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Perspective

Perspective: Implications of Ligand–Receptor Binding Kinetics for Therapeutic Targeting of G Protein-Coupled Receptors

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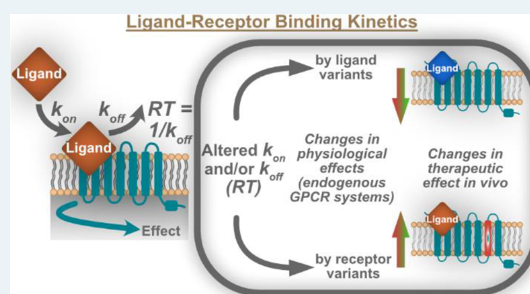
ABSTRACT: The concept of ligand–receptor binding kinetics has been broadly applied in drug development pipelines focusing on G protein-coupled receptors (GPCRs). The ligand residence time (RT) for a receptor describes how long a ligand–receptor complex exists, and is defined as the reciprocal of the dissociation rate constant (k_{off}). RT has turned out to be a valuable parameter for GPCR researchers focusing on drug development as a good predictor of *in vivo* efficacy. The positive correlation between RT and *in vivo* efficacy has been established for several drugs targeting class A GPCRs (e.g., the neurokinin-1 receptor (NK_1R), the β_2 adrenergic receptor (β_2AR), and the muscarinic 3 receptor (M_3R)) and for drugs targeting class B1 (e.g., the glucagon-like peptide 1 receptor (GLP-1R)). Recently, the association rate constant (k_{on}) has gained similar attention as another parameter affecting *in vivo* efficacy. In the current perspective, we address the importance of studying ligand–receptor binding kinetics for therapeutic targeting of GPCRs, with an emphasis on how binding kinetics can be altered by subtle molecular changes in the ligands and/or the receptors and how such changes affect treatment outcome. Moreover, we speculate on the impact of binding kinetic parameters for functional selectivity and sustained receptor signaling from endosomal compartments; phenomena that have gained increasing interest in attempts to improve therapeutic targeting of GPCRs.



KEYWORDS: *association rate constant (k_{on}), dissociation rate constant (k_{off}), residence time (RT), ligand–receptor binding kinetics, G protein-coupled receptors (GPCRs)*

INTRODUCTION

Over the past decade, the kinetics of ligand binding to a receptor (or in short, ligand–receptor binding kinetics) have received increasing awareness and, consequently, been widely utilized in drug development pipelines focusing on G protein-coupled receptors (GPCRs).[1,2] In the early 2000s, Swinney and Copeland suggested that the dissociation rate and the residence time (RT) could be important parameters in the understanding of drug failure during clinical trials.[3,4] Since then, numerous studies for class A GPCRs have highlighted that ligand–receptor binding kinetics are better predictors of *in vivo* efficacy than traditional equilibrium parameters, such as IC_{50}, K_i, or K_D (see Box 1).[8−12] As these traditional parameters are based on measurements in closed systems with fixed concentrations of ligands, equilibrium is often reached. However, the human body is a more complex and "open" system, and the translation from *in vitro* to *in vivo* settings is often challenged by *in vivo* parameters such as circulation, distribution, metabolism, and diffusion of ligands.[13] The RT refers to the time a ligand is bound to its receptor, and hence how long the ligand–receptor complex exists, and is defined as the reciprocal value of the dissociation rate (k_{off}). Thus, the longer the ligand is bound to its receptor by means of a slow dissociation rate the longer the RT will be (and, thereby, the *in vivo* effect). Recently, an increasing awareness has arisen of the association rate constant (k_{on}) as another important parameter to predict *in vivo* efficacy, as a high k_{on} allows for rapid therapeutic action and/or higher receptor occupancy levels and thereby has potential clinical relevance.[14−19] By means of its definition, k_{on} is mostly related to the onset of action.[20] However, it is also related to *in vivo* efficacy as a rapid (and high) occupancy of a receptor by a drug is needed in some diseases, such as inflammatory diseases, where the levels of the endogenous (and competing) ligands can be high.[21]

The involvement of RT as a predictor of *in vivo* drug efficacy and the duration of action is well-established among marketed class A therapeutics. Depending on the disease type, short or long RT ligands are preferred. Examples of antagonists with **long** RT drugs are (1) the neurokinin-1 receptor (NK_1R)

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Box 1. Molecular Pharmacology within Ligand–Receptor Binding Kinetics

Observed Association Rate Constant (k_{obs})

This parameter is a first-order rate constant (in min^{-1}) that describes the observed rate at which a ligand and receptor associate to form a ligand–receptor complex. It comprises of both the association and dissociation rate constants and is highly dependent on the ligand concentration: A higher concentration will result in a faster k_{obs}. The main method to determine k_{obs} is by kinetic binding experiments with a traceable ligand (for instance radioactive or fluorescent labeled) measuring association over time.

Dissociation Rate Constant (k_{off})

This parameter is a first-order rate constant (in min^{-1}) that quantifies the rate at which the ligand–receptor complex dissociates from each other (off-rate). It is independent of the local concentration of free ligand, and in contrast, completely relies on specific interactions between ligand and receptor. The main method for determining the k_{off} is through kinetic binding experiments measuring dissociation over time.

