Drug-target residence time : a case for the adenosine A1 and A2A receptors

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Chapter 7

Conclusions and perspectives

About this chapter

This thesis focuses on an emerging trend in medicinal chemistry, i.e. drug-target residence time. In this concluding chapter, new insights gained from case studies at the adenosine A_1 and A_{2A} receptors are elaborated on, and suggestions for future investigations in this field are presented.

7.1 Conclusions from this thesis

In the present thesis we have characterized the binding kinetics of several series of compounds on the adenosine A_1 and A_{2A} receptors. The spectrum of their residence times varies significantly, ranging from a few seconds to several minutes or hours. For instance, the residence times of 35 A_1R antagonists lasted from 20 sec to 48 min at room temperature (Chapter 3). Likewise, the series of ZM241385 derivatives displayed divergent residence times at the $A_{2A}R$, from 2 min to more than 5 h at 4 °C (chapter 4). Such values, in general, agree well with similar ranges of other ligand-GPCR residence times, which have been summarized in Chapter 2. One example is aprepitant, an NK_1 receptor antagonist for chemotherapy-induced emesis. It has a target-residence time of 222 min at 25 °C.[1] Of note is that the ligands having receptor residence times longer than one or two hours are scarce for the adenosine receptors. So far we have not identified a residence time lasting as long as tiotropium (>24 h at the M_3 receptor, 37 °C) or PF-4850890 (6.79 h at the CRF_1 receptor, 25 °C).[2,3] It is worth mentioning that ZM241385 displayed a target-residence time of 2.1 min at 25 °C, which was nearly 30-fold shorter than its value (72 min) at 5 °C (Chapter 5). We speculate that the observed relatively short RT of adenosine receptor ligands may be derived from the nature of the ligands' binding modes. Both tiotropium and PF-4850890 bind deeply into their respective receptors[4,5] and the binding cavities are covered by relatively large extracellular loops. In contrast, many adenosine receptor ligands, e.g., ZM241385 or UK432,097, are bound closer to the extracellular domain of the receptor, which, in addition, consists of relatively short and flexible extracellular loops.[6,7]

The association rates of the adenosine receptor ligands also vary substantially. Their values are across several orders-of-magnitude. Such information greatly updates our current knowledge, which has taken the on-rate to be diffusion-limited.[8] We have found that this general assumption is not necessarily valid. For instance, the $A_{2A}R$ antagonists' on-rates varied significantly from 0.10 $nM^{-1}\cdot min^{-1}$ to 0.0057 $nM^{-1}\cdot min^{-1}$ (Chapter 4)—more than two orders-of-magnitude from the diffusion-limited value (approximately $10^{10}\,M^{-1}\cdot min^{-1}$).[9] The latter only describes the transport rate of one binding partner (e.g. a ligand) through the reaction solution toward the other (e.g. a receptor), which is quite different from the association rate (k_{on})

of the binding process (formation of the ligand-receptor binary complex). The former depends on the viscosity of the medium, while the latter depends on the reaction mechanism.[10] For the association rate of agonists, their values ranged from 5.0×10^4 $M^{-1}\cdot min^{-1}$ to 1.6×10^7 $M^{-1}\cdot min^{-1}$ at the adenosine A_{2A} receptor (chapter 5), which are far from the diffusion-limited rate as well. Additionally, a ligand for a membrane-bound receptor could also partition into the membrane and then diffuse laterally to the binding site, a proposed mechanism for lipophilic compounds associating with the corticotropin-releasing factor receptor 1.[5] Apparently, an association rate can be far from the diffusion-limited rate. It is therefore necessary to examine both the on- and off-rate of a ligand for an optimized receptor interaction.

