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

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# Chapter 1

General Introduction

About this thesis

"A journey of a thousand miles begins with a single step (千里之行始于足下)". Similarly, pharmacological effects are triggered by the initial step of drug-target binding. This thesis focuses on the kinetics of binding interactions between ligands (drugs) and various G protein-coupled receptors (GPCR). GPCR are one of the largest and most important drug target families; many drugs in use today target these pharmaceutically relevant proteins.[1] However, many candidate drugs are still neither efficacious nor safe.[2] Thus the traditional affinity- or potency-based rationale in the early phases of drug discovery may need to be reconsidered. Although binding kinetics. i.e. the rate with which a ligand associates to and dissociates from the target, has been studied on a few drug target classes, the concept of binding kinetics has been emerging over the last decade as an additional and relevant selection criterion in the drug discovery pipeline.[3, 4]

To describe the importance and relevance of the research performed in this thesis, this chapter serves as a general introduction. First, the GPCR will be introduced. Second, the two families of human cannabinoid and human adenosine receptors will be mentioned; more specifically, both the human cannabinoid receptor 1 (hCB$_1$) and the human adenosine A$_1$ and A$_3$ (hA$_1$ and hA$_3$) receptors will be outlined as prototypical GPCR as well as potential drug targets. These targets are then the main "actors" in the subsequent experimental chapters of this thesis. Furthermore, the concept of binding kinetics will be presented in a chronological way, including some practical requirements for the early phase of drug discovery. Finally, the aim and scope of this thesis will be explained.

What are GPCR ?

GPCR are membrane bound proteins, composed of seven transmembrane helices with extracellular and intracellular loops and an extracellular (N-terminal) and intracellular (C-terminal) tail. They play key roles in physiology by detecting external stimuli (e.g. chemical small molecules, endogenous ligands, or photons) and activating internal signal transduction pathways and eventually physiological responses (**Figure 1A**). An agonist is able to bind to and activate a GPCR to produce a biological response. In contrast, a (neutral) antagonist blocks the action of the (endogenous) agonist, while an inverse agonist causes an action opposite to that of the agonist, i.e. decreasing the basal level of receptor activity (**Figure 1B**). Since GPCR are involved in various critical functions, it may not be surprising that GPCR are the target of about 30% of all drugs on the market.[5] Much of the current understanding of the structure and function of GPCR results from the pioneering research on the rhodopsin and β_2 adrenoceptor. As a result, Robert Lefkowitz and Brian Kobilka were awarded the 2012 Nobel prize in chemistry.[6, 7] However, key questions still remain unanswered in the GPCR field. For instance, although the ligand-GPCR complex can be observed, the detailed molecular recognition or complex stability is unknown.
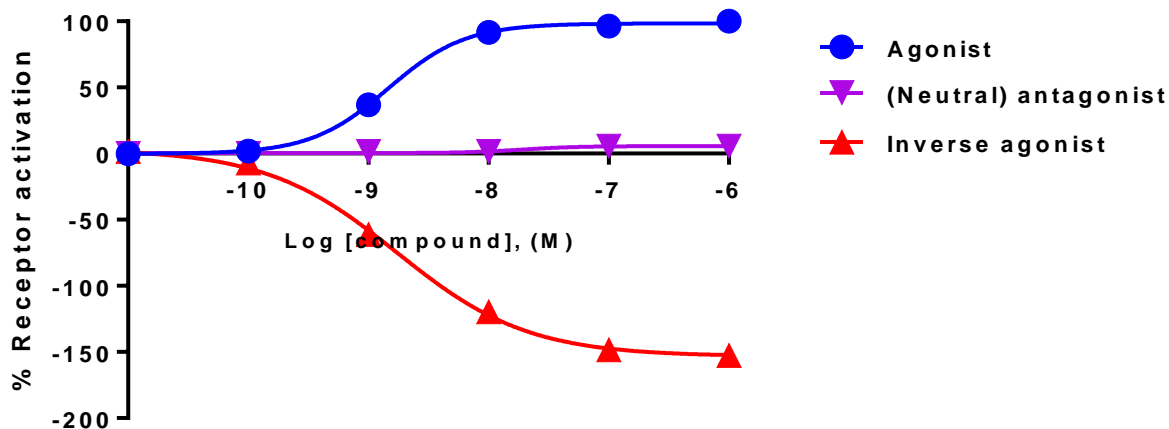
A.
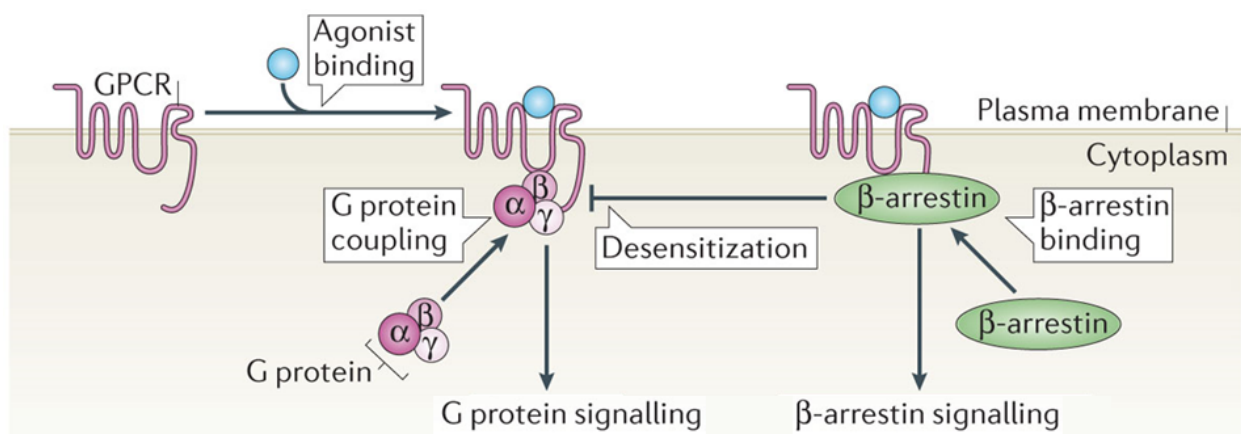
B.