Residence Time (RT)

This parameter is a measure of how long a ligand–receptor complex exists (in min). The main method to determine the residence time is by kinetic binding experiments measuring dissociation, and then calculating RT. The RT is defined as reciprocal value of k_{off}:

$$\mathrm{RT} = \frac{1}{k_{off}}$$

Association Rate Constant (k_{on})

This parameter is a second-order rate constant (often expressed in $min^{-1} \cdot M^{-1}$) that quantifies the rate at which a ligand and receptor associate to form a ligand–receptor complex (on-rate), which is either diffusion- or encounter-limited. The main method to determine the k_{on} is through kinetic binding experiments measuring association and dissociation over time. The k_{on} can be calculated according to the following equation:

$$k_{on} = \frac{k_{obs} - k_{off}}{[\mathrm{L}]}$$

in which [L] is the concentration of the labeled ligand.

Inhibitory Concentration at 50% Maximum Binding (IC_{50})

This parameter is the molar concentration of an unlabeled ligand that inhibits the binding of a traceable ligand (for instance radioactive or fluorescent labeled) by 50%. The method to obtain IC_{50} is, therefore, through competition binding. (IC_{50} may also refer to the concentration of an antagonist responsible for 50% inhibition of a signal elicited by a given agonist.)

Equilibrium Dissociation Constant (K_D)

This parameter describes the affinity of a ligand toward a given receptor under equilibrium conditions (in molar). The main methods to determine the K_D are (1) saturation binding, where K_D equals the ligand concentration where 50% specific receptor binding is achieved, (2) kinetic binding (association and dissociation rate constants determination), where K_D is defined as the ratio between k_{off}/k_{on} and (3) homologous competition binding, where K_D equals $IC_{50} - [\mathrm{L}]$, provided certain experimental conditions are fulfilled.[5] This equation is derived from the Cheng–Prusoff equation (see below) and can be used if (1) the concentration of the labeled ligand [L] is <1/10 of the K_D value and (2) the maximum specific

Box 1. continued

binding is <1/10 of the total added ligand, ensuring that only a small fraction of the ligand is bound to the receptors (i.e., no ligand depletion occurs). Moreover, the traceable ligand and the competitor must be the same compound (homologous binding) to ensure equal affinities for the receptor and avoid any cooperativity between the ligands.[5] From these three main methods, K_D can be calculated according to the following equations:

Saturation binding:

$$K_D = \frac{B_{max} \Delta[\mathrm{L}]}{B_0} - [\mathrm{L}] \quad (1)$$

Kinetic binding:

$$K_D = \frac{k_{off}}{k_{on}} \quad (2)$$

Homologous competition binding:

$$K_D = IC_{50} - [\mathrm{L}] \quad (3)$$

where B_{max} is the total density of receptors in the sample and B_0 is the total specific binding.

Inhibition Constant (K_i)

This parameter describes the affinity of a ligand toward a receptor under equilibrium conditions (in molar). The method for determining K_i is through heterologous competition binding. The K_i can be analyzed according to the Cheng–Prusoff equation:[6]

$$K_i = \frac{IC_{50}}{1 + \left(\frac{[\mathrm{L}]}{K_d}\right)}$$

Motulsky–Mahan Model

This model is used to determine the ligand–receptor binding kinetics (association and dissociation rate constants) of unlabeled ligands in the presence of a previously characterized labeled ligand.[7] The main method to determine these rate constants is through competition association experiments, and analyzed according to the following equation:[7]

$$K_A = k_1[\mathrm{L}] \cdot 10^{-9} + k_2$$

$$K_B = k_3[\mathrm{L}] \cdot 10^{-9} + k_4$$

$$S = \sqrt{(K_A - K_B)^2 + 4 \cdot k_1 k_3 \cdot [L] \cdot [I] \cdot 10^{-18}}$$

$$K_F = 0.5(K_A + K_B + S)$$

$$K_F = 0.5(K_A + K_B - S)$$

$$Q = \frac{B_{max} \cdot k_1[L] \cdot 10^{-9}}{K_F - K_S}$$

$$Y = Q \cdot \left(\frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F \cdot X)} - \frac{k_4 - K_s}{K_s} e^{(-K_s \cdot X)} \right)$$

in which, k_1 is the on-rate of the labeled ligand, k_2 is off-rate of the labeled ligand, k_3 is the on-rate of the unlabeled ligand, k_4 is off-rate of the unlabeled ligand, $[I]$ is the concentration of the unlabeled inhibitor, X the time and Y is the specific binding.

Binding Models

One-Step Binding/Dissociation Model: The one-step binding model is characterized by a one-step association (k_1) and one-step dissociation (k_2) of ligand–receptor interactions:

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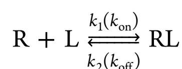
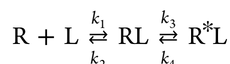
Box 1. continued

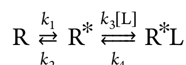


in which R represents the receptor, L is the ligand, and RL is the ligand−receptor complex.

