand-receptor binding kinetics was described in a recently published review (**Table 1**).[38] Currently, the most recognized and most straightforward kinetic profiling approach is applying a radioligand binding filtration assay using membrane proteins, as described throughout the current thesis. Although this method is sensitive and selective, it suffers from constraints imposed by the probe: a) high affinity, b) optimal/fixed experimental temperature (in most cases below physiological 37 °C), c) optimal/fixed incubation duration, d) quantification of competing ligands. In the (near) future better kinetic assays and technologies will be the standard such as a real-time homogeneous assay enabling high-throughput measurements, as exemplified by scintillation proximity assays (SPA) in **Chapter 6.** On the other hand, a few emerging non-radioactive assays (TR-FRET, BRET and SPR) indeed have advantages of real-time measurement of target binding kinetics, even in a high-throughput capacity. However, there are still quite a few disadvantages, such as the use of modified ligands (probes) or targets, fixed temperatures, and half-lives of fluorescent emission (photo bleaching). Advantages and disadvantages of the current techniques to measure drug-target binding kinetics are summarized in **Table 1**.

Table 1. Emerging experimental methods for kinetic profiling. Adapted and modified from ref. 38.

Assays and Technologies	General Performance (in steps)	Advantages	Disadvantages
Radioligand binding	1. Add radioligand (and cold ligand, not shown);	Good sensitivity and selectivity,	Radioactivity, limited throughput,

(filtration)  	2. Incubate with target for different period of time; 3.Harvest receptor/ligand complex on filter and count.	Non-engineered, wild-type receptor a universal tool	time-consuming, labor-intensive
Scintillation proximity assay (SPA)  	1.Add radioligand (and cold ligand, not shown); 2.Incubate with precoupled target SPA-bead complex and count.	High-throughput, homogenous, real-time measurement, accurate and precise measurement	Radioactivity, fixed temperature, limited choices of beads
Time-resolved fluorescence resonance energy transfer (TR-FRET)  	1. Add probe (and ligand of interest, not shown); 2. Add target (as “Donor”) and record signal	Non-radioactive, high-throughput, homogenous, real-time measurement	Fluorescently labeled receptor and ligand, fixed temperature, photo bleaching.
Bioluminescence resonance energy transfer (BRET)  	1. Add probe (and ligand of interest, not shown); 2. Add target (as “Donor”) and record signal	Non-radioactive, high-throughput, homogenous, real-time measurement	Luminescent enzyme tagged receptor, fluorescently labeled ligand
Surface plasmon resonance (SPR)  	1. Immobilize antibody; 2. Capture target; 3. Add ligand of interest and record signal	Non-radioactive, real-time measurement	Immobilized receptor (in large amounts), medium throughput

- From binding kinetics to functional dynamics

According to the medical definitions from the Merriam-Webster Dictionary, “kinetics” literally means the rate of change in a system effected by forces upon motions,[39] whilst “dynamics” describes “forces and their relation primarily to the motion but sometimes also to the equilibrium of bodies”.[40] In other words, *kinetics* emphasizes rates (e.g., ligands with a fast or slow association/dissociation to/from receptors); *dynamics* focuses on patterns of changes in systems (e.g., receptors, signaling pathways, cells, full bodies) after impact from external forces (e.g., all kinds of interactions), and such changes include equilibrium. Thus, there is a causal relation between “kinetics” and “dynamics”. Likewise, this entire thesis hypothesizes that, in GPCR, binding kinetics, more specifically RTs, are indispensable parameters to gauge dynamic functional effects.

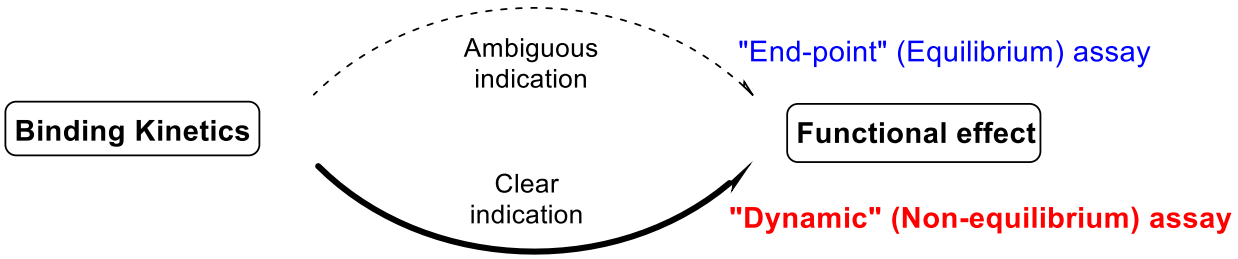


Figure 1. “Dynamic” functional assays are required for better translation of binding kinetics, as opposed to the currently much applied end-point functional assays.

