DRUG-RECEPTOR BINDING KINETICS IN DRUG DISCOVERY: STICKING AROUND AT THE HISTAMINE H₁ RECEPTOR

Reggie Bosma
DRUG-RECEPTOR BINDING KINETICS IN DRUG DISCOVERY
STICKING AROUND AT THE HISTAMINE H₁ RECEPTOR

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Summary

In the last 80 years, a large variety of blockbuster drugs has been developed that are used to alleviate allergy symptoms. These drugs obtain their effect by blocking the interaction between histamine and the histamine H1 receptor (H1R) and are therefore also known as antihistamines. In this work I investigated the kinetics of the binding interactions between antihistamines and the H1R and I explored whether such analyses are useful to incorporate in the development of new drugs. Large differences in H1R binding kinetics were observed between different antihistamines that were not always reflected by the strength of the binding interaction (i.e. binding affinity). It was additionally shown that differences in the binding kinetics of antihistamines at the H1R could affect antagonism of the receptor independent of the binding strength (chapter 5). This, together with evidence presented in the scientific literature, suggests that kinetic analyses of drug-receptor binding have added value for the development of new drugs.

Methodologies to measure drug-receptor binding kinetics were assessed and new methods were developed (chapter 2-5). Some progress was made in the efficiency with which the kinetics of drug-receptor binding could be measured, for example, by using a continuous readout of receptor activation (chapter 3). Moreover, methods that measure the kinetics of drug-receptor binding often employ a competitive ligand that is easy to detect (e.g. radioligand) which allows an indirect quantification of the binding kinetics. For an often-used analysis of competitive binding interactions (i.e. Motulsky-Mahan analysis), limitations were discovered in the range that this analysis yields reliable quantification of the drug-receptor binding kinetics (chapter 4). Hence, this has important implications for the robustness in which binding rate constants (i.e. constants describing the binding kinetics between drug and receptor) can be quantified. Various methodologies were compared to validate the kinetic binding profile of antihistamines in different assays. It was shown that the relative residence time (i.e. length of drug binding at the receptor) of antihistamines, measured by radioligand binding experiments, was indeed reflected in experiments measuring the kinetics of antihistaminergic effects in living cells (chapter 3 and 5). In general, the developed methods are considered to be not only useful for drugs binding the H1R but also for drugs that bind to other members of the G protein-coupled receptor-family.

Another important aspect of this thesis was to investigate the relationship between the drug structure and the drug-receptor binding kinetics (i.e. structure kinetics relationship, SKR). A better understanding of this relationship should provide a handle for the rational drug optimization of the kinetic binding profile of antihistamines. For ligands binding the H1R, several sub-structures were linked to an increase in the receptor residence time (chapter 6-8). Moreover, several of the newer generation antihistamines (e.g. levocetirizine, chapter 6; olopatadine, chapter 6 and 7; rupatadine, chapter 8) contained one or multiple of these chemical structures,
indeed reflected by their long residence time at the H$_2$R. In the last chapter, opportunities and challenges are extensively discussed regarding interpretation of the measured binding rate constants. Several hypotheses are discussed but it is concluded that translation of the observed SKRs to the underlying binding mechanisms warrant further investigation. This should focus on a better understanding of the molecular interactions that are formed between the drug and receptor over time and its relationship with the binding rate constants.
Samenvatting

In de afgelopen 80 jaar zijn er veel medicijnen ontwikkeld voor de behandeling van allergiesymptomen. Deze medicijnen verkrijgen hun effect door de interactie te blokkeren tussen de signaalstof histamine en het membraaneiwit de histamine H₁ receptor (H₁R). De medicijnen zijn daarom ook wel bekend als antihistamines. In dit werk onderzoek ik de snelheid van de bindingsreacties tussen antihistamines en de H₁R en ik onderzoek of zulke analyses nuttig kunnen zijn in de ontwikkeling van nieuwe medicijnen. Tussen de verschillende antihistamines werden grote verschillen gemeten in de snelheid waarmee de bindingsreactie aan de H₁R zich voltrok en deze verschillen kwamen niet naar voren in de bindingssterkte (i.e. affiniteit). In experimenten met levende cellen bleek dat de remmende functie (i.e. antagonisme) van antihistamines op de signaaltransductie die de H₁R in de cel teweegbrengt, sterk beïnvloed wordt door de bindingskinetiek tussen antihistamine en receptor. Samen met bevindingen in de wetenschappelijke literatuur, suggereren de onderzoeksresultaten een meerwaarde van kinetische analyses op medicijn-receptorinteracties in de ontwikkeling van nieuwe medicijnen.

In dit boekje evalueer ik de verschillende methoden die beschikbaar zijn voor kinetische studies naar medicijn-receptorinteracties en ontwikkel ik nieuwe methoden (hoofdstukken 2-5). Voor de nieuwe methoden worden er stappen gezet in de efficiëntie waarmee kinetische studies kunnen worden uitgevoerd. Experimenten waarin receptoractivatie continu gemeten kan worden (zoals in hoofdstuk 3) komen de efficiëntie van kinetische studies ten goede. Om de kinetische bindingseigenschappen van een medicijn te bestuderen wordt vaak gebruik gemaakt van een competitief ligand dat makkelijk gedetecteerd kan worden (e.g. een radioactief ligand) waarmee indirect het binden van het niet-radioactieve medicijn gemeten kan worden. Voor een veelgebruikte analysemethode van competitieve ligandbinding (i.e. Motulsky-Mahan analyse) werden limieten gevonden binnen het gebruikte model, waarbuiten onnauwkeurige voorspellingen werden gemaakt over de bindingskinetiek tussen medicijn en receptor (hoofdstuk 4). Deze bevindingen geven essentiële informatie over de betrouwbaarheid van de gemeten kinetische bindingsconstanten (constanten die de bindingskinetiek tussen medicijn en receptor beschrijven). Verschillende methoden zijn vergeleken om de gemeten bindingskinetiek van medicijnen aan de H₁R te kunnen valideren. Het is aangetoond dat de lengte van receptorbinding (i.e. residence time) voor antihistaminica ook naar voren kwam in experimenten waarin de kinetiek van anti-histaminergic effecten op levende cellen werden bepaald (hoofdstukken 3 en 5). De ontwikkelde methoden hebben ook meerwaarde buiten het onderzoek naar de H₁R om de interacties te bestuderen tussen medicijnen en andere G-eiwit-gekoppelde receptoren.

Een ander belangrijk aspect van dit proefschrift was het bestuderen van de moleculaire structuur van een medicijn in relatie tot de bindingskinetiek aan de H₁R (i.e. structuur kinetiek relatie, SKR). Een beter begrip van deze relatie zou moeten helpen met het gericht optimaliseren van de
receptor bindingskinetiek van antihistaminica. Voor liganden die aan de H₁R binden zijn verschillende moleculaire deelstructuren ontdekt die gelinkt zijn aan een toename in de residence time op de receptor (hoofdstukken 6-8). Meerdere van de nieuwere antihistaminica (e.g. levocetirizine, hoofdstuk 6; olopatadine, hoofdstukken 6 en 7; rupatadine, hoofdstuk 8) zijn opgebouwd uit een of meerdere van deze deelstructuren en dit komt inderdaad tot uiting als een lange residence time voor deze medicijnen op de H₁R. In het laatste hoofdstuk worden de kansen en belemmeringen besproken voor de interpretatie van de gemeten kinetische bindingsconstanten. Meerdere aannemelijke hypotheses worden aangehaald, maar er wordt geconcludeerd dat voor een translatie van de gevonden SKR naar de onderliggende moleculaire interacties tussen medicijn en receptor meer werk nodig is. Dit werk zou zich moeten focussen op de interacties tussen medicijn en receptor in de tijd en de manier hoe dit zich uit in de gemeten kinetische bindingsconstanten.
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Parts are published as:


1 Unmet need in drug discovery

Drug molecules have to fulfill many criteria to be eligible for use in the clinic and its development therefore requires an extensive amount of resources before it is tested on human volunteers during pre-clinical research and even much more in later clinical phase trials (phase I – III). Figure 1 shows a diagram of the average costs made in the various stages of drug development to end up with one new medicine with a total average cost of 873 million euro, based on data that became available in 2007-2008. More recent data from 2013 shows an even higher total average cost per newly developed medicine of 1.4 billion euro. Despite extensive testing in pre-clinical research it cannot be prevented that there is a very low success rate in clinical phase trials (6 – 12%). In phase II studies drugs are for the first time tested in patients and at that stage the highest percentage of drug attrition is observed (71%). The biggest cause is a lack of efficacy (35% – 88%). Moreover, the R&D costs required to develop a single new medicine was put into a model based on, e.g., the success rate and costs of the various stages in drug development. In this study, it was found that the total R&D costs were most sensitive for changes in the success rate of phase II clinical trials. The drug development industry would therefore greatly benefit by a reduced attrition in phase II studies, which seems to depend largely on the in vivo efficacy.

One of the first criteria that is applied to select potential drug molecules is its ability to bind or modulate a biological target. Since it is often unclear what kind of molecules can do this, large amounts of molecules are tested in order to find a few hits that can be further developed. Molecules that are found to bind the biological target or modulate its activity at high concentrations are then further characterized. This is often done by measuring the concentration of the hit compounds that is required to reach half-maximal binding ($K_d$, $K_i$) or half-maximal modulation of the biological function of the target ($IC_{50}$, $EC_{50}$). Hence, molecules are selected that can bind or modulate the target at very low concentration under the assumption that concentrations are stable and there is ample time to bind the target, i.e. equilibrium binding. However, due to the dynamic nature of in vivo drug concentrations as well as endogenous ligand concentrations, this assumption is not always justified. Moreover, of all 85 drugs that were accepted by the FDA between 2001 – 2004, 32% had non-equilibrium kinetics and only 20% of the compounds unambiguously showed a competitive equilibrium mode of action. Therefore, it was suggested to characterize drug-binding not solely in terms of affinity, but rather in terms of the kinetics of binding. Especially a long drug-target residence time, a measure for the drug-target complex life time, was

![Figure 1 - Costs related to the research and development of a new drug entity](image-url)
suggested to relate to an increased therapeutic window, an insurmountable target engagement and less off-target toxicity. However, on-target toxicity has also been described as a result of an insurmountable mode of antagonism on the dopamine D2 receptor, indicating that a long residence time drug will not always be beneficial. It is therefore thought that guiding lead optimization by metrics describing the drug target binding kinetics, with in particular the drug-target residence time, can reduce attrition rates in clinical phase trials.

To incorporate the kinetics of drug-target binding in the drug discovery process required at the start of this work a paradigm shift. Therefore, a consortium of various big pharma and small medium enterprises teamed up with several academic partners under the umbrella of the EUs Innovative Medicines Initiative to investigate how the drug-target binding kinetics should be incorporated in future drug development. This consortium started out in 2012 with a budget of 21 million and goes by the name Kinetics for drug discovery (K4DD) (www.k4dd.eu). The work presented in this thesis was performed within the context of the K4DD program and with support of a number of different K4DD partners.

2 Efficacious drug binding to G protein-coupled receptors: residence time

Receptors within the G protein-coupled receptor (GPCR) family are widely investigated as disease target in drug discovery. Nowadays, 30 percent of all clinically used small-molecule drugs target a GPCR, suggesting therefore the importance of this class of proteins in disease modulation. GPCRs consists of 7 transmembrane spanning α-helical domains (TM-domains) and are known to be responsive to a large variety of different stimuli, leading in most cases to the intracellular activation of heterotrimeric G proteins causing the dissociation of the α and βγ subunits.

Interestingly, quite some clinically used compounds that target GPCRs have been described to possess a long drug-target residence time. In many cases this is considered to result in a longer duration of action in vivo. Tiotropium for example is an antagonist for the muscarinic M3 receptor which is used in chronic obstructive pulmonary disease. The residence time of tiotropium was found to be > 10 hr and it has very long duration of action in vivo, allowing therefore a once-daily dosage for patients. Similarly, the anti-allergy drug, levocetirizine is an antagonist for the H1R and binds the receptor with a residence time of 4 hr, which was also proposed as an explanation for its long duration of action in vivo. Candesartan is used in the treatment of hypertension and exerts its function by antagonizing the angiotensin AT1 receptor. Candesartan binds at the receptor with a residence time of 3 hr, which could explain its insurmountable mode of antagonism as well as its long duration of action in vivo.

As described above, insurmountable antagonism at the dopamine D2 receptor as a result of long residence time is proposed to cause on-target toxicity. Similarly, chronic treatment of rats with the insurmountable H2R antagonist loxididine resulted in gastric tumors, which was not the case for high doses of the surmountable H2R antagonist ranitidine, indicating that the long-lasting inhibitory effect on H2R might lead to on-target toxicity as well. This hypothesis is supported by the fact that an hypoacidic environment of the stomach, a direct result of H2R antagonism, indeed seems to increase the development of gastric tumors.
To summarize, ligand-receptor binding kinetics can have a profound effect on the therapeutic efficacy of drug molecules. Clearly, it will depend on the specific drug target whether long residence time ligands have a clinical advantage over short residence time ligands. An optimized drug discovery strategy should aim for the development of both long and short residence antihistamines in lead optimization to have a diverse set of ligands as starting point to obtain translational efficacy in vivo.

3 Defining ligand binding kinetics

In figure 2A, a ligand binding reaction is schematically depicted. As can been seen, binding is assumed to be a one-step reaction with a single ligand (L) binding a receptor (R) and forming a ligand-receptor complex (RL). Derived pharmacological models interpret this reaction according to law of mass action,

\[ [R] \cdot [L] \cdot k_{on} = [RL] \cdot k_{off} \]

At equilibrium:

\[ [R] \cdot [L] \cdot \frac{k_{on}}{k_{off}} = [RL] \cdot k_{off} \]

Dissociation half life \( t_{1/2} = \frac{\ln 2}{k_{off}} \)

Residence time \( \frac{1}{k_{off}} \)

Possible to incorporate in model:

\[ Y_{max} = \frac{k_{on} \cdot [L]}{k_{off} + [L]} \]

\[ Y = Y_{max} \cdot (1 - e^{-kt}) \]

\[ k_{obs} = k_{on} \cdot [L] + k_{off} \]

\[ k_{on} \cdot e^{kt} \]

Figure 2 - Ligand binding to the receptor over time. (A) Ligand binding to the receptor is a reversible reaction. At equilibrium, the forward rate and reverse rate are equally fast. According to the law of mass action this would mean that the rate of the complex association (product of receptor concentration [R], ligand concentration [L], and association rate constant \( k_{on} \)): [R][L][k_{on}]=[RL][k_{off}]. The ratio of \( k_{off} \) over \( k_{on} \) is equal to the equilibrium dissociation constant \( K_d \), which reflects binding affinity of the ligand for the receptor. Introducing a stable ligand concentration to unbound receptors causes formation of the receptor-ligand complex in time until binding equilibrium is reached (A, blue ‘association’ panel). As can be seen in the blue ‘association’ panel of B, the time to reach equilibrium is dictated by \( k_{obs} \), which can be described as function of \( k_{on}, k_{off} \) and [L]. Proportional receptor occupancy \( (P_{rel}) \) at equilibrium is dependent on ligand concentration and \( K_d \) (A). When unbound ligand is then continuously removed, the rate of formation of the ligand-receptor complex is abolished and only the reverse reaction (ligand dissociation) will be possible (A, green ‘dissociation’ panel). The time needed for ligands to dissociate from the receptor is only dependent on the \( k_{off} \) (B, green ‘dissociation’ panel). Alternatively, the \( k_{off} \) can be expressed as the residence time or dissociation half-life (A). The latter corresponds to the time that is necessary for half of the ligands to dissociate as depicted in the green panel of A.
which states that the reaction rate is proportional to the products of the concentration reactants (i.e. ligand, receptor and ligand-receptor complex) multiplied by a kinetic binding rate constant. This means that the forward rate and reverse rate of the binding reaction can be described as [L]-[R]-k_{on} (binding rate) and [RL]-k_{off} (dissociation rate). Upon addition of a ligand concentration [L] to a receptor population [R], the rate of binding will cause [RL] to increase until the rate of dissociation is the same as the forward rate of the binding reaction. At this state of equilibrium, the ratio between bound and free ligand can be predicted by the ratio of k_{off} over k_{on}, which is a constant called the dissociation constant (K_d). Assuming that the ligand concentrations do not change during the binding reaction (i.e. the bound fraction is negligible compared to the total amount of available ligands), the association of the ligand can be described by the blue panel (figure 2B) and is dependent on both the k_{on} and the k_{off} constants (figure 2A). Moreover, ligand dissociation can be described by the green panel (figure 2B) and is dependent on only the k_{off} value (figure 2A). An often-used binding metric to describe the time a ligand is bound on the target receptor is the residence time, which is defined as the reciprocal of the dissociation rate (1/k_{off}) and is therefore analogous to the dissociation half-life (t_{1/2}=ln2/k_{off}) (figure 2A).

From figure 2 it becomes clear that both the binding affinity (K_d) and the kinetic binding rate constants (k_{on} and k_{off}) are derived from the same model and are directly related to each other. The K_d can be used to determine the occupancy (P_R) of ligand binding upon completion of the binding reaction. However, in situations where ligand concentrations are dynamic, as is typical in vivo, the K_d might not accurately reflect the receptor occupancy. Especially in these cases, the binding rate constants can give a better prediction of efficacy.

4 Ligand binding kinetics at the H1R

In life, timing is everything. Correct spatio-temporal regulation of signaling molecules will determine every physiological response. In allergy for example, mast cells are triggered by allergens to release histamine.25,26 Histamine subsequently induces a variety of different responses like, the recruitment of immune cells, cytokine production and vascular permeability.27–30 Yet, high concentrations of histamine will only be present for a limited amount of time before returning to basal levels.31 This means that if cells are not timely activated by histamine, they have to wait for the next histamine surge. Here lies a possibility to optimize antihistamines based on their dissociation rate, which predicts how fast blocked histamine receptors become available again to respond to histamine. In contrast, optimization on the basis of binding affinity would focus on the predicted level of target binding under the assumption that there is ample time for histamine and antihistamines to have fully associated to, and fully dissociated from the receptor. Yet, when antihistamines dissociate slowly from the receptor, the inhibition of histamine would be underestimated by their equilibrium binding affinity. Since steady-state receptor occupancy is not reached due to the fast changes in ligand concentrations, occupancy might be better estimated by the receptor binding kinetics of the antihistamines.

Histamine is found throughout the body and is involved in the paracrine and autocrine regulation of numerous physiological processes based on the receptor subtype that is activated. Four human GPCRs are responsive to histamine, named the histamine 1-4 receptor (H1R-H4R). The H1R is successfully targeted for the treatment of allergic rhinitis, allergic conjunctivitis, urticaria and motion sickness.32 using, e.g.,
levocetirizine (Xyzal™) and diphenhydramine (Benadryl™). The H₂R has been targeted to treat increased gastric acid secretion 33, by e.g. ranitidine (Zantrac™). The H₂R and H₄R have been implicated as promising targets for the treatment of narcolepsy, Alzheimers disease, depression, obesity (H₃R) 34, pruritus, asthma, allergic rhinitis and dermatitis (H₄R) 35. Recently, pitolisant (Wakix™), a H₄R inverse agonist, was approved as an orphan drug for narcolepsy 36. Since H₁R antihistamines in the treatment of allergic rhinitis and urticaria are suggested to benefit from a long residence time kinetic profile, the H₁R was selected as one of the key targets for further investigation within the K4DD-consortium.

4.1 Molecular understanding of ligand binding to the H₁R

To guide the optimization of drug-receptor binding, it is important to understand the factors that influence these binding properties. Equilibrium binding affinity is routinely measured for ligands binding to the H₁R and is also often used as a measure in mutagenesis studies to determine whether a specific residue is contributing to ligand binding. The doxepin-bound H₁R crystal structure 37 shows that the observed interactions between drug and receptor are indeed found to contribute to the binding affinity of H₁R antihistamines as described in more detail below. This fits with the idea that a ligand makes local stabilizing interactions inside the receptor, leading to a binding mode that is thermodynamically favorable compared to the unbound receptor and ligand. Binding affinity therefore seems an appropriate metric to score the contribution of individual ligand-receptor interactions to the overall binding stability. This is moreover reflected by the success of drug-binding affinity optimization using structural information obtained from, e.g., x-ray crystal structures. One example is the identification and optimization of a new high affinity H₁R scaffold using a virtual screening approach based on the H₁R crystal structure 37–39. The ligand residence times have not been measured routinely in mutagenesis studies or in studies that explore the structure activity relationships of ligand binding. The next paragraphs therefore focus mainly on the evidence based on ligand binding affinity. Moreover, also the molecular aspects currently known to affect the residence time of H₁R antihistamines are discussed below.

4.1.1 Histamine binding

Considering that the four histamine receptors all have to bind histamine, it is likely that the relevant binding interactions are evolutionary conserved. The four histamine receptors are all class-A GPCRs but display distinguished coupling preferences for the Gα subclasses and consequently regulate distinct intracellular signaling pathways 36. The tertiary structure of the seven transmembrane (TM) regions are highly conserved between class A GPCRs and to indicate the specific position of a residue within these TM-domains, the Ballesteros-Weinstein numbering scheme can be used 40. Residues of the four histamine receptors are therefore also compared by using the Ballesteros-Weinstein numbering, which is compiled for all class A GPCRs in the publicly available GPCR-database GPCRdB 41.
Figure 3 - Histamine binding to the four histamine receptors. Conserved (known) interaction positions are denoted by red symbols. For each individual residue, the specific receptor for which residue was implicated to be important for histamine binding is denoted in red. Residues are represented in Ballesteros-Weinstein numbering. 

Figure 4 - H₃R ligand structures. Affinities for the H₃R were taken from 37,42–44.
Although histamine binds and subsequently activates four different histamine receptors it does not do so with equal affinity. Histamine displays highest affinities for hH3R and hH4R, i.e. pKi 8.0 and 7.8 respectively, whereas affinities for hH1R and hH2R are in the micromolar range, i.e. pKi 4.2 and 4.3 respectively 45,46. Like all aminergic GPCRs, the four histamine receptors conserve D3.32 in TM3, which is a key ionic anchor point for ligands that bind these receptors. Substitution of D3.32 with Ala or Asn impaired binding of histamine to all four histamine receptors (figure 3) 47–51. In addition, binding of ligands that contain a basic amine moiety was found to depend on the D3.32 as well, since upon Ala-substitution of this residue, the binding affinity of these ligands were negatively affected 42,48–52. Moreover, the interaction of the basic amine of a ligand binding the H1R with the conserved D3.32 was confirmed in the doxepin-bound crystal structure of the H3R 53.

TM5 is the least conserved among the histamine receptor subtypes, and might therefore shed some light on the higher affinity of histamine for H3R and H4R as compared to H1R and H2R (figure 3) 54. Histamine interacts with residue 5.46 in TM5, which differs between histamine receptor subtypes. The N5.46 and T5.46 in H2R and H4R, respectively, can make less strong ionic/hydrogen bond interaction with the N' of the imidazole ring compared to the negatively charged E5.46 in H3R and H4R, which explains the observed difference in affinity 54. Nonetheless, Ala-substituting residue 5.46 decreased histamine binding affinity for all histamine receptor subtypes 47–50,55,56. Interestingly, Ala-substitution of N5.46 did not impaired affinity of histaprodifen-based agonists for the H1R (figure 4), but decreased their potency, suggesting that this interaction contributes to receptor activation 51.

Mutational analysis of the H1R binding pocket showed that histamine also interacts with W3.28 57, K5.39 15,43, and F6.55 42,51 (see figure 3). Ala-substitution of Y7.43 reduced binding affinity of histamine, which might be the consequence of the loss of a stabilizing interaction between Y7.43 and D3.32 upon histamine binding 42. However, this was not confirmed in another study 58.

Similarly, Ala-substitution of S3.36 decreased histamine affinity for the mutant H1R 59. Further mutational analysis revealed that S3.36 undergoes a rotamer switch upon histamine binding, resulting in receptor activation via the interaction with N7.45 and a consecutive H-bond network involving the N7.49 (NPxxY) and D2.50 59.

For the H3R and H4R, in silico studies suggested that histamine binds similarly in both receptors where hydrophobic residues Y3.33, F5.47 and Y6.51 mediated histamine binding via non-bonding interactions 54. Histamine was finally shown to have reduced efficacy for generating cAMP (but similar potency) upon mutating D5.42 of the H2R into an Ala or Asn, suggesting a possible role for this residue in receptor activation 49.

In order for the body to rapidly attenuate histamine signaling it is probably not beneficial to have prolonged histamine occupancy on the receptor after extracellular histamine levels have already been decreased. This is reflected by the relatively short residence time of histamine for the H1R (0.25 min 60) and the H2R (11 min 61). Likewise, in allergy histamine levels are considered to be elevated in the order of minutes as well. The short residence time of histamine might be in part mediated by the small molecular size of histamine, since this seems to be related to residence time of ligands for their receptor 62.
Table 1 - Residence times of H1R antihistamines. Residence times were calculated from the reported dissociation half-lives ($t_{1/2}/\ln2$) and otherwise from the reported dissociation rate constants ($1/k_{off}$). Measurements were performed on the human H1R or rabbit arteries expressing the H1R (*). Measured on rabbit arteries.

** In another publication, desloratadine had negligible dissociation after 6 h indicating that the residence time might be much longer. **

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<th>Name</th>
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*Measured on rabbit arteries.
** In another publication, desloratadine had negligible dissociation after 6 h indicating that the residence time might be much longer.

Table 2 - Structure kinetic relationship of H1R antihistamines. Dissociation half-lives and binding affinities were determined using radioligand binding studies. Residence times were calculated by dividing the dissociation half-life by the natural logarithm of two.

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4.1.2 Receptor binding by \( \text{H}_1 \text{R} \) antihistamines

Antihistamines for the \( \text{H}_1 \text{R} \) are widely prescribed over the last 8 decades \cite{32} and this success ensured the development of many different antihistamines. Most \( \text{H}_1 \text{R} \) antihistamines are characterized by two aromatic rings that are coupled to a basic amine via a short linear or cyclic spacer (figure 4 and 5). As for histamine, the basic amine of antihistamines interacts with D3.32, whereas their aromatic moiety interacts with other aromatic residues in TM6. Indeed, the ring structures of doxepin interact with hydrophobic residues of TM6 (F6.44, W6.48, Y6.51, F6.52 and F6.55) but also of TM3 (Y3.33 and I3.40) and TM4 (W4.56) in the crystal structure of the doxepin-bound \( \text{H}_1 \text{R} \) \cite{37}. Site-directed mutagenesis revealed the importance of similar interacting residues for both \([^{3}\text{H}]\text{mepyramine}\) and \([^{3}\text{H}]\text{(2S, 4R)PAT}\) with the \( \text{H}_1 \text{R} \) (figure 5) \cite{42,43}. In addition, F5.47 was found to be crucial for \([^{3}\text{H}]\text{mepyramine}\) binding to \( \text{H}_1 \text{R} \).

Doxepin and mepyramine are typical first generation \( \text{H}_1 \text{R} \) antagonists for the treatment of allergic rhinitis, allergic conjunctivitis and urticaria, but causes sedative side effects by their ability to cross the blood brain barrier \cite{66}. Because of their effect on the sleep-wake cycle, first generation antihistamines like diphenhydramine and doxylamine have been used in the treatment of insomnia \cite{67}. Moreover, doxepin has recently been accepted for the treatment of insomnia and even further research of new \( \text{H}_1 \text{R} \) antihistamines with less side effects, or a polypharmacological profile (e.g. 5-HT\(_2\)A receptor) for the treatment of insomnia is ongoing \cite{67,68}. On the other hand, second generation antihistamines targeting the \( \text{H}_1 \text{R} \) for the treatment of, e.g., allergic rhinitis have been developed with reduced brain penetration \cite{44}. One of the strategies, was to develop zwitterionic antihistamines harboring a carboxylic acid moiety, like levocetirizine and fexofenadine, which have reduced penetration of the blood brain barrier \cite{69}. Additionally, several other second generation antihistamines (e.g. desloratadine) are actively transported out of the brain by targeting transporters like P-glycoprotein or ABCB1 \cite{70,71}.

![Figure 5 - Binding interaction of mepyramine with the \( \text{H}_1 \text{R} \). Prototypical interactions of antihistamines within the \( \text{H}_1 \text{R} \) binding site are extrapolated from the doxepin-bound crystal structure and mutagenesis studies \cite{37,42}. Colored shades depict the two conserved structural motifs that are common between ligands binding the \( \text{H}_1 \text{R} \), with the basic amine functional group in blue and the aromatic functional group in red, which often consists of multiple aromatic rings.](image)
Successful therapeutics in the treatment of allergic rhinitis, allergic conjunctivitis and urticaria like desloratadine and levocetirizine have significantly longer residence times on the H1R as compared to some of the first generation antihistamines like mepyramine and diphenhydramine (table 1) \(^{63-65}\). Levocetirizine for example has a residence time of 200 min compared to a residence time of 1.2 min of mepyramine, despite their similar binding affinities of 8.5 (pK\(_i\)) and 8.4 (pK\(_d\)) respectively (table 1) \(^{15}\). The carboxyl group of levocetirizine is important for long residence time on the H1R, as revealed by 5- and 20-fold decreased residence times of analogues with alcohol or methyl ester substituents, respectively (table 2) \(^{15}\). The long residence time of levocetirizine is suggested to be mediated by the interaction of its carboxyl group with K5.39 of the H1R, since levocetirizine has a 10-fold decreased residence time on the H1R-K5.39A mutant (table 2). Moreover, the faster dissociation rates of structural analogues without this carboxyl group were unaffected by this mutation.

A popular explanation for the disconnect between ligand binding affinity and residence time at the drug-target is the transition state model \(^{62,72}\). H1R antihistamines can only reach the most stable binding position by diffusing from bulk solvent into the buried hydrophobic pocket of the H1R. Along this route, the ligand does not necessarily have stabilizing interactions, which together with steric exclusions could make the binding process energetically unfavorable. Ligand binding would then only occur when the ligand has enough energy to bind the receptor. The Eyring equation \(^{73}\) describes the relationship between the transition state energy and the kinetic rate constants, and shows that the difference in free energy between the unbound ligand and receptor (1 in figure 6) compared to the transition state (T in figure 6),

![Figure 6 - A transition state for ligand-receptor binding reactions.](image)

The bound and unbound states are separated by a transition state (T) representing a high free energy coordinate along the reaction path. The difference in free energy between (T) and the unbound state (1), i.e. \(\Delta G^{\text{on}}\), determines the association rate constant \((k_{on})\) and the difference in free energy between (T) and the bound state (2), i.e. \(\Delta G^{\text{off}}\), determines the dissociation rate constant \((k_{off})\). Moreover the energy of binding is determined by the free energy difference between (1) and (2), i.e. \(\Delta G_{\text{on}}\), which determines the ligand binding affinity \((K_d)\) \(^{62}\).
i.e. transition state energy, is related to the \( k_{on} \) constant. Likewise, the difference in free energy between the ligand-receptor complex (2 in figure 6) compared to the transition state (T in figure 6) is related to the \( k_{off} \) value. Finally, the difference in free energy between the bound and unbound receptor state, i.e. binding energy, is related to the binding affinity. Interestingly, this implies that the \( k_{off} \) value is dependent on the sum of the transition state energy and the binding energy, which might explain the disconnect between the binding affinity and residence time.

4.2 Drug-target binding kinetics of H\(_1\)R antihistamines and the in vivo relevance

Local histamine levels are described to vary rapidly over time. Neuronal histamine release (in vitro) and breakdown in rat brain seem to occur in the order of minutes \(^{74-76}\). Moreover, histamine release in the rat stomach can be elevated for hours in response to gastrin, but return to basal levels within minutes when gastrin signaling is inhibited \(^{77}\). After intravenous or intraperitoneal injection histamine levels also return to basal levels within minutes in rats and mice respectively \(^{78,79}\). Likewise, allergen-induced histamine levels in the human skin are elevated for several minutes before returning back to basal levels \(^{31}\). These examples clearly show that degradative enzymes, either histamine-N-methyl transferase or diamine oxidase \(^{80}\), rapidly clear histamine levels and that elevated levels of histamine are usually only transiently present. Consequently, in most instances there will not be a stable equilibrium. If antagonists with a long residence time bind before a histamine surge, the antagonist can occupy the receptor longer then histamine remains available in the tissue due to its rapid clearance. As a result, even high concentrations of histamine would not be able to induce a signal, for the simple reason that no receptor is available for

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Figure 7 - Insurmountable antagonism based on the dissociation rate of antihistamines. (A) Upon allergen exposure, histamine (green) levels are increased for approximately 20 min (7). This histamine surge (green arrow) can only activate the receptor within this timeframe. If pre-bound antihistamine (in red) at \( t = 0 \), does not dissociate from the receptor within this 20 min then the receptor cannot be activated. If antihistamines dissociate readily within this 20 min then histamine can bind the receptor based on the concentration and affinity. Since histamine competes with the antihistamine for the same receptor, higher histamine concentrations are necessary to obtain the same effect. This is reflected by a right shift of the histamine concentration response curve (B, green). If there is hardly antihistamine dissociation in 20 min then small number of receptors becomes available to respond to histamine (B, blue).
binding (i.e. insurmountable antagonism). However, when histamine release is sustained due to a chronic release of histamine, a stable equilibrium situation could eventually be achieved. In this case, long binding antihistamines, when pre-bound to the receptor, would still postpone the onset of histamine binding, but eventually could be displaced if histamine levels are both high and sustained long enough. Indeed, it has been shown that some antihistamines for the H₁R can outlast the availability of high concentrations histamine with e.g. levocetirizine having sustained receptor occupancy of more than

Figure 8 - Prolonged receptor occupancy based on the ligand dissociation rate. In vivo, drug concentrations can decrease rapidly due to metabolism and excretion, this clearance of the drug (A, blue arrow) can be much faster than the rate in which drugs dissociate from the target receptor. If dissociation from the receptor is rapid (A, green), receptor occupancy (B, green line) decreases in line with drug concentration (B, blue line). Hence, receptor occupancy is a function of ligand concentration and affinity (Kᵋ). In contrast, slow dissociating drugs (A, red) will occupy the receptor (B, red line) beyond the decrease in drug concentration.

Figure 9 - hysteresis of biological effect after oral drug administration. After oral administration of a drug, the in vivo free drug concentration changes over time and the biological effect changes with it. The blue arrows represent the time course between data points. The free drug concentration initially increases and then decreases. However, although the drug concentration seems to return back basal levels, a prolonged biological effect remains. Such a disconnect between biological effect and free plasma concentrations of a drug has been described for various antihistamines.
half the receptors for one to three hours and desloratadine for more than 6 hours. Moreover, these long binding antihistamines are indeed shown to display insurmountable antagonism of the histamine induced intracellular calcium release and contraction of isolated guinea pig ileum and trachea. This indicates that in controlled settings effective H\text{1}R inhibition can be predicted based on a slow dissociation rate ($k_{\text{off}}$ value) and not a high affinity (figure 7).

One important argument to optimize the residence time of drugs is that when the dissociation rate is much slower than the rate of clearance from the circulation, biological activity of the drugs (determined by the drug-occupancy of the receptor) will outlast the presence of free ligands (determined by the concentration available drugs) as depicted in figure 8. Such effects have also been described for H\text{1}R antihistamines like levocetirizine and fexofenadine, which in the literature is referred to as hysteresis, i.e., a disconnect between the available drug concentration and biological activity as is depicted in figure 9.

5 Aim of this thesis

There is a general believe that the drug-receptor binding rate constants provide more detailed information about the mechanism of ligand binding than could be obtained from binding metrics that are distilled from simple end-point measurements. Since this information can be obtained already in an early stage of drug-discovery, an early focus on binding rate constants could fundamentally change the characteristics of lead-compounds that are pursued in clinical trials. However, before it is possible to efficiently use drug-receptor binding rate constants as a way to steer drug-optimization, a few essential hurdles have to be overcome.

