Targeting the adenosinergic system: Ligand binding kinetics and label-free assays for the study of SLC29A1 transporter and A2B adenosine receptor

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CHAPTER 7

Conclusions and Future Perspectives

Over the last decades, a great number of ligands binding with high affinity and selectivity on adenosinergic targets have been discovered. However, the number of clinically approved drugs is limited and not representative of the vast efforts that have been made. This discrepancy could be partially explained by the lack of translatability between *in vitro* and *in vivo* experiments. In order to better predict *in vivo* effects from *in vitro* data, an increased understanding of the ***mechanism of action at a molecular level*** is needed, in combination with ***physiologically relevant assays***. Therefore this thesis aimed to explore the concept of binding kinetics for two adenosinergic targets, *i.e.* the A_{2B}AR and ENT1, as well as to develop novel kinetic binding and label-free functional assays. This chapter provides the main conclusions drawn from this thesis, followed by the future perspectives that arise.

Conclusions

Label-free assays for the study of GPCRs and SLCs

Reporter-based assays constitute the great majority of assays used in the study of receptors and transporters (**chapter 1**). However, the use of labels in combination with heterologous cell lines, lead to a limited translational value[1-3]. Physiologically more relevant assays in combination with cell lines endogenously expressing the targets under investigation, are thus required. Label-free cellular assays offer increased physiological relevance compared to the current *status quo* on assays[3]. In this thesis, two novel methodologies using the xCELLigence technology are presented for the study of a GPCR and an SLC. This technology records the cellular impedance generated by the adhesion of cells to gold-coated electrodes.

In **chapter 3**, the development of a label-free assay for the study of A_{2B}AR pharmacology using a heterologous cell line, is described. The non-invasive character of the assay, yielding the integrated receptor-mediated responses on whole cells in real-time recording without the need of labels, increased translatability compared to the typical radioligand binding assays on cell membranes. The methodology was proven a valuable tool to determine the potency of A_{2B}AR (ant)agonists and signal recovery after washing.

The translational value of a label-free assay was further increased with the use of an endogenous cell line for the study of ENT1 pharmacology. **Chapter 4** describes the development and validation of an impedance-based label-free assay for the study of ENT1 inhibitors via the concomitant A_{2B}AR signalling. This is the first case of a label-free biosensor assay applied to the investigation of a non-electrogenic membrane transporter[4]. The sensitive monitoring and accurate quantification of ENT1 inhibition via such an assay, offers the possibility to study any membrane transporter that is or can be linked to a GPCR via their common substrate / ligand, hence creating novel opportunities for the study of membrane transporter pharmacology and drug discovery. This assay was further utilized in **chapter 5** to validate differences in kinetic binding parameters in a functional assay, opening new avenues for the translation of *in vitro* kinetic parameters in *in vivo* functional effects[5].

Kinetic characterization: Drawing a more complete picture of a ligands' pharmacological profile

Current drug discovery programs are mainly investigating thermodynamic drug-target interactions (affinity and potency) under equilibrium conditions, neglecting time-dependent changes in target engagement in open systems (**Chapter 1**). Accumulative data support the importance of binding kinetics as pivotal pharmacological parameters of a drug candidate[6], leading to an imminent need for

kinetic assays. Including the real-time impedance-based assays mentioned above, several kinetic binding assays have been developed throughout this thesis, to assess kinetic binding parameters of A_{2B}AR antagonists and ENT1 inhibitors, next to classical affinity studies.

Chapter 3 describes the development of a radioligand competition association assay for the evaluation of the kinetic binding parameters of a series of novel 8-phenylxanthine-based analogues designed as hA_{2B}AR antagonists. This is the first kinetic study of A_{2B}AR antagonists, leading to the construct of structure-kinetic relationships, next to the typical structure-affinity relationships. Diverse kinetic profiles were obtained and a great spread of target RT was observed. Interestingly, the dissociation rate constant appeared the driving force for affinity, indicating that its optimization is of great importance. Furthermore, the obtained kinetic binding parameters were validated in washout experiments on cell membranes.