It is also of great interest to compare our results at the adenosine receptors with kinases or other enzymes. Of particular note is the induced, significant target isomerization upon ligand binding that is often encountered for enzyme-inhibitor interactions, a state also known as the transition-state. Such alternation of target conformations could yield a tightly bound binary complex, and thus "lock" the ligand for a long duration to exert its inhibitory effect. One example is GW572016 (Lapatinib), a tyrosine kinase inhibitor targeting epidermal growth factor receptor (EGFR) for cancer treatment.[11] It has a very slow off-rate from the purified intracellular domains of EGFR (0.0023 min^{-1}, equivalent to a half-life of 5 h) compared with OSI-774 and another EGFR selective inhibitor, ZD-1839 (Iressa). The latter two have a rapid off-rate, which translates to a half-life of less than 10 min. The difference in their binding kinetics was reflected in the distinct enzyme-inhibitor crystal structures, where GW572016 was in an inactive-like conformation while OSI-774 and ZD-1839 were not. Ligands binding to the adenosine receptors may also select a certain conformation, which comes to mind when considering the fast exchange of receptor states between the G protein-coupled and uncoupled forms. However, it is important to point out that such state transformations of GPCRs are much faster than the 'induced-fit' mechanism observed in the enzyme-inhibitor interactions. As evidence, a plot of k_{obs} values of [^{3}H]-NECA vs [^{3}H]-NECA concentrations displayed a significant linear correlation ($r^2 = 0.96$, $p < 0.0001$) at the adenosine A_{2A} receptor, indicative of a one-step association and dissociation process, quite distinct from the 'induced-fit' mechanism (which would yield a hyperbolic relationship).[8]

Several binding kinetics assays have been used in the present thesis. One of the more traditional assays is the direct kinetic radioligand binding assay. It

is a straightforward method enabling robust and quantitative measurements of ligand-receptor binding kinetics. However, this assay's low-throughput format and the technical difficulty of radiolabeling limit its application in kinetic profiling. The competition association assay based on the model of Motulsky and Mahan is a good alternative for the quantitative measurement of unlabeled ligands' binding kinetics.[12] It has been extensively applied in this thesis from Chapter 3 to Chapter 6 to examine the on- and off-rates of ligands of interest. We have also adopted the competition association assay and modified it into a fast, high-throughput screening assay, requiring only two assay points per compound (Chapter 3). This assay can be of general application for kinetics studies at other drug targets as well.

Kinetic profiling of a ligand-receptor interaction is an important addition to the classic equilibrium-based pharmacological evaluations. Combining both kinetics and equilibrium-based metrics can provide extra information for our understanding of complex pharmacological concepts, such as efficacy/intrinsic activity and allosteric modulation. An attempt to explain different agonists' functional efficacy was presented in Chapter 5, where we found that a sustained target-residence time was important for a higher level of cell signaling, at least for the adenosine A_{2A} receptor. Likewise, we gained more molecular understanding of the phenomenon of allosteric modulation at the A_1R. We learned that an allosteric modulator could alter an orthosteric ligand's affinity not only via influencing its dissociation rate but also via changing its association rate. Additionally, linking an allosteric modulator with an orthosteric ligand into one molecule (a so-called bitopic ligand) displayed a synergistic effect on residence time, which was much longer than the combination of two monovalent ligands. Apparently, the analysis of ligand-receptor binding kinetics can add a wealth of knowledge to current pharmacological receptor concepts.

7.2 Future perspectives

The studies outlined in this thesis aimed to explore ligand-receptor binding kinetics at G protein-coupled receptors, by a careful examination of the adenosine A_1 and A_{2A} receptors. In the (near) future, more knowledge can be obtained by further investigating the molecular basis of receptor kinetics, facilitated by the development of new technologies for rapid, online assessment

of kinetic profiles of new compounds. Furthermore, translating the *in vitro* binding kinetics to *in vivo* effects of future drug candidates targeting the adenosine receptors (ARs) could potentially decrease drug attrition rates and eventually lead to the discovery of more efficacious and safer medicines.

Molecular understanding

a) Physiochemical and hydration properties of dynamic ligand-receptor interactions