Figure 1: Panel **A**) A simplified schematic of GPCR signaling by agonist (activating ligand) binding. One GPCR can be activated through G protein-dependent (G protein coupling) and G protein-independent (β-arrestin binding) pathways, adapted from the review of Ghosh et al.[8] Panel **B**) Theoretical graph showing: 1) dose-dependent receptor activation by an agonist (blue curve); 2) a (neutral) antagonist that in this concept simply prevents the agonist from binding and, hence, from activating the receptor and more downstream signaling pathways (purple curve); and 3) an inverse agonist that in this concept also competes for binding with the agonist, however, it causes a subsequent decrease of basal receptor activity (red curve).

GPCR studied in this thesis

As recorded by the International Union of Basic and Clinical Pharmacology (IUPHAR) Committee on Receptor Nomenclature and Drug Classification, there are about 800 GPCR identified in the human body; The largest GPCR class is the one of rhodopsin-like GPCR, also known as class A GPCR.[8] It would be a daunting task to study all of them. As a result, this thesis is focused on a few, as prototypical examples.

Human cannabinoid 1 (hCB$_1$) receptor

Within the endocannabinoid system (ECS) two human cannabinoid receptor subtypes have been identified: the human CB_1 (hCB$_1$) receptor and the human CB_2 (hCB$_2$) receptor. They are primarily activated by endogenous cannabinoids (endocannabinoids, ECs), including anandamide (or N-

arachidonylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG).[9, 10] The hCB_1 and hCB_2 receptors show 44% overall sequence homology, and display different pharmacological profiles.[11] Due to the long history of *Cannabis sativa* use,[12] a diversity of effects initiated through hCB_1 receptor activation by the plant's main psychoactive component, Δ^9-THC, has been described. These effects include feeling high, alteration of time perception, an increase in body sway and the munchies (i.e. the extreme desire for and enjoyment of food-intake).[13] It has been shown that the latter can also be triggered through activation of the hCB_1 receptor by endocannabinoids (ECs) from breast milk or other food products.[14-16]

The hCB_1 receptor is expressed in the central nervous system (CNS) and is also widely distributed in the peripheral nervous system (PNS) and peripheral tissues,[10, 17] including heart, liver, lung, gastrointestinal tract, pancreas and adipose tissue.[18, 19] The broad presence of the hCB_1 receptor in a variety of complex physiological systems provides numerous opportunities for the development of new medications. In the particular case of obesity, the ECS, including the hCB_1 receptor, is overactive with increased levels of endocannabinoids in plasma, and in central and peripheral tissues.[20] Therefore, blockade of the hCB_1 receptor is a potential approach for the treatment of obesity.