The next target of the adenosinergic system investigated in **chapters 5** and **6** was ENT1. A competition association assay was established and validated as a valuable tool to determine the binding kinetics of unlabeled ENT1 inhibitors. In addition, washout experiments on cell membranes confirmed the results obtained in the competition association assay in both **chapters 5** and **6**. In **chapter 5,** four reference ENT1 inhibitors were characterized for their *in vitro* affinity and binding kinetics. All compounds showed a low nano- to subnanomolar affinity for the target, while one of the compounds, draflazine, presented a significantly longer RT for the target. Kinetic profiling of 9 draflazine analogues revealed that affinity, hence target engagement of draflazine-like ENT1 inhibitors is governed by their dissociation rate constants.

The next step was to study the binding kinetics of a class of compounds structurally unrelated to reference ENT1 inhibitors, as described in **chapter 6**. To do so, a series of spirobenzo-oxazinepiperidinone derivatives were synthesized and tested for their kinetic binding parameters using the competition association assay presented in **chapter 5**. SAR and SKR studies revealed characteristics of the functional groups involved in ENT1 binding. Bulkier substituents at the “right-hand” phenyl ring were well tolerated, suggesting a large binding pocket for ENT1, while substituents hydrophobic in nature or uncharged at physiological pH resulted in high affinity and a long RT. Once again affinity was found to be driven by dissociation rate constants. Interestingly, this trend was reversed when changes in the central scaffold occurred. Reduced polarity in the central scaffold led to faster associating compounds and for these derivatives the association rate constant was governing of affinity. As a result, binding kinetics were found to be scaffold specific and not target specific for ENT1.

All in all, these results validate the significant role of understanding binding kinetics in drug discovery. Lead selection driven exclusively by affinity will result in ligands with potentially “out-of-tune” kinetic characteristics, failing to result in optimal *in vivo* efficacy (driven by k_{off}) or onset of drug action and the duration of *in vivo* target occupancy (driven by k_{on}). Therefore, quantification of the kinetic binding parameters

k_{on}, k_{off} and RT will be an invaluable addition to early drug discovery programs.

Distinct kinetic binding profiles can result in differential *in vitro* functional effects

Next to the investigation of kinetic binding parameters from a binding assay perspective, understanding their translation to *in vitro* functional effect is of essence. Such interpretation brings us a step closer to predict the *in vivo* functional effect, thus comprehending how to fine-tune a drug candidate.

The *in vitro* functional effect of A_{2B}AR antagonists with distinct kinetic profiles were examined in **chapter 3**. Two compounds with long and short RT for the A_{2B}AR were tested in the aforementioned label-free impedance-based assay under non-equilibrium conditions. These conditions were obtained by pre-treatment of the cells with the compounds, followed by washing steps and subsequent activation of the receptor with an agonist. Pretreated with the long RT compound resulted in a lower cell signal. These experimental data verified the hypothesis that the short RT compound would be more readily removed during washing, resulting in an increased number of receptors available for the agonist to bind to and cause a cellular response. Thus, this finding confirmed the link between long RT and an extended pharmacological effect under non-equilibrium conditions.

Similarly, in **chapter 5** the functional effect of ENT1 inhibitors with various kinetic profiles were examined in a label-free assay. Cells were pre-treated for various time periods (30 min and 4 hours) with two structurally similar, yet having distinct binding kinetics for ENT1, followed by a treatment with adenosine. The potency of the ENT1 inhibitors was evaluated by the subsequent AR signal. Interestingly, a time-dependent effect was observed for the long RT compound (compound **4**), as its potency increased after a longer pre-incubation time. This effect was not observed for the shorter RT compound (draflazine), supporting the importance of long target RT for increased target-occupancy.