Induced Fit Model: The induced fit model is characterized by an association (k_1) of L to a low-affinity state of the receptor (R), forming a low-affinity ligand−receptor complex (RL). This process is followed by a conformational change (k_3) in the receptor to form a high-affinity ligand−receptor complex (R*L). This process is reversible, as the R*L can convert back to *RL*, defined by k_4. L can then dissociate (k_2) from RL, to form L and R:



Conformational Selection Model: The conformational selection model dictates that the ligand binds to a certain conformation of the receptor. It is characterized by a conformational change (k_1) in the receptor from a nonselective state (R) to a selective state (R*), in which the ligand (L) is able to bind. Subsequently, L associates with R* and forms a selective ligand−receptor complex (R*L), which is defined by the association rate (k_3) and depends on the value of [L]. This process is reversible, as L can dissociate (k_4) from R*L and convert back to R*. The receptor then changes its conformation back to R, which depends on the rate of the conformational interchange (k_2).



antagonist aprepitant, which shows superior antagonism over the NK_1R antagonists ZD6021 and CP-99994 in treatment of chemotherapy-induced emesis;[8,22,23] (2) maraviroc, a selective CC chemokine receptor 5 (CCR5) antagonist with antihuman immunodeficiency virus type 1 (HIV-1) activity;[24] (3) olmesartan, an angiotensin II type 1 receptor (AT_1R) antagonist targeting hypertension;[25] (4) rupatadine, a histamine H_1 receptor (H_1R) antagonist against allergic disorders;[26] and (5) muscarinic 3 receptor (M_3R) antagonist tiotropium for the treatment of chronic obstructive pulmonary disease (COPD) and asthma.[27] Tiotropium also binds to muscarinic 2 receptor (M_2R), but here its RT is around 10-fold lower than that for M_3R. Moreover, ipratropium, a short RT ligand for the same receptor (with >11-fold lower RT than tiotropium and with 6.5-fold lower affinity), is less effective *in vivo* than tiotropium.[28,29] In the case of marketed agonists, deslorelin has a longer RT than goserelin for the gonadotropin-releasing hormone receptor (GnRHR).[30] For the β_2 adrenergic receptor (β_2AR), the short-acting salbutamol represents a short RT drug and salmeterol represents a long RT drug for the treatment of asthma and COPD.[31,32]

The secretin family of GPCRs (class B1) is activated by endogenous peptide ligands and marketed drugs for these receptors are all characterized by being modified peptides with a high resemblance to the endogenous agonists. Thus, several agonists has been launched for the glucagon-like peptide 1 (GLP-1) receptor for the treatment of type 2 diabetes mellitus (T2DM) and obesity, such as the synthetic variants of GLP-1, lixisenatide, liraglutide, dulaglutide, and semaglutide that in addition to prolonged half-lives all have a longer RT than the endogenous agonist GLP-1.[33,34] In the current perspective, we elaborate on the impact of binding kinetics for drug efficacy *in vivo* exemplified by four class A GPCRs, where detailed structural insight has been provided for ligand−receptor binding kinetics. Moreover, we discuss the role of binding kinetics for functional selectivity and sustained signaling and ultimately how variations in ligand and/or receptors affect binding kinetics.

■ OVERALL STRUCTURAL CONSIDERATIONS FOR LIGAND−RECEPTOR BINDING KINETICS

As a result of the growing number of GPCR structures (reviewed recently in refs 35 and 36), increasing knowledge has been obtained of how ligands bind and how this in turn results in activation of intracellular pathways. It was originally anticipated that the binding of ligands to the receptor and the subsequent receptor activation was regulated in a simple static manner. However, it is now widely appreciated that receptors transit back and forth between different active and inactive conformational states, ultimately resulting in different signaling outcomes (as reviewed by Latorraca et al.).[37] These include activation of heterotrimeric G proteins or the recruitment of G protein-coupled receptor kinases and/or β-arrestins.[38]

The receptor signaling varies between ligands, a phenomenon also referred to as functional selectivity, where different ligands selectively confer one pathway over another pathway through a common receptor. At present, it is not clear how binding kinetics influence and potentially affect functional selectivity between ligands, but it is likely that altered k_{on} and/or k_{off} might contribute. Here, it is of interest to compare overall structural and functional differences between class A and B1,[39,40] for instance, the striking inherent functional selectivity for G protein activation over β-arrestin recruitment for class B1 receptors. This has been shown for both the GLP-1R and the glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR), with ∼1000-fold higher potency in G protein activation (cAMP production) compared to that in β-arrestin recruitment for the endogenous agonists GLP-1 and GIP, respectively.[33,41−46] Moreover, the initial ligand interaction in the so-called two-step activation for the class B1 receptors is with the large extracellular N-terminal domain (ECD) that is not present in class A receptors, and the orthosteric binding pocket is more spacious for class B1 as compared to class A receptors.[39,47−49] Other noticeable differences are the large kink in the end of transmembrane (TM) 6 of the GLP-1R and the calcitonin receptor (CTR) (class B1) compared to the preserved helical propensity of TM6 in β_2AR (class A)[39,40] and the slightly longer helix 8 for class B1 in comparison to class A, supporting the interaction of this helix with the β-subunit of the G protein.[47] Thus, a multitude of structural differences exist between the two classes to explain differences in ligand binding kinetics that in turn could contribute to the functional selectivity in class B1, however this remains to be proven. The rapid increase in GPCR structures will contribute to a larger understanding of the structural features determining ligand−receptor binding kinetics. In the section below, we provide four examples of class A receptors, where detailed analyses have already provided structural insight into ligand−receptor binding kinetics.

