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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Summary

Adenosine is an endogenous ligand which exerts its action by activating adenosine receptors (ARs), while its circulating levels are controlled via a variety of mechanism and proteins, amongst others the equilibrative nucleoside transporters (ENTs). Distortion of the adenosinergic tone is implicated in a multitude of pathophysiological conditions, thus numerous drug discovery efforts have been made to develop drugs targeting ARs and ENTs. Yet, with the exception of adenosine itself and the natural products caffeine, theobromine and theophylline, only an A_{2A}AR agonist and antagonist, as well as two non-selective ENT1 (SLC29A1) inhibitors are currently on the market. Therefore, there is a pivotal need to develop novel concepts that allow us to increase our understanding of the mechanism of action at a molecular level, as well as physiologically relevant assays to evaluate drug candidates in early stages of drug discovery. Hence, this thesis focuses on exploring 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.

Chapter 1 introduces the adenosinergic system and its complexity, in addition to the various "players" in regulating the adenosine levels. Subsequently the need for pharmacological intervention of the adenosinergic targets is discussed, focusing on the study of binding kinetics, and the development of traditional radioligand-based and label-free assays. **Chapter 2** starts with an introduction to the two largest families of membrane proteins, the G protein-coupled receptors (GPCRs) and the Solute Carriers (SLCs), focusing on the ARs and the ENTs. The interplay between GPCRs and SLCs and the possible therapeutic strategies that could be developed is discussed. Especially, this chapter reviews the *in vitro* and *in vivo* evidence of therapeutic effect resulting from the indirect targeting of the four ARs via inhibition of ENT1.

Chapter 3 focuses on the study of A_{2B}AR antagonists. The synthesis of a series of 8-phenylxanthine-based antagonists is described, followed by their biological evaluation. [^{3}H]PSB 603 is introduced and extensively characterized as a tool to be used in radioligand binding assays. The development of a binding and a competition association assay allowed the determination of the affinity for the compounds under investigation, while their binding to the receptor was further evaluated from a kinetic perspective, showing that the dissociation rate constant k_{off} was the driver for affinity. Ultimately, an impedance-based label-free assay was developed for the study of A_{2B}AR pharmacology. Two structurally similar compounds with divergent kinetic profiles, were evaluated in this functional assay and a link between binding kinetics,

especially long residence time (RT), and an extended pharmacological effect under non-equilibrium conditions was shown.

Opposite to the well-studied GPCRs, SLCs are important yet understudied targets due to lack of available assays. **Chapter 4** addresses the need for novel functional assays to study SLCs and describes the development of a label-free, whole-cell method that enables the functional assessment of ENT1 inhibitors. Taking into account that the endogenous ENT1 substrate, *i.e*. adenosine, is also a ligand for ARs, ENT1 inhibition is hypothesized to change the extracellular concentration of adenosine. This change is affecting AR signalling, which is monitored by an impedance-based biosensor. After optimization of the assay conditions, the effects of three reference ENT1 inhibitors were monitored. Interestingly, all inhibitors resulted in an increased apparent potency of adenosine for AR. In addition, all inhibitors concentration-dependently increased the extracellular adenosine concentration, resulting in an indirect quantitative assessment of their potencies. Finally, AR activation was abolished by AR antagonists, confirming that the monitored impedance was AR (especially A_{2B}AR) mediated and validating the assay. Thus, ENT1 transporter is the first SLC characterized by concomitant AR signalling, offering great potential for the method to be broadly applied for the study of a multitude of SLC / GPCR pairs sharing a common substrate / ligand.

Chapter 5 presents the pharmacological characterization of four reference ENT1 inhibitors, from a kinetic perspective. [^{3}H]NBTI is extensively characterized and used as a tool for all ENT1–related radioligand assays. Following the establishment of a competition association assay enabling the quantification of target binding kinetics, the kinetic binding parameters of the reference inhibitors were determined next to the more traditional affinity parameters. One of the reference inhibitors, *i.e.* draflazine, displayed longer RT, hence a series of compounds, sharing the same scaffold, was evaluated. Structure-kinetic relationships (SKR) for ENT1 inhibitors were drawn for the first time, in addition to the more classical structure-affinity relationships (SAR), revealing a compound with a RT of over 10 h. Ultimately, the label-free assay developed in **chapter 4** was used to evaluate the impact of divergent ENT1 inhibitor binding kinetics in a functional assay. The potency of the longest RT compound increased with longer incubation times, an effect not observed for draflazine, supporting the importance of long RT for increased target-occupancy and effect.

Chapter 6 describes the kinetic study of a novel series of spirobenzo-oxazinepiperidinone derivatives as ENT1 inhibitors. Utilizing the radioligand binding assays developed in **chapter 5**, we assessed a series of 29 compounds in displacement, competition association and wash-out assays, to evaluate their affinity and binding kinetics parameters. Hence, an extensive SKR study was performed alongside an SAR analysis with respect to ENT1 binding. We found that the bulkier substituents at the “right-hand” phenyl ring were well tolerated, while hydrophobic or uncharged substituents at physiological pH provided high affinity and a long RT for the target. Additionally, when the polarity of the central scaffold was reduced,

compounds associated faster to the transporter. Correlation plots illustrated that substituents at the “right-hand” phenyl ring may be responsible for long lasting binding and hence a longer pharmacological effect of the inhibitors, whereas the composition of the main ring is responsible for a fast binding to the target and an immediate effect. Taken together, the results of **chapters 3, 5** and **6** show the importance of supplementing the design criteria used in drug discovery with binding kinetics parameters. This kinetic approach may result in a different lead compound selection, and could inspire future drug discovery in the field of adenosinergic targets and membrane proteins in general.

Finally, an overall conclusion summarizing the results described in this thesis as well as the forthcoming future opportunities are presented in chapter 7. Hopefully, the novel insights obtained in this thesis will provide important insight for future drug discovery projects investigating A_{2B}AR and ENT1 as well as other (pairs of) GPCRs and SLCs.