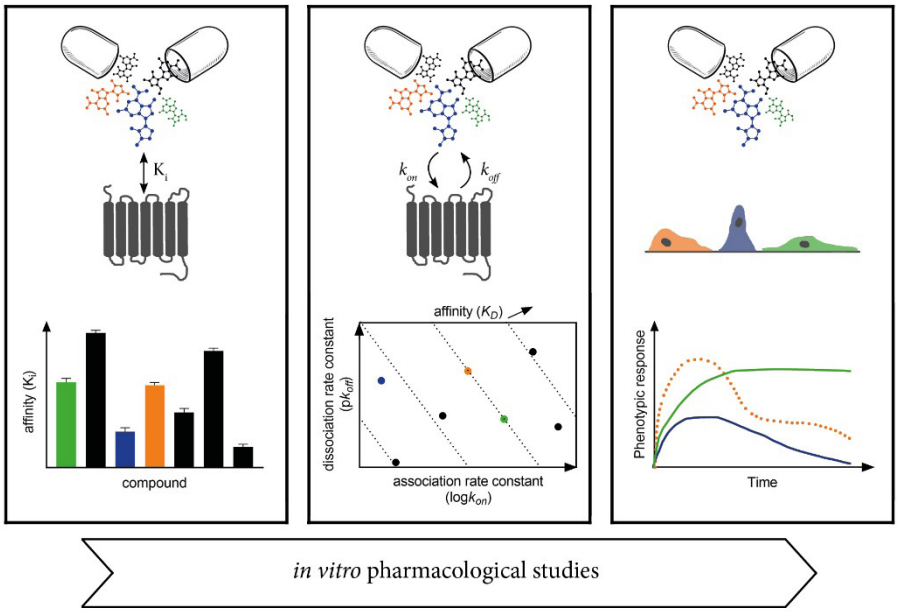

Figure 1: Graphic representation of the type of *in vitro* assays developed in this thesis. Both target-specific (left and middle panel) and cellular phenotypic (right panel) assays were developed for the evaluation of the ligands under investigation. All steps – from the equilibrium affinity determination till the non-equilibrium binding kinetics and functional effect – were found crucial for the characterization of the ligands in order to increase translatability towards an *in vivo* effect.

In summary, by a variety of assays we attempted to shed light on the mechanism of action of ligands for two adenosinergic targets. Specifically, invaluable insights were obtained for the design of A_{2B}AR antagonists (**Chapter 3**) as well as ENT1 inhibitors (**Chapter 5** and **6**). The novel compounds were evaluated in more physiologically relevant assays (**Chapter 3**, **4**, **5**). Taken together, this thesis provides a variety of *in vitro* binding / functional, equilibrium / non-equilibrium, radiolabeled / label-free pharmacological assays (Figure 1) to assess novel ligands for A_{2B}AR and ENT1 and offers a practical guidebook for the initial steps to be taken in drug discovery programs for these adenosinergic targets.

Future prospectives

The potential of drug-target binding kinetics

A retrospective survey in 2004[7] showing that slowly dissociating ligands present better efficacy than relatively faster dissociating equivalents and the introduction of a drug-target residence time (RT) model in 2006[8], has put the concept of drug-target binding kinetics on the map of drug discovery. Since then the concept captured the interest of many researchers, including joint efforts of academic and industrial partners in the form of the IMI project "Kinetics for Drug Discovery" (K4DD)[9], and it has come a long way. Numerous parameters, including rebinding[10] and one- or two-step binding[11], have been investigated. Likewise, possible limitations concerning drug-target binding kinetics in comparison to its clearance[12] and target turnover[13] have been explored. In addition, novel radioligand binding[14,15], FRET[16], SPA[17] and label-free[18] assays have been developed to qualitatively and quantitatively determine *in vitro* kinetic binding parameters. Recently the importance of binding kinetics has been recognized beyond the pure scientific interest. The European Medicines Agency modified the guidelines for first-in-human studies and early clinical trials of new investigational products and requests information on the duration of *in vitro* action, thus binding kinetics[19]. Given that, binding kinetics investigation appears to be more than a soon forgotten trend. It is here to stay and will hopefully transform from an emerging paradigm to a traditional and indispensable drug discovery parameter next to affinity and potency.