First of all, to obtain kinetic information per definition means that the assay throughput decreases compared to end-point measures, because multiple measurements over time will always be required. The radioligand-binding experiments that are conventionally used to measure the drug-target binding kinetics have only limited throughput and it is therefore crucial to develop other methods that can determine the drug-receptor binding rate constants in a robust way with medium to high throughput. Additionally, the relationship between the kinetics of drug-target binding and the biological consequences are still not well understood. Binding studies at the H\text{1}R are often done on homogenates of cells expressing the receptor. Such model systems work well to characterize the equilibrium and kinetic constants of drug-target binding, but do not contain a lot of information regarding the biological consequences of target engagement by drugs with differential binding kinetics. It would be beneficial to incorporate some of the biological parameters that could be influenced by the drug-target binding kinetics already in an early stage in vitro.

If lead compounds with the desired kinetic binding profile for the drug-target are not readily available, compounds will have to be customized. However, the molecular determinants of drug-target binding kinetics are not thoroughly investigated and molecular design strategies to tweak the kinetic binding profile are therefore lacking. When the relation between the molecular features and the kinetic target-binding profile of a drug becomes clear, this would provide a handle for the lead-optimization in drug development.

Aim 1: To develop methods that allow reliable quantification of the drug-target binding kinetics with improved throughput compared to conventionally used methods. (Chapter 2 – 5)
Aim 2: To develop methods that determine the duration of drug action in biological systems in vitro. (Chapter 3 and 5)

Aim 3: To understand how the molecular features of H₁R antihistamines affects their observed binding kinetics to the receptor. (Chapter 6 – 8)
Chapter 2 - Ligand-binding kinetics on histamine receptors

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1 Introduction

Many different methodologies have been described to measure the binding kinetics of a ligand to its receptor. Selecting the appropriate method depends on many different variables and will often differ depending on the specific research question. In this chapter different methods and variables are therefore discussed that are (potentially) important to study the binding kinetics of antihistamines at the histamine receptors. The experimental design is described in detail for the different methods and some overarching concepts in the design of kinetic experiments are highlighted. Finally, a comparison between the different methodologies is made.

2 Quantifying ligand-receptor residence times

2.1 Binding conditions

Ligand-receptor binding rate constants are context dependent and can vary dependent on conditions like the temperature, pH and ionic strength of the buffer. Differences in both temperature and pH have e.g. considerable effects on the kinetic binding rate constants of H1R antihistamines \(^{64}\). Decreased temperature generally decreases association and dissociation rate, but the absolute effect can differ between ligand-receptor pair. Occasionally low temperatures are used to slow down binding, when kinetic binding rates are too fast to experimentally determine \(^{89}\). Mixed effects of the pH on the kinetic binding rate constants are known being receptor and ligand specific \(^{64}\). Moreover, the ionic strength can affect the association rate constant of charged ligands \(^{90-92}\). Interestingly, many binding studies, including studies that measure the kinetic binding rate constants, use low ionic strength buffers based on Tris or HEPES which have much lower ionic strengths than observed \textit{in vivo} (50-250 mM \(^{93}\)). Since ligands targeting aminergic GPCRs typically require a positive charge to make an ionic interaction with the D3.32 in the transmembrane pocket, they might be susceptible for ionic strength effects.

2.2 Radioligand binding studies

Radioligand binding experiments are most frequently used to measure ligand-binding kinetics to GPCRs. To this end, radioligands are incubated with cells or cell homogenate expressing the desired receptor. Next, free radioligand is washed away and bound radioligand is quantified. By isolating the bound fraction of radioligands after different incubation times, the binding of the radioligand over time can be obtained. Radioligand binding can also be used in competition with an unlabeled ligand to indirectly determine the binding rate constants of the latter. An important benefit of radioligand binding is that receptors can be used in their native membrane bound state. A downside of conventional radioligand binding methods is a low throughput, since receptor bound radioligand has to be separated from free radioligand. Limitations of this \textit{stop-flow} based determination of binding are discussed and alternatives are suggested to tackle throughput issues.

2.2.1 Cell homogenate expressing the receptor

Originally, radioligand binding assays were performed on homogenized native tissues expressing the relevant receptor \(^{94,95}\). Nowadays, it is more common to use (homogenized) immortalized cell lines
expressing the receptor of interest either endogenously (often lower expression) or upon stable/transient transfection (often higher expression). Highly expressing transfected cells are often first choice for kinetic binding studies, because it is crucial to have a good signal-to-noise ratio, since differences in curvature that shape the fitted $k_{on}$ and $k_{off}$ rates are often quite subtle. However, it has to be realized that receptor overexpression might increase the number of receptor dimers that might display positive or negative binding cooperativity between their ligand binding sites \(^{96,97}\). Moreover, receptor densities could possibly also affect rebinding when ligands diffusion rates are limited \(^{98}\). Preferably, experiments are designed in such way that rebinding does not affect the measured kinetic binding rate constants (dissociation experiments section 2.2.3, 2.2.5).

**Method (production of cell homogenate from transiently transfected cells)**

- Human embryonic kidney (HEK)293T cells are cultured in culture medium (*i.e.* Dulbecco’s modified eagle medium (DMEM) containing 10 % fetal bovine serum and 1 % penicillin/streptomycin) in a humidified incubator at 37 °C and 5 % CO₂.
- One day before transfection 2×10⁶ HEK293T cells are seeded in 7 mL culture medium in a 10 cm dish.
- The next day, 5 μg DNA encoding the target receptor in 250 μL sterile NaCl solution (150 mM) is mixed with 30 μg 25-kDa linear polyethylenimine in 250 μL sterile NaCl solution (150 mM). The mixture is vortexed and incubated for 15 min. Culture medium is refreshed with 7 mL new medium. After gentle resuspension (*i.e.* no vortexing), the transfection mixture is dropwise added to the cells.
- 48 hours later, cells are washed with PBS, collected and stored as dry pellets at ≤ -20 °C before further experimentation.
- To prepare cell homogenate, the pellets are thawed on ice and resuspended in ice-cold binding buffer (*e.g.* 50 mM Na₂HPO₄/KH₂PO₄ pH 7.4). Cells are subsequently homogenized into fragments by an ultrasonic cell disruptor.
- All models that describe a relation of the ligand concentration in the buffer, assume that this concentration remains constant over time. In reality, however, binding of ligand to the receptor will decrease ligand concentration in solution. This **ligand depletion** is often assumed to be neglectable when bound ligand is less than 10 % of the added radioligand concentration \(^{99}\). For radioligands, this can be easily determined by quantifying concentration added ligand compared to the receptor-bound ligand. As a general rule for experiments with increasing concentrations radioligand the total receptor concentration should be less than 0.1 times the $K_d$ value \(^{100}\).

### 2.2.2 Terminating binding reactions and quantifying activity

Bound radioligand is often quantified using a radioactivity scintillation detector. If free radioligand is not separated from bound radioligand it is normally impossible to quantify the latter. Since kinetic experiments require rapid termination and washing it is advisable to use filtration with ice-cold wash buffer to minimize undesired dissociation. Alternatively, scintillation proximity assays detect bound radioligand more efficiently then free radioligand, and consequently does not require a separation step (section 2.3.1).
Method (terminating binding reactions)

- Half an hour before the binding reaction is terminated, 96-well GF/C filter plates are pre-soaked in 0.5 % 750 kDa branched polyethylenimine.
- At the desired time point (see specific assay format below) terminate the binding reactions over the 96-well filter plate using vacuum filtration, trapping the cell homogenate on the filter.
- Residual free radioligand is removed by three rapid wash steps using ice-cold wash buffer (e.g. Tris-HCl pH 7.4).
- Filter plates are then dried in a stove at 52 °C for >45 min.
- Next, scintillation liquid is added 25 µL per well, and bound radioligand is quantified using a radioactivity scintillation detector after a delay of >200 min to reduce variability in data.

2.2.3 Radioligand binding kinetics

Kinetic binding rate constants of radioligands can be directly quantified by measuring receptor occupancy in time. Ligand binding to a single site on the receptor with a one-step binding mechanism (see chapter 1: figure 2) can be described as a function of time by the following mathematical model:

\[ Y = Y_{max} \cdot \left(1 - e^{-k_{obs}t}\right) \]
Here, $Y$ is the receptor bound radioligand, $Y_{\text{max}}$ is the radioligand binding at equilibrium, and $k_{\text{obs}}$ is the rate in which equilibrium is approached (time$^{-1}$). The $k_{\text{obs}}$ is dependent on the $k_{\text{on}}$, $k_{\text{off}}$ and ligand concentration $[L]$. This relationship is defined by the following equation:

$$k_{\text{obs}} = k_{\text{on}} \cdot [L] + k_{\text{off}} \quad (2)$$

To measure radioligand association, cell homogenate expressing the receptor is incubated with radioligand for various time periods. The time required to reach 50% equilibrium binding equals $\ln 2 / k_{\text{obs}}$ (figure 1A). Since $k_{\text{obs}}$ encompasses both $k_{\text{on}}$ and $k_{\text{off}}$, one cannot extract these binding rate constants from a single association curve. One way to determine $k_{\text{on}}$ and $k_{\text{off}}$ is to use multiple radioligand concentrations, leading to multiple $k_{\text{obs}}$ values (figure 1A). The $k_{\text{obs}}$ increases linearly as function of radioligand concentration with $k_{\text{on}}$ and $k_{\text{off}}$ being the slope and intersection with Y-axis, respectively (figure 1B). Alternatively, kinetic binding rate constants can be determined from an association binding with one concentration radioligand in combination with a dissociation experiment (figure 1C-D). In the association curve the $k_{\text{obs}}$ is obtained and from the dissociation curve the $k_{\text{off}}$ is obtained (see below) so that $k_{\text{on}}$ can be calculated using equation 2.

The radioligand dissociation from the receptor depends only on $k_{\text{off}}$ (see chapter 1: figure 2). Usually cell homogenate is first incubated with radioligand for a certain amount of time before dissociation is initiated. To this end, either free radioligand is removed or excess unlabeled ligand is added so that radioligand rebinding is impossible. In the first method, the radioligand is often diluted to an amount that equilibrium switches entirely to the unbound state of the receptor (‘infinite dilution’). Diluting the ligand concentration below 1% of its respective $K_d$ value, it should be ensured that almost all ligands ($\geq 99\%$) should have dissociated at equilibrium. The second method is probably best equipped to deal with rebinding, since wash steps and infinite dilution strategies might allow rebinding in the case that micro compartments near the receptor (like membrane structures) retain high concentrations of ligand\(^{10}\). Radioligand dissociation from the receptor can be described by an exponential decrease of ligand-bound receptor:

$$Y = Y_0 \cdot e^{-k_{\text{off}}t} \quad (3)$$

Here $Y_0$ is the bound radioligand at $t=0$. The model assumes that radioligand fully dissociates and binding ($Y$) should therefore be corrected for nonspecific binding. The $k_{\text{off}}$ can then be determined by dividing the natural logarithm of two by the time needed for half the receptor-bound radioligand to dissociate (see figure 1C).

However, the assumption that the ligand has a simple one-step binding mechanism to a single site of the receptor is not always valid. For example, the H$_2$R agonists $[^3H]$N-$\alpha$-methylhistamine (NAMH) and $[^3H]$R-$\alpha$-methylhistamine (RAMH) showed biphasic dissociation and biphasic association (for NAMH) curves indicating that their binding mechanism might be more complex than the aforementioned one-step binding mechanism to a single site (table 1)\(^{101,102}\). This could be visualized by plotting the natural logarithm of specific binding over time, from either a radioligand association or dissociation experiment. Normally this would lead to a straight line unless there is biphasic binding. Indeed agonists might display more complicated binding mechanism involving receptor isomerization to distinct conformational states and
cycling to G proteins. If ligand binding is dependent on isomerization of the receptor (e.g. induced fit mechanism and conformational selection) it is expected that a nonlinear relation exists between $k_{\text{obs}}$ and the ligand concentration $103,104$. In these cases, where the binding does not comply with the presumed one-step binding mechanism to a single site of the receptor, it might be best to estimate a global $k_{\text{off}}$ in a dissociation experiment, since this is the parameter that is thought to drive the functional efficacy of a drug $\textit{in vivo}$. Moreover, dissociation is less complex for interpretation than the multifactorial association.

**Method (association binding)**

- Prepare the desired radioligand concentration(s) in binding buffer and dispense 25 µL per well. When using one concentration, prepare triplicate rows on a 96-well plate (4-$K_d$ final concentration in the assay*), when using multiple concentrations radioligand use duplicate rows with 4 different concentrations (0.25-$K_d$, 0.66-$K_d$, 1.5-$K_d$ and 4-$K_d$ final concentrations in the assay*).
- Add 25 µL competitive unlabeled ligand (~4000·K_i final concentration in the assay*) to the first column to quantify nonspecific radioligand binding, and 25 µL binding buffer to all remaining wells.

- After pre-heating all components to 25 °C, start binding reactions at different time points by adding 50 µL receptor-expressing cell homogenate to the wells (section 2.2.1), and incubate under temperature-controlled conditions (25 °C) and gentle agitation (figure 2).
  - Use a different column for each time point with in total 11 time points (columns 2-12). Required incubation time is dependent on the respective radioligand (section 4.1). For [³H]mepyramine the following time points in minutes could be used: 1, 2, 3, 4, 6, 9, 12, 16, 20, 25, 30. Incubations are started in the inverse order with all incubation times coming to an end simultaneously.
  - Cell homogenate can be added at any time to the first column if nonspecific binding does not increase over time**.

Figure 2 - association binding logistics.

Figure 3 - dissociation binding logistics.
• After completion of the desired incubation times, all incubations are simultaneously terminated and bound ligand is quantified (section 2.2.2).

• Determined binding levels are baseline corrected for non-specific binding determined in the first column determined for each concentration. Binding over time is analyzed by nonlinear regression using equation of chapter 1: figure 2B (blue panel) to obtain $k_{on}$ and $k_{off}$ (e.g. using prism 6.0). Since the linear relationship between $k_{obs}$ and $[L]$ is incorporated in the model, it is no longer required to extract the $k_{on}$ and $k_{off}$ from this straight line as in figure 1B. This can be done with the model that describes binding as function of the bound ligand at equilibrium ($Y_{max}$), or a model in which equilibrium bound radioligand is furthermore expressed as the $B_{max}$, ligand concentration and the ratio between $k_{off}$ and $k_{on}$ (which reflects the $K_d$). The latter is recommended. If this experiment is performed in combination with a dissociation experiment, analysis is the same but the $k_{off}$ should be constrained to the pre-determined value of the radioligand.

* Concentration of ligands should be four times the final concentration to correct for dilution.

** Verify that nonspecific binding does not increase over time. If it does increase over time, total binding should be corrected by the non-specific binding determined for each individual time-point.

Method (dissociation binding)

• Prepare 50 µL of the desired radioligand concentration (e.g. 4·$K_d$ final concentration in assay*) on triplicate rows of a 96-well plate.

• All components were pre-heated all to 25 °C before binding reaction was started. 50 µL receptor-expressing cell homogenate is added to the wells (section 2.2.1) and incubated until equilibrium is obtained for radioligand association, as was established with association binding (>3.5/$k_{obs}$) (figure 3A). Alternatively, all samples are pre-incubated for exactly the same time before dissociation is initiated in the next step (figure 3B).

• Dissociation is initiated at different time points, by adding an excess of competitive unlabeled ligand (~4000·$K_i$ final concentration).
  o Use a different column for each time point.
  o To determine the binding at $Y_0$, the first column should not be supplemented with unlabeled competitor.
  o Added volume should be ≤10 % of total volume to prevent dilution of radioligand. In this case use 11 µL with a concentration which is ten times the final concentration.

• After approximately five times radioligand dissociation half-life, dissociation reactions are simultaneously terminated and bound ligand is quantified (section 2.2.2). Binding over time is analyzed with the model in chapter 1: figure 2B (green panel) using non-linear regression. To accommodate non-specific binding, the model can be adjusted to:

$$Y = (Y_0 - NS) \cdot e^{-k_{off} \cdot t} + NS \quad (4)$$

*Concentration should be two times concentrated to compensate for dilution.
2.2.4 Competitive ligand binding kinetics

Kinetic binding rate constants of unlabeled ligands can be indirectly determined using a competitive radioligand. Motulsky and Mahan have developed a mathematical model to describe radioligand association binding to the receptor in the presence of a competitive unlabeled ligand. This model assumes that both ligands are binding the receptor according to the law of mass action in a one-step binding reaction to the same site (i.e. no more than one ligand is bound at the same time) as depicted in figure 4A. The $k_{on}$ ($k_1$) and $k_{off}$ ($k_2$) of the radioligand should be known, in order to determine the $k_{on}$ ($k_3$) and $k_{off}$ ($k_4$) of the unlabeled ligand.

To initiate the experiment, both radioligand and one or more concentrations unlabeled ligand are simultaneously incubated with receptor-expressing cell homogenate for various incubation times. The use of multiple concentrations of unlabeled ligand results in a more robust estimation of $k_3$ and $k_4$. A frequently used way to validate how well the model predicts the ligand binding kinetics, is to calculate the $K_d$ from the obtained $k_{on}$ and $k_{off}$ values and compare it to pre-determined $K_i$ values derived from competition binding at equilibrium using the Cheng-Prusoff conversion. Another internal control would be to compare the $k_1$ and $k_2$ values of the radioligand to the $k_3$ and $k_4$ of the unlabeled analogue.

Figure 4 - Competitive binding of two ligands to a receptor. Receptor is simultaneously subjected to the labeled ligand L and unlabeled ligand I. Binding of the labeled ligand can be measured and is described by depicted mathematical model. In this model $k_1$ ($k_{on}$) and $k_2$ ($k_{off}$) are the association and dissociation rate constants of the radioligand, respectively, whereas the $k_3$ ($k_{on}$) and $k_4$ ($k_{off}$) are the association and dissociation rate constants of the unlabeled ligand. [L] and [I] are concentrations of labeled and unlabeled ligands, respectively. $Y$ is the amount of receptor bound by the radioligand over time (t). $B_{max}$ is the signal of $Y$ when all receptors are occupied. When using this model, [L], [I], $k_1$ and $k_2$ are constrained to constant values.
**Method**

- Equilibrium dissociation constants of unlabeled ligands ($K_i$) and radioligand ($K_d$), and $k_1$ and $k_2$ should be determined before this experiment.

- Prepare duplicate rows on a 96-well plate with 25 µL radioligand (e.g. 3$\cdot$K$_d$ final concentration* (section 2.2.1) without and with three different concentrations unlabeled ligand (1$\cdot$K$_i$, 3$\cdot$K$_i$ and 10$\cdot$K$_i$ final concentration in assay*).

- Add 25 µL competitive unlabeled ligand (~4000$\cdot$K$_i$ final concentration in the assay*) to the first column to quantify nonspecific radioligand binding, and 25 µL binding buffer to all remaining wells.

- After pre-heating all components to 25 °C, start the binding reaction at different time points by adding 50 µL receptor-expressing cell homogenate to the wells (figure 5).
  - Use a different column for each time point with in total 11 time points (columns 2-12). Required incubation time is dependent on the respective radioligand and unlabeled ligand (section 4.1). For $[^3]$Hmepyramine binding to the H$_2$R in competition with its unlabeled analogue the following time points in minutes could be used: 1, 2, 3, 4, 6, 9, 12, 16, 20, 25, 30. Incubations are started in the inverse order with all incubation times coming to an end simultaneously.
  - Cell homogenate can be added at any time to the first column if nonspecific binding does not increase over time**.

- After the desired incubation times, reactions are simultaneously terminated and bound ligand is quantified (section 2.2.2). Binding as function of time is analyzed with the Motulsky-Mahan model using non-linear regression with $k_1$, $k_2$, [L] and [I] as constraint values to obtain $k_3$ and $k_4$ (figure 4).

*Concentrations should be four times concentrated to compensate for dilution.

**Verify that nonspecific binding does not increase over time. If it does increase over time, total binding should be corrected by the non-specific binding determined for each individual time-point.

**2.2.5 Dissociation of unlabeled ligands**

If a complex binding mechanism for the unlabeled ligand is expected (e.g. motulsky and mahan model discussed in section 2.2.4 does not fit the empirical data), the discussed method to determine the kinetic
binding rate constants is likely unfit. In order to still be able to measure a global dissociation rate, it would be easiest to specifically measure the dissociation of the unlabeled ligand. This can be done indirectly using a radioligand. It is required to know the equilibrium dissociation constants of unlabeled ligand and radioligands, since equipotent concentrations unlabeled ligand (as discussed in section 4.1) are used in this assay. First, receptor is pre-equilibrated with 3-Kᵢ concentration unlabeled ligand to yield 75 % receptor occupancy. Subsequent dilution with high concentration radioligand results in a new equilibrium in which almost all unlabeled ligand is displaced. For example by diluting 10 µL of the pre-incubated unlabeled ligand/cell homogenate solution with 190 µL of a 20-Kᵣ concentration radioligand would lead to a situation in which <1 % of the receptors are still occupied with unlabeled ligand, whereas the radioligand will occupy >94 % of the receptors. Since radioligand binding requires first dissociation of the unlabeled ligand from the receptor, the association of radioligand over time reflects the dissociation rate of the unlabeled ligand ¹¹⁷. It is important that the radioligand binding is sufficiently fast, so that almost all binding sites are instantly occupied between interspacing time points. Furthermore, the radioligand should have a high concentration (relative to its Kᵣ) to ensure that rebinding of the unlabeled ligand is impossible. As a final note, caution should be taken with interpreting the initial ‘dissociation’, since the association of radioligand might be more rapid for early time points considering that not all receptors were occupied by unlabeled ligand during pre-equilibration. This is often inevitable, since radioligand concentrations can often not be used at concentrations that would fully displace saturating concentrations unlabeled ligand.

Method

- Equilibrium dissociation constants of unlabeled ligands (Kᵢ) and radioligand (Kᵣ), and k₁ and k₂ should be determined before this experiment.

- 20 µL suspensions cell homogenate are prepared in tubes with a single tube for each unlabeled ligand and time point. Exactly 60 minutes* before dissociation of the unlabeled ligand is planned (figure 6) 20 µL binding buffer or unlabeled ligand (3-Kᵢ final concentration**) are added to the respective tubes. The tubes are sealed, mixed, and incubated at 25 °C. These mixtures are then divided into triplicates of 10 µL per well on a 96-well plate. With one column for each desired dissociation time.

- Dissociation of unlabeled ligand is started by adding 190 µL volume containing a 20-Kᵣ concentration radioligand. Initiate dissociation separately for each column on the desired time points.

- Next, reactions are simultaneously terminated and bound ligand is quantified (section 2.2.2). The radioligand association rate to full (>94 %) receptor occupancy determines the temporal resolution in which dissociation of the unlabeled ligand can be followed. As a control, the amount of bound radioligand in the absence of unlabeled ligand should therefore approximate binding at equilibrium already at the first time point. Higher radioligand concentrations can be used to increase the kₒₛ and consequently the temporal resolution. To quantify the dissociation of the unlabeled ligand, the data can again be fitted by nonlinear regression to equation 4 (section 2.2.3).
*Since for unlabeled ligands it will be unclear whether equilibrium has been reached within the pre-incubation, having a fixed pre-incubation time will ensure that binding levels unlabeled ligands upon dissociation is always the same.

** Concentrations should be two times concentrated to compensate for dilution.

2.2.6 Dual-point competition assay to determine relative binding kinetics of unlabeled ligands

Association competition binding experiment to determine the binding kinetic rate constants for unlabeled ligands are quite laborious considering all required time points and concentrations. To quickly distinguish whether unlabeled ligands dissociate faster or slower from the receptor as compared to the radioligand, a dual-point competition assay was designed. This method requires only two time points and a single

![Diagram of dissociation of unlabeled ligands logistics.](image)

**Figure 6 - Dissociation of unlabeled ligands logistics.**

![Graph of dual point competition binding.](image)

**Figure 7 - Dual point competition binding.** Radioligand binding curves at 3-Kd concentration as function of time in the absence (blue) or presence of equipotent concentration unlabeled ligands. If both radioligand and unlabeled ligand have similar dissociation rates, binding of the radioligand will be described by the red curve. An unlabeled ligand dissociating five times faster than the radioligand will delay binding of the radioligand (green), whereas unlabeled ligands that dissociate slower than the radioligand results in an overshoot for binding of the radioligand at early time points (purple). Binding of the radioligand will be measured after two incubation times: t₁, after which 90% of radioligand in the absence of competitor has bound the receptor, and t₂, after which all ligands are expected to have reached equilibrium. As can be seen, fast dissociating (green) and slow dissociating (purple) ligands will have a different level of binding at t₁, but not t₂ (since equipotent concentrations are used). By taking the ratio of measured radioligand binding at t₁ and t₂, the KRI of unlabeled ligands can be determined.
concentration unlabeled ligand and works on the observation that unlabeled ligands that dissociate slower than the radioligand cause a typical overshoot pattern in radioligand binding (see figure 7, purple), whereas unlabeled ligands with faster dissociation rates than the radioligand will delay the association of the latter to the receptor (see figure 7, green). Two time points are then taken to calculate whether there is an overshoot pattern, or delayed association for the radioligand. The first time point is taken as the time needed for the radioligand, in the absence of unlabeled ligand, to reach ~90% binding of the binding at equilibrium (i.e. \( t_1 \sim 2.3/k_{\text{obs}} \)). This time point would lead to a good discrimination in binding levels as exemplified in figure 7. The second time point \( (t_2) \) should be taken as the point where equilibrium of all ligands is expected. An equipotent concentration (see also 4.1) for all tested unlabeled ligands is used in competition with a constant concentration radioligand. The kinetic rate index (KRI) is then calculated by dividing the amount of bound radioligand at \( t_1 \) by the amount of radioligand bound at \( t_2 \). A drawback of this dual-point competition method is that it only allows an approximation of the dissociation rate and is therefore not reliable when ranking multiple slow dissociating ligands.

**Method**

-The \( k_{\text{obs}} \) of association for a 3·Kd concentration radioligand was determined prior to this experiment.

- Add 25 µL unlabeled ligand (10·Kd final concentration*) to 6 wells of a 96-well plate.
- Add 25 µL radioligand (3·Kd final concentration*) to all wells.
- After pre-heating all components to 25 °C, start binding reaction by adding 50 µL receptor-expressing cell homogenate to the first 3 wells of each unlabeled ligand. Do this with enough time before the planned termination of the binding reaction to allow equilibrium for each ligand. (\( t_2 \) in figure 8)
- Add 50 µL cell homogenate to the 3 remaining wells for each unlabeled ligand corresponding to a ~2.3/\( k_{\text{obs}} \) incubation time. (\( t_1 \) in figure 8)
- After the desired incubation times, reactions are simultaneously terminated and bound ligand is quantified (section 2.2.2). Estimate relative dissociation rate of unlabeled ligands by dividing the amount of bound radioligand at \( t_1 \) by the amount of bound radioligand at \( t_2 \) (KRI). KRI >1 indicates

![Figure 8 - Dual-point competition logistics.](image-url)
lower dissociation of unlabeled ligand as compared to radioligand, whereas $KRI<1$ indicates the opposite.

*Concentrations of ligands should be four times concentrated to compensate for dilution.

2.2.7 Two-step binding to determine relative binding kinetics of unlabeled ligands

Another efficient method compared to conventional analysis (section 2.2.4) estimates the relative dissociation rates for unlabeled competitive ligands. For this method it is first required to determine $IC_{50}$ values of the unlabeled ligands in equilibrium radioligand competition binding experiments. Next, receptor-expressing cell homogenate is pre-incubated with 4 to 8 times $IC_{50}$ concentrations of unlabeled ligand (step 1), followed by a filtration step in which the free ligand was removed and cell homogenate was incubated with the radioligand for a period of $\sim 2.3/k_{obs}$ (step 2). The $k_{obs}$ can be adjusted by varying the concentration radioligand (equation 2). Rapid dissociating unlabeled ligands will hardly delay radioligand association, whereas slow dissociating competitors will make it impossible for the radioligand to bind and consequently delay its association. Although a very useful technique in terms of the throughput, only relative estimates of the dissociation rates can be obtained. This method is unable to discriminate unlabeled ligands that have a $k_{off}$ which greatly exceeds the $k_{obs}$ of radioligand association. In this limiting case, the association of the radioligand would be completely independent of the unlabeled ligand. An additional concern would be that not all free unlabeled ligand is removed leading to overestimation of the residence time, therefore follow-up validation of selected hits is advisable.

**Method**

- The $k_{obs}$ of 3-$K_d$ concentration radioligand was determined prior to this experiment.

- The $IC_{50}$ of all antagonists is determined for inhibition of a 3-$K_d$ concentration radioligand.

- 50 µL receptor-expressing cell homogenate (section 2.2.1) is incubated with and without 50 µL of a 4-$IC_{50}$ final concentration* unlabeled ligand in triplicate at 25 °C. Free unlabeled ligand is removed by filtration over a glass filter. Next, the filter is immediately soaked with 50 µL of a 3-$K_d$ concentration radioligand.

- Radioligand binding is terminated after an incubation time of $\sim 2.3/k_{obs}$ (figure 9) by vacuum filtration and subsequent wash steps with ice-cold wash buffer to remove free radioligand (section 2.2.2).

![Figure 9 - Logistics of two step binding experiments for fast ligands.](image)
• Bound radioligand is quantified by scintillation counting. Binding levels are inversely proportional to the residence time of the unlabeled ligand.

* Concentration unlabeled ligand should be two times final concentration to compensate for dilution.

2.2.8 Two-step binding (unbiased) to determine dissociation rate constants of unlabeled ligands

Dissociation rate of a pre-incubated unlabeled competitive ligand can also be measured without prior knowledge of the unlabeled ligand using an alternative two-step binding approach. However, this approach does require the association and dissociation rate constants ($k_1$ and $k_2$, respectively) of the radioligand. In figure 10 it is shown how this method can be used to calculate the dissociation rate constant of the unlabeled ligand ($k_4$). A concentration series of unlabeled ligand is pre-incubated with receptor-expressing cell homogenate in duplicate (step 1). Next, one of the duplicates is washed after an equilibration step (~30min) to remove free ligand. Radioligand is subsequently added to all samples (step 2) and incubated for ‘t’ minutes before the reaction is terminated. Depending on how fast the unlabeled ligand dissociates, the radioligand will not be able to bind the receptor in the same extend as in the

\[
\frac{[RL]_{\text{L,max displacement}}}{[RL]_{\text{no antagonist}}} = \frac{(a+b)}{a} = \frac{k_1 \cdot [L] \cdot (1 - e^{-(k_1[L]+k_2)t})}{k_1 \cdot [L] + k_2} \\
\frac{b}{k_1 \cdot [L] \cdot (e^{-(k_1[L]+k_2)t} - e^{-k_4t})}{k_1 \cdot [L] + k_2 - k_4}
\]

Figure 10 - Measuring dissociation rate using a two-step binding approach. In (A) a model is depicted which can be used to determine the dissociation rate of unlabeled ligands. This is done by an experiment (B) in which receptors are incubated with an increasing concentration unlabeled competitor. Receptors are subsequently incubated with a radioligand, either immediately (blue curve) or after washing away all free unlabeled ligands (red curve). From the radioligand displacement curve, depicted in blue, a concentration antagonist can be taken which displaces all radioligand (1). For the same concentration antagonist, the bound radioligand after washing away unlabeled ligand (red curve) can be determined (2; [RL]_{L,max displacement}). Bound radioligand in the absence of unlabeled ligand (3; [RL]_{no antagonist}) is determined from the concentration unlabeled ligand, in which no inhibition of the radioligand is observed. To calculate $k_4$, both $k_1$ and $k_2$ have to be known values.
absence of pre-incubated competitor. By increasing the incubation time ‘t’, the more unlabeled ligand will dissociate and the less radioligand binding is affected.

The concentration unlabeled ligand corresponding to 25 times the IC$_{50}$ value of the competition binding curve in which free unlabeled ligand was not washed away (i.e. blue curve in figure 10B), will occupy >95 % of the receptors after pre-incubation. The amount of radioligand bound to the receptor, after washing away this saturating concentration unlabeled ligand, is $[RL]_{max \text{ displacement}}$ (i.e. ‘2’ in figure 10B), whereas radioligand bound receptor in the absence of unlabeled ligand is $[RL]_{no \text{ competitor}}$. The dissociation rate constant ($k_4$) of the unlabeled ligand can then be calculated using the equation in figure 10A and known values $[L]$, $k_1$ and $k_2$ of the radioligand. Moreover, it is assumed that all free unlabeled ligand is removed after the wash step, if not this will lead to an overestimation of the residence time (experiments with adherent cells are easier to wash than cell homogenate).

When pre-incubated unlabeled ligand dissociates completely within the time frame of the experiment or does not dissociate enough, the dissociation rate constant ($k_4$) cannot be determined. Between 20 % and 80 % dissociation of the initially bound unlabeled ligand should allow robust fitting of its $k_{off}$. This might be adjusted for by changing the incubation time. Advantage of using this two-step model compared to the Motulsky and Mahan model (section 2.2.4) is that there are only two different time-points where hands-on work is required. Additionally, both affinity ($K_i$) and residence time of the unlabeled ligand can be determined simultaneously. However, affinity can only be accurately determined if dissociation rate of unlabeled ligand is fast enough to reach equilibrium during the pre-incubation step for all concentrations unlabeled ligand (see also section 4.1). Unlike conventional endpoint competition binding this assay will at least indicate whether binding was indeed at equilibrium, since from the determined dissociation rate it can be calculated whether enough equilibration time was taken into account (>5 times the dissociation half-life of the slowest unlabeled ligand).

Method

-Both the $k_1$ and $k_2$ of the radioligand should be determined before starting this experiment. However, no prior knowledge is required for binding affinity and kinetic binding rate constants of unlabeled ligands.

- Seed receptor-expressing cells in a 96-well plate with a total volume of 100 µL fresh culture medium (cells should preferentially adhere better than HEK293T cells).
- The next day, replace culture medium with 100 µL Hank's balanced salt solution (HBSS) containing increasing concentrations unlabeled ligand with six wells per concentration. Also include a buffer only condition for six wells.
- Remove free unlabeled ligand from 3 wells per used concentration antagonist, after one hour (figure 11), by several wash steps and reconstitute in 100 µL HBSS. Immediately after, add 100 µL radioligand (3-K$_d$ final concentration*) to all wells pre-heated to 25 °C.**
- Incubate for the desired incubation time (figure 11). This should be longer than five times the dissociation half-life of the radioligand. Dissociation half-life of unlabeled ligand can eventually be quantified if this incubation time corresponds to a time of ~0.32 to ~2.32 times the dissociation
half-life of the respective unlabeled ligands (corresponding to 20 % dissociation to 80 % dissociation of the unlabeled ligand).

- Terminate all reactions simultaneously, by washing the cells three times with ice-cold wash buffer (e.g. Tris-HCl pH7.4) to remove unbound ligand. Cells are then lysed using lysis buffer (e.g. 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) and mixed with water permissive scintillation liquid. Bound radioligand is then quantified using a radioactivity scintillation detector.

- The IC_{50} value is determined from the competition binding curve for which unlabeled ligand was not washed away using nonlinear regression. Select a concentration unlabeled ligand ≥25 times the IC_{50} value (concentration I_{max} displacement), and determine for this concentration the amount of bound radioligand after washing away free unlabeled ligand ([RL]_{I_{max} displacement}). Determine the amount of bound radioligand in the absence of unlabeled ligand during pre-incubation ([RL]_{no antagonist}). Use these values together with k_{1} and k_{2} of the radioligand and the radioligand concentration to calculate the k_{4} of the unlabeled ligand (figure 10A).

*Concentration unlabeled ligand should be two times final concentration to compensate for dilution.