Indeed, a positive correlation between agonist efficacy and residence time has been observed in case of the muscarinic acetylcholine M_3 receptor[41] and adenosine A_{2A} receptor.[42] In contrast, no such correlation was observed for the adenosine A_1 receptor.[43] There is a high chance that the effects of binding kinetics may not be perfectly depicted in classic functional assays under equilibrium conditions (e.g., with respect to E_{max}). For instance, E_{max} induced by an agonist can be dynamic over time.[44] Besides, receptor turnover (e.g., hA_3R has a peculiar and rapid desensitization/internalization profile in cultured cells[30]) may further complicate such dynamic processes. For antagonist’s binding

kinetics, insurmountability assays have been described as functional confirmation in this thesis (**Chapters 2, 3** and **4**). Such assays require complicated validation and optimization steps, especially for a long RT antagonist, where different preincubation times can lead to both insurmountable and surmountable effects, which also indicates the existence of functional dynamics. Therefore, dynamics of the functional effect shall be taken into account for further investigations. A simple rationale is presented below (**Figure 1**).

Methods for measuring and quantifying individual signaling pathways in real-time (e.g., G_{α}-protein, β-arrestin, cAMP) are emerging, which depict dynamic functional effects at a specific signaling pathway.[11, 45, 46] Moreover, binding kinetics-driven functional dynamics has been described as "apparent biased signalling".[23] However, the reported methods are performed under distinct experimental conditions due to practical reasons (e.g., temperature, cell type, sampling methods), questioning the robustness of these apparent bias observations. Quite recently, a proximity labeling technique called "APEX" (affinity purification coupled with mass spectrometry) has been introduced which may provide new insights into the kinetics of GPCR signaling.[47] Not only does it enlarge the signaling network,[48] but it also offers high time resolution into GPCR signaling in living cells.[49] In this way the dynamics of multiple signaling pathways can be carefully demonstrated and accurately quantified. Last but not least, by combining such detailed dynamic assessment technology with morphological label-free technology (e.g., xCELLigence™), better insights in cell signaling can be translated from binding kinetics.

- Motulsky-Mahan model, the perfect one?

Throughout the thesis, the Motulsky-Mahan model has been the mathematic foundation for the quantification of binding kinetics.[50] This model has been applied as an "Occam's razor" by simplifying the ligand-target interaction as a single and reversible binding interaction. Indeed such simplification is sufficient for unlabeled (cold) reversible ligands that competitively bind with the radioligand to the same target binding site. However, there are a few issues in its practical application. One example

are GPCR agonists which are able to bind more than one receptor conformation (e.g. active state and inactive state). Another example are bitopic ligands which might bind a target in different orientations.[51] Thus, kinetic quantification of GPCR agonists, irreversible binders and bitopic ligands faces difficulties when applying the Motulsky & Mahan model. In the future, more and more quantification-driven investigations of ligand-receptor binding kinetics, from both theoretical and experimental perspectives, shall be performed to further understand the mechanism of any ligand-target interaction.

- "The great divide" between *in vitro* binding kinetics and *in vivo* pharmacokinetics (PK) and pharmacodynamics (PD)

Although most of the current studies in this thesis have not been translated to *in vivo* studies (except for some ligands from **Chapter 5** by others [52]), one of the more outspoken differences between most *in vitro* and *in vivo* studies is the experimental temperature. Some slowly dissociating ligands were identified in *vitro* at temperatures (much) lower than mammals' body temperature (i.e. 37 ^{o}C), thus, their binding kinetic parameters in *vivo* will be (much) faster. Of note, the ranking of a series of drug candidates should not be changed under such temperature maneuver by translation from *in vitro* to *in vivo.* Still, a slowly dissociating ligand only leads to long-lasting *in vivo* effects, when its dissociation rate from the target exceeds the rate of free drug elimination from the effect compartment (**Figure 2**), ensuring pharmacodynamics (PD) outlasts pharmacokinetics (PK).[53]