To further accelerate the transition towards a drug discovery project implemented with kinetic parameters, a few steps need to be taken. Although drug-target RT's importance is now well-established, ***association kinetics*** still remains neglected. In contrast to the established perception, association kinetics is not diffusion-limited, hence it can be modulated and lead to an adjusted onset of drug action[20]. In addition, mathematical models investigating binding kinetics in conjunction with pharmacokinetics provide evidence that a high target association rate constant leads to an increased (local) concentration of the drug, which slows the decline of target occupancy[12]. For ENT1 in particular, we have shown (**chapter 5**) that k_{on} can be steered with the efforts of medicinal chemists. Thus it would be of great interest to further modify the chemical structure. Due to the recent resolution of the ENT1 3D structure[21], docking studies could be of extreme help to identify and explain possible interactions driving the association kinetics. In combination with the current knowledge on dissociation kinetics, novel inhibitors with easily modulated kinetic characteristics could be achieved. Importantly, except for the binding kinetic characteristics of the synthetic ligands, the ***kinetic profile*** and the ***release rate of the endogenous ligand*** should be reflected upon. Considering that the majority of drug candidates bind to the same site as the endogenous ligand, *i.e.* the orthosteric binding site, there is a constant competition between the ligands, complicating the

drug discovery process. Regarding drug candidates targeting the adenosinergic system, the investigation of adenosine's binding kinetics is essential. Adenosine is an omnipresent nucleoside and is generated via multiple pathways as discussed in **Chapter 1**. As a result, its concentration fluctuates significantly and especially in pathophysiological conditions increases dramatically. Consequently, optimal binding kinetics for a drug candidate is not an absolute value or "a number fits all" situation. Finally, a crucial parameter to increase the impact of binding kinetics is the open access to these data, so that they can be easily incorporated into drug discovery programs. Hence, *publically available databases* are necessary. ChEMBL[22], Binding DB[23], KOFFI-DB[24] and Phenaris Kinetics DB[25] − with the latter one being a commercial, not publicly available database − already include some binding kinetic parameters, further setting a trend towards a kinetic future.

Target binding kinetics are a significant addition to traditional affinity assays. However they are not the single parameter missing during early drug discovery that could explain the lack of translatability between *in vitro* and *in vivo*, or offer a solution to every problem raised during early drug discovery. There are multiple pharmacological parameters contributing to a pharmacological and safety profile of a drug, such as bioavailability, toxicity, metabolism and pharmacokinetic clearance. A recent example of the importance to investigate drug-binding kinetics in a holistic context is the work of Witte *et al.*[26]. Specifically, target saturation was shown to have a significant impact on prolongation of target occupancy. Hence lack of consideration of target saturation may inaccurately attribute this prolonged effect to drug-target binding kinetics alone. Thus, binding kinetics should be investigated in the more general context of pharmacokinetics and not in isolation.

The potential of covalent ligands as tools for A_{2B}AR and ENT1

Extrapolating the concept of long RT ligands, covalent ligands offer a special kinetic profile which presents an "utmost" RT and an "ultimate" target occupancy. Such a ligand contains a reactive group, called warhead, allowing them to bind irreversibly to their target[27]. At least 39 *covalent drugs* targeting a variety of protein families are currently on the market[28], including blockbuster drugs such as aspirin (COX1,2 inhibitor)[29], penicillin (bacterial DD-transpeptidase inhibitor)[30], omeprazole (H^+/K^+ ATPase inhibitor)[31] and clopidogrel (Plavix; $P2Y_{12}$ receptor antagonist)[32]. However, mainly due to safety reasons concerning potential off-target reactivity, covalent ligands have not been considered an option at the initialization of a drug discovery program[28]. In the recent years, however, discovery efforts have gained focus on "targeted covalent inhibitors"[28]. By combining the advantages of non-covalent and covalent approaches, the resulting molecules have high affinity, specificity and long-lasting binding to the target, while off-target toxicity is avoided with the use of a weakly reactive electrophilic warhead[27]. Interestingly, such covalent ligand would be of great interest for ENT1. Reference inhibitor NBTI offers a great starting point for a targeted covalent approach, due to its high affinity and selectivity for the transporter

and the recent 3D structure in which the transporter was co-crystalized.