Muscarinic M_3 Receptor versus M_2. The first example is the drug tiotropium that binds to M_3R and M_2R. In M_3R, the movement of the extracellular loop 2 (ECL2) to a more closed

conformation has suggested the ECL2 of M_3R to be a key regulator of the long RT (35 h) of tiotropium, whereas the more flexible and open ECL2 of M_2R is associated with a shorter RT (5 h).[50,51] When diving deeper into the binding pocket of M_3R to look at individual receptor residues, the RT of tiotropium is dramatically decreased by 583-fold when N508$^{6.52}$ (Ballesteros–Weinstein numbering in superscript)[52] is mutated to alanine.[51] This residue has been suggested to interact through a double hydrogen bond with tiotropium (hydroxy- and ester group) and thereby serve as a so-called "snap-lock" to prevent the ligand from leaving the binding pocket. Another observation is that the group of tyrosine residues in the binding pocket above tiotropium, consisting of Y149$^{3.33}$, Y507$^{6.51}$, and Y530$^{7.39}$, function as mechanical barrier, which hinders tiotropium from leaving the binding pocket of M_3R thereby contributing to the long RT. When these residues are mutated to alanine, the RT is decreased 5208-, 2500-, and 175-fold, respectively. It is, however, noteworthy that under physiological conditions the RT of tiotropium is much shorter and that the onset of action therefore does not strictly derive from the long RT.[53] Instead, the long duration of tiotropium action *in vivo* might originate from rebinding of the drug to its receptor. Here, rather than diffusing away from the local environment, where the receptors are present, tiotropium is more likely to rebind to the same receptor.[54] Moreover, the same study showed that NVA237 (a once-daily dry powder formulation of glycopyrronium bromide) had a more rapid onset of action than tiotropium, as it reached the equilibrium of receptor occupancy levels faster. This could be explained by the difference in k_{on}.[53]

Adenosine A_{2A} Receptor ($A_{2A}R$). The second example is the $A_{2A}R$.[55] Exploration of molecular dynamics simulations of ZM241385 (a high affinity small molecule antagonist) has identified several residues as putative interaction partners with impact on the RT of ZM241385. Certain mutations were found to increase the RT, whereas others decreased the RT of ZM241385 relative to wildtype $A_{2A}R$. Three residues located in the binding cavity (T256$^{6.58}$ and ECL residues E169^{ECL2} and H264^{ECL3}) form a cluster interacting directly or indirectly with ZM241385 through hydrogen bonding. Upon disruption of these bonds by the mutation E169^{ECL2}Q, T256$^{6.58}$A or H264^{ECL3}A, the binding pocket opens up, effectively decreasing the RT of the ligand, by 62-, 17-, and 19-fold, respectively (Figure 1A).[56] Furthermore, short RT ligands with high similarity to ZM241385 do not stabilize the salt bridge between E169 ^{ECL2} and H264^{ECL3}, highlighting that even small changes to a ligand can affect the RT dramatically.[57] Another pocket, formed by I66$^{2.64}$, S67$^{2.65}$, and L267$^{7.32}$, is located slightly more toward the extracellular surface and interacts directly with ZM241385. When mutating I66$^{2.64}$, S67$^{2.65}$, or L267$^{7.32}$ to alanine, the RT is increased with 1.7-, 1.6-, and 2.3-fold, respectively (Figure 1B). These data were supported by another group, using surface plasmon resonance (SPR) to address the off-rate of ZM241385 in several mutations of the A_{2A} receptor including I66$^{2.64}$ (Figure 1B).[58] Here, a similar enhanced RT was observed upon introduction of an alanine at this position.

CC Chemokine Receptor 5. The third example is from the chemokine field, namely CCR5. A study performed by Swinney et al.[59] investigated the impact of mutations in the binding pocket of maraviroc on its binding kinetics, including E283$^{7.39}$ (Figure 2), which is conserved among chemokine receptors.[60,61] E283$^{7.39}$ serves as a key anchor for maraviroc, by interacting with the nitrogen of its tropane group through a salt bridge.[62] The long RT of maraviroc has been ascribed to its multistep binding mechanism. According to this model, the rearrangement of the binding pocket which maraviroc occupies results from a change from a flexible RL state to a more stable long-lasting R*L state through ligand-specific complementary interactions with CCR5 (i.e., as in the induced fit model (see Box 1)).[63] One of the residues supporting the flexible RL state and the stable long-lasting R*L, is the aforementioned E283$^{7.39}$ residue (where alanine substitution heavily impaired K_D). Similar observations were made upon mutations of two other residues directly interacting with maraviroc, namely, W86A$^{2.60}$ and Y108A$^{3.32}$ (Figure 2) (i.e., a dramatically shortened in RT, 11- and 6.7-fold, as well as increased and decreased k_{on}, 1.6- and 2.3-fold, respectively).[62]