For future investigations the combination of binding kinetics, structural biology and molecular dynamics may greatly advance our understanding of the dynamic process of ligand-receptor interactions. For instance, we could compute physicochemical properties and hydration properties of the protein target, especially to map the binding sites that trap water molecules in hydrophobic surroundings. Displacing such less stable, 'unhappy', water molecules could elicit a favorable binding state of the receptor, compared to its (unliganded) apo states, hence influencing a ligand's residence time.[13] It is thus of great interest to take the explicit water molecules into consideration for future molecular understanding of the dynamic process of ligand-receptor interactions. The important role of water molecules was also addressed in recent molecular dynamics simulations at the β_2 receptor and the muscarinic M_3 receptor.[4,14,15] It was revealed that during the binding and unbinding process tremendous hydration and dehydration of both the ligand and the receptor was involved, which appears to be a critical process of great generality for a ligand's association to and egress from its receptor. Structure-based drug discovery aimed for kinetically optimized compounds (e.g. compounds displacing 'unhappy' water molecules) can now be performed at the $A_{2A}R$ given the availability of a high-resolution crystal structure (PDB: 4EIY) and the more than 60 water molecules contained in the transmembrane domains.[6] Moreover, the recent boom of adenosine A_{2A} receptor crystal structures[6,7,16,17] may greatly facilitate the molecular dynamic simulations of the ligand-receptor interactions. It was suggested that for the M_3R and the β-adrenergic receptors their ligands traversed a dominant two-step pathway

as they bind to or unbind from the receptor, initially making contact with a vestibule on the receptors' extracellular surface.[4,14] Such observations could be common for other GPCRs as well. It will be of great interest to perform similar molecular dynamic simulations at the adenosine receptors for an atomic-level description of its kinetic process.

b) New pharmacological models to understand the nature of functional efficacy

In chapter 5, we have illustrated that the intrinsic efficacy of an A_{2A} receptor agonist is inextricably linked to its receptor residence time. This gained knowledge aids us to further understand cell signaling upon ligand stimulation. Interestingly, one of the first attempts to define drug action from a kinetic perspective dates back to the early 1960s, when Paton proposed the 'rate theory' for a mechanistic explanation of efficacy.[18] However, only few studies continued on the track of kinetics since then,[19-21] which was later prevailed by other models that describes efficacy in terms of the ability to stabilize an active receptor conformation[10,11] or a more recently proposed 'stochastic switch' of ligand binding between functionally distinct orientiations.[22] Of note is that the kinetics of agonist binding and the subsequent conformational change of its receptor can influence each other. The binding of an agonist can induce receptor conformational changes and these changes potentially influence the ligand's residence time, for instance, via the receptor isomerizing into a high-affinity state and thus effectively locking the agonist.[23,24] In our view both conformation-stabilization models and kinetic models are complementary to each other rather than contrasting. Thus, an integration of both elements may better explain the nature of functional efficacy.

Furthermore, still little is known about the pharmacological consequences of cell/tissue/body exposure to agonists with a spectrum of receptor residence times. Will long receptor-residence time ligands cause higher levels of receptor desensitization or internalization? What is the connection between agonist-receptor binding kinetics and the downstream signaling kinetics (i.e. link to biased signaling)? These are important questions for future mechanistic investigations. The results thereof will most likely affect the medicinal chemistry strategy aiming for long or short receptor residence time in the future.

c) Mathematical models for theoretical investigations of ligand-receptor binding kinetics

To further increase current molecular understanding of ligand-receptor binding kinetics, new theoretical models are also needed. A mathematical model and simulation have been presented in Chapter 6 for validation of the competition association assay. We used it to determine a bitopic ligand's binding kinetics, for which it provided a powerful theoretical support. The strength of mathematical simulations is not restricted to validating experimental data. It also facilitates our understanding of complicated ligand-receptor interactions especially for those experimental observations otherwise difficult to interpret. For instance, Vauquelin and colleagues reported several case studies where mathematical simulations were extensively used to examine the binding kinetics of a bitopic/bivalent ligand.[25-27] Another example was reported by Charlton and colleagues in which they simulated the different onset of action of two M_3R antagonists that bear distinct kinetic profiles.[28] More theoretical investigations of ligand-receptor binding kinetics should be performed in the future.

New technologies for assessing binding kinetics

An overview of current technologies for accessing ligand-receptor binding kinetics was described in Chapter 2. In general, most of kinetic profiling techniques rely heavily on radioligand binding or functional reversibility assays; the latter in a qualitative manner. New tools are welcome to satisfy the emerging requests in optimizing binding kinetics. Currently, there are several technologies that could be evaluated for future binding kinetics investigations. For instance, several groups recently reported the use of fluorescently labeled ligands in combination with confocal microscopy equipped with high flow perfusion to study ligand-receptor binding kinetics at the level of single living cells.[29,30] Such tools enable the visualization and quantification of time-dependent receptor occupancy at the cellular level. Other assay technologies can also be used for further development including fourier transform infrared spectroscopy (FTIR). This technology simultaneously collects spectral data in a wide spectral range, which can be translated into dynamic conformational