** Instead of using whole cells this experiment can also be performed with cell homogenate, as in section 2.2.7. However, after washing away pre-incubated unlabeled ligand, half of the filtered samples should be reconstituted in buffer containing radioligand with the different concentrations unlabeled ligand, and half should be reconstituted in buffer with only radioligand.

2.3 Real time readouts of ligand binding kinetics

Stop flow experiments are less useful when ligands reach equilibrium very fast due to the logistics of the experiment. However, if binding could be measured continuously it would be easier to have a well-defined association binding for fast compounds especially when incubations can be started and measured simultaneously. Moreover, a big advantage of measuring binding continuously is that all required incubation times can be measured within the same incubation mixture. The same models as discussed for radioligand binding studies (sections 2.2.3-2.2.8) can be used to analyze the data obtained with these real time techniques.

2.3.1 Scintillation proximity assay (SPA)

Scintillation proximity assays use solid scintillation beads on which the target receptor is coupled, for example by linking the cell homogenate expressing the receptor to these beads. Since isotope
disintegrations only converts into light when in very close proximity to these SPA beads, the enrichment of radioligands near the beads as a consequence of receptor binding can be specifically measured. Hence, this assay does not require separation of bound from free ligand and as such it can be measured continuously in a homogenous format. However, temporal resolution might be limited by the specific activity of the radioligand, which determines the counting time per data point. Hitherto, SPA has only been used for equilibrium radioligand binding to histamine receptors.

**Method**

- Cell homogenate (section 2.2.1) expressing the target receptor are incubated with PVT-PEI-WGA SPA, type A beads for 4 hours at room temperature. For cell homogenate expressing the H₂R this is done by combining 1mg of PVT-PEI-WGA SPA beads per 15 µg protein of cell homogenate in 50 µL total volume. For each well, 50 µL of this suspension is added to the respective ligands analogous to the fraction cell homogenate as described in section 2.2.3 and 2.2.4. Difference is that time points are no longer required as separate incubation conditions. After adding the cell homogenate/beads mixture, luminescence is immediately determined at various time points. Temporal resolution is limited by the speed of data acquisition (probably >10 sec/well) and the number of samples.
- Depending on the experiment, data is analyzed according to the respective models as discussed in section 2.2.3 and 2.2.4.

![Figure 12 - Resonance energy transfer as tool to study ligand binding.](image)

**Figure 12 - Resonance energy transfer as tool to study ligand binding.** As alternative to radioligands, fluorescent ligands can also be used to study binding. To do so, a fluorescent or luminescent energy donor is fused to the receptor, which emits light after excitation or by converting a substrate respectively. However, when a fluorescent ligand gets in close proximity to this energy donor, part of the energy will excite the fluorescent moiety of the ligand (acceptor) which will partially be emitted as light at a less energetic wavelength. Light emission of the ligand correlates therefore with a close proximity between ligand and receptor. This will be predominantly receptor-bound ligands.
2.3.2 FRET and BRET based ligand binding assays

Binding of a fluorescent ligand to an N-terminally SNAP-tagged H1R was measured in homogenous format using time-resolved fluorescence resonance energy transfer (TR-FRET) \(^{86}\). Fluorescent ligands are available for H1R, H2R and H3R \(^{122-125}\). This method allows continuous measurement of bound ligand by its FRET signal (figure 12), however photobleaching of fluorescent ligands should be considered. Nevertheless, reasonable kinetic binding rate constants for H1R antihistamines were obtained as compared to radioligand binding assays \(^ {86}\). Another promising technique has also been recently published in which bound fluorescent ligand (acceptor) was not excited by a fluorescent donor but by bioluminescence \(^ {126}\). In this case the receptor N-terminus was fused to NanoLuc, a luciferase which yields bright luminescence upon conversion of the substrate furimazine. Upon binding of the fluorescent ligand this led to a high BRET signal over noise. Advantage of this BRET technique is that photobleaching is prevented, however substrate availability might be limiting for prolonged incubation times.

**Method**

- Requires fluorescent ligand for the target receptor and the receptor should be fused at the N-terminus with a SNAP-tag or NanoLuc.

- Used fluorescent ligand should be compatible with the used donor emission.

**SNAP-tag**

- SNAP-tagged receptor is transfected into HEK293T cells (see section 2.2.1).
- The next day, cells are incubated with 6 mL 100 nM SNAP-Lumi4-Tb in taglite labeling medium for 1 hour at 37 °C and 5 % CO\(_2\). Consecutively cells are detached from the plate using 5 mL enzyme free cell dissociation buffer. Using centrifugation and wash steps (5 min. 500 g), the cells were washed three times in taglite labeling medium.
- Ligands are dissolved in taglite labeling buffer and dispensed in a black 384-well plate in a total volume of 5 µL with a 2 times final concentration. Binding reactions are then started by adding 5 µL containing 5∙10⁴ labeled cells/mL. For dissociation experiments, dissociation is started by adding a small volume competitor (<10 % final volume). It is best when binding can be started by machine injection so that signal can be immediately registered.
- Signal is measured by exciting the SNAP-Lumi4-Tb with a wavelength of 337 nm. TR-FRET signal is then measured by quantifying light intensity for the wavelength of the donor (SNAP-Lumi4-Tb; \(\lambda:490\) nm) and the wavelength of the acceptor (e.g. H1R antihistamine *green mepyramine* \(\lambda:520\)nm). Temporal resolution is limited by the speed of data acquisition (>0.3 sec/well) and the number of samples. FRET signal is expressed as the ratio between emission at 520 nm over the emission at 490 nm wavelengths. Signal should be corrected by subtracting the signal of non-specific binding. Data can then be analyzed using the models discussed previously (section 2.2.3 and 2.2.4).
NanoLuc

- NanoLuc-tagged receptor is transfected into HEK293T cells (see section 2.2.1)
- Seed receptor-expressing cells in a black 96-well plate with a total volume of 100 µL fresh culture medium.
- Upon addition of 10 µM substrate (furimazine), BRET is measured by quantifying light intensity for the wavelength of the donor (NanoLuc; λ:460 nm) and the wavelength of the acceptor (e.g. H1R antihistamine green mepyramine λ:520 nm). Temporal resolution is limited by the speed of data acquisition (>0.3 sec/well) and the number of samples. FRET signal is expressed as the ratio between emission at 520 nm over the emission at 460 nm wavelengths. Signal should be corrected by subtracting the signal of non-specific binding. Data can then be analyzed using the models discussed previously (section 2.2.3 and 2.2.4)

2.3.3 Surface plasmon resonance (SPR)

Surface plasmon resonance is a technique which can measure the mass increase on a gold layered glass chip. When a receptor is caught on this chip it can be used for binding studies, since the additional mass increase of a ligand binding the receptor can be registered by the SPR signal. Ligand is administrated as a continuous flow during which ligand binding will be detected. Protein stability remains a big challenge for using this technique in binding studies against GPCRs. Moreover, coupling strategies for GPCRs to the chip usually requires purifying engineered protein, which was found to lead to discrepancies in ligand binding data. New coupling methods have been developed and some success were reported, though use of SPR for GPCRs has so far been limited 127,128.

2.4 Functional experiments to determine ligand binding kinetics

Although functional experiments are harder to interpret for quantifying the kinetic binding rate constants of ligands, it might give a better representation of their functional consequence under more relevant conditions. The possibilities of using functional experiments as handle in exploring the ligand binding kinetics is therefore discussed.

2.4.1 Re-equilibration experiments (insurmountable antagonism)

A ligand bound to a receptor can only be displaced by another competitive ligand if there is sufficient time for the initially bound ligand to dissociate. This is the basis of insurmountable antagonism, which is frequently observed for different cellular responses. For example in the context of contraction of the guinea pig ileum (H1R), cellular calcium response (H1R), positive chronotropic response (H2R) and stomach acid secretion (H2R) insurmountable antagonism is observed for several histamine receptor antagonists 20,85,129,130. Fundamental for this effect is that receptor is pre-occupied by the antagonist (t=0 in figure 13), after which the cells are stimulated with agonist. For competitive orthosteric ligands it is expected that histamine dose-response curves shift limitless to the right without diminished maximal effect (E_max) in the presence of increasing concentrations antagonist since higher concentrations agonist are needed to initiate the same response. In contrast, if the antagonist does not have the chance to fully dissociate from the receptor in the incubation time of the experiment (incubation ‘t’), then at least part
of the receptors will be unavailable for histamine-induced signaling. This can be seen as a depression of the maximal effect, because even high concentrations of histamine are unable to compete with an antagonist that won’t dissociate from the receptor. Besides a depression of the E_max it is also possible to observe an initial shift to the right if there is a high receptor reserve, however for higher concentrations antagonist the E_max should eventually start to decrease\(^{131}\). A model was developed by Kenakin et al. 2006 that predicts how the depression of the E_max is related to the dissociation rate of the antagonist and the time between agonist stimulation and quantification of the receptor response\(^ {132}\). By pre-incubating with different concentrations antagonist and subsequent stimulating with increasing concentration agonist, dose response curves are obtained that shift to the right and/or show a depression in the E_max for high concentrations antagonist (figure 13). There are three cases that can be distinguished: There is no E_max...

\[\alpha = \frac{[B]}{[A]} + \frac{[A]}{K_B} + 1\]

\[\beta = \frac{[B]}{K_B + 1}\]

\[\gamma = \frac{[A]}{K_A + 1}\]

\[Y = \frac{[A]}{K_A} \cdot \left(1 - (\alpha \cdot (1 - e^{-k_{off} \cdot \tau \cdot t}) + \beta \cdot e^{-k_{off} \cdot \tau \cdot t})) \cdot \tau \cdot E_{max}\right) + 1\]
depression (fully competitive), there is an $E_{\text{max}}$ depression which saturates for high concentration antagonists (hemi-equilibrium) and there is an $E_{\text{max}}$ depression which abolishes all signal for high concentration antagonist (fully insurmountable). For the case in which hemi-equilibrium is obtained, the depicted model (figure 13) can be used to fit the dissociation rate constant of the antagonist, provided that equilibrium dissociation constant ($K_a$) of the agonist for the receptor is known $^{133,134}$. Furthermore, the incubation time ($t$) should be constrained as well. For the situation in which agonist and antagonist dissociate well within the incubation time and equilibrium is therefore obtained, the kinetic dissociation rate cannot be determined. It can only be said that full dissociation from the receptor is possible within the incubation time. Finally, in the case that antagonist is fully insurmountable by the agonist, then dissociation of the antagonist in the incubation time is close to zero and estimation of the dissociation rate is therefore impossible. However, it can be observed that the residence time must be much longer than the incubation time ($t$).

The incubation time is an interesting parameter in this experiment since it will determine what range of antagonist dissociation rates can be evaluated. For example peak responses in intracellular calcium mobilization (see method below) and ERK1/2 phosphorylation were obtained after 15-75 sec and 5 min, respectively $^{134}$. This indicates that in many cases the specific cell response will often determine what incubation time will be applicable to an experiment.

**Method**

-Information is required about the kinetics of the cells response to determine when response should be quantified. Moreover, approximate affinity of antagonist should be known.

- Cells expressing the H1R are seeded 3·10⁴ cells/well in a black, clear bottom 96-well plate.
- Duplicate columns are incubated with 100 µL HBSS supplemented with 20 mM HEPES, containing increasing concentrations antagonist corresponding to 0·Kᵢ to ~1000·Kᵢ in the presence of Fluo4 NW and 4 mM probenecid. This is incubated for 30 min at 37 °C, in which substrate is enzymatically converted intracellular into a calcium sensitive fluorophore (figure 14).
- After 30 min cells are stimulated with 10 µL, containing increasing concentrations* histamine and fluorescence is simultaneously recorded for approximately one minute per well (figure 14). All components were pre-heated to 37 °C. To allow injection and detection at the same time, signal is acquired from the bottom of the plate using excitation at 494 nm and detection at 516 nm wavelengths.
- Next, triton-X-100 is injected to a final concentration of 1.5 % and signal is measured after cells are lysed.
- Signal is normalized to baseline (before histamine injection) and saturated response after cell lysis. Normalized response is then plotted against log concentration agonist in the presence and absence of antagonist. This is analyzed with the discussed model (figure 16) by nonlinear regression in which affinity and ligand concentrations are constrained.

*concentration should be 11 times concentrated to compensate for dilution.
2.4.2 Recovery of receptor signaling after washout

Another way to explore how long an antihistamine remains bound to the receptor is by observing the receptor response at different time points after removing the antagonist from solution. Receptor responsiveness should then return back to the level it had before it was inhibited. For H₁R and H₂R such experiments were performed for example using the chronotropic response of the guinea pig atria (H₂R), guinea pig ileum contraction (H₁R) or human lung contraction (H₁R) [84,88,130]. In these experiments, receptor response was measured at various times after washout of antihistamine or continuously in the presence of histamine. In the washout experiments in the context of the H₁R, recovery of receptor signaling did not seem completely dependent on the dissociation half-life, which was reported to be long (~100 min), but not nearly as long as suggested by the washout data (multiple hours-days) [84,88].

Method

- Information is required about the kinetics of the cells response to determine how response should be quantified over time.

- 96-well plates with CHO cells expressing the target receptor are prepared as in section 2.4.1.
- When cells are strongly adherent to the plate, N wells are pre incubated with a 3·Ki concentration until equilibrium is expected for all used antagonist. N represents the amount of time points for which antagonist is allowed to dissociate. Remove free antagonist by a few rapid wash steps, add buffer and stimulate at different time points after which response is immediately quantified (figure 15). All used components and incubation steps are performed at 37 °C.
- Qualitative information about the recovery speed of receptor signaling is obtained.
3 Choosing the right experiment

Choosing an experiment is very much dependent on the required information and desired throughput. For fragment-based drug discovery, for example, it is likely that the dissociation rate of the ligands are relatively fast, as molecular size is an important determinant in shaping the ligand binding kinetics. In stop flow experiments, the temporal resolution is limited (interspacing time points >30 sec) making this a poor choice for very fast ligands. In this case it might be better to go for a continuous readout like the FRET-based ligand binding experiment (section 2.3.2) which can have a temporal resolution of ~0.3 sec when measured in an individual well over time. Moreover, when evaluating the kinetic binding rates in competition with the endogenous ligand it will be evaluated whether the antagonists has the desired kinetic binding rates observed in a physiologically relevant competition for the receptor. Additionally, functional re-equilibration experiments (section 2.4.1) could help in evaluating whether the respective dissociation rates of a set antagonist could have the potential to inhibit the respective cell response in an insurmountable manner. Table 2 shows a summary of the discussed techniques.

Use an equipotent concentration ligand (x times the affinity value):

\[
[L] = x \cdot K_{d,L} = x \cdot \frac{k_{off}(or \ k_2)}{k_{on}(or \ k_1)}
\]

\[
[I] = y \cdot K_{d,I} = y \cdot \frac{k_{off}(or \ k_4)}{k_{on}(or \ k_3)}
\]

(substitute in formula’s below)

A  Fractional occupancy at equilibrium

\[
P_{RL} = \frac{[L]}{[L] + K_{d,L}}
\]

\[
P_{RL} = \frac{x \cdot K_{d,L}}{x \cdot K_{d,L} + K_{d,L}} = \frac{x}{x + 1}
\]

B  Equilibration speed

\[
k_{obs} = k_{on} \cdot [L] + k_{off}
\]

\[
k_{obs} = k_{on} \cdot x \cdot \frac{k_{off}}{k_{on}} + k_{off} = (x + 1) \cdot k_{off}
\]

C

\[
P_{RL,1} = \frac{[L]}{K_{d,L}} \frac{[L]}{[L] + [I]} K_{d,L}
\]

\[
P_{RL,1} = \frac{x \cdot K_{d,L}}{1 + \frac{x \cdot K_{d,L}}{K_{d,L}} + \frac{y \cdot K_{d,I}}{K_{d,I}}} = \frac{x}{1 + x + y}
\]

D

\[
K_A = (x + 1) \cdot k_2
\]

\[
K_B = (y + 1) \cdot k_4
\]

\[
S = \sqrt{(KA - KB)^2 + 4 \cdot x \cdot y \cdot k_2 \cdot k_4}
\]

\[
K_F = \frac{1}{2} \cdot (KA + KB + S)
\]

\[
K_S = \frac{1}{2} \cdot (KA + KB - S)
\]

\[
Q = \frac{p_{max} \cdot x \cdot k_2}{K_F - K_S}
\]

\[
Y = Q \cdot \frac{k_4 - K_F}{K_F} \cdot \frac{e^{-K_F t}}{Q} - Q \cdot \frac{k_4 - K_S}{K_S} \cdot e^{-K_S t}
\]

\[+ Q \cdot \frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S}
\]

Figure 16 - The use of equipotent concentrations. When using equipotent concentrations ligand concentrations are chosen relative to the ligand affinity. Therefore, ligand concentrations are expressed as x (or y) times the $K_d$ of the ligand. (grey square) As can be seen in equilibrium (blue square), fractional occupancy of the receptor by a ligand (P$_{RL}$) can be expressed using the equations depicted in (A) as a function of ligand concentration ([L]) and the affinity ($K_d$) of L. Moreover, the same fractional occupancy of that ligand in competition with another ligand (I) for the receptor can be described by the equation in (C) as a function of ligand concentrations ([L] and [I]) and the affinity of those ligands. The ligand bound ligand (Y) over time (green square), can be described for a single ligand to the receptor (B) or in competition with another ligand (D). In both cases Y depends on the equipotent concentrations and on the kinetic dissociation rate constants of the respective ligand. However, binding over time is completely independent on the association rate constants when comparing equipotent concentrations.
Table 2 - Experimental formats for quantifying/estimating ligand binding kinetics. An overview is depicted of the discussed different techniques and experimental formats that can assess the kinetic binding rate constants of ligands.

<table>
<thead>
<tr>
<th>section</th>
<th>Temporal resolution</th>
<th>notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop flow</td>
<td>radioligand</td>
<td>&gt;20sec #</td>
</tr>
<tr>
<td>Continuous</td>
<td>SPA</td>
<td>&gt;10sec *</td>
</tr>
<tr>
<td>FRET/BRET</td>
<td></td>
<td>&gt;0,3sec</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Format</th>
<th>section</th>
<th>Quantitative/ Qualitative interpretation of binding rate constants</th>
<th>Throughput</th>
<th>Probe requirement</th>
<th>notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stop‐flow</td>
<td>Continuous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Association binding</td>
<td>2.23</td>
<td>Quantitative information</td>
<td>-</td>
<td>-*</td>
<td>Radioligand† for all ligands binding mechanism</td>
</tr>
<tr>
<td>Dissociation binding</td>
<td>2.23</td>
<td>Quantitative information</td>
<td>-</td>
<td>-*</td>
<td>Radioligand† for all ligands binding mechanism</td>
</tr>
<tr>
<td>Competitive association</td>
<td>2.24</td>
<td>Quantitative</td>
<td>-</td>
<td>+</td>
<td>Radioligand† required</td>
</tr>
<tr>
<td>Competitive dissociation</td>
<td>2.25</td>
<td>Quantitative. For slow dissociating ligands compared to radioligand association (k_{on}).</td>
<td>-</td>
<td>+</td>
<td>Radioligand† required</td>
</tr>
<tr>
<td>Dual‐point competition</td>
<td>2.26</td>
<td>Qualitative. Relative dissociation rate constants compared to radioligand.</td>
<td>++</td>
<td>+</td>
<td>Radioligand† required</td>
</tr>
<tr>
<td>Two step binding (fast compounds)</td>
<td>2.27</td>
<td>Qualitative. Relative dissociation rate dependent on incubation time.</td>
<td>++</td>
<td>+</td>
<td>Radioligand† required</td>
</tr>
<tr>
<td>Two step binding (unbiased)</td>
<td>2.28</td>
<td>Quantitative/qualitative. Dependent on incubation time.</td>
<td>+/-</td>
<td>NA</td>
<td>Radioligand† required</td>
</tr>
<tr>
<td>Re‐equilibration experiments</td>
<td>2.41</td>
<td>Quantitative/qualitative. Dependent on incubation time until signal is, and can be, measured.</td>
<td>-</td>
<td>NA</td>
<td>Agonist required mechanism of inhibition</td>
</tr>
</tbody>
</table>

†) Instead of radioligand, a fluorescent ligand could also be used in the context of BRET/FRET based binding experiments.

*) Throughput still limited, since most ligands will not be labeled. Moreover, fluorescently labeled ligands will likely affect the kinetic binding rate constants and therefore cannot be used to characterize the non-fluorescent ligands.

#) Based on how fast the experimenter can start and stop incubations.

¥) Depends on how high the signal is (available receptors, specific activity of the radioligand). The higher the signal, the faster the wells can be measured. Moreover, the amount of time points is limited, so temporal resolution can only be maintained for very short total incubation times.
4 Experimental design when addressing ligand binding kinetics

In this section some specific issues are discussed that should help in designing experiments meant to interrogate ligand-binding kinetics. Although mostly discussed in the context of radioligand binding experiments, concepts will be broader applicable and therefore might warrant a careful read.

4.1 Equilibration time and equipotent concentrations

For equilibrium saturation binding and competition binding experiments it is very important that reactions have indeed reached equilibrium, since this is required for using the receptor occupancy models to analyze this data. In the mentioned experiments, a wide range of ligand concentrations is used (from very low concentrations to very high concentrations). In the case of low concentrations the product \( k_{on}[L] \) will be negligible compared to the \( k_{off} \) (see equation 2). Experiments that use wide concentration ranges of a ligand will therefore be limited in its approach to equilibrium solely by the \( k_{off} \). A rule of thumb was proposed by Motulsky and Mahan 1984, to wait 5 times the dissociation half-life (3.5/\( k_{off} \)) to ensure an approximation of equilibrium at all used concentrations. This is the case for both the binding of one ligand separately but also for two ligands binding competitively at the same time. In the latter case, the slowest \( k_{off} \) of the two ligands determines the required equilibration time.

In experiments interrogating the effects of ligand binding kinetics it is often hard to make a fair comparison between ligands. One way to make sure that ligands only differ based on the dissociation rate and do not differ in their propensity to compete with another ligand is to use equipotent concentrations. This means that for each tested ligand the same ratio between concentration and affinity ([L]/\( K_d \)) is used. The effect of using equipotent concentration on receptor occupancy at equilibrium (in blue) and equilibration time (green) can be seen in figure 16. When taking \( x \) times the \( K_d \) as ligand concentration, it can be observed that the fractional occupancy at equilibrium will be described by \( x \), independent of the \( K_d \) of the ligand (figure 16A). However, the rate in which equilibrium is obtained (\( k_{obs} \)) will be dependent both on \( x \) and the \( k_{off} \) rate, which will differ between ligands (figure 16B). Note that for equipotent concentration the difference in \( k_{obs} \) between ligands does not depend on the \( k_{on} \) rate. Moreover, in the case of two ligands binding at the same time to a receptor, it can be seen that the fractional occupancy at equilibrium is again dependent on the relative concentrations to their individual \( K_d \) values (figure 16C). Based on the Motulsky and Mahan model, when substituting equipotent concentrations in the formula, it can be seen that binding (Y) becomes independent of the association rates of either ligand. Moreover, when comparing different ligands with equipotent concentrations, the exponential rate constants (KF and KS) only differ between ligands based on the dissociation rate (figure 16D). Therefore, fractional occupancies of the receptor at equilibrium should be the same when using equipotent concentrations of ligands, and differences observed in the onset of equilibrium are dependent solely on the dissociation rate of the used ligands.

4.2 Choosing concentrations and time points

When measuring the binding kinetics of ligands it is most practical to not use too many concentrations of ligand or different incubation times, because all concentrations will also have to be measured on all chosen time points. In stop-flow based experiments this could easily lead to a high number of conditions...
that need to be tested. It is therefore important to wisely choose concentrations and time points. In order to choose the required time points it helps if an educated guess can be made concerning the dissociation rate of the ligand. When information concerning the dissociation rate is known, then it can be calculated what would be a useful timeframe for the experiment. Probably 0 up to 5 times the $t_{1/2}$ would be a good pick for both association and dissociation experiments (figure 17A and B), as this will allow a close approximation of equilibrium for all relevant concentrations within the time frame of the experiment (see also section 4.1). Moreover, it is useful to have more time points in the early phase binding/dissociation, since for this phase there are the biggest differences in binding.

In order to choose the right concentrations of radioligand it is helpful to know the affinity of the radioligand. When association binding is performed to determine the $k_{on}$ and $k_{off}$ kinetic rate constants (section 2.2.3) it is important to ensure that there is enough difference between the measured $k_{obs}$ values to get a reliable fit of the $k_{on}$ and $k_{off}$. This can be done by choosing sufficient differences in ligand

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**Figure 17 - Time points and concentrations in stop-flow based experiments.** Lines represent the theoretical models describing the association and dissociation of ligands to the receptor. **A)** depicts the association of ligand to its target receptor. It can be seen that equipotent concentrations will always lead to the same curves when binding is expressed as function of the dissociation half-life. To best describe the theoretical curves, most data points should describe the early phase of binding where the biggest differences in binding are observed. Late phase time points are important to describe the amount of binding at equilibrium. **B)** depicts the dissociation of a ligand from the receptor. Curve will be completely dependent on the dissociation half-life and can be best described by measuring mostly early time points. Most ligand should have been dissociated after five times the dissociation half-life. **C)** describes the association of a ligand in the presence of another ligand. It can be seen that binding of $L$ is dependent on the presence of a concentration $[I]$. Equilibrium will be reached based on the slowest dissociation half-life. In this case this is the unlabeled ligand $I$. Since the labeled ligand binds much faster, an increase in binding of the labeled ligand can be seen for early time points. Therefore, the measured early time points should be adjusted to the labeled ligand and the measured late time points should be adjusted to the slowest of the two ligands.
concentrations that will result in variances in the \( k_{\text{obs}} \) (see equation 1 and 2). Moreover, by varying the \( Y_{\text{max}} \) sufficiently there is a more stringent fitting for the ratio between the \( k_{\text{off}}/k_{\text{on}} \) (i.e. the \( K_d \)) when this is incorporated in the mathematical model (chapter 1: figure 2B blue). Therefore, it is probably good to aim for a diversity in \( Y_{\text{max}} \) values to obtain well separated curves (figure 17A).

For competitive association binding experiments, both concentrations and time points are dependent on both ligands. Again, the equilibrium situation for the applied ligand concentrations can be predicted using the affinities of both ligands. In figure 17C, an example of concentrations is shown (expressed as equipotent concentrations). It is important that antagonist displaces a substantial amount (but not fully) the radioligand binding at equilibrium compared to radioligand binding in the absence of antagonist. When multiple concentrations unlabeled ligands are used, clear differences between the different curves should be attained (e.g. 25, 50 and 75 % inhibition of the radioligand at equilibrium). As for the chosen time points, the slowest ligand should be detrimental for how long the experiment lasts. Moreover, enough data points in the early phase of binding are also important and is dependent mostly on the dissociation half-life of the radioligand (time points between 0-3 times the \( t_{1/2} \)). An example for possible concentrations and time points are depicted in figure 17C.

4.3 Conclusion

Ligand binding kinetic constants have been implicated to be an important drug parameter and would be valuable information for discriminating potential drug candidates. It was therefore discussed what experiments would be appropriate for discriminating ligands based on their residence time. Discussed experimental formats were advantages in either their level of accuracy, throughput or physiological relevance. This overview should be a tool for researchers to design their experiment in a way that appropriate information is obtained. Additionally, it could provide insight and help in the design of new experimental formats.
Chapter 3 - BRET-based β-arrestin2 recruitment to the histamine H₁ receptor for investigating antihistamine binding kinetics

Co-authors: Ryo Moritani, Rob Leurs and Henry F. Vischer

1 Introduction

The histamine H₁ receptor (H₁R) belongs to the superfamily of membrane bound G protein-coupled receptors (GPCRs) and has been successfully targeted by many blockbuster drugs. H₁R antagonists are prescribed for treatment of allergic rhinitis, allergic conjunctivitis, urticaria, insomnia and motion sickness.

H₁R activates heterotrimeric Gq/11 proteins, resulting in increased intracellular calcium levels via the phospholipase C (PLC) and inositol 1,4,5-trisphosphate (IP₃) signaling pathway. This subsequently leads to a myriad of responses like transcriptional activity induced by nuclear factor-kB and nuclear factor of activated T-cells (NFAT). In contrast, it has been found that β-arrestin2 (arrestin3) desensitizes the H₁R induced Ca²⁺ response but increases signaling via extracellular signal-regulated kinases (ERK). Arrestins have often been described to bind phosphorylated GPCRs. Indeed, G-protein coupled receptor kinase 2 desensitizes H₁R-mediated Ca²⁺ response by phosphorylating the receptor suggesting that β-arrestin2 might be recruited to the H₁R after phosphorylation.

In drug discovery many potential drug candidates are screened in order to find a suitable drug. Ligand binding affinity for the receptor is inversely proportional to the equilibrium dissociation constant (Kₐ), which together with ligand concentration determines occupancy of the receptor at binding equilibrium (i.e. the ligand-receptor association rate equals its dissociation rate). The Kₐ is an important pharmacological parameter that significantly guides ligand optimization efforts. However, it is now increasingly acknowledged that Kₐ cannot always predict the effectiveness of a drug in vivo. In the human body, endogenous and drug ligand concentrations are dynamic and consequently a binding equilibrium is never or only very transiently reached. It has therefore been proposed that the dissociation rate constant (kₐoff) sometimes is additionally important to predict ligand-receptor occupancy, since this relates to the prolonged ligand-receptor occupancy after ligand clearance from the blood. A frequently used parameter related to the kₐoff is ligand-target residence time, which is defined as the reciprocal of kₐoff. It has been reported that some antihistamines for the treatment of allergic rhinitis and allergic conjunctivitis have a long receptor residence time. For example, levocetirizine and desloratadine were both measured to have a residence time on the H₁R exceeding an hour.

Ligand binding kinetics to GPCRs are often measured in radioligand membrane-binding experiments. However, it is unclear to what extent this accurately reflects target residence time of antagonists in agonist-induced responses on living cells. In this research we set up a bioluminescence resonance energy transfer (BRET)-based assay to monitor histamine-induced β-arrestin2 recruitment to H₁R. Real time measurement of BRET in living cells upon stimulation with histamine allowed us to distinguish slow from fast binding antihistamines.

2 Materials and Methods

2.1 Material

Dulbecco’s Modified Eagles Medium and poly-L-lysine was acquired from Sigma-Aldrich (St. Louis, MO, USA) and cell culture supplements fetal bovine serum and penicillin/streptomycin were bought from GE

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healthcare (Uppsala, Sweden). Trypsin, Hanks Balanced Salt solution, BCA protein assay were acquired from Thermo Fischer scientific (Waltham, MA, USA). 25-kDa linear polyethylenimine for transfection was purchased from Polysciences (Warrington, PA, USA). Luciferase substrates D-luciferin and coelenterazine-h (CTZ-h) were obtained from Promega (Madison, WI, USA). The dounce homogenizer was bought from Tamson (Bleiswijk, the Netherlands). GF/C plates, Microscint-O, [3H]mepyramine, the cell harvester and the Wallac Microbeta counter were all acquired from Perkin Elmer (Waltham, MA, USA). Histamine-2HCl (TCI, Portland, OR, USA), levocetirizine-2HCl (Biotrend chemicals, Zurich, Switzerland), desloratadine (Haichang industry, Jinan, China) and mepyramine maleate (Research Biochemicals International, Natick, MA, USA) and other used chemicals were from analytical grade quality.

2.1.1 Constructs

The reporter gene construct pNFAT-luc was obtained from Agilent Technologies (Santa Clara, CA, USA). All other constructs were in the mammalian expression vector pcDEF3 expressing the N-terminal HA-tagged hH1R, hH1R fused at the C-terminus with Rluc8 or β-arrestin2 fused at the C-terminus with mVenus. Rluc8 was cloned in frame with the H1R behind the C-terminus as has been described 147. Other constructs have been described previously 147–149.

2.2 Methods

2.2.1 Cell culture

Human embryonic kidney cells transformed with large T antigen (HEK293T) were sub-cultured at 37 °C and 5 % CO2 in Dulbecco’s Modified Eagles medium supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin.

2.2.2 NFAT induced reporter gene assay

1 µg HA-hH1R, 2 µg pNFAT-luc, 2 µg empty pcDEF3 and 30 µg lineair polyethylenimine was mixed in a 150 mM NaCl solution and incubated for 25 min at 22 °C. The transfection mix was added to 2·10^6 HEK293T cells in suspension, subsequently followed by seeding 3·10^4 cells per well on a white 96-well plate. The next day, cells were treated with increasing concentration histamine (10^{-3} - 10^{-10} M) for 5 h under standard culturing conditions. Medium was then decanted and wells were supplemented with 25 µL luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM MgCl2, 0.78 µM Na2H2P2O7, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 µM dithiothreitol). Luminescence was measured using a Wallac 1420 Victor3 Microplate Reader plate reader for 1 sec/well (PerkinElmer, Waltham, MA). EC_{50} values of histamine were determined by fitting the data to equation 1 131.

2.2.3 BRET based proximity determination between β-arrestin2 and the H1R

Two million cells were seeded in a 10 cm² dish and transfected the next day with 1 µg H1R-Rluc8, 4 µg β-arrestin2-mVenus and 30 µg linear polyethylenimine as previously described 149. After 24 h, cells were collected and seeded 5·10^4 cells/well in a poly-L-lysine coated white 96-well plate. Cells were used for experimentation the following day.
2.2.3.1 β-arrestin2 recruitment

Transfected cells were washed with assay buffer (Hanks Balanced Salt solution with 20 mM HEPES at pH 7.4) and pre-incubated for 15 min with 10 μM CTZ-h and consecutively stimulated with an equal volume histamine (10^{-3}-10^{-9} M) or vehicle at 37 °C. Luminescence was measured every two minutes (1 sec/well) for 60-100 min using a BRETplus1 luminescence module (535 nm and 475 nm) of the PHARStar FS (BMG labtech GmbH, Ortenberg, Germany). Between adding the ligands and measuring the luminescence in the first cycle was an approximated delay of 2 min. BRET signal is depicted as the ratiometric signal of the luminescence of the energy acceptor (mVenus, 535 nm) over that of the energy donor (Rluc8, 475 nm). Signal was baseline corrected and EC50 values of histamine were determined by fitting the data to equation 1. Moreover, the observed rate constants of β-arrestin2 recruitment were estimated by fitting the data to equation 2. The entire experiment was performed at 37 °C and all solutions were prepared in assay buffer and pre-heated before being supplemented to the cells. In experiments to monitor receptor reserve, cells were pre-incubated with or without 25-75 nM phenoxybenzamine (PBZ), prior to CTZ-h treatment, for 30 min after which cells were replenished with fresh buffer.

2.2.3.2 β-arrestin2 recruitment by histamine in competition with antihistamines

Transfected cells were pre-incubated for 15 min with 10 μM CTZ-h and consecutively stimulated with an equal volume containing 10^{-5} M histamine combined with increasing concentrations mepyramine (1∙10^{-7}-2∙10^{-6} M), desloratadine (1∙10^{-9}-2∙10^{-7} M) or levocetirizine (1∙10^{-7}-3∙10^{-6} M). BRET signal was measured as described above. Graphs depict baseline corrected data.

2.2.3.3 Inhibition of β-arrestin2 recruitment

Transfected cells were first incubated with stimulation buffer (50 μL 10^{-5} M histamine and 20 mM HEPES in Hanks Balanced Salt solution at pH 7.4) in the presence and absence of 10^{-5} M mepyramine for 60 min. Cells were then supplemented with 25 μL 20 μM CTZ-h in stimulation buffer and incubated for 15 min. Consecutively, mepyramine (1∙10^{-7}-2∙10^{-6} M), desloratadine (6∙10^{-9} M) or levocetirizine (1.2∙10^{-7} M) in stimulation buffer was additionally supplemented (25 μL) and signal was measured as described above. Graphs depict normalized data to the histamine response in the absence of inhibitor (1) and presence of 10^{-5} M mepyramine (0).