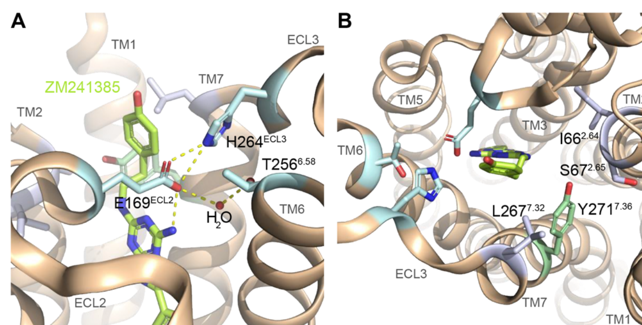


Figure 1. Receptor residues in the binding pocket of $A_{2A}R$ that lead to altered RT of ZM241385 upon mutation to alanine (Protein Data Bank (PDB) accession number: 4EIY). (A) Side view of the hydrophobic pocket in the receptor, formed by E169^{ECL2}, T256$^{6.58}$, and H264^{ECL3}, which is also further stabilized by a water molecule. Mutagenesis of residues that are located in this hydrophobic pocket <u>decreases</u> the RT of ZM241385. (B) Top view of $A_{2A}R$; above Y271$^{7.36}$ (essential for ZM241385's binding and decreasing RT when mutating to alanine (~10-fold)),[55,58] there exists another hydrophobic pocket that is formed by I66$^{2.64}$, S67$^{2.65}$, and L267$^{7.32}$. Upon mutagenesis of these residues, the RT of ZM241385 is <u>increased</u>.

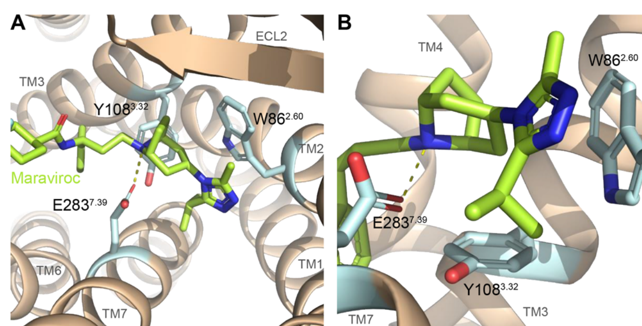


Figure 2. Receptor residues within the CCR5 binding pocket that are crucial for maraviroc's long RT (PDB accession number: 4MBS). Top view (A) and side view zoom (B) of maraviroc's binding pocket. E283$^{7.39}$ is crucial for the transition from the flexible RL state to the long-lasting R*L state. Other receptor residues, such as W86$^{2.60}$ and Y108$^{3.32}$, have been shown to be detrimental in shortening the RT when mutated to alanine.

β_2 Adrenergic Receptor. The fourth example is salmeterol, which possesses a long aryloxyalkyl tail that allows for additional receptor interactions to a exosite in β_2AR (a second high-affinity binding site for salmeterol) in comparison to salbutamol and epinephrine that both bind to the orthosteric binding site (Figure 3).[64] These additional binding interactions might be responsible for the 5- to 7-fold longer RT of salmeterol compared to those of salbutamol and epinephrine. Also, for this

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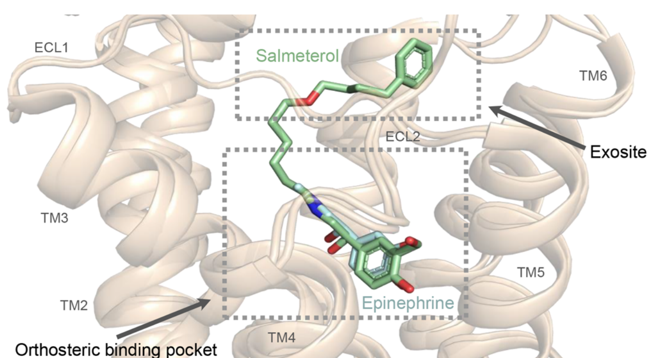

Figure 3. Orthosteric binding pocket and exosite of β_2AR (PDB accession numbers: 6MXT and 4LDO). Superimposed salmeterol−β_2AR and epinephrine−β_2AR structures highlight the additional binding interactions of salmeterol with β_2AR contributing to the longer RT.

receptor, k_{on} has been ascribed to predict *in vivo* efficacy. Thus, during an asthma attack, k_{on} plays an essential role in the (fast) onset of the therapeutic response, such as observed for abediterol, a ligand currently in phase II clinical trials targeting β_2AR.[18]

As exemplified above, the implementation of structural data to support the binding kinetics of drugs has been very useful in highlighting the relevant receptor residues. Today, many drugs with improved and different k_{on} and k_{off} have been published; however, structural information is still lacking for the majority. It is worth mentioning that a shift in k_{on} and/or k_{off} for a certain ligand−receptor pair does not necessarily have the same impact as a similar shift in a different ligand−receptor pair. Ultimately, the goal is to tailor the ligand−receptor binding kinetics and the subsequent *in vivo* efficacy, thereby improving the therapeutic outcome. Therefore, we propose that the increasing amount of structural information from MD simulations as well as that of receptor structures should be used to guide ligand k_{on} and k_{off} optimizations.