Another overlooked pharmacological effect is target rebinding (**Figure 2)**, which means that a freshly dissociated ligand re-associates to the same or neighboring target. Prominent rebinding might be an important factor in dictating the duration of a ligand's *in vivo* effect, even if a ligand-target complex's lifetime is short. In this thesis, the RT of rimonabant, a hCB_1 receptor antagonist, was determined to be 14 min (**Chapters 2** and **3**). However in a previous study its RT was reported to be much longer (>80 min) in intact cells, which was explained by its prominent target rebinding.[54] Thus, despite its

relatively short RT determined *in vitro,* one could speculate that *in vivo* the rimonabant-related CNS on-target toxicity could be unavoidable.

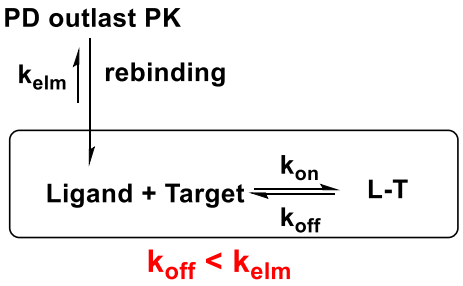


Figure 2. The binding kinetics *in vivo* is influenced by the rates of both free drug elimination (k_{elm}) from the effect compartment (the box) and rebinding. A slow dissociation rate (k_{off}) of a ligand exceeding its k_{elm} shall result in a long lasting *in vivo* effect, ensuring pharmacodynamics (PD) outlasts pharmacokinetics (PK). k_{on} is the association rate constant of a ligand to its target, k_{off} the dissociation rate constant.

Besides, recent theoretical studies on PK/PD has shown that such rebinding could persist by increased (local) concentrations of the drug, resulting from a high target association rate constant.[55, 56] On the other hand, an increased drug concentration can also lead to increased off-target binding and thus decreased selectivity.[56] Therefore, it is necessary to examine both association and dissociation rate constants of a ligand to its target for better translating *in vitro* binding kinetics to *in vivo* PK/PD. In this thesis, rimonabant has the slowest k_{on} among the antagonists tested in **Chapter 2**, but the fastest in **Chapter 3**, and thus is it appealing to further compare these antagonists' *in vivo* PK profile, concerning their rebinding and target occupancy. By combining data from different perspectives, the quality of the translation from *in vitro* to *in vivo* could be evaluated thoroughly.

Last but not least, species differences are another issue when translating *in vitro* findings to *in vivo* effects, which has been reported for both hCB_1 receptor and hA_3 receptors. [57-59] Thus, in binding kinetics, we suggest to perform an *in vitro* investigation of the "kinetic hits" in the species that will be used in later *in vivo* studies, next to obtaining data for the human receptor.[60] In this way, species variability can be predicted before the actual *in vivo* studies, and if needed, another lead compound can be selected which lacks these differences.

Final notes

The entire thesis evolves around the concept of ligand-receptor binding kinetics for the early phase of drug design and discovery. We extensively investigated this concept at two potential drug targets, the cannabinoid 1 receptor and adenosine A_3 receptor. We provide evidence that binding kinetics investigations on GPCR add indispensable information on these drug-target interactions. Besides, we propose that structure-kinetics relationship studies, next to the more traditional structure-affinity relationship studies, can improve the final decision and selection process of new chemical entities. Finally, we validated the scintillation proximity assay as a robust tool for high-throughput binding kinetics determination.

Hopefully, all findings from this thesis have brought new insights at a molecular understanding of ligand-receptor binding kinetics, and will offer suggestions for the design of better ligands with an appropriate kinetic profile, new technologies for rapid kinetic assessment, and ultimately suitable evaluation schemes for a better translation towards effective and safe drugs.