2.2.4 Radioligand binding experiments

2.2.4.1 Preparation of cell homogenates

Two million cells were seeded in a 10 cm² dish and transfected the next day with 5 μg HA-H1R DNA using 30 μg 25-kDa linear polyethyleneimine, as previously described. Two days after transfection, cells were collected in ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4). The cell suspensions were centrifuged at 1900 g for 10 min at 4 °C. Pellets were washed twice with ice-cold PBS and subsequently stored at -20 °C until further experimentation.

2.2.4.2 Saturation binding
Cell pellets were resuspended in binding buffer (50 mM Na₂HPO₄ and 50 mM KH₂PO₄, pH 7.4) and dounce homogenized by plunging the pestle 10 times with 1500 rpm. Protein content of the cell homogenates was determined with a BCA kit. Homogenates (± 0.5-3 μg/well) were incubated in the presence of increasing concentrations (0.1–50 nM) [³H]mepyramine in triplicate for 4 h. In addition, nonspecific [³H]mepyramine binding was determined in the presence of 10 μM mianserin. Incubations were terminated by rapid filtration over GF/C plates using a cell harvester, subsequently followed by three rapid filtrations using ice cold wash buffer (50 mM Tris-HCl pH 7.4). Filter plates were dried at 52 °C for at least 45 min before adding 25 μL Microscint-O. Bound [³H]mepyramine was quantified using the Wallac Microbeta counter. Equilibrium dissociation constant (Kᵦ) of [³H]mepyramine for the H₁R was subsequently determined from the nonspecific and total binding, by fitting the data to equation 3. Graph depicts the total binding corrected for the non-specific binding. All binding reactions were performed at 25 °C with gentle agitation. Moreover, preparation of cell homogenate, filtration and [³H]mepyramine quantification steps were executed in the same fashion for all binding experiments.

2.2.4.3 Equilibrium competition binding

Equilibrium dissociation constants for unlabeled ligands (Kᵦ) were determined by incubating cell homogenate with ±3 nM [³H]mepyramine and increasing concentrations (1∙10⁻⁴-1∙10⁻¹¹ M) unlabeled ligands in triplicate for 4 h. Inhibitory concentrations cold ligand preventing 50 % of the [³H]mepyramine binding (IC₅₀) were determined from the displacement curves by fitting the data to equation 4. Displacement curve of histamine was fitted together with the displacement curve of mepyramine in which the BOTTOM was shared. Depicted graphs are normalized to the TOP and BOTTOM of the mepyramine displacement curve. The Kᵦ was calculated from the IC₅₀ values by using the Cheng-Prusoff equation.

2.2.4.4 [³H]mepyramine binding kinetics

Four different concentrations of [³H]mepyramine (0.2-4 nM) were incubated with the cell homogenate for various time points (0-41 min.) in duplicate. All zero time points were additionally incubated with 10 μM mianserin to prevent specific binding of [³H]mepyramine. Non-specific binding did not increase with time, therefore residual binding at zero time points was considered to be the base-line. Binding rate constants of [³H]mepyramine (kₜ₉ and k₉ₜ) were determined by fitting the data to equation 5.

2.2.4.5 Competitive binding kinetics

For cold ligands, binding rates were determined by co-incubating a single concentration [³H]mepyramine of 1.5-12 nM in duplicate for various time points [0-81 min.] with cell homogenate in the absence and presence of three different concentrations cold ligand corresponding to 1-100 times the Kᵦ of the respective ligand. Incubations were held under gentle agitation at 25 °C. Binding rate constants of the cold ligands were then determined by fitting the data to equation 6, based on a model derived by Motulsky and Mahan.

2.2.5 Data analysis
All experiments are depicted by representative graphs. Values in tables are the mean ± SEM from 3 or more experiments. All analysis were done using prism 6 (GraphPad Software, San Diego, USA). The following models were used for analysis:

logEC$_{50}$

$$\text{Response} = \frac{\text{Top} - \text{Bottom}}{1 + 10^{\text{logEC}_{50} - \text{log}[A]}} + \text{Bottom} \quad (1)$$

One-phase association

$$\text{Response} = \text{Plateau} \cdot (1 - e^{-k_{obs}t}) \quad (2)$$

Total and non-specific saturation binding

$$\text{Non-specific binding} = NS \cdot [L^*] + C$$

$$\text{Total binding} = \frac{B_{\text{max}} \cdot [L^*]}{K_d + [L^*]} + NS \cdot [L^*] + C \quad (3)$$

logIC$_{50}$

$$RL^* = \frac{\text{Top} - \text{Bottom}}{1 + 10^{\text{log}[I] - \text{logIC}_{50}}} + \text{Bottom} \quad (4)$$

Association kinetics using multiple concentrations ligand

$$RL^* = \frac{B_{\text{max}} \cdot [L^*]}{[L^*] + \frac{k_2}{k_1}} \cdot (1 - e^{-k_{obs}t}) \quad (5)$$

$$k_{obs} = k_1 \cdot [L^*] + k_2$$

Kinetics of competitive binding

$$K_A = k_1 \cdot [L^*] + k_2$$

$$K_B = k_3 \cdot [I] + k_4$$

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

$$K_F = \frac{(K_A + K_B + S)}{2}$$

$$K_S = \frac{(K_A + K_B - S)}{2}$$

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

$$RL^* = Q \cdot \left( \frac{(k_4(K_F - K_S)}{K_F K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_Ft)} - \frac{k_4 - K_S}{K_S} e^{(-K_St)} \right) \quad (6)$$

RL$^*$ is the specific binding and the B$_{\text{max}}$ is the maximum specific binding of $[^3]H$ mepyramine. [A] stands for the concentration histamine, [L$^*$] for that of $[^3]H$mepyramine and [I] for the concentration cold ligand.
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NS is the slope in which non-specific binding increases with [L*] and C is the background in the absence of any radioligand. Association rate constants are denoted by \( k_1 \) or \( k_3 \) and the dissociation rate constants by \( k_2 \) or \( k_4 \) for \(^3\text{H}\)mepyramine or cold ligand respectively. For the kinetics of competitive binding model, binding rate constants of \(^3\text{H}\)mepyramine are required to fit the binding rate constants of the unlabeled ligand.

### 3 Results

HEK293T cells were transiently transfected with the N-terminally HA-tagged H\(_1\)R together with the firefly luciferase gene under transcriptional control of NFAT. A subsequent stimulation for 5 h at 37 °C with increasing concentrations histamine demonstrated a dose-dependent increase in NFAT-mediated transcription with a pEC\(_{50}\) of 6.9 ± 0.0 (figure 1 red curve). B-arrestin2 recruitment to the H\(_1\)R was determined by means of BRET\(^{149}\). To this end, the C-terminal tail of the H\(_1\)R was fused with an energy donor, the engineered *Renilla luciferase* Rluc8 (\( \lambda_{\text{emission}} \): 487 nm upon CTZ-h conversion)\(^{151,152}\), whereas \( \beta \)-arrestin2 was fused on the C-terminal tail to the green fluorescent protein mVenus (\( \lambda_{\text{excitation}} \): 515 nm; \( \lambda_{\text{emission}} \): 528 nm)\(^{153}\). Close proximity (<10 nm) between Rluc8 and mVenus\(^{154}\), as a consequence of \( \beta \)-arrestin2 recruitment to H\(_1\)R, results in bioluminescence resonance energy transfer from Rluc8 to mVenus. HEK293T cells co-expressing H\(_1\)R-Rluc8 and \( \beta \)-arrestin2-mVenus were stimulated with histamine for 40 min, inducing a dose-dependent increase in BRET between Rluc8 and mVenus with a pEC\(_{50}\) of 5.7 ± 0.1 (figure 1, blue curve). The potency of histamine for the recruitment of \( \beta \)-arrestin2 to the H\(_1\)R is 10-fold lower than the potency in which histamine elicits NFAT mediated transcription.

The kinetics of \( \beta \)-arrestin2 recruitment to the H\(_1\)R was evaluated by continuously measuring the BRET ratio in time (figure 2). BRET ratios increased over time upon stimulation with histamine and settled at an equilibrium state after approximately one hour (figure 2A). Histamine-induced recruitment of \( \beta \)-arrestin2 to the H\(_1\)R was analyzed using an exponential association model (equation 2). This resulted in a decent, but not perfect data fitting and an estimated association rate (\( k_{\text{obs}} \)) of 0.21 min\(^{-1}\). Interestingly, this \( k_{\text{obs}} \) did

![Figure 1](image.png)

**Figure 1** - Histamine H\(_1\) receptor activation induces NFAT mediated transcription and \( \beta \)-arrestin2 recruitment in HEK293T cells. (red curve) HEK293T cells expressing the N-terminally HA-tagged H\(_1\)R and an NFAT reporter gene were stimulated with histamine leading to NFAT mediated transcription of firefly luciferase in a concentration dependent manner. Quantity of the expressed luciferase gene was measured by an enzymatic light reaction. A representative graph is shown (N=5) with mean and SD of duplicate values. (blue curve) HEK293T cells expressing \( \beta \)-arrestin2-mVenus and H\(_1\)R-Rluc8 induced by histamine exhibit increased translocation of \( \beta \)-arrestin2 to the H\(_1\)R in a concentration-dependent matter as measured by BRET. Signal was baseline corrected by the signal of cells treated with 10\(^{-9}\) M histamine. A representative graph is shown (N=4) with mean and SD of triplicate values.
not change between the histamine concentrations 10⁻³ M and 10⁻⁶ M (supplementary table 1). This is in contrast to what is expected to happen with the \( k_{\text{obs}} \) for ligand binding, which typically increases with higher concentrations ¹⁰³. Uncoupling of \( \text{H}_1\text{R} \)-associated \( \beta\)-arrestin2 was rapidly induced by increasing concentrations mepyramine after a 60 min pre-incubation with histamine (figure 2B). It can be observed that inhibition of \( \beta\)-arrestin2 recruitment was dose dependent and fully reversible for high concentration mepyramine. Moreover, upon addition of high concentrations mepyramine, full uncoupling of the interaction between \( \text{H}_1\text{R} \) and \( \beta\)-arrestin2 was completed within the time needed to start detection of the BRET ratios (i.e. approximately 2 min). This shows that both histamine dissociation and \( \beta\)-arrestin2 uncoupling from the \( \text{H}_1\text{R} \) occurs rapidly, implying fast dissociation rate constants for both binding steps. Moreover, for all tested concentrations mepyramine, histamine inhibition reached equilibrium within 8 min indicating a fast dissociation rate constant for this antihistamine as well.

Table 1 - Potency and efficacy of histamine induced \( \beta\)-arrestin2 recruitment to the \( \text{H}_1\text{R} \) after pre-incubation with phenoxybenzamine.

<table>
<thead>
<tr>
<th>pre-treatment</th>
<th>( p_{\text{EC50}} ) (M)</th>
<th>( \text{E}_{\text{max}} ) (% vehicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>5.7 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>25 nM</td>
<td>5.6 ± 0.1</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>50 nM</td>
<td>5.6 ± 0.1</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>75 nM</td>
<td>5.7 ± 0.1</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>
Moreover, since histamine binding to H1R seems to have a rapid equilibration and β-arrestin2 recruitment depends on H1R occupancy by histamine, the (p)EC₅₀ value of histamine to recruit β-arrestin2 should stay constant over time. Indeed, the potency of histamine remains constant between 10 and 60 min although there is a slight increase in the maximal BRET signal during this time period (figure 3A and supplementary table 2).

If there is a linear relationship between histamine occupancy of H2R and the level of response, then reducing available H2R numbers will only affect the maximum response. The EC₅₀ value (i.e. half maximum activation) should not change. To interrogate this, H2R numbers available for histamine binding were reduced by a 30 min pre-incubation with and without 25-75 nM of the irreversible antagonist phenoxybenzamine (PBZ) ¹⁵₀,¹⁵⁵. After removal of unbound PBZ, cells were stimulated with increasing concentrations histamine for 40 min (figure 3B). The maximal β-arrestin2 recruitment was diminished upon pre-treatment with PBZ in a concentration-dependent manner, whereas the potency of histamine to recruit β-arrestin2 was unaffected (table 1).

Ligand binding kinetics of the unlabeled antihistamines are typically measured in competition with a radioligand ⁶¹,⁶⁴,⁸⁸,¹⁴⁶,¹⁵⁶. However, the prognostic value might be higher when this is measured from the functional response using a real-time assay on living cells in competition with the endogenous agonist. Binding to the H1R by one fast dissociating antihistamine (mepyramine ⁶⁴), two slow dissociating antihistamines (levocetirizine ⁶⁴; desloratadine ⁶⁴,⁶⁵) and histamine were first characterized by radioligand binding experiments. HEK293T cells transiently expressing the N-terminally HA-tagged H1R were homogenized and the cell homogenate was incubated with the respective ligands at 25 °C. The kinetic binding rate constants of radiolabeled mepyramine were measured by incubating increasing
concentrations [3H]mepyramine with cell homogenate for 0-40 min (figure 4A). Binding rate constants of [3H]mepyramine were then derived from the association curves using non-linear regression. The association rate constant ($k_{on}$) was determined at $110 \pm 6 \times 10^{6}\cdot \text{M}^{-1}\cdot \text{min}^{-1}$, the dissociation rate constant ($k_{off}$) was $0.22 \pm 0.01 \text{min}^{-1}$ and the B_{max} of expressed H1R was $43 \pm 12 \text{pmol/mg protein content of the cell homogenate. The equilibrium dissociation constant (K_d) could be calculated from these binding rate constants and the pK_{d,calc} was found to be $8.7 \pm 0.0$. This value is in agreement with the pK_{d} of [3H]mepyramine ($8.7 \pm 0.1$), measured by incubating increasing concentrations radioligand with cell homogenate expressing the H1R for 4 h (figure 4B). Additionally to the measured dissociation rate constant, the target residence time was calculated as the reciprocal of the $k_{off}$ and was found to be $4.7 \pm 0.3$ min. Equilibrium dissociation constants (pK_{i}) of desloratadine, mepyramine, levocetirizine and histamine for H1R were determined by incubating cell homogenate with increasing concentrations of ligand in competition with a single concentration [3H]mepyramine for 4 h (figure 4C and table 2). Next, binding rate constants for the same H1R ligands were determined from competitive association binding experiments against [3H]mepyramine to cell homogenate for 0-80 min (figure 5). Competitive binding by [3H]mepyramine to H1R against unlabeled mepyramine or histamine leads to apparently normal association binding of the radioligand with only a suppression of the maximal radioligand binding (figure 5A and D). In contrast, levocetirizine and desloratadine bind much slower to H1R as compared to mepyramine and when co-incubated with [3H]mepyramine this results in a kinetic advantage for the binding of [3H]mepyramine to the H1R, reflected by an overshoot during early time points (figure 5B and C). From the competitive association curves the kinetic binding rate constants for the unlabeled ligands were determined using a model derived by Motulsky and Mahan (113) (figure 5). Moreover, from these binding rate constants affinity and residence time values were derived (table 2). It indeed appears that mepyramine and histamine have a much faster $k_{off}$ ($0.28 \pm 0.05 \text{min}^{-1}$ and $0.38 \pm 0.07$ respectively)

Figure 4 - Binding of [3H]mepyramine and cold ligands to a homogenate of HEK293T cells expressing the H1R. Binding experiments were performed by incubating ligands with a homogenate of HEK293T cells expressing the N-terminally HA-tagged H1R and bound [3H]mepyramine was quantified after set incubation times. (A) Increasing concentrations of [3H]mepyramine were incubated with cell homogenate expressing the H1R at 25 °C for various incubation times. Graph depicts mean and SD of duplicate values. (B) Increasing concentrations of [3H]mepyramine were incubated with cell homogenate for 4 h at 25 °C. (C) A single concentration [3H]mepyramine was co-incubated with increasing concentrations unlabeled ligands with cell homogenate for 4 h at 25 °C. Data was normalized to the top and bottom of the fitted mepyramine displacement curve and bottom was shared during the fitting of the histamine displacement curve. Graphs (B) and (C) depicts mean and SD of triplicate values. Representatives for all graphs are shown of N≥3.
compared to levocetirizine and desloratadine (0.008 ± 0.001 min⁻¹ and 0.008 ± 0.003 min⁻¹ respectively). Moreover, kinetic binding rate constants of unlabeled mepyramine were similar to the kinetic binding rate constants determined for [³H]mepyramine.

Analogous to competitive association binding experiments, β-arrestin2 recruitment to the H₁R was measured in time upon stimulation with histamine in competition with mepyramine, levocetirizine and desloratadine for 100 min (figure 6A-C). A similar trend is observed between competitive radioligand binding and competitive β-arrestin2 recruitment experiments. Histamine in competition with mepyramine shows a gradual increase in β-arrestin2 recruitment (figure 6A) whereas histamine induces an overshoot in β-arrestin2 recruitment when in competition with the slow dissociating levocetirizine (figure 6B) and desloratadine (figure 6C).

Because the dissociation of histamine and uncoupling of β-arrestin2 from the H₁R is relatively fast compared to the binding of antihistamines it was hypothesized that experiments in which histamine-induced β-arrestin2 binding was inhibited would mostly reflect the binding kinetics of the antihistamines. Kinetics of β-arrestin2 uncoupling was therefore determined by co-incubating cells with antihistamines after a 60 min pre-incubation with 10⁻⁵ M histamine. Concentrations of mepyramine, levocetirizine and desloratadine were chosen to obtain 50-75 % inhibition of histamine-induced β-arrestin2 recruitment to H₁R (figure 6D). Mepyramine-induced β-arrestin2 uncoupling from the H₁R readily reached equilibrium unlike levocetirizine and desloratadine, which in line with their receptor binding kinetics did not reach a stable level of β-arrestin2 uncoupling within 100 min.

4 Discussion

For many GPCRs it has been established that β-arrestin2 is recruited upon agonist activation ¹⁵⁷. Real-time β-arrestin2 recruitment to the H₁R was measured using a BRET-based detection method. β-arrestin2 recruitment to an agonist-bound receptor requires first activation of the receptor, G protein coupling and activation, GRK recruitment, and finally receptor phosphorylation. These consecutive events caused a
delay between ligand binding and detection of β-arrestin2 recruitment to the receptor by BRET \(^{158,159}\). As a consequence of this delay, β-arrestin2 recruitment rate (\(k_{\text{obs}}\)) to \(H_1R\) was independent of the used histamine concentrations. Despite this lag time between the binding of histamine and the recruitment of β-arrestin to the \(H_1R\), it was shown that there was a dependency of β-arrestin2 for the agonist bound receptor state in a transient manner. Histamine has a fast dissociation rate constant for \(H_1R\) binding as shown by radioligand binding experiments (table 2). Indeed, inhibition of histamine binding led to full β-arrestin2 uncoupling within 2 min, reflecting the short lifetime of the histamine-\(H_1R\)-β-arrestin2 complex, in line with previous reports \(^{160,161}\). This allowed us to use β-arrestin2 recruitment as an indirect measure of agonist binding.

Histamine has a 10-fold higher potency to induce NFAT activation as compared to β-arrestin2 recruitment to the \(H_1R\) (figure 1). G-protein mediated \(H_1R\) signaling to downstream NFAT activity is subject to signal amplification and consequently low histamine occupancy of the \(H_1R\) can already induce a maximal response. This receptor reserve increases the potency in which histamine activates NFAT relative to its binding affinity for the \(H_1R\) \(^{131,150}\). For β-arrestin2 recruitment to the \(H_1R\) as measured by BRET it is not expected to detect any signal amplification since the interaction is thought to have a 1:1 stoichiometry \(^{162}\). Consequently, the potency of the histamine induced BRET-signal should be close to the \(H_1R\) binding affinity of histamine. Treatment with PBZ to reduce the levels of \(H_1R\) on the cell, led to a reduction in the maximal response but not potency of histamine induced β-arrestin2 recruitment. This indeed implies that in our studies there is no receptor reserve for β-arrestin2 recruitment to the \(H_1R\) by histamine \(^{132,150,163}\).

Figure 5 - Competitive binding kinetics of [\(^{3}H\)]mepyramine and unlabeled \(H_1R\) ligands to the histamine \(H_1R\) receptor. [\(^{3}H\)]mepyramine was incubated in the presence and absence of mepyramine (A), levocetirizine (B), desloratadine (C) and histamine (D) with a homogenate of HEK293T cells expressing the N-terminally HA-tagged \(H_1R\). This was performed at 25°C for various incubation times before quantification of bound [\(^{3}H\)]mepyramine. Representative graphs are shown of \(N\geq3\) depicting mean and SD of duplicate values.
and accordingly, the histamine-induced BRET signal is a measure of histamine binding for the entire H1R population. We subsequently investigated the usefulness of the H1R-induced β-arrestin2 recruitment to study ligand-binding kinetics in real-time on living cells.

Comparison between radioligand association and real-time histamine-induced β-arrestin2 recruitment assays in the presence of competitive antihistamines showed similar traces of radioligand binding and BRET, respectively (figure 5). A clear overshoot pattern was observed when cells were co-incubated with levocetirizine and desloratadine, which is characteristic for their relative slow association to the H1R compared to that of the radioligand and histamine-induced β-arrestin2 recruitment. Moreover, this overshoot pattern in the radioligand binding and BRET was not observed for the fast H1R associating mepyramine. Histamine induced β-arrestin2 recruitment to H1R was not solely dependent on the binding of the agonist. Therefore, BRET traces of β-arrestin2 recruitment could not be analyzed as was done for the radioligand binding using the Motulsky and Mahan model, which describes the competitive receptor association of two competing ligands only. This limits interpretation of the data to clear qualitative differences in the antihistamine dissociating rate constants. Nonetheless, this real-time BRET-based β-arrestin2 recruitment assay exceeds radioligand-binding experiments in experimental throughput and kinetic resolution. The level of bound radioligand can be quantified by scintillation liquid only if unbound
radioligand is removed, consequently terminating the binding reaction. Therefore, for each time point incorporated in radioligand binding experiments, an additional binding reaction is required. In contrast, BRET can be measured continuously upon stimulation, reaching therefore a much higher kinetic resolution and throughput. Recently, a format to detect radioligand binding continuously was developed using SPA beads, which potentially could level the throughput between experiments \(^{117,164}\). However, kinetic resolution is still lower than could be obtained with the BRET readout, since a reasonable signal accumulation time for scintillation counting is required to obtain a robust signal. Throughput and kinetic resolution is therefore limited by the amount of individual detectors that can simultaneously measure binding. Moreover, SPA beads are used to measure the affinity in a high throughput homogeneous format \(^{121,146}\), but have not been routinely used for ligand binding kinetics. Its application for different drug targets like the H\(_1\)R remains to be seen.

Another method has recently been developed in which binding of a fluorescent ligand is continuously measured by time-resolved fluorescent resonance energy transfer \(^{86}\). Although this approach has similar throughput and kinetic resolution, experiments require a fluorescently labeled ligand for the H\(_1\)R rather than the endogenous agonist. Moreover this assay characterizes binding of ligands to the H\(_1\)R, whereas the BRET-based \(\beta\)-arrestin2 recruitment determines ligand-binding kinetics in a functional assay.

Functional experiments measuring recovery of H\(_1\)R activity subsequent to a washout of pre-incubated antihistamine as a measure of their relative residence times can also be performed using the H\(_1\)R mediated contraction of guinea pig ileum \(^{84}\) and human bronchus \(^{88}\). The \(\beta\)-arrestin2 recruitment assay would be a substitute for such functional experiments with much higher throughput due to the homogeneous 96-well format in which it is performed. Moreover, the linear relation between histamine induced \(\beta\)-arrestin2 recruitment and binding to the H\(_1\)R does not apply for the histamine induced contraction of guinea pig ileum, which has shown to have a receptor reserve \(^{163}\), therefore complicating interpretation of the binding kinetics of antihistamines.

Competitive inhibition of histamine-induced \(\beta\)-arrestin2 recruitment to H\(_1\)R in real time to estimate receptor-binding kinetics of antihistamines, assumes that these ligands display no efficacy towards H\(_1\)R-mediated \(\beta\)-arrestin2 recruitment. However, the last decade it became apparent that ligands can be biased in their efficacy towards G protein activation and/or \(\beta\)-arrestin recruitment. For example, the well-known H\(_4\)R antagonist JNJ7777120 and other indolecarboxamides appeared to be biased agonists on \(\beta\)-arrestin2 recruitment to H\(_4\)R, and downstream ERK1/2 activation \(^{165,166}\). Hence, antihistamines that have been previously identified to antagonize H\(_1\)R-mediated G protein signaling in response to histamine, should first be tested on their own for biased agonism in \(\beta\)-arrestin2 recruitment.

5 Conclusion

BRET-based histamine-induced \(\beta\)-arrestin2 recruitment reflects agonist-receptor occupancy and can consequently be used as an efficient approach to indirectly estimate binding kinetics of agonist and antagonists to the H\(_1\)R. With the current trend to incorporate ligand-receptor residence time in drug development, BRET-based functional experiments can be used both as end-point or continuous readout of receptor response, capturing all required information to steer drug optimization.
6 Acknowledgements

Marjolein Verhoeven and Susanne I. Kuiper are acknowledged for their help with the NFAT reporter gene experiments.

Supplementary material

Supplementary Table 1 - Observed binding rate of histamine induced β-arrestin2-mVenus recruitment to H1R-Rluc8 as measured by the real-time BRET signal in HEK293T cells.

<table>
<thead>
<tr>
<th>[histamine]</th>
<th>One-phase association rate constant</th>
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<tr>
<td>$10^{-3}$ M</td>
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</tr>
<tr>
<td>$10^{-4}$ M</td>
<td>$0,21 \pm 0,03 \text{ min}^{-1}$</td>
</tr>
<tr>
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<tr>
<td>$10^{-6}$ M</td>
<td>$0,19 \pm 0,05 \text{ min}^{-1}$</td>
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Supplementary Table 2 - Potency of histamine to induce β-arrestin2-mVenus recruitment to H1R-Rluc8 as measured by the BRET signal in HEK293T cells after different incubation times.

<table>
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</tr>
<tr>
<td>60 min</td>
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Chapter 4 - Probe dependency in the determination of binding rate constants via competition association experiments

Co-authors: Henry Vischer and Rob Leurs

Manuscript is in preparation for submission.
Introduction

The pharmacodynamics of a drug is often expressed as the required concentration to obtain half-maximal target binding (Kᵢ, Kᵣ) or half-maximal modulation of target function (IC₅₀, EC₅₀). However, it is increasingly debated whether this provides sufficient information to estimate the *in vivo* effect of a ligand ⁵⁻⁸. Drug-target binding kinetics have therefore received increased interest the last decade, with in particular the drug-target residence time for which a correlation with the *in vivo* drug efficacy has been proposed for an number of important target classes, including the large family of membrane-bound G protein-coupled receptors (GPCRs) ⁶,¹¹,¹³,¹⁸,¹⁴³.

Radioligand binding is routinely used to determine ligand binding kinetics to GPCR targets ¹⁴,¹⁵,¹¹⁸,¹⁴⁵,¹⁶⁷⁻¹⁷⁰. This endpoint assay can be readily used to measure radioligand binding kinetics at the GPCR of interest. To determine the binding kinetics of unlabeled ligands, the competitive effect on the association binding of a GPCR radioligand is analyzed using the Motulsky-Mahan model ¹¹³. Although the Motulsky-Mahan analysis is often performed in the context of GPCR ligands, it was never investigated whether the quantified binding rate constants of unlabeled ligands are affected by the binding properties of the used radioligand probe. The histamine H₁ receptor (H₁R) is a prototypical GPCR drug-target which is therapeutically targeted by several antagonists in the treatment of, e.g. allergic rhinitis and urticaria. The success of the H₁R as a disease target has resulted in a rich repertoire of ligands that can bind the receptor, including different radioligands ³⁲,⁴⁴,⁶⁴,¹⁰⁸,¹⁷². Two radioligands ([³H]mepyramine and [³H]levocetirizine) with similar binding affinity at the H₁R but a large difference in their binding kinetics were therefore employed to determine and compare the binding kinetics of a set of unlabeled H₁R ligands ⁶⁴. The results of our study show that the radioligand-specific datasets with unlabeled ligand binding rate constants mostly have a similar trend, but the observed range and accuracy of the determined binding rate constants are depended on the used radioligand probe.

Methods

Materials

[³H]mepyramine (20 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA, USA) and [³H]levocetirizine (25.9 Ci/mmol) was a kind gift from AstraZeneca (Alderley, Cheshire, United Kingdom). Commercially acquired unlabeled ligands were triprolidine hydrochloride monohydrate (Tocris Bioscience, Bristol, United Kingdom), doxepin hydrochloride (Tocris Bioscience, E/Z mixture with a ~85:15 ratio), Olopatadine hydrochloride (BOC Sciences, Shirley, NY, USA), acrivastine (BOC Sciences), levocetirizine dihydrochloride (Biotrend, Cologne, Germany), S-cetirizine dihydrochloride (TLC PharmaChem, Mississauga, Canada), Mepyramine maleate (Research Biochemicals International, Natick, MA, USA), R-fexofenadine (Sepracor Inc., Marlborough, MA, USA), S-fexofenadine (Sepracor Inc.), desloratadine (HaiHang Industry, Jinan City, China), Terfenadine (MP biomedicals, Santa Ana, CA, USA). Additionally, VUF14454, VUF14544, VUF14506 and VUF14493 were synthesized in house as described (chapter 6) ³⁹.
Radioligand binding experiments

Radioligand binding experiments were performed as described before (chapter 3) with minor alterations as summarized below. HEK293T cell pellets were produced expressing the N-terminally HA-tagged H1R and stored at -20°C. Cells were thawed, resuspended in radioligand binding buffer (50 mM Na2HPO4 and 50 mM KH2PO4, pH 7.4) and homogenized with a Branson sonifier 250 (Branson Ultrasonics, Danbury, CT, USA). Homogenates (0.5 – 3 mg/well) were then incubated with the respective ligands at 25°C under gentle agitation. For equilibrium saturation binding, increasing concentrations [3H]mepyramine or [3H]levocetirizine were incubated for 4 h in the absence or presence of mianserin. For equilibrium competition binding, 3 nM [3H]mepyramine was used in the presence of increasing concentrations unlabeled ligands. In radioligand association binding experiments, four concentrations [3H]mepyramine (0.2 – 5 nM) and [3H]levocetirizine (1 – 60 nM) were used. For competitive association binding experiments 1 – 100x Ki concentrations of the respective unlabeled ligand was co-incubated with a single concentration radioligand ranging between 1.5 – 12 nM for [3H]mepyramine or 5 – 15 nM for [3H]levocetirizine. Kinetic ligand binding was performed for the depicted incubation times. Binding reactions were terminated with the cell harvester (Perkin Elmer) using rapid filtration and wash steps over PEI-coated GF/C filter plates. Filter bound radioligand was then quantified by scintillation counting using Microscint-O and the Wallac Microbeta counter (Perkin Elmer). Binding curves were analyzed by GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) using non-linear regression as described before (chapter 3). The relative error was calculated for $k_{on}$ and $k_{off}$ values by dividing the reported error of the non-linear regression (SD) by the fitted mean value.
Results

Equilibrium binding of increasing concentrations \([3H]\)mepyramine (figure 1A) and \([3H]\)levocetirizine (figure 1B) to H1R-expressing HEK293T cell homogenates, revealed that both radioligands saturably bind to the H1R with high affinities, resulting in pK\(_d\) values of 8.6 ± 0.1 and 8.1 ± 0.1, respectively (table 1). Moreover, in these saturation binding experiments similar B\(_{max}\) values (~30 pmol receptor per mg protein) were observed using both radioligands, showing that \([3H]\)mepyramine and \([3H]\)levocetirizine interact with the same H1R population. To determine the binding rate constants of the radioligands at the H1R, four different concentrations of \([3H]\)mepyramine (figure 1C) or \([3H]\)levocetirizine (figure 1D) were co-incubated with cell homogenate for increasing incubation times. The rate constants \((k_{on} and k_{off})\) of \([3H]\)levocetirizine binding to the H1R are a 100-fold lower than the binding rate constants of \([3H]\)mepyramine binding (table 1), as was previously described \(^{64}\). Moreover, equilibrium dissociation constants calculated from the binding rate constants \((pK_{d,kin} = k_{off}/k_{on})\) were in good agreement with equilibrium dissociation constants determined by saturation binding experiments \((pK_d)\) (table 1).

A chemical diverse set of unlabeled H1R ligands, including reference molecules with known differences in their H1R binding kinetics, was selected for characterization of their H1R binding kinetics using either of the two radioligand probes \(^{15,173}\)(chapter 3 and 6). To guide the design of competitive association experiments, binding affinities \((K_i)\) of the unlabeled ligands were first determined by equilibrium competition binding. Cell homogenates were therefore co-incubated with \([3H]\)mepyramine and increasing concentrations of the unlabeled ligands (figure 2). Binding affinities \((K_i)\) for H1R were calculated from the determined IC\(_{50}\) values using the Cheng-Prusoff equation \(^{115}\).

The binding rate constants of unlabeled ligands at the H1R were determined by competitive association binding experiments, in which H1R binding of the radioligand probes is quantified over time in the absence or presence of 3 different concentrations of each of the unlabeled ligands. Concentrations of unlabeled ligand were varied a tenfold between the lowest and highest used concentration which was within an equipotent range of 1 – 100 times the respective K\(_i\) of the ligands at the H1R. From the resulting radioligand association binding curves, the binding rate constants of unlabeled ligands can be determined by Motulsky-Mahan analysis (figure 3, table 2) \(^{113}\). Since each time point requires a new binding reaction, the kinetic resolution for quantifying radioligand binding is limited and dependent on the number of parallel reactions that can be performed. Therefore, incubation times were adjusted for the individual radioligands to best capture their kinetic profile of binding. For the rapidly binding radioligand

<table>
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<th></th>
<th>([3H])mepyramine</th>
<th>([3H])levocetirizine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK(_d)</td>
<td>8.6 ± 0.1</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td>pK(_{d,kin})</td>
<td>((k_{off}/k_{on}))</td>
<td>8.7 ± 0.0</td>
</tr>
<tr>
<td>k(_{on})</td>
<td>(10^2)min(^{-1})M(^{-1})</td>
<td>112 ± 5</td>
</tr>
<tr>
<td>k(_{off})</td>
<td>min(^{-1})</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>RT</td>
<td>min</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>B(_{max})</td>
<td>(pmol/mg)</td>
<td>26 ± 4</td>
</tr>
</tbody>
</table>

Table 1 - H1R binding parameters of radioligand probes. Values represent the mean ± SEM of ≥ 3 experiments.
[3H]mepyramine a relatively short 80 min incubation time was chosen (figure 3A – 3C), whereas a 360 min incubation time for the slowly binding probe [3H]levocetirizine (figure 3D – 3F) was employed. The association of the radiolabeled probes to the H1R in the presence and absence of three competing unlabeled ligands with (from left to right) fast, intermediate, and slow binding kinetics, is depicted in figure 3 and cover the diversity in binding kinetics observed within the full set of unlabeled ligands.

Competitive binding of [3H]mepyramine in the presence of unlabeled mepyramine leads to a gradual increase in radioligand binding until binding equilibrium has been established after ± 10 min (figure 3A). In the presence of doxepin and levocetirizine there is first a transient overshoot in the binding of [3H]mepyramine which results from the relative slow binding (lower \( k_{off} \) value) of unlabeled ligands compared to the rapid binding of [3H]mepyramine (figure 3B and C). Conversely, since [3H]levocetirizine binds much slower than [3H]mepyramine \( (k_{off}[3H]levo << k_{off}[3H]mep) \), no overshoot pattern is observed for [3H]levocetirizine binding to the H1R in the presence of the same three unlabeled ligands (figure 3D – 3F).