■ IMPACT OF BINDING KINETICS ON THE SUSTAINED SIGNALING FROM INTERNALIZED RECEPTORS

Most GPCRs undergo internalization, after which some recycle back to the cell surface and others undergo degradation. Some receptors remain active from their endosomal compartments after internalization (sustained signaling) as described for class A vasopressin type 2 receptor (V2R) and the class B1 parathyroid hormone receptor (PTHR), GLP-1R, and GIPR.[65−69] As speculated by Hothersall et al.,[70] this sustained signaling could be linked to increased RT and co-internalization of receptor in complex with ligand. Consequently, a ligand with a short RT will dissociate from the receptor prior to internalization and thereby lack prolonged signaling, whereas a ligand with longer RT will remain bound to the receptor and enable signaling from intracellular localizations. Notably, the effect of changing the pH to a more acidic endocytic environment could cause a change in affinity, thereby changing the RT of the ligand−receptor complex and highlighting that the RT of the ligand may not be the same, as when measured at the cell surface.[71,72]

Along those lines, it has been shown that allosteric modulators can prolong intracellular signaling. In case of PTHR, where Ca^{2+} act as an allosteric modulator for PTH, the presence of Ca^{2+} significantly increases the RT of PTH, and thereby enhancing the cAMP production from endosomal compartments.[73] The C-terminal half of PTH (residues 15−34) has been identified as essential for this endosomal signaling, as exemplified by the naturally occurring single nucleotide polymorphism (SNP) in PTH (PTH^{R25C}), which is linked to hypocalcemia in humans.[74] In this SNP, a decreased binding of Ca^{2+} is observed that consequently leads to a lower RT and a more transient receptor activation at the cell surface.[73] Thus, it seems possible to improve the therapeutic outcome of GPCR targeting though a regulation of the duration of endosomal signaling. Whether short or long sustained signaling from intracellular localizations is warranted for a given drug depends on the disease and the pharmacodynamic profile of the drug (agonist, antagonist, neutral ligand or allosteric modulator).

■ NATURALLY OCCURRING RECEPTOR VARIANTS WITH IMPACT ON LIGAND−RECEPTOR BINDING KINETICS

As described above, SNPs of endogenous ligands may affect binding kinetics. Likewise, naturally occurring variants of receptors can alter binding kinetics and/or receptor signaling and thereby alter the phenotype in affected individuals. We recently showed that the SNP GIPR E354$^{6.53b}$Q (Wootten numbering in superscript,[75] b refers to class B GPCRs) was associated with altered binding of the natural hormone GIP(1−42) that in turn resulted in an increased RT (but unaltered k_{on}), increased signaling and a higher internalization rate.[76] At the molecular level, the carboxylic acid of residue E354$^{6.53b}$ (Figure 4A) is predicted to form a salt bridge with the

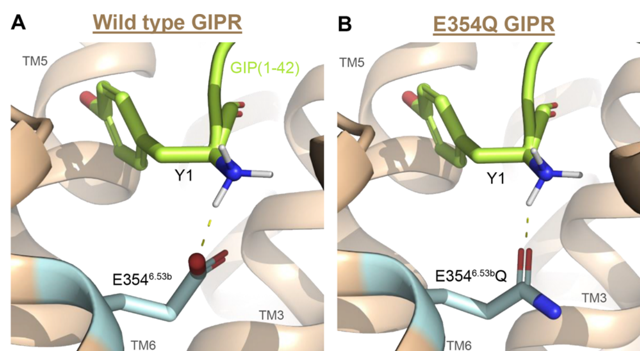

Figure 4. E354$^{6.53b}$Q increases the RT of GIP(1−42) on GIPR.[76] Both E354$^{6.53b}$ (A) and E354$^{6.53b}$Q (B) interact with the N-terminal nitrogen (Y1) of GIP(1−42). E354$^{6.53b}$ forms a salt bridge, while the loss of anionic properties by glutamine at this position in (E354$^{6.53b}$Q) still allows for a hydrogen bond in the same place.

N-terminal nitrogen of GIP(1−42), explaining why the change to glutamine (Q) impacts RT (Figure 4B). Other groups have described decreased receptor recycling to the cell surface for this variant; accordingly, this mutation resulted in a long-term receptor downregulation, leading to slightly impaired blood glucose control and compromised bone strength with higher risk of fractures in carriers of E354$^{6.53b}$Q.[77,78]

■ LIGAND VARIANTS AFFECTING LIGAND−RECEPTOR BINDING KINETICS

In contrast to the considerable knowledge of ligand−receptor binding kinetics for drugs acting on GPCRs, less is known about the kinetics of the endogenous ligands. Some GPCRs

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have several endogenous agonists, including the majority of chemokine receptors (class A)[79] or in the case of class B1, GLP-1R, where at least three endogenous agonists have been identified, namely, GLP-1 itself, oxyntomodulin, and glucagon.[80,81] For GLP-1, ligand−receptor binding kinetics (including RT) have been characterized in several studies, albeit with variable findings.[82−84] For instance, Jones et al.[84] described a RT for GLP-1(7−36) at the GLP-1 receptor of less than 10 min, which is notably shorter than the one described by Roed et al.[83] (several hours), a difference that could be caused by assay variations. Analogs of GLP-1, such as exendin-4 (derived from the Gila monster), have been described to have a longer RT than GLP-1,[33,84] with synthetic variations in the N-terminus of exendin-4 either decreasing (substitution of the first histidine to a phenylalanine (ex-phe1)) or increasing (introduction of an aspartic acid on the third position (ex-asp3)) the RT. Moreover, the k_{on} of both ex-phe1 and ex-asp3 were lower than for exendin-4. In comparison to exendin-4, lixisenatide and liraglutide have longer RTs, whereas the RTs of dulaglutide and semaglutide were reported to be comparable to exendin-4. Recently, it was shown that extendin-4 partially competes with a nonpeptidic agonist, and the presence of this ligand affected the rapid ligand−receptor binding kinetics of extendin-4, ultimately slowing it down.[85]