References

1. Casarosa, P.; Bouyssou, T.; Germeyer, S.; Schnapp, A.; Gantner, F.; Pieper, M. Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs. *J. Pharmacol. Exp. Ther.* **2009,** *330*, 660-668.
2. Smith, G. F. Medicinal chemistry by the numbers: the physicochemistry, thermodynamics and kinetics of modern drug design. In *Progress in Medicinal Chemistry*, Lawton, G.; Witty, D. R., Eds. Elsevier B.V.: Burlington, **2009**; Vol. *48*, p 1.
3. Guo, D.; Dorp, E. J. H.; Mulder-Krieger, T.; Veldhoven, J. P. D.; Brussee, J.; IJzerman, A. P. Dual-point competition association assay: a fast and high-throughput kinetic screening method for assessing ligand-receptor binding kinetics. *J. Biomol. Screen* **2012,** *18*.
4. Doornbos, M. L. J.; Cid, J. M.; Haubrich, J.; Nunes, A.; van de Sande, J. W.; Vermond, S. C.; Mulder-Krieger, T.; Trabanco, A. A.; Ahnaou, A.; Drinkenburg, W. H.; Lavreysen, H.; Heitman, L. H.; IJzerman, A. P.; Tresadern, G. Discovery and kinetic profiling of 7-aryl-1,2,4-triazolo[4,3-a]pyridines: positive allosteric modulators of the metabotropic glutamate receptor 2. *J. Med. Chem.* **2017,** *60*, 6704–6720.
5. Yu, Z.; van Veldhoven, J. P. D.; Louvel, J.; 't Hart, I. M. E.; Rook, M. B.; van der Heyden, M. A. G.; Heitman, L. H.; IJzerman, A. P. Structure–affinity relationships (SARs) and structure–kinetics relationships (SKRs) of Kv11.1 blockers. *J. Med. Chem.* **2015,** *58*, 5916-5929.

6. Yu, Z.; IJzerman, A. P.; Heitman, L. H. K(v)11.1 (hERG)-induced cardiotoxicity: a molecular insight from a binding kinetics study of prototypical K(v)11.1 (hERG) inhibitors. *Br. J. Pharmacol.* **2015,** *172*, 940-955.
7. Motulsky, H. J.; Mahan, L. C. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol. Pharmacol.* **1984,** *25*, 1-9.
8. van Veldhoven, J. P. D.; Liu, R.; Thee, S. A.; Wouters, Y.; Verhoork, S. J. M.; Mooiman, C.; Louvel, J.; Ijzerman, A. P. Affinity and kinetics study of anthranilic acids as HCA_2 receptor agonists. *Bioorg. Med. Chem.* **2015,** *23*, 4013-4025.
9. Morales, P.; Goya, P.; Jagerovic, N.; Hernandez-Folgado, L. Allosteric modulators of the CB_1 cannabinoid receptor: a structural update review. *Cannabis and Cannabinoid Research* **2016,** *1*, 22-30.
10. Abood, M. E. Allosteric Modulators: a Side Door. *J. Med. Chem.* **2016,** *59*, 42-43.
11. Cawston, E. E.; Redmond, W. J.; Breen, C. M.; Grimsey, N. L.; Connor, M.; Glass, M. Real-time characterization of cannabinoid receptor 1 (CB_1) allosteric modulators reveals novel mechanism of action. *Br. J. Pharmacol.* **2013,** *170*, 893-907.
12. Cawston, E. E.; Connor, M.; Di Marzo, V.; Silvestri, R.; Glass, M. Distinct temporal fingerprint for cyclic adenosine monophosphate (cAMP) signaling of indole-2-carboxamides as allosteric modulators of the cannabinoid receptors. *J. Med. Chem.* **2015,** *58*, 5979-5988.