Rimonabant, a hCB_1 receptor inverse agonist, was developed by Sanofi-Aventis and introduced on the market in Europe in 2006. However, it was quickly withdrawn from the market due to the risk of unacceptable psychiatric side effects.[21-23] Many other hCB_1 receptor antagonists entered clinical trials, such as taranabant [24] and otenabant,[25] however, they were not developed further due to similar psychiatric side effects despite their different chemical structures. Although researchers have experienced many setbacks on this drug target, the intensive drug discovery efforts have never been terminated. Since 2016, a series of crystal-structures of the hCB_1 receptor have been resolved with antagonists (i.e. AM6538 and taranabant, [26, 27] **Figure 2A** and **2B**) or agonists (i.e. AM11542 and AM841,[28] **Figure 2C** and **2D**). Therefore, those hCB_1 receptor structures provide a molecular basis for

predicting the binding modes of hCB1 ligands, and may help in the future development of better drug candidates for the hCB$_1$ receptor.
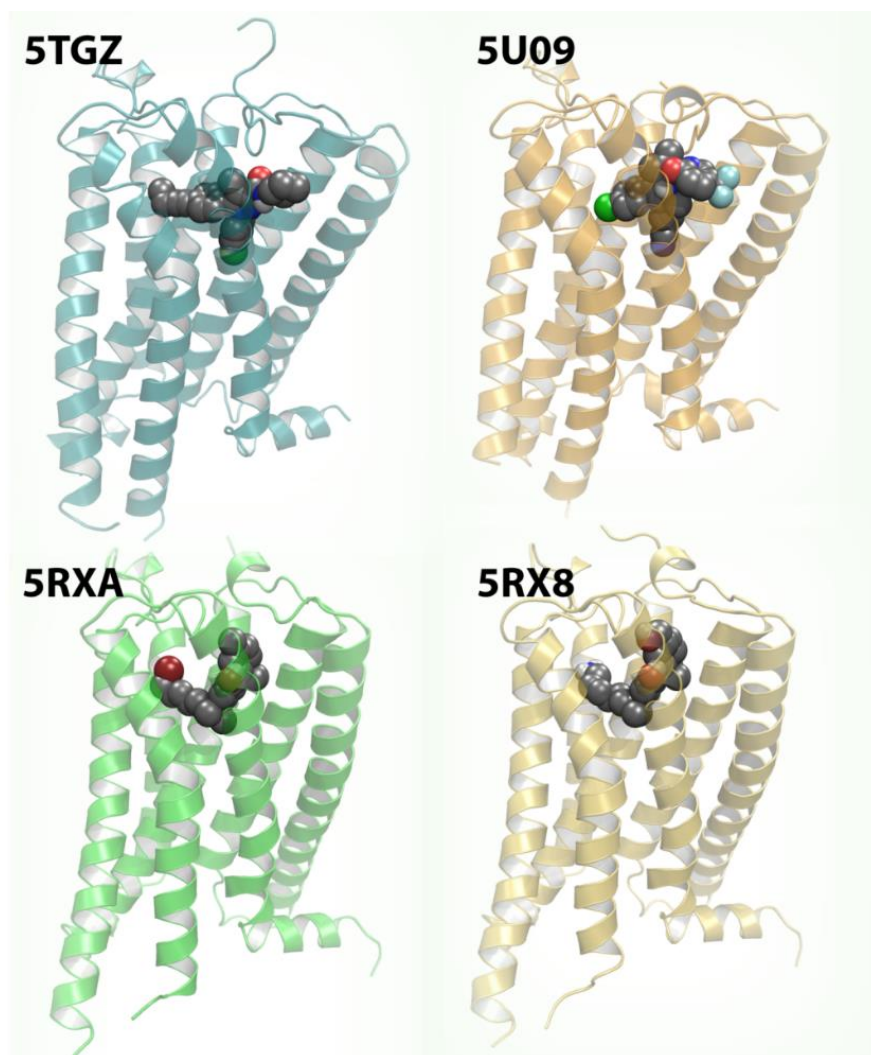