The observed association binding data of both radioligands (figure 3A – F) agreed well with the fitted non-linear regression line based on the Motulsky-Mahan model from which binding rate constants \((k_{on} \text{ and } k_{off})\) could be calculated (table 2). Binding rate constants of unlabeled ligands correlated between datasets obtained by using either [3H]mepyramine or [3H]levocetirizine as probe which is depicted for the \( k_{on} \) values in figure 4A \((R^2 = 0.69, P < 0.001)\) and the \( k_{off} \) values in figure 4B \((R^2 = 0.77, P < 0.001)\). However, the regression lines (solid lines) deviate from unity (dashed line) and some unlabeled ligands showed differences in binding kinetics between the two datasets. More than 10-fold differences in the \( k_{on} \) values were e.g. observed for VUF14454 and VUF14493 between both datasets. The differences in the \( k_{on} \) values between datasets were largest for ligands with a relatively high \( k_{off} \) value (table 2). Additionally, a probe dependent difference for the range in \( k_{off} \) values was observed, with [3H]mepyramine discriminating unlabeled ligands over a range in \( \log k_{off} \) between -2.2 and 0.1 and [3H]levocetirizine discriminating unlabeled ligands over a range in \( \log k_{off} \) between -3.2 and -0.7 (figure 4B). These data suggest that [3H]mepyramine better distinguishes fast binding unlabeled ligands (high \( k_{off} \) values), whereas [3H]levocetirizine better distinguishes slow binding unlabeled ligands (low \( k_{off} \) values). From the determined binding rate constants, the binding affinity (\( pK_{d,kin} = k_{off}/k_{on} \)) and the residence time (RT =

![Figure 2 - Competition binding between [3H]mepyramine and unlabeled ligands at the H1R. Binding of [3H]mepyramine to H1R-expressing cell homogenates was determined in the presence of increasing concentrations of the unlabeled ligands doxepin (blue), mepyramine (green) or levocetirizine (red). Representative graphs are shown of ≥ 3 experiments and the depicted data points represent the mean ± SEM of triplicate values.](image-url)
1/k_{off}), a proposed metric for the in vivo drug efficacy, were calculated (table 2). The pK_{d,kin} values correspond well with the respective pK_i values (figure 4C), with a good correlation for both the [3H]mepyramine-dataset (R^2 = 0.93, P < 0.0001) and [3H]levocetirizine-dataset (R^2 = 0.87, P < 0.0001). Furthermore, the pK_{d,kin} values correlate nicely between the probe specific datasets as well (R^2 = 0.87, P < 0.0001, data not shown).

To investigate the accuracy of the obtained k_{on} and k_{off} values, the relative error that was obtained by non-linear regression for the fitted binding rate constants were calculated for each individual experiment. The relative errors of the k_{on} and the k_{off} values were pooled and plotted against the corresponding mean k_{off} value that was derived from the same competitive association curve (figure 5). A probe-dependent accuracy is observed for high k_{on} values (figure 5A) as well as low and high k_{off} values (figure 5B). The accuracy of the fitted binding rate constants decreases for unlabeled ligands with a higher k_{off} at the H1R (figure 5). Moreover, the accuracy for fitting specifically the k_{off} additionally decreases for unlabeled ligand with a lower k_{off} at the H1R (figure 5B). The k_{on} value seems to be generally more accurate when determined in a [3H]mepyramine binding experiment, whereas the k_{off} value is more accurate for the [3H]levocetirizine-dataset in the case of unlabeled ligands with a logk_{off} < -2 and less accurate when the logk_{off} > -2.
Figure 4 - Comparison of the pharmacodynamics of unlabeled ligands as measured by using two different probes. Binding rate constants of unlabeled ligands were determined in radioligand binding studies, using either \(^{3}H\)mepyramine or \(^{3}H\)levocetirizine as competitive probe. A correlation plot is depicted for the \(k_{on}\) (A) and \(k_{off}\) (B) as determined from competitive association experiments using \(^{3}H\)mepyramine (x-axis) or \(^{3}H\)levocetirizine (y-axis). (C) The correlation plot between the affinity calculated from the kinetic binding rate constants (pK_{d,kin}) and the affinity from competition binding experiments (pK_i) is depicted. Dashes lines represent a perfect correlation respective to the X-axis values and solid lines represent the linear regression lines.

Discussion

When characterizing the pharmacodynamics of new drug molecules, it is advocated to include the target-binding kinetics, in particular the residence time, since this is considered to have a predictive value for the \textit{in vivo} efficacy of a drug\textsuperscript{6,9,11,18,143}. For GPCRs, ligand binding kinetics is often measured by filtration based, low throughput, radioligand binding experiments\textsuperscript{15,64,72,87,88}. Novel methods that allow a continuous detection of ligand binding with e.g. fluorescently labelled ligands as probes, have recently been

Figure 5 - Probe dependency for the accuracy of unlabeled ligand \(k_{on}\) and \(k_{off}\) at the H_1R. The accuracy in which the Motulsky-Mahan model fitted the \(k_{on}\) (A) and \(k_{off}\) (B) by non-linear regression was examined for the different experimental conditions that were employed in this study. To compare the accuracy of the fitted mean \(k_{on}\) and \(k_{off}\) values over a broad range, the relative magnitude of the error (SD), as derived from non-linear regression, was calculated for each individual replicate experiment and pooled for all ligands. The relative error was calculated by normalizing the SD by the mean (relative error = SD / mean). Subsequently, the relative error for the \(k_{on}\) and \(k_{off}\) were plotted against the corresponding mean \(k_{off}\) determined from the same competitive association curve. Data points derived from competitive association experiments that employed \(^{3}H\)levocetirizine are depicted in red. Additionally, data points from competitive association experiments with \(^{3}H\)mepyramine are shown, in which the total incubation time was either 80 min (blue) or 300 min (black). The arrows depict the \(k_{off}\) of the used probes with \(^{3}H\)levocetirizine in red and \(^{3}H\)mepyramine in blue as reported in table 1. Dashed lines represent a relative error of 1 (mean = SD).
introduced to increase the throughput of measuring competitive ligand association \(^{86,126,164,174}\). Despite methodological advances, all methods employ the Motulsky-Mahan approach of competition association binding to investigate unlabeled ligand binding kinetics.

Despite the strong interest in drug-target binding kinetics, it is unclear whether the receptor binding kinetics of the used probe might influence the obtained kinetic binding parameters of unlabeled ligands when measured in competition association experiments. Therefore, in this study we use two radioligand probes ([\(^{3}\)H]mepyramine or [\(^{3}\)H]levocetirizine) for the H\(_1\)R, that differ in their H\(_1\)R binding kinetics, to measure the binding rate constants of a diverse set unlabeled antagonists. Understanding the potential probe-dependency for quantifying the binding kinetics of unlabeled ligands is crucial for a proper cross-comparison of different methods (which inherently use different labeled ligands) and for a realistic estimation of the drug-receptor residence time.

In our experiments we observed that both \(k_{\text{on}}\) and \(k_{\text{off}}\) values obtained for unlabeled ligands binding at the H\(_1\)R, correlates when using either [\(^{3}\)H]mepyramine or [\(^{3}\)H]levocetirizine as probe (figure 4A,B). However, the \(k_{\text{off}}\) values among the same set unlabeled ligands are higher when measured using [\(^{3}\)H]mepyramine as probe (figure 4B). Moreover, although > 10-fold differences in the residence time (1/ \(k_{\text{off}}\)) are observed among (S)-cetirizine, triprolidine, mepyramine, VUF14454, VUF14493 and VUF14544 when using [\(^{3}\)H]mepyramine as probe, no difference is observed when the residence times is measured for the same set unlabeled ligands with [\(^{3}\)H]levocetirizine as probe. The comparison of these two datasets therefore suggests a probe dependent limit for discriminating the \(k_{\text{off}}\) of unlabeled ligands.

A probe-dependent effect is also apparent from the relative errors in the \(k_{\text{on}}\) (figure 5A) and \(k_{\text{off}}\) values (figure 5B). The relative error on the fitted binding rate constants increases when the corresponding mean \(k_{\text{off}}\) value (i.e. from the same competitive association curve) increases. Interestingly, a pronounced increase in the relative error on the mean binding rate constants is observed when the unlabeled ligands bind faster than the respective radioligand (\(k_{\text{off}}\) unlabeled > \(k_{\text{off}}\) radioligand, see arrows figure 5A and 5B).

Table 2 - Binding rate constants and calculated \(pK_{d,\text{kin}}\) and RT of unlabeled ligands at the H\(_1\)R. Values represent the mean ± SEM of ≥ 3 experiments, unless stated otherwise. Competitive association experiments were performed with [\(^{3}\)H]mepyramine (80 min incubation time) or [\(^{3}\)H]levocetirizine (300 min incubation time) as probe, with representative graphs shown for a subset unlabeled ligands in figure 3.

<table>
<thead>
<tr>
<th>Unlabeled Ligand</th>
<th>(pK_{d,\text{kin}}^a)</th>
<th>(k_{\text{on}}) (10^6\text{min}^{-1}\text{M}^{-1})</th>
<th>(k_{\text{off}}) min(^{-1})</th>
<th>RT min</th>
<th>(pK_{d,\text{kin}}^a)</th>
<th>(k_{\text{on}}) (10^6\text{min}^{-1}\text{M}^{-1})</th>
<th>(k_{\text{off}}) min(^{-1})</th>
<th>RT min</th>
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<tr>
<td>olonatadine</td>
<td>8.5 ± 0.0</td>
<td>1.8 ± 0.1</td>
<td>0.0061 ± 0.0004</td>
<td>170 ± 10</td>
<td>9.3 ± 0.3</td>
<td>1.6 ± 0.5</td>
<td>0.0007 ± 0.0002</td>
<td>1600 ± 400</td>
</tr>
<tr>
<td>levocetirizine</td>
<td>8.2 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.008 ± 0.001</td>
<td>140 ± 20</td>
<td>8.5 ± 0.0</td>
<td>1.4 ± 0.1</td>
<td>0.0049 ± 0.0008</td>
<td>230 ± 30</td>
</tr>
<tr>
<td>desloratadine</td>
<td>9.5 ± 0.0</td>
<td>30 ± 10</td>
<td>0.008 ± 0.003</td>
<td>160 ± 50</td>
<td>9.3 ± 0.1</td>
<td>9 ± 2</td>
<td>0.0044 ± 0.0006</td>
<td>240 ± 30</td>
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<tr>
<td>(S) fexofenadine</td>
<td>7.4 ± 0.1</td>
<td>0.23 ± 0.03</td>
<td>0.011 ± 0.003</td>
<td>110 ± 30</td>
<td>7.9 ± 0.0</td>
<td>0.19 ± 0.01</td>
<td>0.0022 ± 0.0002</td>
<td>450 ± 40</td>
</tr>
<tr>
<td>(R) fexofenadine</td>
<td>7.3 ± 0.1</td>
<td>0.24 ± 0.03</td>
<td>0.013 ± 0.003</td>
<td>90 ± 20</td>
<td>7.7 ± 0.1</td>
<td>0.18 ± 0.01</td>
<td>0.0040 ± 0.0006</td>
<td>260 ± 40</td>
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<tr>
<td>doxepin</td>
<td>9.1 ± 0.1</td>
<td>70 ± 10</td>
<td>0.06 ± 0.02</td>
<td>22 ± 7</td>
<td>9.7 ± 0.1</td>
<td>80 ± 30</td>
<td>0.014 ± 0.003</td>
<td>80 ± 20</td>
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<tr>
<td>(S) cetirizine</td>
<td>6.4 ± 0.1</td>
<td>0.21 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>11 ± 1</td>
<td>6.8 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.05 ± 0.02</td>
<td>27 ± 8</td>
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<td>triprolidine</td>
<td>8.1 ± 0.1</td>
<td>36 ± 5</td>
<td>0.30 ± 0.03</td>
<td>3.5 ± 0.4</td>
<td>8.7 ± 0.0</td>
<td>30 ± 10</td>
<td>0.05 ± 0.02</td>
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<td>mepyramine</td>
<td>8.8 ± 0.1</td>
<td>200 ± 50</td>
<td>0.28 ± 0.05</td>
<td>3.9 ± 0.6</td>
<td>8.8 ± 0.1</td>
<td>40 ± 10</td>
<td>0.07 ± 0.02</td>
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<tr>
<td>VUF14454</td>
<td>8.6 ± 0.1</td>
<td>250 ± 90</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.3</td>
<td>8.7 ± 0.0</td>
<td>11 ± 2</td>
<td>0.026 ± 0.005</td>
<td>41 ± 7</td>
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<tr>
<td>VUF14493</td>
<td>8.4 ± 0.1</td>
<td>300 ± 100</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>8.6 ± 0.0</td>
<td>80 ± 70</td>
<td>0.2 ± 0.2</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>VUF14544</td>
<td>7.8 ± 0.1</td>
<td>100 ± 40</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>7.9 ± 0.0</td>
<td>2.4 ± 0.4</td>
<td>0.032 ± 0.004</td>
<td>33 ± 5</td>
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</table>

\(^{a}pK_{d,\text{kin}} = k_{\text{off}} / k_{\text{on}}\)

\(^{b}\)RT = residence time = 1/ \(k_{\text{off}}\)
This implies that \(^{3}H\)mepyramine, which has a 100-fold higher \(k_{\text{off}}\) than \(^{3}H\)levocetirizine, is better suited to discriminate the binding kinetics of fast binding (high \(k_{\text{off}}\)) unlabeled ligands at the H\(_{2}\)R. Moreover, in our dataset the \(k_{\text{on}}\) (5A) and \(k_{\text{off}}\) (5B) are fitted with a higher accuracy when using \(^{3}H\)mepyramine as probe for unlabeled ligands with a residence time less than 100 min (log \(k_{\text{off}} > -2\)).

As can be seen in figure 3, the differences in curvature between competition association graphs are relatively small when the unlabeled ligands bind faster than the radioligand (3D – 3E; \(k_{\text{off}}\) unlabeled ligand \(> k_{\text{off}}\) radioligand). This suggests that the differences in the binding kinetics of unlabeled ligands have only small effects on the competition association curve when using a relatively slow binding radioligand probe. Quantifying the binding rate constants will therefore be quite error-prone and this can explain a poor differentiation of the short residence times (high \(k_{\text{off}}\)) of unlabeled ligands, using \(^{3}H\)levocetirizine as probe.

In contrast to the fitted \(k_{\text{on}}\)-values which are less accurate for unlabeled ligands that rapidly bind the H\(_{1}\)R (\textit{vide supra}; figure 5A), the fitted \(k_{\text{off}}\)-values are additionally inaccurate when the unlabeled ligands bind increasingly slow to the receptor (low \(k_{\text{off}}\); figure 5B). This accuracy again seemed probe-dependent, since the \(k_{\text{off}}\) of slow binding unlabeled ligands (residence time of more than 100 min; log \(k_{\text{off}} < -2\)) is fitted with a better accuracy when using \(^{3}H\)levocetirizine as probe in competition association experiments, compared to when \(^{3}H\)mepyramine was employed (figure 5B). It should be noted however that the probe-dependent accuracy for the determined binding rate constants likely depends partially on the used incubation time and kinetic resolution in competitive association experiments which were also dependent on the used probe.

In conclusion, by using different radioligand probes to measure the binding kinetics of unlabeled ligands, a correlation in the binding rate constants is observed, however, with a probe dependent disconnect in the measured absolute \(k_{\text{off}}\) and \(k_{\text{on}}\) values. Based on these data with H\(_{2}\)R ligands, it is recommended to use a fast binding probe (high \(k_{\text{off}}\) value) to measure the binding kinetics of fast binding unlabeled ligands. Moreover, when comparing the binding kinetics of unlabeled ligands with a long residence time (low \(k_{\text{off}}\) value) a long incubation time in combination with a slow binding probe is found to be more accurate (low \(k_{\text{off}}\) value). In general, caution should be taken for interpreting the binding rate constants when the unlabeled ligands bind the receptor faster than the labeled probe (\(k_{\text{off}}\) unlabeled ligand \(> k_{\text{off}}\) labeled ligand) or with very slow ligands that have a residence time that exceeds the total incubation time of the experiment. Considering that the Motulsky-Mahan model is by far the most frequently used way to derive the residence time of GPCR ligands, this study provides important considerations for the study of drug-target binding kinetics at GPCRs. Moreover, our conclusions can most likely also be translated to other target classes.

\textbf{Acknowledgement}

N. Bushby, M.J. Waring and R.J. Sheppard from AstraZeneca are acknowledged for their kind contribution of \(^{3}H\)levocetirizine.
Chapter 5 - The target residence time of antihistamines determines their antagonism of the G protein-coupled histamine H1 receptor

Co-authors: Gesa Witt, Lea Vaas, Ivana Josimovic, Philip Gribbon, Henry F. Vischer, Sheraz Gul, Rob Leurs

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1 Introduction

Despite the wide utilization of binding affinity and related drug binding metrics in drug discovery, it is increasingly debated whether these values provide sufficient information to allow prediction of in vivo efficacy. It is currently recognized that the binding rate constants of drug-target interactions may bring additional prognostic value in scenarios, in which there are no stable ligand concentrations, as is typical for in vivo systems. In particular the target residence time, which is the reciprocal of the dissociation rate constant of a bound ligand, is thought to be an important metric for drug optimization. A long residence time is, for example, related to an insurmountable mode of antagonism when endogenous agonist concentrations are transiently increased, as is observed in the case of neuronal signaling. Additionally, compounds with a long residence time show prolonged drug-target occupancies beyond the point at which pharmacologically active drug concentrations are present in the blood. A long residence time (>1 h) was e.g. observed for several clinically used antihistamines that bind to the histamine H₁ receptor (H₁R), an important drug target for the treatment of e.g. allergic rhinitis. In analogy with insurmountable antagonism observed in neuronal signaling, it has been described that histamine levels after allergen challenge are only transiently increased, implying that an insurmountable mode of antagonism could effectively block high concentrations of histamine. Moreover, in an ex vivo organ bath experiment in which antagonists were continuously removed, it was observed that the long residence time compounds azelastine and GSK1004723 both retained a long inhibition of the histamine-induced, H₁R-mediated bronchial contraction. In vivo, the clinically used antihistamine levocetirizine with a long residence time, also shows hysteresis of efficacy after depletion of free levocetirizine concentrations in the blood. Together, these data suggest that residence time is an important predictor of in vivo efficacy of H₁R antihistamines.

The H₁R is a prototypic member of the therapeutically relevant family of G protein-coupled receptors (GPCRs). The kinetic binding rate constants of unlabeled ligands for GPCRs are often measured using radioligand or fluorescent binding techniques. In these experiments, the effect of the unlabeled ligand on the binding of the labeled ligand is measured over time and kinetic binding rate constants are determined. Such experiments are often done using cell membranes as a source of the receptor. In this study, we developed methods to measure the kinetics of H₁R antagonism upon depletion of the free concentration antihistamine, by measuring recovery of the histamine-induced response over time in a physiologically relevant cell system. To this end, a fluorescent based calcium mobilization assay and a label-free, dynamic mass redistribution based assay were evaluated for the measurement of histamine-induced responses in human HeLa cells, cervical cancer cells known to endogenously express low levels of the H₁R. Using these assay formats, it is shown that the receptor recovery time is correlated to the residence time of antihistamines, hence, this parameter might therefore have predictive value for the in vivo efficacy of such ligands. The described orthogonal assays will also be very relevant for future GPCR drug discovery projects, as both calcium signaling, as well as DMR-responses, can be measured for a large number of GPCRs.
2 Materials and methods

2.1 Materials:

Fetal bovine serum (FBS) was from Bodinco (Alkmaar, the Netherlands). Penicillin/streptomycin 100x was purchased from GE healthcare (Uppsala, Sweden). Hank’s balanced salt solution (HBSS), BCA protein assay kit and Fluo-4 NW dye were from Thermo Fisher Scientific (Waltham, MA, USA). A 1x trypsin solution, Dulbecco’s modified medium/Ham’s F-12 (DMEM/F12) and Dulbecco’s modified medium (DMEM) were from Sigma Aldrich (St. Louis, MO, USA). Sterile, black and clear bottom 96-well plates were from VWR (Radnor, PA, USA). Polypropylene 384 well microplates were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany). Transfection reagent linear 25kDa polyethyleneimine (PEI) was from Polysciences (Warrington, PA, USA). Branson sonifier 250 homogenizer was from Emerson (St. Louis, MO, USA). From Perkin Elmer (Waltham, MA, USA) the following were obtained: [3H]mepyramine; GF/C plates; Microscint-O; the Cell Harvester; the Wallac microbeta; the EnSpire® Multimode Plate Reader equipped with Corning® Epic® Label-free technology; the JANUS MDT Automated Workstation; EnSpire®-LFC 384–fibronectin coated plates. The NOVOstar plate reader was from BMG Labtech (Ortenberg, Germany). Pharmacological tool compounds were acquired from the following commercial sources: Probenecid, olopatadine hydrochloride and acrivastine from Sigma Aldrich (St. Louis, MO, USA); Histamine hydrochloride from TCI chemicals (Portland, OR, USA); mepyramine maleic acid from Research Biochemicals International (Natick, MA, USA); levocetirizine dihydrochloride from Biotrend (Köln, Germany); doxepin hydrochloride and triprolidine hydrochloride from Tocris Bioscience (Bristol, UK); desloratadine from HaiHang Industry Co., Ltd. (Jinan City, China). HeLa cells and HEK293T cells were from an in-house eukaryotic cell biobank as described in previous publications 57,173. Compounds VUF14454, VUF14493, VUF14506 and VUF14544 were synthesized at the Vrije Universiteit Amsterdam and were fully characterized with respect to purity and identity. All pharmaceutical compounds were dissolved in DMSO to a stock concentration of 10⁻² M unless otherwise specified in the method section. Moreover, materials from a deviating source are stated explicitly in the respective section of the methods. All other chemicals are of analytical grade quality.

2.2 Radioligand binding experiments

2.2.1 Cell culture

HEK293T cells and HeLa cells were cultured in a humidified atmosphere at 37 °C with 5% CO₂ in DMEM and DMEM/F12 medium respectively which was supplemented with 10% FBS and 1x penicillin/streptomycin. Cell pellets of HEK293T cells transiently expressing the HA-hH₁R were derived as described before 173. Cell pellets of HeLa cells were derived by flushing cells from a sub confluent 10 cm² dish. Cells were then washed with PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄] and consecutively pelleted by centrifuge steps. Cell pellets were then stored until further experimentation at -20 °C.

2.2.2 Radioligand binding
Characterization of $[^3H]$mepyramine and unlabeled ligands using radioligand binding experiments was described extensively before with minor changes. In short, cell pellets were reconstituted in binding buffer [50 mM Na$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.4] and homogenized.

For saturation binding experiments $1 – 15$ nM $[^3H]$mepyramine in the absence or presence of $10^{-5}$ M mianserin was incubated with either $0.5 – 3$ µg cell homogenate of HEK293T cells or $20 – 60$ µg of HeLa cells for $4$ h at $37$ °C. Binding affinity ($K_d$) and $B_{max}$ was determined from the total (without mianserin) and non-specific (with mianserin) binding of $[^3H]$mepyramine using non-linear regression in Prism 6.0 (GraphPad Software, San Diego, USA). Reported $pK_d$ and $B_{max}$ values represent the mean and SEM of ≥ $3$ experiments.

Competition binding experiments were performed by incubating ($0.5 – 3$ µg) HEK293T cell homogenate transiently expressing the H$_1$R with a single concentration ($3 – 6$ nM) $[^3H]$mepyramine and increasing concentrations unlabeled ligand ($10^{-11} – 10^{-4}$ M) for $4$ h at $37$ °C. From the resulting radioligand displacement curves, the $IC_{50}$ was determined and binding affinity ($K_i$) was then derived by using the Cheng-Prusoff equation. Reported $pK_i$ represent the mean and SEM of ≥ $3$ experiments.

Kinetic binding rate constants of $[^3H]$mepyramine were determined to be $0.22$ min$^{-1}$ ($k_{off}$) and $1.1 \times 10^8$ M$^{-1}$min$^{-1}$ ($k_{on}$). To determine the kinetic binding rate constants of unlabeled ligands, ($0.5 – 3$ µg) a HEK293T cell homogenate transiently expressing the H$_1$R was co-incubated with a single concentration $[^3H]$mepyramine $1.5 – 12$ nM for various incubation times ($0 – 81$ min) at $25^\circ$C in the presence and absence of three concentrations unlabeled ligand. Final concentrations unlabeled ligands were selected to have various levels of inhibition of $[^3H]$mepyramine binding within the total incubation time. To have well separated binding curves over the course of the experiments, ligands with a fast $k_{off}$ were used on approximately $1 – 10$ times the $K_i$ value whereas the slow dissociating ligands had to be used on approximately $10 – 100$ times the $K_i$ value. The resulting binding of $[^3H]$mepyramine to the H$_1$R was analyzed using the Motulsky-Mahan model yielding the $k_{on}$ and $k_{off}$ of the unlabeled ligands. Moreover the binding affinity and residence time could be calculated from the binding rate constants, with $K_d,calc = k_{off} / k_{on}$ and residue time (RT) = $1 / k_{off}$. Reported $k_{on}$, $k_{off}$, $K_d,calc$ and RT values represent the mean and SEM of ≥ $3$ experiments.

2.3 Histamine-induced intracellular calcium mobilization

2.3.1 Fluorescent detection of calcium mobilization using Fluo4NW

HeLa cells were cultured as described above. HeLa cells were lifted from a subconfluent dish by incubating with a 1x trypsin solution for $4$ min. Cells were then seeded in a black, clear bottom 96-well plate, with $2 \times 10^4$ cells per well. Subsequently, the cells were pre-incubated overnight in culture medium with a range of antagonist concentrations or without antagonist, as is specified per assay format below. After 18-20 hours, assay buffer was prepared by supplementing HBSS with $20$ mM HEPEs pH 7.4 and $2.5$ mM probenecid (from a $2.5 \times 10^{-1}$ M stock in water). Dye solution was then prepared by dissolving one aliquot of Fluo4 NW in $22$ mL assay buffer. Medium was aspirated and Fluo4 NW dye solution was supplemented to the cells and incubated for an hour at $37^\circ$C in the presence of the respective antagonist. After labeling the cells with Fluo4 NW, readout of the calcium response was measured per individual well in series. For
each measurement, histamine was injected to induce a peak calcium response and consecutively, triton-
X-100 was injected to lyse the cells for a saturated Fluo4 NW calcium response. Fluorescence at $\lambda_{\text{excitation}}$
494 nm and $\lambda_{\text{emission}}$ 516 nm was measured with the NOVOstar, which was set to 37°C, once per second
for three segments: firstly, the background signal was quantified as the average fluorescence before
histamine injection ($F_b$); secondly, the histamine induced calcium mobilization was quantified as the
fluorescent intensity between the histamine injection and triton-X-100 injection ($F_{HA}$); finally, the
saturated calcium response was quantified as the maximum signal after triton-X-100 injection ($F_t$). The
histamine-induced response ($F_{HA}$) was then normalized according to equation 1.

$$\text{normalized fluorescent response} = \frac{F_{HA} - F_b}{F_t - F_b} \quad (1)$$

2.3.2 Histamine dose response relationship

Cells were pre-incubated with or without increasing concentrations of the antagonists mepyramine ($10^{-8.3} \text{–} 10^{-6.7}$ M), doxepin ($10^{-8} \text{–} 10^{-9.2}$ M), olopatadine ($10^{-9} \text{–} 10^{-7.8}$ M) or levocetirizine ($10^{-8.1} \text{–} 10^{-6.9}$ M). Furthermore, for every concentration antihistamine, cells were stimulated with increasing concentrations histamine ($10^{-8} \text{–} 10^{-3.1}$ M with 0.7 log-unit steps; prepared from a $10^{-1}$ M stock in deionized water) using
duplicate wells per experiment. Per well fluorescence was detected for 60 s as described with a 20 µL
histamine injection at the 20 s mark and a 50 µL triton-X-100 (final concentration 1.5 %) injection at the
50 s mark. Either the normalized peak fluorescence or the area under the curve (AUC) for 30 s after
histamine stimulation was then used as a measure for the calcium mobilization, which was plotted against
the respective histamine concentration. The AUC was quantified using GraphPad Prism (GraphPad
Software, San Diego, USA). Settings were as such that peaks were ignored when there was less than 1%
difference between min and max fluorescence and/or peaks shaped by fewer than 10 data points. The
resulting dose-response curves were analyzed by non-linear regression according to equation 2.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log EC50 - \log [histamine]}} \quad (2)$$

2.3.3 Receptor recovery of calcium mobilization after antagonist washout

One 96-well plate with HeLa cells was separated into four sections and each section was pre-incubated
with a respective 10 times $K_i$ concentrations of antihistamine or vehicle condition. Hence, three different
antihistamines were used per 96-well plate. Following Fluo4 NW loading in the presence of the respective
concentration antihistamine, cells were washed and reconstituted in 100 µL assay buffer that was pre-
heated to 37°C ($t_0$). Subsequently, the histamine induced calcium mobilization was measured per well,
alternating between the four conditions (pre-treatment with vehicle and each of the three
antihistamines), for 24 cycles. The first measurement was started approximately 2 min after washing the
cells and subsequent wells were measured with 75 s intervals. Fluorescence was detected during this 75
s as described and 20 µL histamine (final concentration $10^{-5}$ M) was injected at the 10 s mark and 50 µL
trition-X-100 (final concentration 1.5%) at the 65 s mark. The normalized peak response following histamine stimulation was then plotted against the washout time (t) defined as the time between \( t_0 \) and histamine injection in the respective well. The data was analyzed using non-linear regression with a one-phase association model in GraphPad Prism according to equation 3.

\[
Y = Y_0 + (Y_{\text{max}} - Y_0) \times (1 - e^{-k_{\text{rec}}t})
\]  

Here \( Y_0 \) was constrained to be 0, \( Y_{\text{max}} \) is the histamine-induced response upon reaching a steady-state and \( k_{\text{rec}} \) is the recovery rate of the histamine induced response. For cells pre-treated with olopatadine, insufficient receptor recovery was observed within the time-frame of the experiment and \( Y_{\text{max}} \) was therefore constrained to be the average histamine response measured in cells pre-treated with vehicle condition. For all other antihistamines a free fit of \( Y_{\text{max}} \) was allowed, reported in the results as the steady state recovery (%). Receptor recovery times (RecT) were calculated for each experiment as the reciprocal of the \( k_{\text{rec}} \). Reported \( k_{\text{rec}} \) and RecT values are the mean ± SEM of ≥ 3 experiments.

2.4 Dynamic mass redistribution (DMR)

For the measurement of dynamic mass redistribution in HeLa cells upon treatment by agonists and antagonists, a resonant waveguide grating (RWG) biosensor method was used. This is a widely used method for non-invasive quantification of G protein-coupled receptor (GPCR) modulation in living cells and details of the technology are described elsewhere 180,181.

2.4.1 DMR receptor recovery after washout

HeLa cells were grown in DMEM High Glucose medium with L-Glutamine supplemented with 10% FBS and 1x penicillin/streptomycin (all reagents were obtained from Capricorn Scientific GmbH (Ebsdorfergrund, Germany)) and incubated in humidified atmosphere at 37 °C and 5% CO2 in air. For DMR experiments, culture medium was transferred to an EnSpire-LFC 384– fibronectin coated plate (10 µL/ well) and incubated for 30 min at room temperature. Afterwards antagonists were diluted in cell culture medium to a 10 x Ki final assay concentration and 10 µL per well were transferred to the LFC plate. All compounds were diluted from a 100 mM stock in DMSO, except for desloratadine (25 mM), VUF14454 (2.5 mM) and acrivastine (5 mM). 1.5 \( 10^4 \) HeLa cells were seeded per well, resulting in a final assay volume of 30 µL/ well. As vehicle control wells with medium/DMSO treated cells were included. The LFC plate was incubated overnight in a humidified atmosphere at 37 °C and 5% CO2 in air. On the next day assay buffer [HBSS (Sigma Aldrich), 20 mM HEPES (Sigma Aldrich), 0.5% (v/v) DMSO] was adapted to room temperature before use. 22-24 h after cell seeding antagonists were removed from the LFC plate and wells were washed four times with label-free assay buffer (25 µL/ well). The total assay volume after the washing step was 30 µL/ well.

A dilution series of histamine (from \( 10^{-1} \) M stock in deionized water) was prepared in label-free assay buffer and dispensed into an intermediate microplate. In all experiments a DMSO concentration of 0.5% (v/v) was not exceeded. The LFC plate was placed in an EnSpire® Multimode Plate Reader equipped with Corning® Epic® Label-free technology. 1 h and 2 h after antagonist washout a baseline was recorded (5 minutes) and histamine was transferred from the intermediate microplate into the LFC plate using a
JANUS® MDT Automated Workstation and a 30 min kinetic DMR measurement was recorded on the EnSpire® Multimode Plate Reader at room temperature. Data was analysed individually for each quadrant of the 384 well LFC plate using GraphPad Prism. Peak response values of vehicle-treated cells were plotted against the log_{10} histamine concentration and data was fitted using a four-parameter sigmoidal fit. The maximum (100% effect) and minimum (0% effect) values of the control dose response curve were used to normalize the histamine induced peak responses of antagonist treated cells, to give a relative peak response (%).

The recovery rate (k_{rec}) of the histamine induced DMR-response after antagonist washout was estimated using equation 3 when the effect was significantly different at both time points. For estimation of the recovery rate the response induced by a saturating concentration histamine (60 µM) was used as determined for: vehicle treated cells (Y_{max}); antagonist treated cells 1 h after washout (Y_{0}); antagonist treated cells 2 h after washout (Y). Depicted values represent mean and standard deviation of two independent experiments. Moreover, for antihistamines that allowed full recovery within 1 h after washing away unbound ligands, RecT was estimated to be < 30 min. This estimation is based on a > 90% inhibition right after washout (t = 0) and a functional recovery of > 90% after 1 h.

2.4.2 DMR agonist/antagonist co-incubation

An LFC plate was activated with 10 µL culture medium. Afterwards 1.5 \times 10^4 HeLa cells per well were seeded and incubated over night as described above. 22-24 h after cell seeding medium was removed from the LFC plate and wells were washed four times with label-free assay buffer (25 µL/well). The LFC plate was placed in an EnSpire® Multimode Plate Reader. A dilution series of antagonist was prepared in label-free assay buffer and dispensed together with histamine (Selleck Chemicals, Munich, Germany) into an intermediate microplate (Polypropylen 384 well microplate). 2 h after washing a baseline was recorded (10 minutes) on an EnSpire® Multimode Plate Reader. Afterwards, compounds were transferred from the intermediate microplate into the LFC plate and a 60 min kinetic DMR measurement was started. For data analysis the area under der curve (AUC) was determined from 1-20 min and plotted against the antagonist concentration. Data was fitted using a sigmoidal four-parameter fit and EC_{50} values were determined.

3 Results

It is well known that the human adenocarcinoma HeLa cell line endogenously expresses functional histamine H_{1}Rs, coupled to the mobilization of calcium and changes in cell morphology. As such, this

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Expression of the H_{1}R and binding affinity of [3H]mepyramine. Average values are shown ± SEM of ≥ 3 experiments.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H_{1}R expression</td>
<td>pK_{d}</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>endogenous</td>
</tr>
<tr>
<td>HEK293T cells</td>
<td>transient</td>
</tr>
</tbody>
</table>
cell line was selected as a physiological relevant test model for the proposed kinetic receptor recovery studies. Expression levels of the H1R were quantified in HeLa cells as well as in transiently transfected HEK293T cells using a radioligand saturation binding experiment (table 1). Increasing concentrations of the H1R antagonist [3H]mepyramine were incubated with cell homogenates of the respective cell line for 4 h at 37 °C. [3H]mepyramine displayed comparable affinity for H1R on HeLa cells and HEK293T cells with pKd values of 8.5 ± 0.2 and 8.1 ± 0.1, respectively. A very low window of specific [3H]mepyramine binding was observed for the HeLa cell line, in sharp contrast to the HEK293T cells, which transiently expressed the human H1R at >100-fold higher expression levels (table 1). The observed expression of the H1R on HeLa cells was in the same order of magnitude as described in literature (55 - 130 fmol/mg protein) [177,178].