Other examples, in which endogenous ligands were studied in term of binding kinetics include the cannabinoid 2 receptor (CB2R), CB1R, H_1R, NK_1R, GnRHR, the atypical chemokine receptor 3 (ACKR3), CCR1 and CCR5.[26,30,86−89] In the case of CB2R, anandamide as well as 2-arachidonoylglycerol had shorter RTs than the synthetic full agonists.[86] Moreover, the k_{on} of these endogenous ligands were, in most cases, also slower. Antagonists for CB1R such as rimonabant, initially approved but later withdrawn from the market due to severe side effects, have been used to improve therapeutic targeting of this receptor by investigating derivatives of this ligand on their RT.[89] It was postulated that certain parts of the scaffold of rimonabant could be used to optimize affinities, whereas others improve RT. As a result of this, a potential candidate (with a longer RT) based on rimonabant had insurmountable properties, whereas rimonabant did not. The longer RT of this potential candidate could be traced back to the rearrangement of water molecules according to the simulations.

In the case of H_1R, desloratadine, a shorter RT ligand but with a higher affinity and the same scaffold as rupatadine, was less effective after functional recovery of histamine-induced calcium mobilization.[26] The addition of its methylpyridin-3-ylmethyl moiety for rupatadine made it possible to make additional interactions with the receptor. Besides, variations of desloratadine without a spacer group show a relatively similar RT, whereas a one-carbon spacer prolongs the RT (as for rupatadine).

For NK_1R, the kinetic characterization of the endogenous ligands led to an excellent correlation between the ligand's RT and *in vitro* efficacy, as well as between the ligand's k_{on} and the *in vitro* potency, as determined via a label-free assay.[87] Moreover, a follow-up study showed that antagonists display different inhibitory potencies depending on the binding kinetics of the endogenous agonist.[23]

In case of the GnRHR, synthetic GnRH analogs displayed RTs ranging from 6 to 111 min in radioligand binding assays, whereas the RT measured by time-resolved fluorescence resonance energy transfer (FRET) varied from 2 to 61 min.[30] Interestingly, the RT of GnRH itself was around 6 min, indicating that most of these synthetic analogs had a longer RT. Moreover, these data underline discrepancies between the different assay techniques that were used; therefore, careful consideration is required to select a suitable technique.

In the situation of ACKR3, CXCL12 had a slower off-rate (and thereby, a longer predicted RT) than that of CXCL11. However, due to the experimental setup employed, the off-rate (and thereby the RT) could not be accurately quantified.[88] Interestingly, on the homologous CXCR4 receptor, CXCL12 had a shorter RT and faster k_{on} than it had for ACKR3, indicating that the k_{on} and k_{off} for CXCL12 on ACKR3 are unusually slow.[90] Whether this slow binding kinetics contributes to the preferred arrestin coupling over G protein signaling for ACKR3 remains to be determined. As for another chemokine receptor (CCR1), by studying the molecular mechanism behind the allosteric action of metal ion chelators on CCL3 binding to CCR1, we discovered that an increased k_{on} of CCL3 (in the presence of the allosteric modulators) was the main denominator for the improved binding of CCL3.[91] For CCR5, the higher RT of AOP-CCL5, as compared to that of CCL5, led to a lower recycling and higher degradation of the receptor and thereby competing more effectively with the HIV envelope.[92]

Post-translational modifications (PTMs) can also alter ligand binding and the subsequent downstream signaling, as observed for the peptide hormone that activates the GIP receptor. The endogenous agonist GIP(1−42) is cleaved by dipeptidyl peptidase-IV (DPP-IV), resulting in GIP(3−42), that acts as an antagonist at supraphysiological concentrations.[93] Another naturally occurring antagonist, GIP(3−30), originates from the metabolite of GIP(1−30) through DPP-IV cleavage.[94] In addition to our recent investigation of the k_{on} and RT of GIP(1−42), so far only a monoclonal antibody antagonist for GIPR has been characterized for its ligand−receptor binding kinetics.[76,95] In comparison, the monoclonal antibody had around a 10-fold shorter RT and a much higher k_{on} as compared to GIP1−42. Taken together, these examples above emphasize that kinetic parameters should be taken into account when developing novel drugs targeting GPCRs, next to examining the kinetic parameters of the endogenous agonist(s) and their variants.

■ PROBE DEPENDENCY IN THE DETERMINATION OF LIGAND−RECEPTOR BINDING KINETICS

Most often, ligand−receptor binding kinetics are investigated using radiolabeled ligands. However, other methods exist such as SPR, FRET, and bioluminescence resonance energy transfer (BRET), in which receptors and/or ligands are modified (for a review of these methods, see refs 96−98). The challenge of using radiolabeled ligands is the necessity of labeling each ligand of interest with a radioisotope, making it time-consuming and expensive. One way to circumvent this issue is by applying the Motulsky−Mahan model,[7] which allows measurements of ligand−receptor binding kinetics in heterologous systems. Here, ligand−receptor binding kinetics of an unlabeled ligand can be determined in competition with a high-affinity radioactive- or fluorescent-labeled probe. So far, this model has been applied successfully for several class A and B GPCR targets, including the corticotropin-releasing factor 1 receptor (CRF_1R).[12,30,33,99−101] However, it is important to note that the choice of radioligand to study ligand−receptor kinetics is crucial, as the displacement (and thereby dissociation) will depend on the radioligand in the heterologous competition situation. This was illustrated in 1994, where we described mutations in TM2 of the NK_1 receptor that compared to the wild type receptor resulted in a