Figure 2: Crystal structures of hCB$_1$ receptor with various ligands. The typical seven helices of hCB$_1$ receptors are shown as ribbons and the intracellular and extracellular domains are present as loops. Panel **A** (upper left): Global structure of the hCB$_1$ receptor in complex with the antagonist AM6538[26] (PDB: 5TGZ). The receptor is shown in cyan cartoon representation. Panel **B** (upper right): Global structure of the hCB$_1$ receptor in complex with the antagonist taranabant[27] (PDB: 5U09). The receptor is represented as a yellow cartoon. Panel **C** (lower left): Global structure of the hCB$_1$ receptor in complex with the agonist AM11542[28] (PDB: 5XRA). The receptor is represented as a green cartoon. Panel **D** (lower right): Global structure of hCB$_1$ receptor in complex with the agonist AM841 [28] (PDB: 5XR8). The receptor is represented as a light yellow cartoon. All the ligands are presented in a space-filling style and their atoms color coded: black = carbon, red = oxygen, blue = nitrogen, green = chlorine, cyan = fluorine.

Human adenosine A₁ and A₃ (hA₁ and hA₃) receptor

Human adenosine A_1 and A_3 receptors belong to the family of human adenosine receptors with four distinct subtypes (hA_1, hA_{2A}, hA_{2B} and hA_3).[29] Although all subtypes are activated by the endogenous and ubiquitous local hormone adenosine, these purinergic receptors differ in their distribution and to which G protein they are coupled. Classically, following agonist activation hA_1 and hA_3 receptors induce a decrease in cyclic adenosine monophosphate (cAMP) levels due to their primary coupling to G_i proteins, while hA_{2A} and hA_{2B} receptors couple to G_s proteins and stimulate cAMP formation.[30]

Both hA_1 and hA_3 receptors have similar clinical potential for the treatment of ischemia-reperfusion injury,[31] renal disease,[32] and neuropathic pain.[33, 34] Besides, two hA_3 agonists have moved forward into a series of clinical trials: for IB-MECA, in total twelve completed or planned trials have been reported related to inflammatory conditions (e.g. keratoconjunctivitis sicca, rheumatoid arthritis, psoriasis, uveitis, etc.); for 2-Cl-IB-MECA, four trials have been registered for liver diseases (e.g. hepatocellular carcinoma, hepatitis C).[35, 36] Nevertheless, distinct pharmacological effects related to hA_1 and hA_3 receptors (i.e. cardioprotection[31, 37, 38] and neuroprotection[39]) can be ascribed to their different downstream effectors. For cardioprotection, the hA_1 receptor is linked to the atrial cardiac myocyte potassium channel and to phospholipase C, while the hA_3 receptor has been found to couple to not only phospholipase C but also phospholipase D; such plausible "hA_1 receptor – PLC" and "hA_3 receptor – PLD" signaling pathways may indicate distinct effects in the heart.[40, 41] For neuroprotection, the hA_1 receptor is activated acutely to inhibit neuronal calcium transients;[42] whereas hA_3 receptor activation in astrocytes results in a release of neuroprotective CCL2.[43]