Except for the ultimate goal of a safe and therapeutic drug, covalent ligands are mainly used as *pharmacological tools* for the systematic research into functional and structural properties[33]. A characteristic example is the use of covalent ligands in an effort to stabilize the target and determine its X-Ray structure. Such an approach has been effective for many GPCRs, including the cannabinoid receptor CB1[34], β_2-adrenoceptor[35], and A_1AR[36]. A covalent antagonist targeting $A_{2B}AR$ could get us a step closer to stabilize and crystallize the receptor, hence provide further insight in the mechanism of inhibition of $A_{2B}AR$ antagonists. Our collaborators (Kenneth A. Jacobson lab) and we have taken initial steps towards a covalent $A_{2B}AR$ antagonist, using compound **8** described in **Chapter 3** as a template and incorporating a warhead, leading to promising primary results (data not shown). Finally, a covalent ligand could be equipped with a ligation / "click" handle and after its covalent binding to the target it could be coupled to a fluorescent or biotin reporter group in order to generate a detectable signal. *Affinity-based probes*, as these compounds are referred to, have been used for the study of a handful of GPCRs[37-40] and a single SLC transporter[41]. Based on these studies, affinity-based probes proved to be useful tools to study target expression profiles, drug-target engagement, identification of off-targets, and target visualization in biological systems, such as primary human immune cells[38]. Therefore, their development for the study of $A_{2B}AR$ and ENT1 represents a promising approach to advance drug discovery in the field of the adenosinergic system.

The promise of indirectly targeting adenosine receptors

Traditionally, drug discovery efforts have focused on the development of selective agonists that target directly the orthosteric binding site of the receptor. However, in the case of ARs such an approach has resulted in a limited number of approved drugs acting as agonists[42], a number that is not representative of the vast efforts of the scientific community. Hence, a number of complementary approaches have been developed. Positive allosteric modulators[43,44], *i.e.* ligands binding on a distinct binding site from the orthosteric one and having no activity by themselves, yet enhancing the activity of an (endogenous) agonist, as well as enzymatic[45,46] and nucleoside transporter[47] inhibitors have drawn the attention of researchers in order to increase the ARs activity.

Nucleoside transporter inhibition, especially ENT1, has been in depth discussed in **Chapter 2** showing the promising *in vitro, n vivo* and clinical results concerning subsequent therapeutic effect of AR activation. In our perspective such a concept has even greater potential for a therapeutic result, when combined with a multitarget (or polypharmacological) ligand approach, hence ligands acting on more than one target. There are ample examples where ENT1 inhibition alone was not sufficient for the therapeutic effect, thus ENT2 inhibition or depletion was needed[48,49]. In addition,

a dual ENT1/2 inhibitor could suppress potentially compensatory mechanisms of the cell, leading to a further augmentation of the extracellular concentration of adenosine. As a similar concept, a multitarget ligand could inhibit any combination of ENTs and CNTs. Next to ligands only inhibiting NTs, multitarget ligands both inhibiting ENT1 and activating ARs have been reported. For example, ligands reported to be an inhibitor of ENT1 and an agonist of A_{2A}AR have shown promising results in Huntington's disease[50,51]. These dual-target ligands open up opportunities to design multitarget ligands showing a variety of roles against dissimilar proteins. As such, another interesting pair of proteins targeted by the same ligand would be ENT1 and A_3AR. Since there is limited availability of data on A_3AR activation resulting from ENT1 inhibition as discussed in **Chapter 2**, a multitarget ligand would assist initially in the understanding of the underlying mechanism and the potential therapeutic effect.