13. Kulkarni, P. M.; Kulkarni, A. R.; Korde, A.; Tichkule, R. B.; Laprairie, R. B.; Denovan-Wright, E. M.; Zhou, H.; Janero, D. R.; Zvonok, N.; Makriyannis, A.; Cascio, M. G.; Pertwee, R. G.; Thakur, G. A. Novel electrophilic and photoaffinity covalent probes for mapping the cannabinoid 1 receptor allosteric site(s). *J. Med. Chem.* **2016,** *59*, 44-60.
14. Laprairie, R. B.; Bagher, A. M.; Kelly, M. E. M.; Denovan-Wright, E. M. Cannabidiol is a negative allosteric modulator of the cannabinoid CB_1 receptor. *Br. J. Pharmacol.* **2015,** *172*, 4790-4805.
15. Baillie, G. L.; Horswill, J. G.; Anavi-Goffer, S.; Reggio, P. H.; Bolognini, D.; Abood, M. E.; McAllister, S.; Strange, P. G.; Stephens, G. J.; Pertwee, R. G.; Ross, R. A. CB1 Receptor Allosteric Modulators Display Both Agonist and Signaling Pathway Specificity. *Mol. Pharmacol.* **2013,** *83*, 322-338.
16. Pertwee, R. G. Inverse agonism and neutral antagonism at cannabinoid CB_1 receptors. *Life Sci.* **2005,** 76, 1307-1324.
17. Erdozain, A. M.; Diez-Alarcia, R.; Meana, J. J.; Callado, L. F. The inverse agonist effect of rimonabant on G protein activation is not mediated by the cannabinoid CB1 receptor: evidence from postmortem human brain. *Biochem. Pharmacol.* **2012,** 83, 260-268.
18. Storr, M. A.; Bashashati, M.; Hirota, C.; Vemuri, V. K.; Keenan, C. M.; Duncan, M.; Lutz, B.; Mackie, K.; Makriyannis, A.; Macnaughton, W. K.; Sharkey, K. A. Differential effects of CB_1 neutral antagonists and inverse agonists on gastrointestinal motility in mice. *Neurogastroenterol. Motil.* **2010,** *22*, 787-e223.
19. Tudge, L.; Williams, C.; Cowen, P. J.; McCabe, C. Neural effects of cannabinoid CB_1 neutral antagonist tetrahydrocannabivarin on food reward and aversion in healthy volunteers. *Int. J. Neuropsychopharmacol.* **2015,** *18*, pyu094.
20. Delgado-Peraza, F.; Ahn, K. H.; Nogueras-Ortiz, C.; Mungrue, I. N.; Mackie, K.; Kendall, D. A.; Yudowski, G. A. Mechanisms of biased *β*-arrestin-mediated signaling downstream from the cannabinoid 1 receptor. *Mol. Pharmacol.* **2016,** *89*, 618-629.
21. Kenakin, T. Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* **2011,** *336*, 296-302.
22. Kenakin, T.; Christopoulos, A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discovery* **2013,** *12*, 205-216.
23. Klein Herenbrink, C.; Sykes, D. A.; Donthamsetti, P.; Canals, M.; Coudrat, T.; Shonberg, J.; Scammells, P. J.; Capuano, B.; Sexton, P. M.; Charlton, S. J.; Javitch, J. A.; Christopoulos, A.; Lane, J. R. The role of kinetic context in apparent biased agonism at GPCRs. *Nat. Commun.* **2016,** *7*, 10842.
24. Lane, J. R.; May, L. T.; Parton, R. G.; Sexton, P. M.; Christopoulos, A. A kinetic view of GPCR allostery and biased agonism. *Nat. Chem. Biol.* **2017,** *13*, 929-937.