A set H1R antihistamines was selected based on the diversity in chemotype and expected diversity in receptor residence times (supplementary table 1) [15,173]. First, the equilibrium (i.e. steady-state) binding of antihistamines to the H1R was characterized using radioligand binding experiments. To determine the binding affinity (pKc) of antihistamines for the H1R, a homogenate of HEK293T cells transiently expressing the H1R was co-incubated with a single concentration [3H]mepyramine (3 – 6 nM) and increasing concentrations of unlabeled antihistamines for 4 h at 37 °C. Examples of four [3H]mepyramine displacement curves for the prototypical H1R antihistamines mepyramine, doxepin, levocetirizine and olopatadine are depicted in figure 1. More than 2 log unit differences in pKc values were observed between antihistamines (table 2) and the respective Kc-values were comparable to those described previously [15,39,52,184]. Subsequently, the kinetic binding rate constants of antihistamines were determined in [3H]mepyramine competitive association experiments. Antihistamines were therefore co-incubated with [3H]mepyramine and a homogenate of HEK293T cells, transiently expressing the human H1R, for 0 – 81 min at 25 °C (figure 2; table 2). In such competitive association binding experiments a typical overshoot pattern in the binding of the radioligand is observed when it has a higher koff than the unlabeled ligand at the receptor [113]. In the presence of unlabeled mepyramine (figure 2A) there is no overshoot pattern in the

Figure 1 - Competition binding for the histamine H1 receptor by [3H]mepyramine and unlabeled antihistamines. An homogenate of HEK293T cells transiently expressing the H1R was incubated with increasing concentrations unlabeled antihistamines and 5.6 nM [3H]mepyramine for 4 h at 37 °C in 50 mM Na2HPO4/KH2PO4 pH 7.4. Representative graphs of ≥ 3 experiments are shown with mean ± SD of triplicate values (n = 3).
binding of the radioligand to the H1R, indicating that the ligand $k_{off}$ at the H1R is similar (or higher) for mepyramine as compared to [$^3$H]mepyramine. In the presence of doxepin (figure 2B), levocetirizine (figure 2C) and olopatadine (Fig 2D) an overshoot in [$^3$H]mepyramine binding to the H1R is observed which reflects the relative low $k_{off}$ of these unlabeled ligands at the H1R compared to the $k_{off}$ of [$^3$H]mepyramine. The kinetic binding rate constants ($k_{on}$ and $k_{off}$) are determined from these kinetic binding traces using the Motulsky-Mahan model.113 The representative antihistamines mepyramine, doxepin, levocetirizine and olopatadine, depicted in figure 2A-D, illustrate the range in $k_{off}$ values that were obtained from competitive association experiments. From the kinetic binding constants, the respective binding affinity for the H1R could be calculated ($pK_{d,calc}=-\log(k_{off}/k_{on})$). Additionally, the residence time was calculated ($RT = 1/k_{off}$), which is a measure for the length of H1R engagement by the antihistamine. The binding characteristics of all tested ligands are reported in table 2.

Based on the kinetic and equilibrium [$^3$H]mepyramine binding experiments, it is shown that the tested set of antihistamines display >100 fold differences in $K_i$, binding rates and residence times for their binding to the H1R (table 2). The >35-fold differences in residence time at the H1R between levocetirizine ($RT = 140$ min), desloratadine ($RT = 160$ min) and mepyramine ($RT = 3.9$ min) agreed with previously reported data showing a 50 – 140 fold longer residence time for levocetirizine and desloratadine compared to mepyramine ($RT$, mepyramine: $0.8$ – $8$ min)15,64,86. Interestingly, the differences in residence time at the H1R between antihistamines were not reflected by the respective differences in the $pK_i$. For example, levocetirizine has a similar H1R binding affinity but much longer residence time at the H1R as compared to mepyramine (table 2).

The H1R is known to activate Gαq, leading to increased intracellular calcium levels via activation of phospholipase C183,185. Indeed, in HeLa cells, an histamine-induced dose dependent increase in the calcium mobilization can be measured as an increase in the fluorescence of the calcium-sensitive Fluo4 NW dye ($\lambda_{excitation}$ 494 nm and $\lambda_{emission}$ 516 nm). As can be seen in figure 3A, a peak increase in fluorescence

---

Table 2 - Characterization of the binding of antihistamines to the H1R using competitive [$^3$H]mepyramine binding experiments. Average values are shown ± SEM of ≥ 3 experiments.

<table>
<thead>
<tr>
<th>Antihistamine</th>
<th>pKi (37 °C)</th>
<th>pKd,calc (25 °C)</th>
<th>$k_{on}$ (25 °C) (10^6∙min^{-1}∙M^{-1})</th>
<th>$k_{off}$ (25 °C) (min^{-1})</th>
<th>RT* (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mepyramine</td>
<td>8.5 ± 0.0</td>
<td>8.8 ± 0.0</td>
<td>200 ± 50</td>
<td>0.28 ± 0.05</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>levocetirizine</td>
<td>8.1 ± 0.1</td>
<td>8.2 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.008 ± 0.001</td>
<td>140 ± 20</td>
</tr>
<tr>
<td>Doxepin</td>
<td>9.3 ± 0.1</td>
<td>9.1 ± 0.1</td>
<td>70 ± 10</td>
<td>0.06 ± 0.02</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>Olopatadine</td>
<td>9.1 ± 0.0</td>
<td>8.5 ± 0.0</td>
<td>1.8 ± 0.1</td>
<td>0.006 ± 0.000</td>
<td>170 ± 10</td>
</tr>
<tr>
<td>Triprolidine</td>
<td>8.3 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>36 ± 5</td>
<td>0.30 ± 0.04</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Acrivastine</td>
<td>7.2 ± 0.0</td>
<td>7.0 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.065 ± 0.004</td>
<td>15.6 ± 0.9</td>
</tr>
<tr>
<td>desloratadine</td>
<td>9.1 ± 0.1</td>
<td>9.5 ± 0.0</td>
<td>25 ± 12</td>
<td>0.008 ± 0.003</td>
<td>160 ± 50</td>
</tr>
<tr>
<td>VUF14544</td>
<td>7.6 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>100 ± 40</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>VUF14454</td>
<td>8.2 ± 0.1</td>
<td>8.6 ± 0.0</td>
<td>250 ± 90</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>VUF14506</td>
<td>7.7 ± 0.1</td>
<td>7.9 ± 0.0</td>
<td>3.6 ± 0.7</td>
<td>0.05 ± 0.01</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>VUF14493</td>
<td>8.3 ± 0.0</td>
<td>8.4 ± 0.1</td>
<td>300 ± 100</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

*pRT = residence time (1/k_{off})*
Figure 2 - Competitive association of [3H]mepyramine and unlabeled antihistamines to the H1R. An homogenate of HEK293T cells transiently expressing the H1R was incubated with a 2.4 nM concentration [3H]mepyramine in the absence or presence of 1 to 100-fold Ki concentrations of the respective unlabeled antihistamines for the indicated time at 25 °C in 50 mM Na2HPO4/KH2PO4 pH 7.4. Representative graphs of ≥ 3 experiments are shown with mean ± SD of duplicate values (n = 2).

Figure 3 - Histamine-induced intracellular calcium mobilization in HeLa cells endogenously expressing the H1R. (A) Calcium dependent fluorescence was measured over time after injection of increasing histamine concentrations at t=0. Representative graphs are shown of ≥ 3 experiments depicting the mean ± SD of duplicate values (n = 2). (B) For comparison, peak response and area under the curve (AUC) were determined from the kinetic calcium mobilization traces and plotted against the histamine concentration.
is obtained within seconds after stimulation of HeLa cells with histamine. The fluorescence signals then decrease to a steady state level for at least 30 s. The H1R response was quantified both by the histamine-induced peak response or the area under the curve (AUC) observed between 0 and 30 s after stimulating with histamine. Plotting the log concentration histamine versus the relative calcium mobilization leads to overlapping histamine-dose-response curves. Analyzing both curves with a 4-parameter sigmoidal fit resulted in similar pEC50 values of 6.1 ± 0.1 and a Hill slope of 1.1 ± 0.1 (figure 3B).

Pre-incubated long-residence-time antagonists can display insurmountable antagonism of receptor signaling if agonist-induced responses are measured before the establishment of a binding equilibrium between agonist, antagonist and the receptor. The slow dissociation of the antagonist from the receptor will reduce the number of receptors available for agonist-induced activation, which is reflected by a decreased E_max that might be preceded by an initial rightward shift of the dose response curve in the presence of a receptor reserve. It was therefore tested whether insurmountable antagonism of histamine-induced calcium mobilization could be observed when cells were pre-incubated with the antihistamines mepyramine, doxepin, levocetirizine and olopatadine. This series of H1R antagonists reflects a wide range in ligand residence times at the H1R (table 2). HeLa cells were pre-incubated with

Figure 4 - Insurmountable antagonism of histamine-induced calcium mobilization in HeLa cells. HeLa cells were pre-incubated overnight with mepyramine (A), levocetirizine (B), doxepin (C) or olopatadine (D). The next day calcium mobilization was measured upon injection of histamine. Representative graphs are shown of ≥ 3 experiments with mean ± SD of duplicate peak responses (n = 2) normalized to the E_max of histamine.
various concentrations of antagonists overnight and cells were subsequently stimulated with increasing concentrations histamine (figure 4). For all four inhibitors the maximal histamine-induced response in HeLa cells was reduced compared to vehicle treated cells. For mepyramine, the H1R antagonist with a short residence time at the H1R, some decrease in the E_max was observed next to a rightward shift of the histamine dose-response curves (figure 4A). For doxepin (figure 4B), levocetirizine (figure 4C), and olopatadine (figure 4D), a full insurmountable inhibition of the histamine-induced calcium mobilization was observed. The insurmountable antagonism was marked by a clear drop in the E_max for each subsequent increase in antagonist concentration up to a (near) complete block of any histamine-induced effect at the highest concentrations. These data are in line with the expectations for H1R antagonists with a long residence time, but this assay format does not easily allow one to obtain quantitative data on the kinetic properties of the tested H1R antagonists. Moreover, the insurmountable antagonism by doxepin could not be differentiated from the insurmountable antagonism of levocetirizine and olopatadine, despite the 6 – 8 fold difference in residence time that was measured in radioligand binding experiments (table 2).

An alternative assay format was therefore evaluated in which the relative blockade of the receptor was quantified over time in order to discriminate antihistamines with relatively long drug target engagement times (figure 5A). HeLa cells were incubated overnight with a 10 x K_i concentrations of the respective antihistamine. The next day antihistamine dissociation was induced by two rapid wash steps followed by injection of histamine at the indicated time points and calcium mobilization was measured. The resulting calcium-dependent fluorescent-traces (B) were quantified by the peak fluorescence. (C) Peak fluorescence was plotted against the time between antagonist removal and histamine injection. Representative graphs are shown of ≥ 3 experiments, with single measurements per time point. The histamine-induced peak fluorescence was normalized to the average peak fluorescence of buffer pre-treated cells.

Figure 5 - Recovery rate of histamine-induced calcium mobilization upon dissociation of antihistamines from the H1R. (A) HeLa cells were pre-incubated overnight with 10 x K_i concentrations of the respective antihistamine. The next day antihistamine dissociation was induced by two rapid wash steps followed by injection of histamine at the indicated time points and calcium mobilization was measured. The resulting calcium-dependent fluorescent-traces (B) were quantified by the peak fluorescence. (C) Peak fluorescence was plotted against the time between antagonist removal and histamine injection.

Representative graphs are shown of ≥ 3 experiments, with single measurements per time point. The histamine-induced peak fluorescence was normalized to the average peak fluorescence of buffer pre-treated cells.
antagonist, the functional response of histamine was expected to recover with rates that would mirror the $k_{off}$ of the tested H3R antagonists. The receptor recovery over time after washing away unbound antagonist was fitted to a one-phase association model (equation 3; figure 5C). This one-phase exponential model is the same as the dissociation model used for describing drug-target dissociation and was observed to fit the functional response over time reasonably well. As a measure for the absolute time-scale in which recovery rate takes place, the $k_{rec}$ was transformed into the receptor recovery time (RecT) by taking the reciprocal of the $k_{rec}$. This is analogous to the way in which residence time is calculated from the $k_{off}$ values for the dissociation of ligand binding. The histamine induced response after olopatadine incubation did not approach a steady state within the time-frame of the experiment. To make an estimate of the receptor recovery rate for olopatadine, the steady state was constrained to the average histamine induced calcium levels measured in vehicle treated cells. Additionally, after pre-treatment with levocetirizine the functional H3R did not fully recover within the time-frame of the experiment either, but the average steady state recovery (%), reflected by the $Y_{max}$, suggests proper fitting of the curves. The determined $k_{rec}$, RecT and steady state recovery was quantified for all antihistamines (table 3). As can be seen, analyses allowed for good discrimination between antihistamines over a 40-fold range in $k_{rec}$ and RecT. However, full recovery of histamine induced calcium mobilization was already obtained after 1 or 2 data points following a pre-incubation with, e.g., VUF14454 and VUF14493. The kinetic resolution of this assay cannot readily discriminate between antihistamines with very fast recovery rates of the receptor responses.

As an orthogonal assay to measure the histamine-H3R induced response in HeLa cells, dynamic mass redistribution (DMR) was employed. In this assay format cells are grown on resonant waveguide grating (RWG) biosensors. This allows to detect changes in cellular mass distribution (e.g. translocation of proteins, cytoskeleton rearrangements) close to the biosensor surface. DMR was shown to be an effective way to quantify histamine induced signaling mediated by the H3R. Upon stimulating HeLa cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>$k_{rec}$ (min$^{-1}$)</th>
<th>RecT (min)</th>
<th>Steady state recovery (%)</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mepyramine</td>
<td>0.12 ± 0.03</td>
<td>10 ± 2</td>
<td>103 ± 1</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>levocetirizine</td>
<td>0.014 ± 0.003</td>
<td>87 ± 20</td>
<td>104 ± 10</td>
<td>140 ± 20</td>
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<tr>
<td>doxepin</td>
<td>0.032 ± 0.003</td>
<td>32 ± 3</td>
<td>78 ± 5</td>
<td>22 ± 7</td>
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<tr>
<td>olopatadine$^a$</td>
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<td>226 ± 60</td>
<td>NA$^a$</td>
<td>170 ± 10</td>
</tr>
<tr>
<td>triprolidine</td>
<td>0.06 ± 0.01</td>
<td>19 ± 2</td>
<td>92 ± 4</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>acrivastine</td>
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<td>13 ± 2</td>
<td>102 ± 2</td>
<td>15.6 ± 0.9</td>
</tr>
<tr>
<td>desloratadine</td>
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<td>30 ± 1</td>
<td>83 ± 4</td>
<td>160 ± 50</td>
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<tr>
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<td>100 ± 2</td>
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<td>96 ± 5</td>
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</tr>
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<td>0.24 ± 0.06</td>
<td>5 ± 2</td>
<td>98 ± 1</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ Maximal signal (i.e. full recovery) was constrained during analysis, to the average histamine-induced response observed for vehicle treated cells. $^b$ $k_{rec}$ = observed recovery rate of H3R activity. $^c$ RecT = recovery time (1/$k_{rec}$). $^d$ RT = residence time (1/$k_{off}$). Taken from table 2.
with increasing concentrations of histamine, DMR response reached a maximum after approximately 5 min (figure 6A), as previously reported for Gaq mediated signaling by other G protein-coupled receptors (GPCRs) \(^{181}\). After fitting the data with a 4-parameter sigmoidal fit a pEC50 of 6.1 was obtained from both curves with a Hill slope of 0.88 (AUC) and 0.92 (peak response) (figure 6B).

Co-incubating HeLa cells with histamine at a concentration of approximately EC80 and increasing concentrations of antihistamines reduced the DMR response concentration-dependently (figure 7) with the following pIC50 values determined from AUC analysis (mean ± SD of 2 experiments): mepyramine 8.1 ± 0.2, doxepin 8.0 ± 0.4, olopatadine 7.0 ± 0.1 and levocetirizine 6.3 ± 0.2. It was observed for all
inhibitors that the DMR-signal was not fully reduced to background level at the highest concentration. From the full kinetic traces it was observed that the peak response from co-incubated cells stabilized at different levels compared to untreated cells (supplementary data). Incubation of cells with antagonists alone resulted in a negligible DMR response, comparable to the background traces (data not shown).

The RecT of the histamine-induced DMR response after removal of unbound antihistamines was subsequently quantified. HeLa cells were incubated with a 10 x Kᵣ concentration of each of the antagonists to ensure high occupancy of the receptor. On the following day cells were washed with assay buffer (HBSS, 20 mM HEPES and 0.5% (v/v) DMSO). At two different time points (1 h, 2 h) after antagonist washout cells were treated with histamine and the DMR response was recorded over 30 min. The histamine induced DMR response was normalized against the response of DMSO treated cells. Results from two independent experiments are shown, depicted as mean ± SD of six values (n = 6).

Figure 8 - Histamine-induced DMR-response after washout of pre-incubated antihistamines. HeLa cells were incubated overnight in the presence of antagonist (10 x Kᵣ concentration) or DMSO (control). On the following day the antagonist was removed by washing the cells with assay buffer (HBSS, 20 mM HEPES and 0.5% (v/v) DMSO). At two different time points (1 h, 2 h) after antagonist washout cells were treated with histamine and the DMR response was recorded over 30 min. The histamine induced DMR response was normalized against the response of DMSO treated cells. Results from two independent experiments are shown, depicted as mean ± SD of six values (n = 6).
Table 4 - Suppression of the histamine induced DMR-$E_{\text{max}}$ after removal of unbound antihistamines. A dose-dependent DMR response was observed for increasing concentrations histamine as was evaluated 1 h and 2 h after washing away unbound antihistamines. The $EC_{50}$ was determined as before (figure 8) and the $E_{\text{max}}$ value was estimated from the response of $10^{-4.2}$ M histamine and normalized to the $E_{\text{max}}$ of vehicle treated cells. Values represent mean and SD of n=6 measured over two different days.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Suppression of the histamine induced DMR-$E_{\text{max}}$ after removal of unbound antihistamines.</th>
<th></th>
<th>Log $[\text{ligand}]$</th>
<th>1 h after washout</th>
<th>2 h after washout</th>
<th>$k_{\text{rec}}^{a}$</th>
<th>Rec$^{b}$</th>
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<tr>
<td>Vehicle Control</td>
<td>NA</td>
<td>100 ± 2</td>
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<td>5.4 ± 0.1</td>
<td>0.0030 ± 0.0001</td>
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<td>5.8 ± 0.1</td>
<td>0.011 ± 0.001</td>
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<td>0.0024 ± 0.0005</td>
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<td>99 ± 3</td>
<td>103 ± 5</td>
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<td>VUF14506</td>
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<td>87 ± 2</td>
<td>98 ± 2</td>
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<td>6.2 ± 0.1</td>
<td>0.029 ± 0.003</td>
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<td>6.1 ± 0.1</td>
<td>NA</td>
<td>&lt;30</td>
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</tbody>
</table>

$a k_{\text{rec}}$ = observed recovery rate of H1R activity

$b$ Rec$^{T}$ = recovery time ($1/k_{\text{rec}}$)

maximal response of histamine was not suppressed by pre-incubation with the fast-dissociating antihistamine mepyramine (figure 8A). In contrast, the slow-dissociating antihistamines levocetirizine and olopatadine (figure 8C, D), suppressed the $E_{\text{max}}$ up to 2 hours after removing unbound antihistamines. Pre-incubation with the medium-slow-dissociating doxepin suppressed $E_{\text{max}}$ but to a lower extent than olopatadine and levocetirizine (figure 8B).

Insurmountable antagonism was pronounced for olopatadine, levocetirizine, desloratadine, doxepin, acrivastine and VUF14506. Triprolidine also showed insurmountable antagonism, but this was the same at 1 h and 2 h after washing away unbound antagonist, indicating that binding equilibrium had been reached. As such, the observed level of insurmountable antagonism is most likely not caused by prolonged drug-target occupancy, but might be due to a yet unidentified mechanism of action of triprolidine affecting the DMR response in HeLa cells. The receptor recovery rate after preincubation with each of the antihistamines was estimated from the relative effect of a saturating concentration histamine, obtained at 1 and 2 h after removal of unbound antihistamines (see Materials & Methods).

A comparison between the receptor recovery rates and kinetic dissociation rate constants of the set of antihistamines from tables 2 – 4 are depicted in figure 9 and supplementary table 1. The $ln k_{\text{off}}$ of the antihistamines correlated with the $ln k_{\text{rec}}$ of the histamine-induced DMR (not shown) and calcium mobilization upon washout of these antihistamines (figure 9A). Moreover, the $ln k_{\text{rec}}$ as determined by both methods correlated to each other also very well (figure 9B). Although the receptor recovery times and residence times seem to be related, the absolute values of the various metrics are not in the same range. In general, recovery time of the DMR response seem to take longer than for the calcium mobilization (tables 3, 4; supplementary table 1).
4 Discussion

Drug-target residence time has received a lot of attention over the last decade and has now been suggested as a way to better predict drug-efficacy in vivo in an early stage of drug development. It has been postulated that a long drug-target residence time can prolong the in vivo effects of such drugs compared to drugs with a shorter residence time. Moreover, long-residence antagonists might exert insurmountable antagonism of the receptor, which is independent on the concentration agonist and can therefore be much stronger than competitive antagonism. However, whether a long drug-target residence time is beneficial for the in vivo efficacy, is dependent on the specific biology of the drug-target. For example, a long drug-target residence time only leads to a prolonged receptor occupancy in vivo if the receptor turnover is low. Moreover, an insurmountable mode of antagonism is also highly dependent on the cellular context, like e.g. the expression level of the target-receptor. For several GPCR-targets the importance of a prolonged drug-occupancy has however been advocated and the residence time of GPCR ligands are therefore currently explored as a way to increase the therapeutic window.

The determination of drug-target residence time for GPCR-ligands is usually performed by radioligand binding experiments, or more recently, by fluorescent ligand binding experiments. The
residence time of unlabeled ligands can be quantified by measuring their competitive effects on the probe binding-kinetics, using the Motulsky-Mahan model \[113\]. Although the residence time values obtained in these experiments describe the kinetics of GPCR binding at the molecular level, these parameters do not necessarily directly reflect the functional effects in a physiological relevant setting. In order to bridge the gap between GPCR binding studies on membrane preparations and the in vivo evaluation of the action of long residence time GPCR ligands, we evaluated the kinetics of GPCR antagonism for a set of H\(_1\)R antagonists with different ligand binding kinetics in two cell-based assays. The potential success of such a strategy is exemplified for the Neurokinin 1 Receptor, for which it was shown that prolonged antagonism after a washout of unbound antagonists, corresponded with the respective therapeutic window of these antagonists in vivo \[188\]. Moreover, the functional effects observed for the set of H\(_1\)R antagonists were compared to the observed residence times, as determined in \[^3\text{H}\]mepyramine competition binding assays.

In this study, we employed two GPCR assays that can be performed without altering the genetic cellular context and which can be broadly applied to measure GPCR signaling. The calcium mobilization assay has been widely used to measure signaling of GPCRs that couple to the G\(_{\alpha_q/11}\) protein, but also GPCRs that couple to G\(_{\alpha_z}\) subunits can still induce a calcium mobilization response via released \(\beta\gamma\)-subunits \[98\]. The DMR-response relies on the morphological changes of the cell, which has been linked to all canonical GPCR-signaling cascades \[179\]. HeLa cells, endogenously expressing the H\(_1\)R at low, but physiological relevant densities, were used in both assays.

Stimulation of HeLa cells with histamine induced both a robust calcium-mobilization (figure 3), as well as DMR-response (figure 6) in the HeLa cells with similar potencies (pEC\(_{50}\) 6.1 – 6.2). The rise in intracellular calcium concentrations was clearly more rapid (within seconds, figure 3), than the induction of DMR response (within 5-10 min, figure 6), in line with previous reports \[183,186\]. The calcium response was effectively antagonized by the tested H\(_1\)R antagonists, but whereas co-incubation with the short residence time antagonist mepyramine resulted in rightward shifts of the histamine dose response curves with some depression of the maximal response, the long residence time antagonists like doxepin, levocetirizine and olopatadine (figure 4) showed substantial insurmountable antagonism. The slow dissociation of these long residence time antagonists from the H\(_1\)R strongly suggests a temporal insurmountable mode of antagonism by engaging to the orthosteric binding site. An allosteric binding mechanism might theoretically also explain the depression of the maximal histamine response \[132\]. Yet, this seems unlikely in view of the overlapping binding sites of the tested antagonists and histamine, as experimentally determined by x-ray crystallography \[37\], site-directed mutagenesis studies \[15,42,43,52\] and molecular modeling experiments \[37,42,54\]. Recently, insurmountable antagonism of the muscarinic acetylcholine receptor and orexin-2 receptor was used to calculate \(k_{\text{off}}\) rate constants of antagonists \[133,134\]. The observed insurmountable antagonism of the histamine induced calcium response (figure 4) could potentially also be used to discriminate antihistamines based on their relative \(k_{\text{off}}\) at the H\(_1\)R. However, the used hemi-equilibrium model \[132\] is only applicable in the case of a partial insurmountable effect, as was observed for mepyramine at the highest concentrations (figure 4A). Since saturating concentrations doxepin (figure 4B), levocetirizine (figure 4C) and olopatadine (figure 4D) completely block the response to histamine, it is not possible to distinguish these antihistamines by this means.
As alternative assay-format, the functional recovery time (RecT) of the H1R responsiveness was measured after washing away pre-bound antihistamines. In this study, 40-fold differences in the RecT were observed for the tested H1R antihistamines, including 5-fold (DMR) to 8-fold (calcium mobilization) differences in RecT between doxepin, levocetirizine and olopatadine. Hence, the functional recovery time allows the discrimination of antihistamines with a broad range of H1R residence times. For both the calcium mobilization and the DMR-assay, a similar trend in the $k_{rec}$ of the H1R was obtained after pre-incubating with the various antihistamines (figure 9B). Moreover, the measured $k_{rec}$ values nicely correlated with the $k_{off}$ values of the respective H1R antihistamines, as measured by [3H]mepyramine competition binding kinetics (figure 9A). Our data suggests that the drug-target residence time indeed prolongs the antagonism by H1R-antihistamines following washout and hence a rapid and strong reduction of its free concentration ligand. Moreover, our observations are also in line with the prolonged therapeutic window in vivo that has been described for some long residence time antihistamines.

The presented methods effectively determine the length of receptor antagonism using a functional readout that is broadly applicable to GPCR research. The measured non-equilibrium antagonism (RecT), which e.g. reflects the level of insurmountable antagonism (figure 4), is proposed to affect the in vivo efficacy of drugs. Insurmountable antagonism is described to be affected by the transduction coefficient of the functional receptor response and is therefore dependent on the cellular context. Since the described methods are applicable to cell lines endogenously expressing the target receptor, RecT does not only reflect the relative residence time, it has the potential to reflect which antagonists have a long enough residence time to impose prolonged non-equilibrium antagonism. Recently, a different functional readout of the H1R was employed to measure the relative residence time of antihistamines on the H1R. This BRET based β-arrestin2 recruitment assay allows a continuous readout of the H1R response that was stable in time, improving therefore the throughput in which the relative residence time was determined. As a downside, this method requires genetic manipulation of cells by introducing a tagged H1R and tagged β-arrestin2, resulting therefore in e.g. non-physiologically relevant expression levels.

One limitation of the washout experiments is the possibility that unbound antihistamines are not completely removed by the wash step. An incomplete recovery of receptor signaling was e.g. observed after pre-incubation with doxepin and desloratadine (figure 5B, table 3). During the washout, only the unbound ligands are removed and the dissociation of H2R-bound ligands might provide enough ligands after the initial washout to result in residual receptor occupancy. However, this would only happen if the binding affinity (pK_i) is high enough to allow binding at these low ligand concentrations, especially as HeLa cells express low numbers of H2Rs. Interestingly, doxepin and desloratadine have the highest pK_i values of all the antihistamines that were tested in this study (table 2). Another confounding factor in the assay might be the potential partitioning of H1R ligands in the cell or membranes, resulting in ligands accumulation and H1R rebinding after washout of free ligands. Yet, this issue has also been described to affect the determination of residence times via the kinetic competition binding method.

The kinetic information in the DMR washout experiments was low and to prevent over-interpretation of the data, the $k_{rec}$ values were only estimated when there was a significant difference in the histamine induced DMR response between 1 h and 2 h after washout of the antihistamines. As a drawback, this
could exclude compounds which have a very low \(k_{rec}\) value, but this seems unlikely for all ligands that were excluded here, since in all cases a >70% recovery of the histamine induced effect was observed already 1 h after the wash step. For the evaluated H\(_2\)R ligands the calculated \(k_{rec}\) values obtained in the DRM assay correlate well with the values obtained in the orthogonal calcium-mobilization assay, despite the fact that the kinetic resolution of the measurements was much lower.

In conclusion, in this study we describe two orthogonal, functional kinetic assays for GPCR ligands, that can be performed with cells that have endogenous expression of the receptor without the need for any genetic manipulation. The obtained RecT values for H\(_1\)R antagonists correlate well with their H\(_1\)R residence times as determined by radioligand binding. Hence, the use of RecT as a drug metric for the kinetics of antagonism might be valuable to steer the lead optimization of GPCR, as the described generic assays can potentially be used to measure the kinetics of antagonism of ligands for a large variety of GPCRs. Moreover, the assays can additionally be employed with clinically relevant cell lines (e.g. primary cells).

5 Acknowledgments
Marjolein Verhoeven and Susanne I. Kuiper are acknowledged for their help with the calcium mobilization experiments.

Supplementary information

Supplementary Figure 1 - Statistical differences in DMR peak responses. Generalized linear model with Gamma family and identity link (Additive arithmetic mean model) of the form \(Relative\_peak\_response.. ~ treatment\_combi + Experimental\_Repeat\) was build. The outcome variable is highly skewed, but always positive and the effects of treatment-wash-out-time and experimental repetitions are assumed to be additive on the original scale. Multiple comparisons of means with type I error rate of 5% were calculated for comparing the effect of antihistamines in comparison to the DMSO-control for both wash-out-times (A) and the effect of the wash-out-time for each respective antihistamine (B). All computations were performed in R \(^{190}\) and MCPs were computed using the functionality provided by the add-on package multcomp \(^{191}\) R Core Team (2016).
Supplementary Figure 2- DMR traces of agonist/antagonist co-incubation assay. HeLa cells were incubated in parallel with histamine and increasing concentrations of antagonist. An additional control condition was included without antagonist (wo AT) and a condition without either agonist or antagonist (wo AG/wo AT). Area under the curve was determined in the first 20 min after compound addition and plotted against the antagonist concentration to determine an apparent IC$_{50}$ (figure 7 in the manuscript).
Chapter 6 - Probing the histamine H1 receptor binding site to explore ligand binding kinetics

Co-authors: Sebastiaan Kuhne, Albert J. Kooistra, Rick Riemens, Marc C.M. Stroet, Henry F. Vischer, Chris de Graaf, Maikel Wijtmans, Iwan J.P. de Esch, Rob Leurs

Manuscript is in preparation for submission.
Abstract
Analysis of structure-kinetic relationships (SKR) leads to an improved understanding of receptor-ligand interactions. Here, fragment 1 (4-(2-benzylphenoxy)-1-methylpiperidine) was used in different fragment growing approaches to mimic the putative binding mode of the long residence time (RT) ligands olopatadine, acrivastine, and levocetirizine at the histamine H₁ receptor (H₁R). SKR analyses reveal that introduction of a carboxylic acid moiety can increase RT at the H₁R up to 11-fold. Ligand efficiency (LE) decreases upon the introduction of the negatively charged group, whereas kinetic efficiency (KE) increases up to 8.5-fold. Different olopatadine/acrivastine mimics displayed up to 15-fold differences in RT. Interestingly, the levocetirizine mimics appear to be less sensitive to structural changes with only a 3-fold differences in RT. This study illustrates that for the H₁R, there are several ways to increase RT but the different strategies differ significantly in SKR.

Introduction
G protein-coupled receptors (GPCRs) modulate a wide range of cellular activities and form one of the largest protein classes in humans. These membrane bound receptors play an important role in many disease areas and around 30% of the currently marketed drugs target GPCRs. In particular aminergic GPCRs have been extensively studied by structure-activity relationships (SAR) using ligand binding affinity and activity data. More recently, there has been a growing interest in studying ligand binding kinetics as they describe different aspects of the molecular recognition process. Receptor-ligand binding kinetics are considered to have a remarkable impact on the clinical efficacy of drug candidates. Consequently, studying binding kinetics early on in the drug discovery process is considered to provide a new strategy to decrease the late stage attrition rate. Receptor-ligand binding is characterized by the association rate constant (k_{on}) and dissociation rate constant (k_{off}), which together determines the equilibrium dissociation constant K_{d} (=k_{off}/k_{on}). A parameter that describes how long the ligand-receptor complex exists is the target residence time (RT) of a ligand, defined as the reciprocal of the k_{off} value. Structural and molecular determinants of binding affinity (K_{d}) to aminergic GPCRs are well investigated, but the molecular features that govern kinetic binding rate constants are still poorly understood.

In this study, the structure-kinetic relationships (SKR) for a set of ligands that bind the H₁R are investigated. Historically, H₁R ligands have been classified into two groups, i.e., the first- and second-generation (figure 1A). First-generation ligands (e.g., doxepin and tripolidine) most often have two (fused) aromatic rings and a basic amine. Many second-generation ligands possess an additional carboxylic acid moiety, either attached to one of the aromatic rings (olopatadine and acrivastine) or to the basic amine (levocetirizine). The first generation ligand doxepin has been co-crystallized bound to the orthosteric binding site of the H₁R protein (figure 1B). The structure reveals that doxepin binds between the transmembrane domains 3, 4, 5, and 6. The amine moiety of doxepin interacts with the H₁R residue D107, a hallmark feature of all biogenic amine receptors. The aromatic moieties of the ligand are accommodated by two distinct hydrophobic pockets that are formed by (mostly) aromatic residues. In the H₁R crystal structure, a phosphate ion was also identified, binding between the orthosteric pocket and the extracellular vestibule, coordinated by the residues K191, K179, H435, and Y431. It has been suggested by molecular modeling and site-directed mutagenesis studies, that some of the second-generation antihistamines, e.g., levocetirizine, occupy this phosphate pocket with their anionic
The second generation antihistamines olopatadine, acrivastine, and levocetirizine have decreased ligand efficiencies (LE, defined as the binding energy divided by the number of heavy atoms of a ligand \(^{201}\)), but remarkably longer RTs \(^{64,146,173}\) and higher kinetic efficiencies (KE, defined as the RT divided by the number of heavy atoms of a ligand \(^{202}\)) than some of the first generation ligands such as doxepin and triprolidine (figure 1A). Therefore, targeting residues in the phosphate pocket with anionic moieties, such as carboxylic acids represent a promising design strategy to increase RT for H1R receptor ligands.

We have previously identified fragment 1 as a high affinity ligand for the human H1R \(^{39}\). This fragment has a high LE and is devoid of any (stereo)isomerism that could complicate assessing the SAR and SKR. The fragment has been used to systematically probe different binding regions, including the amine-binding region (D1073.32, W4286.48, Y4316.51, I4547.39, and Y4587.43), the upper (Y1083.33, W1584.56, Y4316.51, F4326.52) carboxylate group \(^{43,54,64,146,200}\). The second generation antihistamines olopatadine, acrivastine, and levocetirizine have decreased ligand efficiencies (LE, defined as the binding energy divided by the number of heavy atoms of a ligand \(^{201}\)), but remarkably longer RTs \(^{64,146,173}\) and higher kinetic efficiencies (KE, defined as the RT divided by the number of heavy atoms of a ligand \(^{202}\)) than some of the first generation ligands such as doxepin and triprolidine (figure 1A). Therefore, targeting residues in the phosphate pocket with anionic moieties, such as carboxylic acids represent a promising design strategy to increase RT for H1R receptor ligands.