Last but not least, the crystal structure of hA_1 receptor has been resolved quite recently (**Figure 3**).[44] Such progress suggests that the binding interaction of ligands on the hA_1 receptor will be better understood in the near future. Moreover, this structure also aids in understanding the binding modes of hA_3 receptor ligands, since this receptor is quite similar to hA_1 receptors.[29]
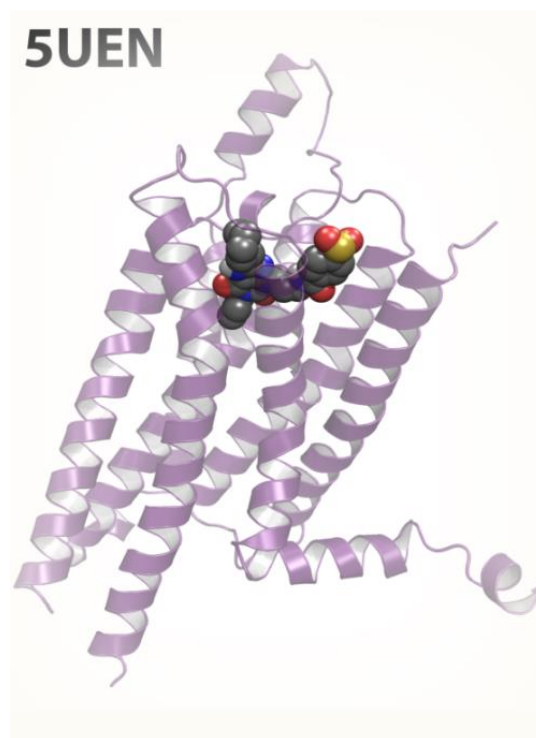
Figure 3: Global crystal structure of hA_1 receptor in complex with the antagonist DU172 (PDB: 5UEN).[44] The typical seven helices of hA_1 receptors are shown as ribbons and the intracellular and extracellular domains are present as loops. The receptor is represented as a purple cartoon. DU172 is presented in a space-filling style and its atoms are color coded: black = carbon, red = oxygen, blue = nitrogen, yellow = sulfur.

The concept of binding kinetics at these representative GPCR is not completely new. For example, a hCB_1 antagonist and negative allosteric modulators have been studied in this context,[45, 46] as are the human adenosine receptors.[47] However, key questions are remaining:

- Can we characterize ligands with a "good" kinetic profile on these receptors?

- Can we establish structure-kinetics relationships (SKR) of ligands for these receptors?

- Can we improve the technology of the kinetic assays on these receptors?

- Can we confirm that a ligand with "good" kinetic profile leads to target selectivity and clinical efficacy and/or safety?

To answer these questions, the concept of binding kinetics shall be introduced.

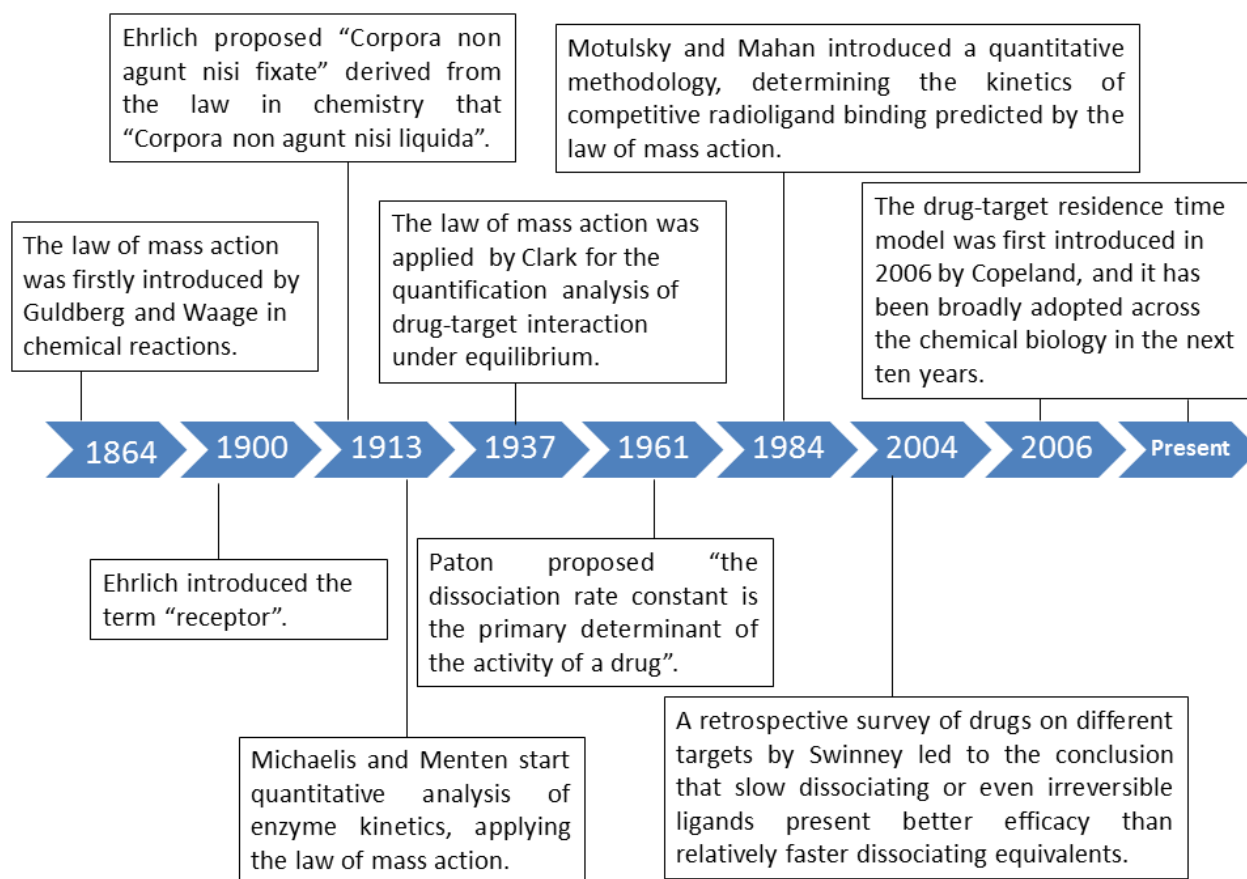## Historical aspects of drug-target binding kinetics