25. Borea, P. A.; Varani, K.; Vincenzi, F.; Baraldi, P. G.; Tabrizi, M. A.; Merighi, S.; Gessi, S. The A_3 adenosine receptor: history and perspectives. *Pharmacol. Rev.* **2015,** *67*, 74-102.
26. Jacobson, K. A.; Gao, Z.-G. Adenosine receptors as therapeutic targets. *Nat. Rev. Drug Discovery* **2006,** *5*, 247-264.
27. Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Merighi, S.; Varani, K.; Borea, P. A.; Spalluto, G. A_3 adenosine receptor ligands: history and perspectives. *Med. Res. Rev.* **2000,** *20*, 103-128.
28. Baraldi, P. G.; Preti, D.; Borea, P. A.; Varani, K. Medicinal chemistry of A_3 adenosine receptor modulators: pharmacological activities and therapeutic implications. *J. Med. Chem.* **2012,** *55*, 5676-5703.
29. Hasko, G.; Linden, J.; Cronstein, B.; Pacher, P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discovery* **2008,** *7*, 759-770.
30. Klaasse, E. C.; IJzerman, A. P.; de Grip, W. J.; Beukers, M. W. Internalization and desensitization of adenosine receptors. *Purinergic Signalling* **2008,** *4*, 21-37.
31. Jacobson, K. A.; Moro, S.; Kim, Y.-C.; Li, A.-H. A_3 adenosine receptors: protective vs. damaging effects identified using novel agonists and antagonists. *Drug Dev. Res.* **1998,** *45*, 113-124.
32. Gessi, S.; Merighi, S.; Varani, K.; Leung, E.; Mac Lennan, S.; Borea, P. A. The A_3 adenosine receptor: an enigmatic player in cell biology. *Pharmacol. Ther.* **2008,** *117*, 123-140.
33. Trincavelli, M. L.; Tuscano, D.; Cecchetti, P.; Falleni, A.; Benzi, L.; Klotz, K.-N.; Gremigni, V.; Cattabeni, F.; Lucacchini, A.; Martini, C. Agonist-induced internalization and recycling of the human A_3 adenosine receptors. *J. Neurochem.* **2000,** *75*, 1493-1501.
34. Stoddart, L. A.; Vernall, A. J.; Briddon, S. J.; Kellam, B.; Hill, S. J. Direct visualisation of internalization of the adenosine A_3 receptor and localization with arrestin3 using a fluorescent agonist. *Neuropharmacology* **2015,** *98*, 68-77.
35. Hill, S. J.; May, L. T.; Kellam, B.; Woolard, J. Allosteric interactions at adenosine A_1 and A_3 receptors: new insights into the role of small molecules and receptor dimerization. *Br. J. Pharmacol.* **2014,** *171*, 1102-1113.
36. Corriden, R.; Kilpatrick, L. E.; Kellam, B.; Briddon, S. J.; Hill, S. J. Kinetic analysis of antagonist-occupied adenosine-A_3 receptors within membrane microdomains of individual cells provides evidence of receptor dimerization and allosterism. *The FASEB Journal* **2014,** *28*, 4211-4222.
37. May, L. T.; Bridge, L. J.; Stoddart, L. A.; Briddon, S. J.; Hill, S. J. Allosteric interactions across native adenosine-A_3 receptor homodimers: quantification using single-cell ligand-binding kinetics. *The FASEB Journal* **2011,** *25*, 3465-3476.
38. Schuetz, D. A.; de Witte, W. E. A.; Wong, Y. C.; Knasmueller, B.; Richter, L.; Kokh, D. B.; Sadiq, S. K.; Bosma, R.; Nederpelt, I.; Heitman, L. H.; Segala, E.; Amaral, M.; Guo, D.; Andres, D.; Georgi, V.; Stoddart, L. A.; Hill, S.; Cooke, R. M.; De Graaf, C.; Leurs, R.; Frech, M.; Wade, R. C.; de Lange, E. C. M.; Ijzerman, A. P.; Müller-Fahrnow, A.; Ecker, G. F. Kinetics for drug discovery: an industry-driven effort to target drug residence time. *Drug Discovery Today* **2017,** *22*, 896-911.
39. Kinetics. https://www.merriam-webster.com/dictionary/kinetics#medicalDictionary
40. Dynamics. https://www.merriam-webster.com/dictionary/dynamics#medicalDictionary
41. Sykes, D. A.; Dowling, M. R.; Charlton, S. J. Exploring the mechanism of agonist efficacy: a relationship between efficacy and agonist dissociation rate at the muscarinic M_3 receptor. *Mol. Pharmacol.* **2009,** *76*, 543-551.
42. Guo, D.; Mulder-Krieger, T.; Ijzerman, A. P.; Heitman, L. H. Functional efficacy of adenosine A_{2A} receptor agonists is positively correlated to their receptor residence time. *Br. J. Pharmacol.* **2012,** *166*, 1846-1859.
43. Louvel, J.; Guo, D.; Agliardi, M.; Mocking, T. A. M.; Kars, R.; Pham, T. P.; Xia, L.; de Vries, H.; Brussee, J.; Heitman, L. H.; IJzerman, A. P. Agonists for the adenosine A_1 receptor with tunable residence time. A case for nonribose 4-amino-6-aryl-5-cyano-2-thiopyrimidines. *J. Med. Chem.* **2014,** *57*, 3213-3222.
44. Cheng, J.; McCorvy, J. D.; Giguere, P. M.; Zhu, H.; Kenakin, T.; Roth, B. L.; Kozikowski, A. P. Design and discovery of functionally selective serotonin 2C (5-HT_{2C}) receptor agonists. *J. Med. Chem.* **2016,** *59*, 9866-9880.