Figure 1. (A) Antihistamines targeting the H1R and binding affinities (pK\(_a\)) and RT as determined in this study. Ligand efficiency (LE) = (ΔG\(_d\))/N, where ΔG\(_d\) = −R·T·ln(K\(_i\)) and N is the number of heavy atoms (HA, i.e., non-hydrogen atoms), and R = (8.31447215 J K\(^{-1}\) mol\(^{-1}\) and 4184 J = 1 kcal), and T = 298.15 K \(^{201}\). Kinetic efficiency (KE) = RT/N, where RT is the residence time, and N is the number of HAs \(^{202}\). (B) The H1R X-ray structure (PDB-code: 3RZE) with the co-crystallized ligand doxepin (green carbon atoms) and the proposed binding mode of fragment 1 (magenta carbon atoms) \(^{54,200}\). Important binding site residues are represented as ball-and-sticks with light gray carbon atoms. Nitrogen, oxygen, and hydrogen atoms are colored blue, red, and cyan, respectively. Polar hydrogen atoms of the ligands are shown, but are absent for the binding site residues. The H1R binding site surface is shown and colored to designate the four different regions of the binding site, i.e., the amine binding region, the lower aromatic binding region, the upper aromatic binding region, and the phosphate binding region. The UniProt numbers (first) and the Ballesteros-Weinstein numbering (second in superscript) are reported for each residue throughout this manuscript \(^{40}\). (C) Schematic 2D representation of fragment 1, binding affinity and kinetic parameters (k\(_{on}\), k\(_{off}\), RT) as determined in this study, and the three different design strategies to probe the H1R SKR.
and F435.55) and lower (F199.47, F424.64 and W428.48) aromatic region, and was also used to explore the role of solvation in the H2R binding site on ligand binding interactions. In this study well-defined binding hot spots were identified and it was shown that fragment 1 was targeting those hot spots optimally 39. The aim of the current study is to build upon this molecular understanding of H1R-ligand interaction and to gain understanding of H2R SKR. To this end, hit fragment 1 (RT = 2.0 ± 0.1 min) was used as a scaffold to design and synthesize a set of derivatives to explore H2R binding kinetics. Ligands interrogating distinct binding regions of the H1R receptor (figure 1B, C) were designed and synthesized. Three different fragment growing and modification strategies were employed to modulate the binding kinetics by introducing carboxylic acid moieties at 1) the 5-position of the aromatic ring, 2) the 6-position of the aromatic ring, both mimicking olopatadine and acrivastine, and 3) the basic amine, mimicking levocetirizine (figure 1C).

Results
Design and synthesis of tool compounds to investigate H1R SAR and SKR
To modulate H1R binding kinetics and hence investigate the SKR, we designed and synthesized a diverse set of 15 analogs of 1 (figure 2A). Fragment 1 is believed to target the same aromatic regions and amine-binding region as doxepin in the H1R crystal structure, but not the anionic phosphate pocket. The designed ligands mimic the binding mode of the long RT H1R reference compounds olopatadine, acrivastine, and levocetirizine as suggested by molecular modeling studies (figure 2B–G).

Binding mode predictions. Binding modes of ligand 2b, 3b, 2c, 3c, 4c, olopatadine, acrivastine, and levocetirizine were obtained using PLANTS 203 docking in the doxepin-bound H1R crystal structure (PDB: 3RZE)200 (figure 2B–G). As designed, the poses of ligand 2b and 3b have a similar positioning of the acetic acid moiety compared to the docking pose of olopatadine (figure 2B,E), justifying growing from both the 5- and 6-position of the aromatic ring of fragment 1. The docking poses of 2c and 3c show a slight shift of the acrylic acid moiety compared to the docking pose of acrivastine (figure 2C,F). The docking pose of 4c shows that the carboxylic acid moiety can be positioned in a similar position as levocetirizine (figure 2D,G), justifying growing from the basic nitrogen atom of fragment 1. Furthermore, the docking pose of levocetirizine is in line with the binding mode described by Kooistra et al 54.

Probing the olopatadine and acrivastine binding region. To mimic the binding mode of olopatadine and acrivastine, zwitterions 2b/3b bearing an acetic acid moiety and 2c/3c bearing an acrylic acid moiety (figure 2A–C,E,F) were synthesized (Scheme 1 and 2). In addition, compounds with alternative spacer length (2a/3a) and small non-anionic polar groups (2d, 2e, 3d) (figure 2A) were synthesized (Scheme 1 and 2). The compounds probing the olopatadine and acrivastine binding region, growing from the 5-position (2a–e), were synthesized as outlined in Scheme 1. Friedel-Craft alkylation of benzene by commercially available benzyl bromide 4 gave intermediate 5 (Scheme 1). Demethylation using BBr3 gave phenol 6 that was subsequently used in a Mitsunobu reaction to afford tertiary amine 7. Hydrolysis of 7 gave zwitterion 2a. Ester 5 was reduced with LAH to benzyl alcohol 8, and subsequent oxidation with MnO2 yielded aldehyde 9. Demethylation using sodium ethanethiolate afforded phenol 10, and subsequent reaction with tert-butyl 4-((methylsulfonyl)oxy)piperidine-1-carboxylate yielded aldehyde 11 39. A Wittig reaction of aldehyde 11 with (methoxymethyl)triphenylphosphonium chloride gave after column chromatography an inseparable mixture of E/Z isomers of enol ether 12. Subsequent reduction of
the Boc group in 12 with LAH gave tertiary amine 13 as an inseparable mixture of E/Z isomers. Enol ether deprotection using HCl gave aldehyde 14, which owing to the presence of the aryl-acetaldehyde moiety, was immediately subjected to an in situ Pinnick oxidation to afford after reversed-phase column chromatography zwitterion 2b. Aldehyde 11 was also used to synthesize benzyl alcohol 2d applying LAH to reduce both the aldehyde and Boc-group to an alcohol and methyl group, respectively. Oxidation of benzyl alcohol 2d with MnO2 gave crude aldehyde 15, which was subjected to a tandem Wittig reaction and saponification reaction to afford zwitterion 2c. To further investigate the role of the 5-position, a methoxy substituent was introduced (2e). Treatment of ketone 16 with Et3SiH and TFA gave phenol 17. Coupling with tert-butyl 4-((methylsulfonyl)oxy)piperidine-1-carboxylate and subsequent reduction of the Boc group afforded 2e 39.

**Figure 2.** (A) Fragment growing of hit fragment 1, mimicking the H1R reference compounds olopatadine, acrivastine, and levocetirizine, to study the H1R SKR. Proposed binding mode of the H1R reference compounds (B) olopatadine (gray carbon atoms), (C) acrivastine (gray carbon atoms), and (D) levocetirizine (gray carbon atoms) in the H1R. Proposed binding mode of the (E) olopatadine mimics 2b (blue carbon atoms) and 3b (orange carbon atoms), (F) acrivastine mimics 2c (blue carbon atoms) and 3c (orange carbon atoms), and (G) levocetirizine mimic 4c (green carbon atoms) in the H1R. Binding modes were obtained by docking the compounds in the H1R crystal structure (pdb: 3RZE) 200. Rendering and color-coding are the same as in figure 1.
Scheme 1 - Synthesis of ligands to probe the olopatadine and acrivastine binding region: growing from the 5-position of the aromatic ring. Reagents and conditions: (a) AlCl3, benzene, rt, 165 min, 73%. (b) BBr3, DCM, 0 °C to rt, 3 h, 70%. (c) 1-methylpiperidin-4-ol, PPh3, DEAD (40 wt% in toluene), THF, 0 °C to rt, 24 h, 31%. (d) 1.0 M NaOH (aq), MeOH, reflux, 33%. (e) 1.0 M LAH in THF, THF, −78 °C to rt, 2.5 h, 96%. (f) MnO2, DCM, rt, 42 h, 93%. (g) EtSNa, DMF, 120 °C, 3 h, 61%. (h) tert-butyl 4-((methylsulfonyl)oxy)piperidine-1-carboxylate, Cs2CO3, DMF, 60 °C, 16 h, 74%. (i) t-BuOK, (methoxymethyl)triphenylphosphonium chloride, THF, rt, 6 h, 66%. (j) 1.0 M LAH in THF, THF, 0–45 °C, 8.5 h, 92%. (k) 1.0 M HCl in dioxane, H2O, rt, 1 h. (l) NaH2PO4·H2O, 2-methylbut-2-ene, NaClO2, t-BuOH, H2O, rt, 1 h, 6% over two steps. (m) 1.0 M LAH in THF, THF, 0 °C to reflux, 1.5 h, 38%. (n) MnO2, DCM, rt, 16 h. (o) Methyl (triphenylphosphoranylidene)acetate, toluene, 0 °C to rt, 48 h. (p) 2.0 M NaOH (aq), MeOH, reflux, 6 h, 7% over three steps. (q) Et3SiH, TFA, DCM, rt, 17 h, 52%. (r) tert-butyl 4-((methylsulfonyl)oxy)piperidine-1-carboxylate, Cs2CO3, DMF, 65 °C, 22 h. (s) 1.0 M LAH in THF, THF, 0–50 °C, 4 h, 54% over two steps. Compound 2d was converted to a hemifumaric acid salt and compound 2e to a fumaric acid salt.
The compounds probing the olopatadine and acrivastine binding region, growing from the 6-position (3a–d), were synthesized as outlined in Scheme 2. Ortho-formylation of commercially available phenol 18 using paraformaldehyde, MgCl₂ and TEA gave aldehyde 19 (Scheme 2). Compound 19 was reacted with tert-butyl 4-((methylsulfonyl)oxy)piperidine-1-carboxylate to afford aldehyde 20. The aldehyde and Boc group in compound 20 were reduced with LAH to an alcohol and methyl group, respectively, to give benzyl alcohol 3d. Oxidation of 3d with MnO₂ yielded aldehyde 21, a versatile building block. It was used in a Pinnick oxidation to give zwitterion 3a and in a Wittig reaction with (methoxymethyl)triphenylphosphonium chloride to give enol ether 22 as an inseparable mixture of E/Z isomers after column chromatography. The enol ether mixture was deprotected with HCl to give aldehyde 23 and subsequent in situ Pinnick oxidation furnished after reversed-phase chromatography zwitterion 3b. Aldehyde 21 was also used for a Wittig reaction with methyl (triphenylphosphoranylidene)acetate to obtain zwitterion 3c.

Probing the levocetirizine binding region. To mimic the binding mode of levocetirizine, zwitterions 4a–f with an increasing number of methylene units (figure 2A,C,G) were synthesized (Scheme 3). Compound 1
was alkylated on the amine with the corresponding bromo esters to afford intermediates 25a–f. Subsequent ester hydrolysis yielded the desired zwitterions 4a–f, which were isolated as the HCl salts.

**Pharmacological evaluation** Equilibrium dissociation constants (Kᵢ) of the compounds (tables 1 and 2) for the human H₁R were determined using [³H]-mepyramine radioligand-binding studies on HEK293T cell homogenates that transiently express the H₁R. The affinity of [³H]-mepyramine for the H₁R was determined by incubating increasing concentrations radioligand with cell homogenate in the absence or presence of 10⁻⁵ M mianserin, reflecting the total (red) and non-specific binding (black) of radioligand, respectively (figure 3A). The affinity (pKₐ) and Bₘₐₓ values of specific [³H]-mepyramine binding were determined to be 8.6 ± 0.1 and 72 ± 12 pmol/mg protein. Kinetic binding rate constants were determined by incubating four different concentrations [³H]-mepyramine with cell homogenate for increasing incubation times (figure 3B). From the observed association binding the kinetic binding rate constants were determined for [³H]-mepyramine (kₐ: 110 ± 6 10⁶ M⁻¹·min⁻¹; kₐff: 0.22 ± 0.01 min⁻¹). The calculated affinity of [³H]-mepyramine from its binding rate constants (pKₐ,calc) was 8.7 ± 0.0, which agreed well with the affinity determined in saturation binding experiments. Competition binding experiments were performed by incubating ± 3 nM [³H]-mepyramine with increasing concentration unlabeled ligand for 4 h and is depicted for a set of representative ligands in figure 3C. Concentrations that displaced half maximal binding of [³H]-mepyramine were used to calculate the binding affinity (pKᵢ) of the unlabeled ligands using the Cheng-Prusoff equation (tables 1 and 2). Finally, association binding of ± 3 nM [³H]-mepyramine was measured in competition with a concentration of ± 10 times the Kᵢ of unlabeled test ligand (figure 3D). For the unlabeled ligands levocetirizine (blue), 2c (green) and 4c (brown), but not for 1 (red), an overshoot in [³H]-mepyramine binding for early time points is clearly observed. This overshoot reflects the relatively low kₐff of levocetirizine, 2c and 4c compared to [³H]-mepyramine, explaining the observed kinetic advantage of the radioligand for binding to the H₁R. Analysis of these curves using the Motulsky and Mahan model indeed showed that levocetirizine has the lowest dissociation rate constant (0.011 ± 0.001 min⁻¹), followed by 4c (0.045 ± 0.004 min⁻¹) and 2c (0.055 ± 0.003 min⁻¹) and a high kinetic dissociation rate constant of 1 (0.50 ± 0.03 min⁻¹). Kinetic binding rate constants and binding affinities for these and other unlabeled ligands are shown in tables 1 and 2.
SKR of ligands targeting the olopatadine and acrivastine binding regions The zwitterionic molecules 2a–c and 3a–c show a large decrease in binding affinity (and hence lower LE) and association rate constant compared to amine 1 (table 1). This observation is in line with the lower affinities and association rate constants of the zwitterions olopatadine and acrivastine compared to their first generation analogs doxepin and tripolidine, respectively (table 1). Zwitterions 2a–c and 3a–c show a wide range of RTs (and KEs) at the H1R, ranging from 1.2 min for 2a to 18 min for 2c. Thus, a 9-fold increase in RT was achieved for compound 2c compared with first generation scaffold 1. The RT of olopatadine increases 7-fold compared to doxepin, whereas a 5-fold increase in RT is observed for acrivastine compared to tripolidine. Benzyl alcohol 2d has a binding affinity similar to 1, resulting from a 2-fold decrease of the association rate and 2-fold increase in RT. On the other hand, benzyl alcohol 3d has a 10-fold lower binding affinity. Methoxy analog 2e displays a decrease in binding affinity and association kinetics, but has a similar RT compared to 1. The calculated pKd values (i.e., based on the kinetic rate constants; figure 3D) for the tested ligands correspond well with the affinity determined by competition binding experiments (pKi; figure 3C).

Figure 3 - Characterization of ligand binding to a homogenate of HEK293T cells expressing the H1R using [3H]-mepyramine. (A) A HEK293T cell homogenate expressing the H1R was incubated with increasing concentrations [3H]-mepyramine for 4 h in the absence (red) or presence (black) of 10 μM mianserin. (B) Cell homogenate was incubated with 2.9 nM (blue), 1.3 nM (red), 0.5 nM (green) and 0.3 nM (brown) of [3H]-mepyramine for various incubation times. (C) Cell homogenate was incubated with a single concentration [3H]-mepyramine with increasing concentrations unlabeled ligand for 4 h. Ligands are depicted by the red curve (1), green curve (2c), brown curve (4c) and blue curve (levocetirizine). (D) A single concentration [3H]-mepyramine was co-incubated for various times with cell homogenate in the absence (black) or presence of an approximate concentration of 10 times the respective Ki of these unlabeled ligands (red curve (1), green curve (2c), brown curve (4c) and blue curve (levocetirizine)). Representative graphs are shown of N≥3 experiments with mean and SD of triplicate values (A, C) or duplicate values (B, D).
Table 1 - Pharmacological evaluation of the H1R reference compounds olopatadine, doxepin, acrivastine, triprolidine and ligands probing the olopatadine and acrivastine binding region. Data was obtained from competition binding experiments to the H1R using [3H]mepyramine as radioligand. The $k_{on}$ and $k_{off}$ were obtained from the competitive binding of the radioligand over time, and pK_d and RT values were calculated for individual experiments from these binding rate constants. The pKa values were obtained from radioligand displacement curves after 4 h incubation with the radioligand. Data represents the mean and SEM of N≥3. a LE calculated as described in figure 1. b KE calculated as described in figure 1.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$pK_i$</th>
<th>$pK_{d,calc}$</th>
<th>LE</th>
<th>$k_{on}$</th>
<th>$k_{off}$</th>
<th>RT</th>
<th>KE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxepin</td>
<td>9.6 ± 0.1</td>
<td>9.2 ± 0.1</td>
<td>0.62</td>
<td>64 ± 9</td>
<td>0.043 ± 0.007</td>
<td>26 ± 5</td>
<td>1.24</td>
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<tr>
<td>Olopatadine</td>
<td>8.3 ± 0.2</td>
<td>8.5 ± 0.1</td>
<td>0.45</td>
<td>1.9 ± 0.2</td>
<td>0.006 ± 0.001</td>
<td>190 ± 50</td>
<td>7.6</td>
</tr>
<tr>
<td>triprolidine</td>
<td>8.3 ± 0.2</td>
<td>8.0 ± 0.1</td>
<td>0.50</td>
<td>36 ± 6</td>
<td>0.32 ± 0.03</td>
<td>3.2 ± 0.3</td>
<td>0.15</td>
</tr>
<tr>
<td>acrivastine</td>
<td>7.7 ± 0.2</td>
<td>6.9 ± 0.0</td>
<td>0.44</td>
<td>0.58 ± 0.05</td>
<td>0.067 ± 0.006</td>
<td>15 ± 1</td>
<td>0.58</td>
</tr>
<tr>
<td>2a</td>
<td>&lt;5</td>
<td>4.8 ± 0.1</td>
<td>0.28</td>
<td>0.06 ± 0.02</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>2b</td>
<td>6.9 ± 0.1</td>
<td>6.8 ± 0.2</td>
<td>0.38</td>
<td>1.3 ± 0.5</td>
<td>0.17 ± 0.02</td>
<td>6.0 ± 0.5</td>
<td>0.24</td>
</tr>
<tr>
<td>2c</td>
<td>6.5 ± 0.1</td>
<td>7.0 ± 0.0</td>
<td>0.34</td>
<td>0.62 ± 0.06</td>
<td>0.055 ± 0.003</td>
<td>18 ± 1</td>
<td>0.69</td>
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**SKR probing the levocetirizine binding region.** Upon introduction of a carboxylic acid functionality (4a–f, table 2) to ligand 1, the binding affinity (and hence the LE) was decreased as a consequence of a decrease in the association rate constants (40 to 65-fold), which was larger than the decrease in the dissociation rate constants. Concomitantly with a decreased $k_{\text{off}}$, the RT (and hence KE) was increased 4.5 to 11-fold. Table 2 shows that the calculated $pK_d$ for all ligands (4a–f) correspond well with their equilibrium binding affinity values ($pK_i$).

Table 2 - Pharmacological evaluation of the H1R reference compound levocetirizine and ligands probing the levocetirizine binding region. Data was obtained from competitive binding experiments to the H1R using [3H]-mepyramine as radioligand. The $k_{\text{on}}$ and $k_{\text{off}}$ were obtained from the competitive binding of the radioligand over time, and $pK_d$ and RT values were calculated for individual experiments from these binding rate constants. The $pK_d$ values were obtained from radioligand displacement curves after a 4 h incubation with the radioligand. Data represents the mean and SEM of N≥3. $^a$ LE calculated as described in figure 1. $^b$ KE calculated as described in figure 1.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$pK_i$</th>
<th>$pK_{d,\text{calc}}$</th>
<th>LE$^a$</th>
<th>$k_{\text{on}}$ ($10^6$ M$^{-1}$ min$^{-1}$)</th>
<th>$k_{\text{off}}$ (min$^{-1}$)</th>
<th>RT (min)</th>
<th>KE$^b$</th>
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<tr>
<td>levocetirizine</td>
<td>8.1 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>0.41</td>
<td>1.4 ± 0.2</td>
<td>0.011 ± 0.001</td>
<td>92 ± 7</td>
<td>3.41</td>
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<tr>
<td>4a</td>
<td>8.6 ± 0.1</td>
<td>8.5 ± 0.0</td>
<td>0.5</td>
<td>160 ± 30</td>
<td>0.50 ± 0.03</td>
<td>2.0 ± 0.1</td>
<td>0.10</td>
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<tr>
<td>4b</td>
<td>7.5 ± 0.1</td>
<td>7.4 ± 0.0</td>
<td>0.41</td>
<td>2.9 ± 0.3</td>
<td>0.11 ± 0.02</td>
<td>9 ± 1</td>
<td>0.36</td>
</tr>
<tr>
<td>4c</td>
<td>7.6 ± 0.1</td>
<td>7.5 ± 0.0</td>
<td>0.40</td>
<td>2.9 ± 0.3</td>
<td>0.10 ± 0.02</td>
<td>10 ± 1</td>
<td>0.38</td>
</tr>
<tr>
<td>4d</td>
<td>7.8 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>0.39</td>
<td>3.2 ± 0.3</td>
<td>0.045 ± 0.004</td>
<td>23 ± 2</td>
<td>0.85</td>
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<tr>
<td>4e</td>
<td>7.8 ± 0.0</td>
<td>7.7 ± 0.1</td>
<td>0.38</td>
<td>4 ± 1</td>
<td>0.060 ± 0.006</td>
<td>17 ± 2</td>
<td>0.61</td>
</tr>
<tr>
<td>4f</td>
<td>7.7 ± 0.2</td>
<td>7.5 ± 0.0</td>
<td>0.36</td>
<td>2.5 ± 0.2</td>
<td>0.076 ± 0.007</td>
<td>13 ± 1</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>7.6 ± 0.1</td>
<td>7.5 ± 0.0</td>
<td>0.35</td>
<td>2.37 ± 0.08</td>
<td>0.069 ± 0.005</td>
<td>15 ± 1</td>
<td>0.50</td>
</tr>
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</table>
A set of 15 analogs based on fragment 1 (figure 2A) has been used to study and modulate H1R binding kinetics, by mimicking the binding mode of the long RT binders olopatadine, acrivastine, and levocetirizine (figure 2B–G). The ligands 2b–e, 3a–d, 4a–f, and the H1R reference compounds olopatadine, acrivastine, and levocetirizine are plotted in figure 4 to illustrate their SKR. Probing H1R binding kinetics by mimicking the olopatadine and acrivastine binding mode. A carboxylic acid moiety was introduced directly on the phenyl ring (2a and 3a), with a methylene linker in order to mimic the binding mode of the H1R reference compound olopatadine (2b and 3b), and with an ethylene linker to mimic the binding mode of acrivastine (2c and 3c). This results in compounds that have a similar or higher RT at the H1R ranging from 1.2 min to 18 min compared to ligand 1. The compounds with the longest RTs at the H1R are 2c (RT: 18 ± 1 min) and 3b (RT: 12.1 ± 0.5), mimicking the binding mode of acrivastine and olopatadine, respectively. This 6–9 fold increase in RT relative to 1 (RT: 2.0 min) is similar to the relative increase in RTs of the second generation antihistamines olopatadine and acrivastine compared to their first generation analogs without a carboxylic acid moiety, i.e. doxepin and triprolidine. Mimicking the acrivastine binding mode, i.e., 2c, results in a similar RT and KE to acrivastine. However, the ligands that mimic the binding mode of olopatadine (i.e., 2b and 3b) do not reach the RT and KE of olopatadine. We speculate that the remarkably long RT and high KE of doxepin and olopatadine is (partially) related to their rigid tricyclic core structure. The current set of compounds also showed that ligand-binding kinetics optimization is more delicate in the aromatic region, in line with the findings of...
Kuhne et al.\textsuperscript{39} which showed that optimal positioning of the aromatic rings is essential for binding the H\(_1\)R with high affinity. The proposed binding modes of 2a and 3a suggest that the absence of a linker results in compounds which cannot simultaneously bind favorably in the upper aromatic region and interact with the phosphate pocket residues, and hence show a lower binding affinity and RT. The vector to grow from (i.e., the 5- or 6-position) is also important for binding kinetics as exemplified by the compounds 2b and 3b as well as 2c and 3c having different RTs and binding affinities. Furthermore, polar non-anionic groups (2d, 2e, and 3d) growing from the aromatic ring hardly changed RT at the H\(_1\)R (<2 fold).

\textit{Probing H\(_2\)R binding kinetics by mimicking the levocetirizine binding mode.} Compounds 4a–f were designed to mimic the levocetirizine binding mode. By introducing carboxylates linked with spacers of various lengths to the amine group of 1, the acidic moiety is able to interact with residues within the phosphate pocket (K191\textsuperscript{5.39}, K179\textsuperscript{4.49} and H435\textsuperscript{7.35}) of the H\(_1\)R. Interestingly, all ligands (4a–f) have comparable binding kinetics as illustrated in figure 4, with less than a 3-fold difference in their RT at the H\(_1\)R. Apparently, when decorating this vector of the fragment, having a carboxylate results in low binding rate constants but the position of the carboxylate is not as strict (compare figure 4A and 4B). This could potentially be ascribed to protein-ligand interactions that are made during ligand egress from the orthosteric binding site.

On a structural level the relatively flat SKR for the levocetirizine mimics (figure 4B) could be explained by the more accessible channel towards the phosphate pocket growing from the basic nitrogen (figure 2G), compared to growing in the more narrow upper aromatic pocket (figure 2E,F), which results in a pronounced SKR for the olopatadine and acrivastine mimics. This is further supported by a previous study showing that analogs of 1 require optimal placement of the aromatic rings in the aromatic regions for a high binding affinity\textsuperscript{39}.

Ligand 4c shows the highest RT at the H\(_1\)R (23 min), mimics the sidechain of the levocetirizine, and has a similar proposed binding mode as levocetirizine (figure 2D,G). However, the low \(k_{\text{off}}\) and hence high RT and KE of levocetirizine binding to the H\(_2\)R was not achieved (figure 4). Gillard et al.\textsuperscript{19} showed that analogs of levocetirizine with an alcohol or ester group instead of the carboxylic acid moiety also have an RT exceeding that of 1 by >5-fold, indicating that the core scaffold of levocetirizine itself is partially responsible for its long RT.

For all but one (2a) of the carboxylic acid moiety-containing compounds, the dissociation rate is decreased, and hence the RT at the H\(_1\)R is increased (figure 4). By a thermodynamic interpretation of a one-step binding reaction between ligand and receptor, the \(k_{\text{on}}\) and \(k_{\text{off}}\) are dependent on the difference in free energy between the transition state with that of the unbound (\(\Delta G_{\text{on}}^{\dagger}\)) and the bound state (\(\Delta G_{\text{off}}^{\dagger}\)), respectively. The \(\Delta G_{\text{off}}^{\dagger}\) is therefore dependent on the \(\Delta G_{\text{on}}^{\dagger}\) together with the difference in free energy between the bound and unbound state (\(\Delta G_{\text{d}}\))\textsuperscript{62}. Interestingly, all analogs bearing a carboxylic acid moiety have a reduced affinity for the H\(_1\)R compared to fragment 1, implying a smaller \(\Delta G_{\text{d}}\). The prolonged RT at the H\(_1\)R, a consequence of the increased \(\Delta G_{\text{off}}^{\dagger}\), is therefore likely caused by the increased \(\Delta G_{\text{on}}^{\dagger}\). Indeed, upon substituting 1 with a carboxylic acid moiety this resulted without exception in a smaller \(k_{\text{on}}\) for the H\(_1\)R therefore suggesting a higher free energy of the transition state\textsuperscript{62,206}. Analogs containing an alcohol (2d and 3d) or a methoxy group (2e) instead of a carboxyl acid moiety had much smaller differences in
their $k_{on}$ values compared to 1. This further exemplifies that the negatively charged moieties could destabilize the transition state consequently leading to a longer RT of ligand binding to the H1R. This could for instance be rationalized as an increase in the required desolvation energy for ligand-receptor binding. Desolvation was for example rate limiting in silico for ligand association to the β2-adrenergic receptor. Alternatively, the increased transition state energy could be explained, e.g., by (repulsive) interactions and conformational changes along the access/egress pathway.

This study furthermore indicates that optimal placement of the ligand in the different binding regions is essential to maximize the H1R binding affinity as well as the RT of H1R ligands. Based on equilibrium binding affinity values the zwitterions would be deprioritized in a SAR-based ligand optimization, whereas based on their binding kinetics the zwitterions would be prioritized. This study clearly shows that LE is not a preferred metric to describe the quality of the zwitterionic compounds for the zwitterionic compounds (2a–c, 3a–c and 4a–f). Our study emphasizes the importance to measure binding kinetics early on in a drug discovery program and to use the KE metric as an additional selection criterion for subsequent hit and lead optimization, next to the standard equilibrium binding affinity determinations and their corresponding LE values.

In conclusion, a variety of ligands mimicking the well-established H1R antihistamines olopatadine, acrivastine, and levocetirizine were designed and synthesized. This resulted in 15 compounds of which 12 with longer RTs and larger KEs (e.g., 2c and 4c) for binding H1R than hit fragment 1. Introducing carboxylic acid moieties at the 5- and 6-position of the aromatic ring (2a–e and 3a–d), and hence mimicking the binding mode of olopatadine and acrivastine, resulted in large differences in RT (up to 15-fold) for binding to the H1R. However, for compounds that mimic levocetirizine by growing a carboxylate group from the basic nitrogen atom (4a–f), the differences in RTs at the H1R were less pronounced (up to 3-fold). Ligand 4c, with four methylene units between the carboxylic acid and the basic nitrogen atom, has the highest RT (23 min) for binding to H1R, which is more than a 10-fold increase compared to hit fragment 1. The obtained SKR shows that the introduction of a carboxylic acid moiety was sufficient to reduce the $k_{on}$, which often corresponded with a lower LE but higher KE. However, for the ligands containing a carboxylic acid moiety on the aromatic ring, an optimal KE was only achieved when a high LE was maintained. This could only be accomplished by putting a carboxylic acid group in exactly the right position of the aromatic ring as exemplified by the scattered SKR for the olopatadine and acrivastine mimics (figure 4A). Interestingly, the introduction of a carboxylic acid group at the basic amine of 1 resulted in less variation in pKd and RT at the H1R. The latter is illustrated by the ligands 4a–f (figure 4B), which have a different number of methylene units between the basic nitrogen atom and the carboxylic acid. A structure-based explanation for the observed difference in SKR between the levocetirizine mimics vs the olopatadine and acrivastine mimics is that the latter mimics need to fit in a relatively narrow pocket, whereas the pocket to accommodate the levocetirizine mimics is larger. This study illustrates that carefully designed tool compounds growing from different vectors, and using a variety of linkers, can be used to investigate the structural requirements for binding to GPCRs with an elongated RT, as exemplified here for the H1R.

Method
Pharmacology.

General information. Cell culture medium DMEM (Dulbecco's Modified Eagle Medium) and 1× trypsin solution were obtained from Sigma-Aldrich (St. Louis, USA). Medium supplements fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GE healthcare (Uppsala, Sweden). Transfection reagent linear 25 kDa polyethylenimine (PEI) was bought from Polysciences (Warrington, USA). The Branson sonifier 250 was obtained from Branson Ultrasonics (Danbury, USA). BCA protein assay kit was purchased from Thermo scientific (Waltham, USA). Radioligand binding materials and equipment: [3H]-mepyramine, GF/C plates, Microscint-O, the Cell Harvester and the Wallac microbeta were obtained from Perkin Elmer (Waltham, USA). The H1R reference compounds were obtained from commercial suppliers: doxepin hydrochloride (Tocris Bioscience, E/Z mixture with a ~85:15 ratio), triprolidine hydrochloride monohydrate, (Tocris Bioscience), Olopatadine hydrochloride (BOC Sciences), acrivastine (BOC Sciences) and levocetirizine dihydrochloride (Biotrend).

DNA constructs. Mammalian expression vector pcDEF3 containing the N-terminal HA-tagged human H1R (GenBank entry AB041380.1), as previously described 147.

Cell culture and membrane. HEK293T cells were cultured in DMEM medium supplemented with 10% FBS and 1× penicillin/streptomycin at 37 °C and 5% CO2. Cells were lifted using a 1× trypsin solution after which 2·10⁶ cells were seeded in a 10 cm² dish. Cells were left overnight and then transfected using a mixture containing 150 mM NaCl, 30 µg linear 25 kDa PEI and 5 µg pcDEF3 encoding the human H1R as previously described 149. After two days, cells were collected and washed with a PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) and consecutively stored as dry pellets at −20 °C until further experimentation.

Radioligand binding experiments. Saturation binding. A frozen cell pellet expressing the human H1R was reconstituted in 5.5 mL binding buffer (50 mM Na₂HPO₄/KH₂PO₄, pH 7.4) and homogenized using a Branson sonifier 250 set to a constant duty cycle and a microtip limit of 2, until a homogeneous suspension was obtained (4 – 8 s). Protein content of membrane homogenates was determined using a BCA protein assay. Increasing concentrations [3H]-mepyramine (0 nM – 230 nM) were then incubated with 0.5 µg – 10 µg membrane homogenate, depending on the expressed receptor, for 4 h at 25 °C under gentle agitation. This was done both in the absence and presence of 10⁻⁵ M mianserin, which blocks the specific binding of [3H]-mepyramine to human H1R. Incubation was terminated by three rapid washing steps using the Cell Harvester with ice-cold Tris-HCl solution (50 mM Trizma base set to pH 7.4 using HCl) over GF/C plates that were coated with 0.5 % branched PEI for at least 30 min. Filter plates were then dried in a stove at 52 °C before supplementing microscint-O to the filter plates and measuring radioactivity using a Wallac Microbeta counter. Membrane preparation, termination of the binding reaction and quantifying radioactivity as described here, were performed for all binding experiments. Total binding and non-specific binding (i.e., in the presence of mianserin) was analyzed by nonlinear regression using equation 1 (non-specific binding) and 2 (total binding).

Competition binding. Cell homogenate was incubated with a single concentration 2.5 nM – 5.5 nM [3H]-mepyramine together with increasing concentration compounds (10⁻³ M – 10⁻¹¹ M) for 4 h at 25 °C under
gentle agitation. Binding was analyzed by nonlinear regression using equation 3 to obtain concentrations that displaced 50% of the radioligand (IC₅₀). These values were converted to Ki values using the Cheng-Prusoff equation ¹¹⁵.

**Association binding.** Cell homogenate was incubated with four concentrations between 0.2 nM – 4 nM [³H]-mepyramine at 25 °C under gentle agitation for (0–41 min) in the absence or presence of 10⁻⁵ M mianserin. Binding in the presence of mianserin was assumed to be the baseline (i.e., t=0 min). Binding over time was baseline corrected and then analyzed by nonlinear regression using equation 4.

**Competition association binding.** Membrane homogenate was incubated with a single concentration 2.5 nM – 5.5 nM [³H]-mepyramine at 25 °C under gentle agitation for (0–81 min) with either a ±10 times Ki equipotent concentration unlabeled ligand or 10⁻⁵ M mianserin. Binding in the presence of mianserin was assumed to be the baseline (i.e., t=0 min). Binding over time was baseline corrected and then analyzed by nonlinear regression using equation 5.