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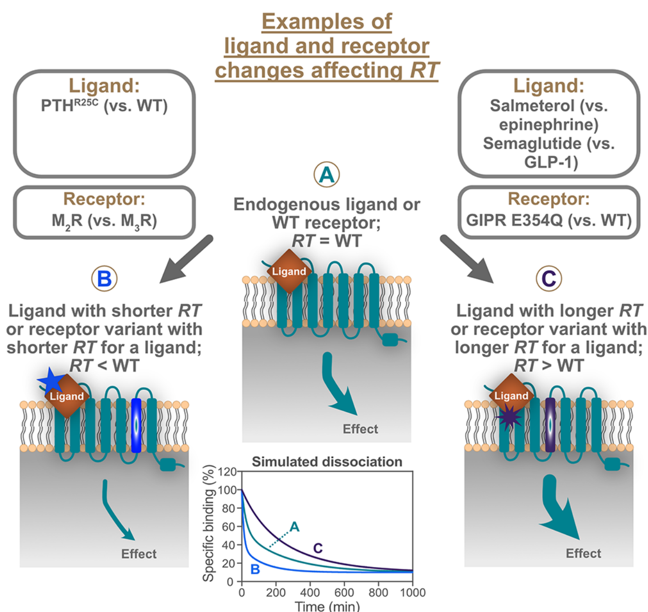

Figure 5. Graphical overview of how variations in ligand and receptor can alter ligand−receptor RT. Receptor changes affecting ligand−receptor binding kinetics can lead to a shorter RT (B) or to a longer RT (C) for a certain ligand as compared to the wild type (WT) receptor (A). Besides post-translational modifications (PTM) of the same ligand, different endogenous ligands and analogs of endogenous ligands) can also lead to a shorter RT (B) or to a longer RT (C) as compared to an endogenous ligand (A).

conformational constraint that prevented the small-molecule antagonist CP96345 from competing with radiolabeled endogenous agonist ([^{125}I]-Substance P), but still allowed high-affinity binding of [^{3}H]-CP96345).[102] Along the same lines, a study by Guo et al.[103] on the adenosine A_1 receptor (A_1AR) pointed out that the kinetics of ligand binding to a receptor can be altered by the addition of an allosteric modulator in a probe-dependent manner. Another example comes from the metabotropic glutamate 2 receptor (mGlu$_2$R; class C GPCR), in which the k_{on} and k_{off} of the endogenous ligand glutamate were affected by a negative allosteric modulator, whereas this was not the case for the agonist LY354740.[104]

An interesting phenomenon was discovered for H_1R, dopamine D_2 receptor (D_2R), and A_1AR, in which large differences in on- and off-rate were observed for a set of unlabeled ligands using two different probes.[105−108] The usage of [^{3}H]mepyramine as a radioligand for H_1R appeared to be easier to discriminate between unlabeled ligands with a fast k_{off} than with [^{3}H]levocetrizine. The reason for this was that levocetrizine had a 100-fold lower k_{off} than mepyramine; therefore, the accuracy of both k_{on} and k_{off} of unlabeled ligands can be predicted better with mepyramine as a radioligand.[105] Similarly, for D_2R, the slow k_{off} of spiperone was less accurate in predicting the k_{off} of fast dissociation ligands as compared to the relatively fast k_{off} probe PPHT-red.[108] Moreover, the accuracy of kinetic measurements is dependent on the probe concentration. As such, the importance of using a suitable probe for ligand−receptor binding kinetics calls for careful consideration, and ideally one with a fast binding is required. This especially applies to the measurement of kinetics in heterologous systems for ligands that do not share the same binding mode.

Moreover, in competition-binding experiments, where a filtering approach is often used, the unbound radioligand is washed away from the bound radioligand. Consequently, during this process it is of high importance that the bound radioligand is not washed away as a result of short RT (high k_{off}). However, this is not the case in fluorescent-based competition experiments, where no washing steps are involved as they are homogeneous and real-time in nature, meaning that the k_{off} of a probe can be higher in fluorescent-based competition versus radioligand competition assays. Recently, SPA-based competition experiments have been introduced as another approach to circumvent the filtration of the radioligand.[99,109]

According to Sykes et al.[108] the initial machine read time is also important, as *on-line* (addition of ligands directly into sample wells) and *off-line* (addition of ligands in sample wells prior to the insertion of reader) additions may dictate the resolution and affect the accuracy of the measurement. This especially applies to ligands with fast k_{off}, where it is critical to measure before equilibrium is reached.

CONCLUDING REMARKS

Studying ligand−receptor binding kinetics to perceive the role of GPCRs in physiology and their potential targeting from a therapeutic point of view is still underappreciated. Here we emphasize that kinetic parameters such as k_{off} and RT but also k_{on} are important for predicting efficacy and describe how alterations in ligands and/or receptors may impact binding kinetics (collected in Figure 5). From a translational perspective, the more we understand of how ligand efficacy is regulated, the better our chances will be for successful discovery of novel and improved drug candidates in the future.

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Notes

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