45. Bosma, R.; Moritani, R.; Leurs, R.; Vischer, H. F. BRET-based β-arrestin2 recruitment to the histamine H_1 receptor for investigating antihistamine binding kinetics. *Pharmacol. Res.* **2016,** 111, 679-687.
46. Michela, B.; Claudia, S.; Piero, A.; Diego Dal, B.; Catia, L.; Ajiroghene, T.; Rosaria, V.; Gabriella, M. Overview on radiolabel-free in vitro assays for GPCRs. *Mini-Rev. Med. Chem.* **2017,** *17*, 3-14.
47. Doerr, A. Biochemistry: GPCR interactions in space and time. *Nat. Methods* **2017,** *14*, 551-551.
48. Lobingier, B. T.; Hüttenhain, R.; Eichel, K.; Miller, K. B.; Ting, A. Y.; von Zastrow, M.; Krogan, N. J. An approach to spatiotemporally resolve protein interaction networks in living cells. *Cell 169*, 350-360.e312.
49. Paek, J.; Kalocsay, M.; Staus, D. P.; Wingler, L.; Pascolutti, R.; Paulo, J. A.; Gygi, S. P.; Kruse, A. C. Multidimensional tracking of GPCR signaling via peroxidase-catalyzed proximity labeling. *Cell* **2017,** *169*, 338-349.e311.
50. Motulsky, H. J.; Mahan, L. C. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol* **1984,** 25.
51. Wittmann, H.-J.; Strasser, A. Competitive association binding kinetic assays: a new tool to detect two different binding orientations of a ligand to its target protein under distinct conditions? *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2017,** *390*, 595-612.
52. Jacobson, K. A.; Merighi, S.; Varani, K.; Borea, P. A.; Baraldi, S.; Aghazadeh Tabrizi, M.; Romagnoli, R.; Baraldi, P. G.; Ciancetta, A.; Tosh, D. K.; Gao, Z.-G.; Gessi, S. A3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy. *Medicinal Research Reviews* **2017**, 10.1002/med.21456.
53. Dahl, G.; Akerud, T. Pharmacokinetics and the drug–target residence time concept. *Drug Discovery Today* **2013,** *18*, 697-707.
54. Wennerberg, M.; Cheng, L.; Hjorth, S.; Clapham, J. C.; Balendran, A.; Vauquelin, G. Binding properties of antagonists to cannabinoid receptors in intact cells. *Fundam. Clin. Pharmacol.* **2011,** *25*, 200-210.
55. de Witte, W. E. A.; Vauquelin, G.; van der Graaf, P. H.; de Lange, E. C. M. The influence of drug distribution and drug-target binding on target occupancy: The rate-limiting step approximation. *European Journal of Pharmaceutical Sciences*.
56. de Witte, W. E. A.; Danhof, M.; van der Graaf, P. H.; de Lange, E. C. M. In vivo Target Residence Time and Kinetic Selectivity: The Association Rate Constant as Determinant. *Trends in Pharmacological Sciences* **2016,** 37, 831-842.
57. Gamage, T. F.; Farquhar, C. E.; Lefever, T. W.; Thomas, B. F.; Nguyen, T.; Zhang, Y.; Wiley, J. L. The great divide: separation between in vitro and in vivo effects of PSNCBAM-based CB_1 receptor allosteric modulators. *Neuropharmacology* **2017,** 125, 365-375.
58. Du, L.; Gao, Z.-G.; Paoletta, S.; Wan, T. C.; Gizewski, E. T.; Barbour, S.; van Veldhoven, J. P. D.; IJzerman, A. P.; Jacobson, K. A.; Auchampach, J. A. Species differences and mechanism of action of A3 adenosine receptor allosteric modulators. *Purinergic Signalling* **2017**.
59. Alnouri, M. W.; Jepards, S.; Casari, A.; Schiedel, A. C.; Hinz, S.; Müller, C. E. Selectivity is species-dependent: Characterization of standard agonists and antagonists at human, rat, and mouse adenosine receptors. *Purinergic Signalling* **2015,** *11*, 389-407.
60. Bot, I.; Ortiz Zacarías, N. V.; de Witte, W. E. A.; de Vries, H.; van Santbrink, P. J.; van der Velden, D.; Kröner, M. J.; van der Berg, D.-J.; Stamos, D.; de Lange, E. C. M.; Kuiper, J.; Ijzerman, A. P.; Heitman, L. H. A novel CCR_2 antagonist inhibits atherogenesis in apoE deficient mice by achieving high receptor occupancy. *Sci. Rep.* **2017,** *7*, 52.