Figure 4: The emergence of binding kinetics studies.

The concept and study of drug-target binding kinetics have emerged since the evolution of modern pharmacology (**Figure 4**). In the 19th century, the law of mass action was firstly described in chemical reactions, while in the year 1900, Paul Ehrlich introduced the term "receptor", symbolizing the era of modern pharmacology. Later in 1913, Ehrlich also coined the phrase "corpora non agunt nisi fixata" (a drug will not work unless it is bound).[48, 49] From then on, drug action in the human body is described as a kinetic event, not only in terms of transport of drug molecules to the environment of targets (e.g., receptors), but also with respect to the drug-target interaction itself. In other words, the lifetime of the drug-target complex is relevant to drug action. More specifically, the law of mass action is the fundamental descriptor of drug-target interactions [50-52]:
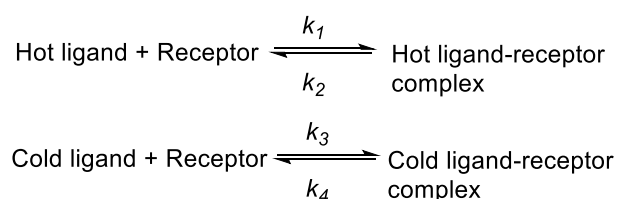
$$\text{Drug + Target} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Drug-target complex} \longrightarrow \text{Effects}$$
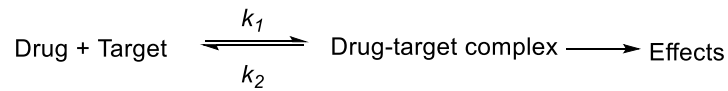
in which k_1 and k_2 represent the association (k_{on}) and dissociation (k_{off}) rate constant of a drug to and from its target, respectively. This mathematical application was first reported in enzyme kinetics (since 1913) and, later (1937) more emphasis was placed on equilibrium parameters of drug-target binding interactions. It lasted until 1961, when Paton proposed that "the dissociation rate constant is the primary determinant of the activity of a drug".[53, 54] Kinetic and affinity parameters were intertwined as drug activity (i.e. affinity), which is the ratio of both association and dissociation rate constants:[55]

$$\text{Kinetic } K_D \text{ (an affinity parameter)} = k_2/k_1$$

Methodologies to determine kinetics (presented as various rate constants) have been explored and improved over the decades. One example is the invention of patch clamp technology in the late 1970s which allowed (gating) kinetics of ion channels to be measured.[56] Another active research field is enzyme kinetics which was pushed forward by the study of catalytic mechanisms.[57] However, kinetics in the field of GPCR has often been overlooked or neglected. In 2004, a retrospective survey of 50 drugs on 17 different drug targets led to the conclusion that slowly dissociating or even irreversible ligands present better efficacy than relatively faster dissociating equivalents.[58] Noteworthy, GPCR were only a small fraction (~18%) among the investigated drug targets, in contrast to the fact that GPCR are one of the largest classes of drug targets.