However, multitarget ligands could turn into a double-edged sword, causing clinical problems if they are not fully understood[52]. Hence, there are a few challenges that need to be addressed and overcome. A main challenge is the selection of the right combination of targets. Depending on the disease and the tissue of interest, different combinations of ENTs and CNTs may need to be targeted. In addition, in the case of ENT1 inhibitor / ARs activator multitarget ligands, their use in a set-up beyond the lab mandates further investigation. The ENT1 inhibitor 'feature' of the multitarget ligand would increase the extracellular concentration of adenosine, resulting in non-selective AR activation, while the AR agonist feature would result in selective AR activation. Finally, optimization of selectivity for the desired targets remains a challenge, as for "monotarget" ligands. Fundamental research by investigating multiple ligands, and by performing structure-affinity/activity relationship studies as well as target structure-based studies is needed[53].

Label-free cellular phenotypic assays: simulating a physiological relevant condition *in vitro*

Label-free cellular-based detection methods have been shown to be physiological relevant, hence crucial for the advancement of both GPCR- and transporter-related drug discovery[54]. Accordingly, the number of biosensor-based label-free cellular technologies has been increasing over the last decade[55,56]. Especially phenotypic cell-based assays are setting the trend[57]. In phenotypic assays, instead of focusing on engaging one specific target, drug discovery aims at identifying molecules with a specific biologic effect without making assumptions about a specific target or mechanism of action[57]. Such an approach is very useful in a multifactorial disease, rather than a monogenic one where molecule selection is target-based.

In the case of ligands targeting the adenosinergic system and especially ARs, a great challenge is faced as adenosine has a multitude of roles. Therefore, demonstrating effects of a potential drug on ARs *in vitro* under an "isolated"

experimental setting, measuring a specific signaling pathway is not sufficient to prove *in vivo* or clinical effectiveness. Hence, the development of cellular label-free phenotypical assays simulating a pathophysiological condition could be a great addition in the assay armamentarium for the ligand evaluation at an early stage, in combination with target-based drug discovery.

In **Chapter 4**, a novel label-free phenotypic assay has been developed. Adapting such an assay in order to mimic pathophysiological situations where the adenosinergic system seems to play a determining role is highly relevant and interesting. Based on the diseases adenosinergic targets are involved in, and in combination with patient-derived cells expressing the target of interest (like lymphoblastoid cell lines; LCLs[58], and human induced pluripotent stem cells; iPSCs[59]), a number of relevant label-free assays were developed. For example, A_{2B}AR has been found a relevant target in cancer migration and angiogenesis[60]. Hence development of a migration assay for the screening of A_{2B}AR antagonists on a physiological relevant model system might reveal lead compounds, for which we could better predict the *in vivo* efficacy from the *in vitro* one. Similarly, cytotoxicity assays and assays with a co-culture of diverse cell types could offer a range of possibilities in the investigation of adenosinergic signalling, by mimicking complex physiological conditions.

Altogether, development of label-free assays based on patient-derived cell lines simulating the pathophysiological condition under investigation will result in more focused insights in disease mechanisms. Implementing the complexity of adenosine's role into the parameters of the assays holds great promise for the study of the ARs and NTs, as well as other GPCRs and SLCs.

Final notes

In a nutshell, this thesis intends to explore the concept of binding kinetics for the adenosinergic system, in order to provide a better understanding of the mechanism of action at a molecular level, and to offer tools that assist in a better prediction of *in vivo* effects from *in vitro* experiments. We investigated two adenosinergic targets belonging to the SLC and GPCR superfamilies, *i.e.* the ENT1 and A_{2B}AR, respectively. To obtain these insights, a number of ENT1 inhibitors and A_{2B}AR antagonists have been studied and *novel labeled and label-free pharmacological assays* have been developed. Altogether, we hope that the data and the novel assays presented in this thesis will contribute to the advancement of drug discovery in the adenosinergic field, and GPCRs and SLCs in general.

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