changes upon ligand-receptor or protein-protein interactions at an atomic level.[31] One application of the FTIR technology in the field of GPCRs was reported by Elgeti *et al*. to study the conformational diversity of rhodopsin in a membrane environment—a study that extended the static picture provided by the available crystal structures.[32] Cell-based label-free technologies, such as xCELLigence described in Chapter 5, enable real-time, online signal readout upon ligand addition—a feature that might be used in binding kinetics characterizations, as well. Additionally, surface plasmon resonance (SPR) is also a good tool to measure ligand-receptor binding kinetics, which has been well reviewed by Nunez *et al*.[33] This assay is complementary to classical radioligand binding assays when a radioligand is not available. Of note is the current difficulty in the purification of target proteins from a membrane environment for the assay set-up—a technical bottleneck that needs to be overcome for its general application. It is also worth mentioning that applications of the above-mentioned new technologies might be limited to low throughput assay formats due to inherent technical difficulties. For a high-throughput format testing, one could consider scintillation proximity assay (SPA) or the recently developed Tag-lite® platform, avoiding a wash or filtration step as in traditional radioligand binding studies. Both assays allow continuous, multiple reads from one sample. The application of these two technologies has been exemplified at the GnRH receptor (SPA) and at the dopamine D_2 receptor (Tag-lite® platform), respectively.[34,35]

Translation of *in vitro* binding kinetics to *in vivo* evaluation

Translating *in vitro* binding kinetics into an *in vivo* setting is essential for the prediction of drug efficacy and safety. Such a progress, needless to say, is quite difficult and complex. Two major issues need to be taken into account, which are addressed as follows.

1) There are mismatches in binding kinetics between human and laboratory animal species. An expected margin of safety in one species could possibly disappear in human if on- /off-rates at the target vary in different species. On the other hand, a compound discarded due to a poor margin of safety or selectivity in animal models could be adequately selective for the desired over the unwanted effects in human. Such variation in ligand-receptor binding kinetics would be particularly important when efficacy and safety

studies are conducted in different species. Thus, we suggest including the investigation of ligand-receptor binding kinetics—at least for those candidate compounds progressing into (pre-) clinical phases—in those species that are anticipated in later proof-of-concept testing.

2) The *in vivo* duration of drug action is not only dependent on macroscopic pharmacokinetic properties and long-lasting target binding but is also influenced by target rebinding. The local environment of many effect compartments may largely prevent the diffusion of free drug molecules moving away from their target. This could result in consecutive (re-)binding to the same target and/or targets nearby even when the drug's concentration in the bulk phase has already dropped to near-zero levels.[36,37] In light of this, one could consider examining ligand-receptor binding kinetics in more physiological relevant conditions, for instance, by using intact (primary) cells and assay conditions reflecting those *in vivo* (e.g. buffer, temperature, pH).[28,38] Data obtained under such circumstances could establish a bridge between the *in vitro* and *in vivo* setting and thus help their translation.

It is worth mentioning that there is not always a direct link between slow dissociation and long-lasting *in vivo* target engagement, as the rate of free drug elimination from the effect compartment is also a key influencing factor—a finding commented on by several groups.[10,36,39,40] A collection of data sets from isolated cells, tissues, and animal models will provide more information.

7.3 Final note

Ligand-receptor binding kinetics is increasingly recognized to play a pivotal role in the early phase of drug design and discovery. In this thesis, we extensively investigated the ligand-receptor binding kinetics, particularly residence time, at the adenosine A_1 and A_{2A} receptors. Our case studies at these two prototypical GPCRs demonstrate that binding kinetics as an emerging paradigm can provide additional information of drug-target interactions at the molecular level.

We propose that a better molecular understanding of binding kinetics is needed to aid the development of drug candidates with optimized kinetics. We also anticipate the ensuing emergence of new technologies enabling rapid assessment of a compound's binding kinetics in the future. Linking

these findings in *in vitro* settings to an existing data set of *ex vivo* / *in vivo* experiments may provide an evaluation scheme that is more predictive for drug efficacy and safety. Hopefully, this thesis will be an impetus for further binding kinetics-directed research in the (near) future.

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