Steady-state potency metrics (i.e. K_i and IC_{50}) are often used to characterize drugs targeting GPCR in a traditional early-phase drug discovery endeavor. However, there is mounting evidence to show that high affinity drug candidates cannot prevent high attrition rates in clinical trials.[2] This may, among others, be due to the dynamic, non-equilibrium, conditions *in vivo* (like in the human body) that often are in contrast to the equilibrium conditions applied in *in vitro* assays.[59] Therefore, other, better *in vivo* effect-predicting parameters should be taken into account. Binding kinetics as a term

collectively stands for the association (k_{on}) and dissociation (k_{off}) rate constants of a drug to and from

its target. Particularly, over the last ten years, drug-target residence time (RT) has emerging as an

additional parameter to assess the therapeutic potential of drug candidates with respect to drug

efficacy and safety. RT is defined as the reciprocal of k_{off} and is an indication for the life-time of a

drug-target complex. [59-61] In the field of GPCR, a number of structure-kinetics relationships (SKR)

studies has been published, and the results suggest that including binding kinetics data (or kinetics

profiles) when triaging compounds can improve the resulting decision process.[62-64] Following such

rationale, appropriate binding kinetics experiments shall be incorporated in an early stage (*in vitro*) of

drug design and discovery. Classical radioligand kinetic association and dissociation assays enable

straightforward determination of association ($k_{on} = k_1$) and dissociation ($k_{off} = k_2$) rate constants.

However, (radio)labeling every ligand of interest is impractical. In 1984, Motulsky and Mahan

introduced an alternative strategy in which the kinetics of unlabeled ligands (cold ligands) can be

quantified by using only one labeled tracer (hot ligand).[65] Such so-called competition association

assay was validated by a further developed mathematic model obeying the law of mass action:

$$\text{Hot ligand + Receptor} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Hot ligand-receptor complex}$$

$$\text{Cold ligand + Receptor} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{Cold ligand-receptor complex}$$

In brief, both a cold ligand of interest and a well-characterized (i.e. k_1 and k_2) hot ligand can

competitively bind to the same receptor; the competitor may delay the time-dependent ascent of

hot ligand binding or even produce a time-dependent decrease in hot ligand binding after an initial

'overshoot'.[65] This method has been used to quantify the binding kinetics of cold ligands (i.e. k_3 and

k_4) to several GPCR.[62, 63, 66, 67] However, the competition association assay described above is quite

laborious and time-consuming. Therefore, a so-called "dual-point competition association assay" was

established, and validated for the hA_1 receptor.[68] By using only two time points of hot ligand specific

binding, a semi-quantitative measurement yielded a so-called kinetic rate index (KRI) of the cold ligand. Thereby, the throughput of kinetics assays can be improved greatly.

Objectives and outline of this thesis

Investigating ligand-GPCR binding kinetics is a leading theme in this thesis, which will be explored using hCB_1, hA_1 and hA_3 receptors. Currently, the drug discovery paradigm is shifting from studying structure-affinity relationships (SAR) alone, to a combination of SAR and extensive structure-kinetics relationships (SKR). This strategy is firstly exemplified in a study of hCB_1 receptor antagonists (**Chapter 2**). We provide evidence that, next to affinity, additional knowledge of binding kinetics is useful for selecting new hCB_1 receptor antagonists in the early phases of drug discovery. Following this, another selection of hCB_1 receptor antagonists with divergent residence times (RTs) was observed to have distinct modes of functional antagonism, in both G-protein dependent and G protein-independent signaling (**Chapter 3**).

The other research chapters (**Chapter 4-6**) are devoted to adenosine receptors. Firstly, an SKR study on a series of hA_3 receptor antagonists is performed in **Chapter 4**; furthermore, a "k_{on}-k_{off}-K_D" kinetic map enables the division of the antagonists into three subgroups, providing a possible direction for further development of hA_3 receptor antagonists. On the other hand, binding kinetics of hA_3 receptor agonists is reported in **Chapter 5**, providing binding kinetics insights of GPCR agonists. In **Chapter 6**, the application of a novel radio-isotopic technology in binding kinetics is described for the hA_1 receptor. Its robustness and potential for high-throughput screening may render this technology a preferred choice for further kinetics studies.

Last but not least, **Chapter 7** provides a general conclusion of the novel findings presented in this thesis, and future perspectives and opportunities for this field of research. Hopefully, this thesis will contribute to broadening the implementation of kinetics studies in drug discovery.

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