**Analysis.** Analysis was performed in GraphPad Prism 6.0. Nonlinear regression was performed using the following models:

**Total and non-specific saturation binding**

\[ RL_{NS}^* = NS \cdot [L^*] + \text{background} \quad (1) \]

\[ RL_{total}^* = \frac{B_{\text{max}} \cdot [L^*]}{K_d \cdot [L^*]} + NS \cdot [L^*] + \text{background} \quad (2) \]

\[ \log IC_{50} \]

\[ RL_{total}^* = \frac{\text{Top} - \text{Bottom}}{1 + 10^{\text{log}[I] - \log IC_{50}}} + \text{Bottom} \quad (3) \]

**Association kinetics using multiple concentrations ligand**

\[ k_{obs} = k_1 \cdot [L^*] + k_2 \]

\[ RL_{specific}^* = \frac{B_{\text{max}} \cdot [L^*]}{[L^*] + \frac{k_2}{k_1} \cdot (1 - e^{-k_{obs} \cdot t})} \quad (4) \]

**Kinetics of competitive binding (constrain k₁ and k₂)**

\[ K_A = k_1 \cdot [L^*] + k_2 \]

\[ K_B = k_3 \cdot [I] + k_4 \]

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

\[ K_F = \frac{(K_A + K_B + S)}{2} \]

\[ K_S = \frac{(K_A + K_B - S)}{2} \]

\[ Q = \frac{B_{\text{max}} \cdot [L^*] \cdot k_1}{K_F - K_S} \]

\[ RL_{specific}^* = Q \cdot \left( \frac{k_4 (K_F - K_S)}{K_F K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F \cdot t)} - \frac{k_4 - K_S}{K_S} e^{(-K_S \cdot t)} \right) \quad (5) \]

\( RL^* \) is the bound [³H]-mepyramine and \( B_{\text{max}} \) is the maximally possible specific binding of [³H]-mepyramine. [L*] stands for the concentration of [³H]mepyramine and [I] for the concentration unlabeled ligand.
Association rate constants are denoted by \( k_1 \) or \( k_3 \) and the dissociation rate constants by \( k_2 \) or \( k_4 \) for \([3H]\)-mepyramine or unlabeled ligand respectively.

**Computational methods.**
*Preparation of the ligands, docking and scoring.* The 2D-structures of the ligands 2b, 2c, 3b, 3c, 4c and the reference compounds olopatadine, acrivastine and levocetirizine were built in ChemBioDraw Ultra 14.0.0.117, and subsequently transformed to SMILES format. Protonation (pH = 7.4) of the SMILES was performed using Chemaxon’s Calculator (version 5.1.4 [208]) and successively converted to MOL2 format using CORINA (version 3.49 [209]). PLANTS (version 1.2) [203] docking was performed in 6-fold at search speed 2 and 25 generated poses per ligand with a clustering RMSD of 2.0. The H1R binding site was defined as a radius of 10.8 around the co-crystallized compound Doxepin in the 3RZE crystal structure [200]. As previously reported, a filter was applied that only selects the docking poses possessing an H-bond and ionic interaction with D1073.32 [38,39]. The docking pose with the lowest PLANTS ChemPLP score (i.e., the energetically most favorable pose) was selected [210].

**Chemistry.**
Chemical procedures were described extensively elsewhere [211].

**Abbreviations**
Acri, acrivastine; DMEM, Dulbecco’s Modified Eagle Medium; dox, doxepin; FBS, fetal bovine serum H1R, HA, non-hydrogen atoms; Histamine H1 receptor; KE, kinetic efficiency; levo, levocetirizine; LE, ligand efficiency; mep, mepyramine;olo, olopatadine; PEI, polyethylenimine; RT, residence time; SKR, structure-kinetic relationship; TEA, trimethylamine; trip, triprolidine.

**Acknowledgment**
The authors gratefully acknowledge the technical assistance of Hans Custers.
Chapter 7 - Intramolecular rigidity increases residence time of ligands targeting the histamine H1 receptor

Co-authors: Sebastiaan Kuhne, Jelle van den Bor, Henry F. Vischer, Iwan J.P. de Esch, Rob Leurs.
Abstract

There is an increasing interest to guide hit optimization by considering the binding kinetics of ligands. Yet, in contrast to conventional structure-activity relationships (SAR), structure-kinetics relationships (SKR) are not as thoroughly explored, even for well-studied archetypical drug targets such as the histamine H₁ receptor (H₁R). Previously, it was shown that the residence time (RT) of H₁R ligands can be increased by introducing a carboxylic acid group (Chapter 6). In this study we show that the RT is dependent on the rigidity of the aromatic head-group. It was shown that fusing the rings of an antihistamine containing two separate aromatic rings into one tricyclic-aromatic head group, prolonged the RT of the rigid analog at the H₁R. Interestingly, introducing a carboxylic acid moiety on a tricyclic scaffold, thereby combining both structural features that are shown to increase the RT for H₁R ligands, did not always lead to a further increase in RT. This study shows that restraining the conformational freedom of the aromatic head group can be an effective strategy to increase the drug-target RT.

Introduction

The drug-target residence time (RT), defined as the reciprocal of the kinetic dissociation rate constant $k_{off}$ is increasingly acknowledged to be an important metric for drug-binding, which is linked to the in vivo efficacy of drugs. SAR-based hit and lead optimization programs rely on the equilibrium dissociation constant (Kₐ) as a measure for the drug binding affinity. Often, the binding kinetics of ligands are ignored, despite the fact that there is not always a good correlation between the Kₐ and RT of ligands for a drug target, as was also confirmed for the histamine H₁ receptor (H₁R). The RT of ligands for the H₁R can be increased by introducing a carboxylic acid at various positions of prototypical H₁R ligands (Chapter 6). A similar SKR has been established for the well-known antihistamines doxepin (RT= 26 min) and triprolidine (RT= 3 min) and their respective analogs that contain a carboxylic acid group, i.e., olopatadine (RT= 190 min) and acrivastine (RT= 15 min), respectively (figure 1). It is noted that the doxepin series has a much higher RT at the H₁R compared to the triprolidine series, indicating that in addition to the carboxylate group there are other features that influence the drug-target RT.

Moreover, the tricyclic antihistamine desloratadine, which does not contain a carboxylic acid moiety, was also reported to possess a remarkably long RT at the H₁R. One apparent difference in chemotype is the presence of a tricyclic aromatic head group for doxepin and desloratadine compared to triprolidine.

![Figure 1 - Antihistamines and their corresponding binding affinities (pKᵢ) and RTs (Chapter 6).](image)
(figure 1A). Doxepin and desloratadine (figure 1A) both have a tricyclic ring system, resulting in a so-called butterfly-shaped conformation of the ligands 39,54. Notably, the butterfly shape of doxepin is confirmed in the H1R crystal structure (figure 4B). Moreover, both compounds have the same number of rotatable bonds. Ligands binding to a variety of drug-targets (mostly GPCRs and kinases) from the Pfizer database were compared in a retrospective study 212. For ligands with a similar molecular weight (300–500 Da), it is shown that ligands with ≤5 rotatable bonds more often have a longer drug-target RT than ligands with >5 rotatable bonds. Furthermore, the relative RT of >1800 ligands was determined for the dopamine D2 receptor by Tresadem et al. and they showed that ligands with a long RT have on average a higher number of ring structures 119. These findings suggest that the number of rotatable bonds 213 and the number of rings can influence the drug-target RT.

The aim of this study is to further investigate how ligand rigidity and ring structures influence ligand RT at the H1R. The role of a rigid tricyclic head group is explored by determining the RT at the H1R of a diverse set of ligands (figure 2, table 1). A subsequent dataset (table 2) allows the direct comparison of ligands with non-fused aromatic ring systems with ligands in which these rings were fused by an ethane or ethylene bridge. Finally, ligands were synthesized that combined the tricyclic-aromatic head group together with a carboxylic acid group (Scheme 1, table 3) in order to examine if these structural motifs have an additive effect on the H1R-RT.

**Results**

Since ligands with higher molecular mass tend to have an increased RT 212, a variety of chemotypes targeting the H1R with similar molecular weight were selected and characterized for their kinetic binding rate constants (table 1, supplementary table 1). The ligands in table 1 all contain an aromatic head group and a basic amine, characteristic features of H1R antihistamines 39. Despite these similarities, two groups

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Table 1 - Characterization of ligand binding at the H1R. Equilibrium binding affinity values and kinetic binding rate constants were determined using radioligand binding studies against a homogenate of HEK293T cells transiently expressing the HA-H1R as described in figure 1 and the methods section. RT and the equilibrium binding affinity constants (pK_d,calc) were calculated from the kinetic binding rate constants. All values represent mean ± SEM of ≥ 3 experiments.
of ligands can be distinguished, i.e., the tricyclic ligands 8–12 and the non-tricyclic ligands 1–7 (figure 2A). To determine the equilibrium dissociation constants (pKₐ) of the ligands, increasing concentrations of unlabeled ligand were co-incubated with a single concentration [³H]mepyramine and with an homogenate of HEK293T cells transiently expressing the H₁R (figure 3A). To determine the kinetic binding rate constants, the binding of [³H]mepyramine in competition with a single concentration of unlabeled ligand was measured for different incubation times. From the resulting competitive radioligand association curves (figure 3B), the kₐ and kₐ of were determined (table 1, supplementary table 1) 113. It was observed that low kₐ and kₐ values were often observed for compounds with a low number of rotatable bonds (figure 1B). However, binding kinetics cannot be explained by the low number of rotatable bonds alone, as exemplified by ligand 7. This ligand has a low number of rotatable bonds but has one of the highest kₐ and kₐ values among the tested ligands. Interestingly, it was found that the tricyclic ligands 8–12 generally have a much higher binding affinity for the H₁R than the non-tricyclic ligands 1–7 (table 1, figure 2A). Furthermore, the tricyclic ligands are also shown to have a lower kₐ and hence higher RT at the H₁R. Among the tricyclic compounds was desloratadine, for which we confirm its reported long RT (190 ± 40 min) 64,65,173.

Figure 2 - Ligands that contain a tricyclic-aromatic head group have a low kₐ and kₐ at the H₁R. (A) A diverse set of H₁R ligands with comparable molecular weight can be divided in non-tricyclic molecules (1–7, black dots) and tricyclic molecules (8–12, red dots). (B) The non-tricyclic H₁R ligands have a higher kₐ and kₐ than the tricyclic ligands and this related with the number of rotatable bonds in the molecules.
Thus, table 1 shows that the ligands containing a tricyclic head group have a longer RT at the H1R than the ligands that lack the tricyclic head group. To systematically investigate whether a tricyclic ring system is indeed causing a decrease in the $k_{\text{off}}$ value, and thus increased ligand RT at the H1R, pair-wise comparisons of molecules are required. Therefore, diphenhydramine (1), cyclizine (4) and ligand 14, all containing the prototypical basic amine and two separate aromatic rings, were directly compared with the ligands 13, 15–17 (table 2). The latter ligands contain an ethane or ethylene linker between the aromatic rings, thereby creating ligands with a tricyclic ring system, and this resulted in a 4-40 fold increase in RT at the H1R (table 2). This increase in RT for 13, 15–17 is higher than the increase in binding affinity that was only 2-10 fold compared to the analogs without this tricyclic ring system 1, 4 and 14. Especially for the tricyclic ligands 15 and 17, for which the aromatic rings are fused with an ethylene linker, there was a large increase in RT. The 3-fold increase in RT at the H1R for ligand 16 compared to ligand 4 is in line with the 3-fold increase in binding affinity. However, fusing the rings with an ethylene bridge (17) instead of an ethane bridge (16) gave an additional 3-fold increase in RT while leaving the binding affinity unaffected.

From the tested ligands in tables 1 and 2, desloratadine (10) and clozapine (11) have the longest RT at the H1R. Besides the rigid tricyclic head group, both ligands contain a chlorine group on one of their aromatic rings. Previous work did not suggest large effects on the binding affinity upon chlorine substitution of H1R ligands 39, but the effect on RT was not explored. Therefore, the effect on RT by introducing a chloro-moietiy on a tricyclic ring system was investigated as outlined in table 3. To investigate the role of a chlorine group on the RT of a ligand, the drug azatadine (9) was selected and analog 18 with a chlorine

![Figure 3 - Competitive binding of [3H]mepyramine and unlabeled ligands to the H1R. Equilibrium binding affinity constants and kinetic binding rate constants were determined using radioligand binding studies against a homogenate of HEK293T cells transiently expressing the H1R. (A) Equilibrium binding affinity values (pK$_i$) were determined using radioligand displacement experiments in which a single concentration [3H]mepyramine was co-incubated with increasing concentrations unlabeled ligand for 4 h. (B) The kinetic association ($k_{\text{on}}$) and dissociation rate ($k_{\text{off}}$) constants were determined by co-incubating a single concentration [3H]mepyramine with a ±10·K$_i$ concentration of unlabeled ligand for various incubation time. Representative graphs are shown of ≥ 3 experiments and depicted data points represent the mean ± SD of triplicate values (A) or duplicate values (B).](image-url)
moiety on the 8-position of the ring was synthesized as illustrated in Scheme 1. Furthermore, it was previously shown for ligand 6 that an additional carboxyl group gave an 11-fold increase in RT at the H1R when growing from the basic amine atom with a four-methylene spacer (Chapter 6). The same approach was used for compound 18 and a carboxylic acid group was introduced to obtain ligand 20 (table 3, Scheme 1).

Table 3 shows that the chlorine moiety of ligand 18 did not affect the RT at the H1R, nor did it affect the binding affinity compared to azatadine. Subsequent introduction of a carboxylic acid moiety (i.e., 20) by growing the structure from the basic amine resulted in a decreased pKi compared to 18, but a comparable RT at the H1R. Interestingly a large decrease in the $k_{on}$ (50-fold) was observed (table 3) which was also reported when introducing the same group on the basic amine of ligand 6 (Chapter 6). Therefore, the carboxylate moiety alters the association rate of compound 18, but not so much the dissociation rate of this particular compound.

Notably, for the entire set of ligands the binding affinity determined by competition binding (pK) was in agreement with the calculated affinity from the kinetic binding rate constants (pK_{d,calc}), as depicted in supplementary table 1 and supplementary figure 1.

**Table 2 - Tricyclic H1R ligands have a longer RT at the H1R than their corresponding analogs without this tricyclic ring system.** Equilibrium binding affinity constants and RTs were determined using radioligand binding studies against a homogenate of HEK293T cells transiently expressing the H1R. All values represent mean ± SEM of ≥ 3 experiments.

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<td>0.43 ± 0.03</td>
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<td>13, VUFH1607</td>
<td>8.9 ± 0.0</td>
<td>7.8 ± 0.2</td>
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<td>3.0 ± 0.4</td>
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<tr>
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<td>9.5 ± 0.1</td>
<td>113 ± 2</td>
</tr>
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<td>4, VUFH1896</td>
<td>8.2 ± 0.1</td>
<td>2.5 ± 0.4</td>
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<tr>
<td>17, BS7617</td>
<td>8.7 ± 0.1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>16, VUFH1896</td>
<td>8.7 ± 0.1</td>
<td>30 ± 4</td>
</tr>
</tbody>
</table>
Discussion

It was previously shown that there can be significant differences between SAR and SKR for ligands binding the H1R (Chapter 6)\textsuperscript{15}. In this study, we have shown that ligands with increased rigidity in the aromatic head group, i.e., bearing a tricyclic structure, have a higher binding affinity and a higher RT at the H1R (tables 1 and 2, figure 2B). A closer look at the SKR shows that increasing rigidity in the aromatic head group has a positive effect on the H1R binding affinity (pK\textsubscript{i}) and often reduces the k\textsubscript{on}. Consequently (see also equation 1; \textsuperscript{143}), the increase in RT was often larger than the increase in affinity for H1R ligand binding, as e.g., illustrated by the compounds 4 (RT = 2.5 min, pK\textsubscript{i} = 8.2) and 17 (RT = 30 min, pK\textsubscript{i} = 8.7) for which there was a 3-fold difference in H1R binding affinity (K\textsubscript{i}), but more than a 10-fold difference in RT. This exemplifies that the introduced rigidity in the tricyclic ring system might not only affect the binding stability, which is related to the binding affinity, but also increases the transition state energy barrier for ligand dissociation. Moreover, this once again demonstrates that SAR-studies that rely on the binding affinity do not always capture the differences in binding kinetics.

Scheme 1 - Synthesis of VUF15007 (18), VUF15008 (20) and VUF14451 (7). Tertiary amine 18 was obtained via a reductive amination with desloratadine (10) and formaldehyde in the presence of acetic acid and sodium triacetoxyborohydride as a reducing agent. Alkylation of desloratadine (10) with 5-bromopentanoate afforded ester 19 and subsequent saponification with NaOH gave zwitterion 20. Compound 7 was obtained by reaction 1-chloroisoquinoline with N-methylpiperazine, using microwave-assisted heating. Compound 7 was obtained as a fumaric acid salt. Reagents and conditions: (A) (a) CH\textsubscript{2}O (37 wt\% in water), AcOH, Na(CH\textsubscript{3}COO)\textsubscript{3}BH, DCM/MeOH 2:1, rt, 1.5 h. (b) 5-bromopentanoate, K\textsubscript{2}CO\textsubscript{3}, DMF, 80 °C, 4 h. (c) 2 M aqueous NaOH, EtOH, reflux, 4 h. (B) (a) N-methylpiperazine, 160 °C, 5 min, microwave.
Table 3 - Exploring the effects on ligand RT at the H1R by introducing a chloro atom (18) and a subsequent carboxylic acid moiety (20) on the tricyclic antihistamine azatadine. Equilibrium binding affinity constants and kinetic binding rate constants were determined using radioligand binding studies against a homogenate of HEK293T cells transiently expressing the H1R. RT and the equilibrium binding affinity constants (pKd,calc) were calculated from the kinetic binding rate constants. All values represent mean ± SEM of ≥ 3 experiments.

<table>
<thead>
<tr>
<th>ligand</th>
<th>pKi</th>
<th>pKd,calc</th>
<th>k_on (10^6·M⁻¹·min⁻¹)</th>
<th>k_off (min⁻¹)</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>9.7 ± 0.2</td>
<td>9.6 ± 0.0</td>
<td>32.0 ± 0.3</td>
<td>0.0088 ± 0.0001</td>
<td>114 ± 2</td>
</tr>
<tr>
<td>18, VUF15007</td>
<td>9.6 ± 0.1</td>
<td>9.6 ± 0.1</td>
<td>28 ± 1</td>
<td>0.008 ± 0.003</td>
<td>150 ± 40</td>
</tr>
<tr>
<td>20, VUF15008</td>
<td>7.6 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>0.44 ± 0.05</td>
<td>0.011 ± 0.001</td>
<td>100 ± 10</td>
</tr>
</tbody>
</table>

\[ RT = \frac{1}{K_d \times k_{on}} \quad (1) \]

The introduced ligand rigidity in the aromatic head group by locking the aromatic rings into a tricyclic ring system, clearly leads to longer RTs at the H1R (table 2). However, comparing the different tricyclic ligands shows that there are still large differences in RT between the different chemotypes (table 1 and 2). The ligands with the highest RT at the H1R, i.e., azatadine (9), desloratadine (10), and cyproheptadine (15) all have a constrained connection (i.e., double bond) between the tricyclic aromatic head group and the basic amine which is incorporated in an aliphatic ring. The RT of those ligands is a 3–10 fold longer compared to the tricyclic ligands doxepin (8), 13 and 17 for which there is a less constrained connection between the tricyclic aromatic head group and the basic amine moiety. This suggests that additional ligand rigidity apart from the tricyclic core might further enhance ligand RT at the H1R. Understanding of the above identified SKR on a molecular level remains difficult at this time, since structural insights in ligand-H1R binding remain limited to the doxepin-bound H1R crystal structure (figure 4B). This static X-ray structure does not explain why doxepin with its tricyclic scaffold has a relative long RT. However, the available H1R structural data can be compared with other relevant GPCR structural data. For example, in a study by Tautermann et al. 194 that explores muscarinic M3 receptor (M3R) ligands, it was shown that tiotropium (figure 4A), was enclosed by the aromatic residues Y1493.33, Y5076.51 and Y5307.39 (Ballesteros-Weinstein numbering 40), referred to as the hydrophobic cage (figure 4A). Mutating either of these residues to...
alanines led to a 175 to 5200-fold decrease in RT of tiotropium at the M3R. Furthermore, molecular dynamic simulations suggested that an open conformation of the aromatic cage was necessary to enable dissociation of tiotropium from the receptor 194. Analogous to the M3R, the H1R crystal structure also shows an aromatic binding region (figure 4B), which include among others the conserved residues Y1083.33 and Y4316.51. These two residues were shown to affect the RT of tiotropium at the M3R (vide supra) and, moreover, enclose doxepin (8) in the H1R crystal structure 37. Ligands such as 1 and 5 might readily bind to and dissociate from such a rigid binding pocket since the high number of rotatable bonds allows adaptation of their conformation to the binding site. On the contrary, compounds containing a rigid tricyclic head group, like the co-crystallized ligand doxepin, and the ligands desloratadine and clozapine, might depend more on the conformational changes of the receptor to pass the aromatic cage during association and dissociation events. The aromatic isoquinoline ring system in ligand 7 is relatively small and cannot adopt the H1R-prototypical butterfly shape, which might prevent a locked binding mode in the aromatic cage. This could explain why ligand 7 despite its rigid isoquinoline moiety does not have a long RT at the H1R. Extensive molecular dynamic (MD) simulations, using the obtained SAR and SKR described in this chapter might support this hypothesis.

Upon substitution of 6, all analogs with a carboxylic acid group were previously shown to have a lower binding affinity for the H1R but a comparable or longer RT (Chapter 6). Notably, the RT for these analogs correlated with the affinity, suggesting that substitution with the carboxylic acid group increases RT the most when the loss in binding affinity is minimized. Similarly, in an attempt to increase the drug-target RT, the rigid compound 18 was substituted with a carboxylic acid group. This resulted in a decreased $k_{on}$, which could potentially prolong the RT according to equation 1. However, this decreased $k_{on}$ was paired with a 100-fold increase in the $K_i$. Effectively, this resulted in a small decrease in RT. In contrast, when comparing olopatadine with doxepin, it was shown that antihistamines with a tricyclic head group can have an increased RT at the H1R upon introducing the carboxylic acid moiety (figure 1). Optimization of ligand RT at the H1R can therefore be achieved by different design strategies, i.e., 1) introducing carboxylic acid moieties (Chapter 6, 15) and introducing ligand rigidity. Yet, combining these two strategies is not straightforward, since increasing the molecular rigidity might interfere with optimal placement of the essential pharmacophoric features (i.e., aromatic head group and basic amine) together with the carboxylic acid group in the H1R.

In conclusion, it was shown in this study that ligands with a tricyclic head group increase RT at the H1R compared to their non-tricyclic analogs. This therefore provides a new handle for drug discovery to optimize the drug-target RT of H1R antihistamines. The static aromatic character of the H1R binding site might explain why rigid ligands such as desloratadine, azatadine and clozapine have a long RT at the H1R. Increasing RT by introducing ligand rigidity might be a strategy which is applicable to other drug targets with a highly aromatic binding site.

Materials and Methods

Pharmacology.

Materials. Dulbecco’s Modified Eagle’s Medium was acquired from Sigma-Aldrich (St. Louis, MO, USA). Medium was supplemented with fetal bovine serum and penicillin/streptomycin from GE healthcare.
(Uppsala, Sweden). 25-kDa linear polyethylenimine was acquired from Polysciences (Warrington, PA, USA). HBSS, trypsin and the BCA protein assay were bought from Thermo Fischer scientific (Waltham, MA, USA). The Branson sonifier 250 homogenizer was bought from Emerson (St. Louis, MO, USA). GF/C plates, Microscint-O, [3H]mepyramine, the cell harvester and the Wallac Microbeta counter were all bought from Perkin Elmer (Waltham, MA, USA). Diphenhydramine hydrochloride was purchased from Sigma Aldrich. Mepyramine maleate was obtained from Research Biochemicals International. Triprolidine hydrochloride and doxepin hydrochloride (mixture of E/Z isomers (ratio: 85:15)) were purchased from Tocris. Azatadine dimaleate and desloratadine were purchased from HaiHang Industry Co., Ltd. Cyclizine hydrochloride was purchased from Toronto Research Chemicals (TRC). Clozapine was purchased from Tokyo Chemical Industry Co., Ltd. GBR30388 (mianserin hydrochloride), VUFH1607 (maleate salt), VUF5577 (hydrochloride salt), BS7316 (cyproheptadine hydrochloride), VUFH1896 and BS7617 were obtained from the in-house library of the Department of Medicinal Chemistry of the Vrije Universiteit Amsterdam. VUF14454, VUF14493, VUF14451, VUF15007 and VUF15008 were synthesized at the Department of Medicinal Chemistry of the Vrije Universiteit Amsterdam. The synthesis of VUF14451, VUF15007 and VUF15008 is described below. Unless specified otherwise, all compounds have a purity of ≥95%, calculated as the percentage peak area of the analyzed compound by UV detection at 230 nm (see methods section and supplementary table 2).

**Cell culture and radioligand binding.** Production of cell homogenates expressing the HA-H1R and the performed radioligand binding experiments conducted were previously described with minor changes. In short, HEK293T cells were transiently transfected using 25kDa polyethylenimine with a pcDEF3 vector encoding the N-terminally HA tagged H1R. Cells were collected and frozen two days post-transfection. Upon conducting a radioligand binding experiment, a frozen aliquot of cells was reconstituted in binding buffer [50mM Na2HPO4/KH2PO4, pH 7.4], homogenized and then co-incubated with [3H]mepyramine with or without an additional unlabeled ligand at 25°C under gentle agitation. Binding reactions were terminated by filtration and three rapid consecutive wash steps using ice-cold wash buffer [50mM Tris-HCl, pH 7.4]. Filter-bound radioactivity was quantified using scintillation counting using the Wallac Microbeta.

Previously it was determined for the radioligand [3H]mepyramine binding the H1R, that the equilibrium dissociation constant (Kd) is 2.29 nM, the kinetic dissociation rate constant (koff) is 0.22 min⁻¹ and the kinetic association rate constant (kon) is 1.1-10⁸ min⁻¹M⁻¹ (Chapter 6). In radioligand displacement experiments single concentration 1–5 nM [3H]mepyramine was co-incubated with increasing concentrations (10⁻¹¹ – 10⁻⁴ M) unlabeled ligands for 4 h at 25 °C. Kᵢ values could be determined from the displacement curves by converting the obtained IC₅₀ values using the binding affinity and concentration of [3H]mepyramine. For competitive association experiments a single concentration 1–5 nM [3H]mepyramine was co-incubated with a single concentration unlabeled ligand for increasing incubation times of 0 – 80 min at 25 °C. Concentration antagonist was chosen to be 10·Kᵢ, or fine-tuned to have a similar level of radioligand displacement after 80 min (>40%). Kinetic binding rate constants of the unlabeled ligands were determined from the resulting radioligand binding over time by fitting the data to the Motulsky and Mahan model using non-linear regression. In this model the concentrations of both
ligands and the $k_{\text{on}}$ and $k_{\text{off}}$ of $[^3]$H)mepyramine at the H1R were constrained. From the fitted kinetic binding rate constants, the equilibrium dissociation constant ($pK_{d,\text{calc}}$) and residence time (RT) could be calculated.

**Chemistry.**

Chemical procedures were described extensively elsewhere 211.

**Acknowledgements**

The authors gratefully acknowledge the contribution of Philipp Fronik.

**Supplementary Information**

**Supplementary Table 1 - Characterization of ligand binding to the histamine H1 receptor (H1R).** Equilibrium binding affinity constants and kinetic binding rate constants were determined using radioligand binding studies against a homogenate of HEK293T cells transiently expressing the H1R. RT and the equilibrium binding affinity constants ($pK_{d,\text{calc}}$) were calculated from the kinetic binding rate constants. All values represent mean ± SEM of N experiments.

<table>
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<tr>
<th>#</th>
<th>Ligand name</th>
<th>$pK_i$ (±)</th>
<th>N</th>
<th>$pK_{d,\text{calc}}$</th>
<th>$k_{\text{on}}$ (10^6 min^-1 M^-1)</th>
<th>$k_{\text{off}}$ (min^-1)</th>
<th>RT (min)</th>
<th>N</th>
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<tbody>
<tr>
<td>1</td>
<td>diphenhydramine</td>
<td>8.0 ± 0.1</td>
<td>3</td>
<td>8.1 ± 0.2</td>
<td>300 ± 200</td>
<td>2.3 ± 0.2</td>
<td>0.43 ± 0.03</td>
<td>3</td>
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<tr>
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<td>mepyramine</td>
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<td>3</td>
<td>8.8 ± 0.0</td>
<td>150 ± 30</td>
<td>0.23 ± 0.03</td>
<td>4.6 ± 0.6</td>
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</tr>
<tr>
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<td>3</td>
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<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
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<td>0.430 ± 0.007</td>
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<td>0.043 ± 0.007</td>
<td>26 ± 5</td>
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<td>32.0 ± 0.3</td>
<td>0.0088 ± 0.0001</td>
<td>114 ± 2</td>
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<td>0.035 ± 0.004</td>
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<td>28 ± 1</td>
<td>0.008 ± 0.003</td>
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<td>0.44 ± 0.05</td>
<td>0.011 ± 0.001</td>
<td>100 ± 10</td>
<td>3</td>
</tr>
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</table>
Supplementary Figure 1 - Correlation of binding affinity derived from radioligand displacement (pKi) and competitive association (pKd,calc) studies. The dashed line represents a perfect correlation of unity, whereas the solid line represents the regression line indicating the observed correlation between the pKi and pKd,calc. The coefficient of determination (R^2) of the observed correlation is indicated in the graph.

Supplementary Table 2. Purities: calculated as the percentage peak area of the analyzed compound by UV detection at 230 nm.

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Chapter 8 - The route to an elongated residence time at the histamine H1 receptor: growing from desloratadine to rupatadine

Co-authors: Zhiyong Wang, Albert J. Kooistra, Sebastiaan Kuhne, Jelle Bor, Chris de Graaf, Henry F. Vischer, Iwan J.P. de Esch, Maikel Wijtmans and Rob Leurs

Manuscript is in preparation for submission.
ABSTRACT

Drug-target binding kinetics is increasingly acknowledged to be an important predictor of *in vivo* drug efficacy. Consequently, it is of interest to gain a better understanding of the relationships between the molecular ligand structure and the binding kinetics of a drug with the target receptor. For an archetypical G protein-coupled receptor, the histamine H₁ receptor (H₁R), several antagonists with a long *in vivo* duration of action have been described, which is suggested to be the consequence of their long receptor residence times. Rupatadine (1) is an antagonist with a long duration of action *in vivo* but an unknown residence time at the H₁R.

Here, we show that rupatadine has a more than 10-fold longer residence time than desloratadine (2), a prototypical long residence time H₁R antagonist and a structural analog of rupatadine. We explored the structure-kinetics relationship (SKR) of a series of rupatadine and desloratadine analogs, which differ in the substituent on the piperidine ring. To this end, structural analogs were synthesized which together embody a stepwise deconstruction of the (5-methylpyridin-3-yl)methyl substituent of rupatadine.

Aliphatic N-substitutions on the piperidine of desloratadine increased the residence time at the H₁R. Moreover, it was shown that all analogs containing aromatic variations on the (5-methylpyridin-3-yl)methyl group of rupatadine retained a very long residence time at the H₁R. Interestingly, large aliphatic substitutions that were directly substituted on the basic amine (i.e. lacking the spacer) decreased both binding affinity and residence time at the H₁R, most likely caused by a steric clash with the H₁R protein. In all, substituents of various sizes on the piperidine, provided that a steric clash with the receptor is avoided, allow optimization of the H₁R residence time for desloratadine and potentially other antagonists.

1 Introduction

Drugs have to bind a therapeutically relevant target to obtain a biological effect and, as such, target binding is in many cases well characterized in the drug development process. The binding affinity is an often-used parameter to measure drug binding to targets (quantified as Kᵅ or Kᵣ value), implicitly assuming ligand binding to occur under equilibrium conditions. Yet, drug pharmacodynamics can also be characterized by the drug-target binding kinetics, which provides important details about the mechanism of target binding, unexplained by solely the binding affinity ⁶–⁸. Especially the drug-target residence time, which is a measure for the lifetime of a drug-target complex, has been suggested to be important for the biological efficacy of drugs *in vivo* ⁶,⁹–¹¹,¹³,¹⁸,¹⁴³.

An important class of proteins in drug discovery are G protein-coupled receptors (GPCRs), which are targeted by > 30% of all small-molecule drugs ¹². The histamine H₁ receptor (H₁R) is an archetypical GPCR and is successfully targeted by antagonists for the treatment of, for example, allergic disorders ³². A long duration of action has been observed *in vivo* for second generation H₁R antagonists, like levocetirizine and fexofenadine, which have a long residence time at the H₁R ⁸¹,¹⁴⁶. It has been postulated that a suitably long drug-target residence time might increase the therapeutic window *in vivo* when clearance of the drug is faster than the dissociation of the drug from the receptor ²¹⁴. In such cases, drug action would last longer than the presence of free drug plasma concentrations (i.e. hysteresis), as was indeed the case for levocetirizine and fexofenadine. A strong hysteresis of H₁R antagonism was also shown for rupatadine
which antagonizes the histamine-induced flare response up to 72 hours after oral administration, whereas plasma levels could only be detected up to 12 hours after administration. Desloratadine is a close structural analog of rupatadine (figure 1), which has been shown to have a long H₁R residence time, as well as, a long duration of action in vivo. The long duration of action for rupatadine in vivo is therefore hypothesized to result from its long drug-target residence time.

Here, we report the residence time measurements of rupatadine and desloratadine at the H₁R. It was shown that rupatadine has a ≥ 10-fold longer residence time at the H₁R compared to desloratadine. As a consequence, rupatadine completely antagonized the histamine-induced calcium mobilization in HeLa cells for > 2 hr after removal of unbound antagonist, whereas in the same experiment, desloratadine allowed a time-dependent gradual recovery of the histamine-induced response. To understand the structure-kinetics relationship (SKR) for rupatadine and desloratadine in more detail, the binding kinetics at the H₁R were characterized for newly synthesized analogs (3-24) that retain the core scaffold of 1 and 2 but contain a diverse set of aromatic and aliphatic N-substituents on the piperidine ring. It was shown that relatively small aliphatic N-substitutions were sufficient for a prolonged H₁R residence time compared to desloratadine, unless this was negated by steric interference in the binding pocket.

2 Results

2.1 Binding properties of rupatadine and desloratadine at the H₁R.

Based on the long duration of action in vivo, we hypothesized a long residence time of rupatadine at the H₁R. Binding to the human H₁R by rupatadine and its structural analog desloratadine was therefore investigated by standard H₁R radioligand binding experiments using [³H]mepyramine. First, the H₁R affinity of both ligands was determined using standardized competition binding experiments. These experiments show that rupatadine has a high nanomolar binding affinity (pKᵢ 8.4 ± 0.1) at the H₁R. This value is lower than the H₁R binding affinity of desloratadine (pKᵢ 9.1 ± 0.1), which agrees with previously reported affinity values in the literature (pKᵢ = 8.8 – 10).

Competitive association experiments were subsequently performed to examine the binding kinetics of rupatadine and desloratadine at the H₁R. First, [³H]mepyramine was selected as radioligand and
experiments were performed at 25°C and an 80 min incubation time as described previously (figure 2A)\(^{173}\). A clear overshoot in \(^{3}H\)mepyramine binding was observed for both unlabeled ligands, which is indicative of the relatively long residence time of the unlabeled ligands compared to \(^{3}H\)mepyramine\(^{113,118}\). Both binding curves showed a similar overshoot pattern, the difference in binding kinetics of rupatadine and desloratadine were therefore difficult to discriminate based on the Motulsky-Mahan analysis. Desloratadine was found to have a residence time of 190 ± 40 min (similar to what was reported in the literature\(^{64,173}\)) but for rupatadine, the \(k_{\text{off}}\) value (and thus the residence time) could not be accurately constrained by the model. It was speculated that the residence times of desloratadine and rupatadine at the H\(_1\)R could be better discriminated when the radioligand has a long residence time, similarly to desloratadine and rupatadine. \(^{3}H\)levocetirizine is known to have a 100-fold longer residence time at the H\(_1\)R than \(^{3}H\)mepyramine and was therefore employed in competitive association experiments. Under the employed experimental conditions complete kinetic binding traces of \(^{3}H\)levocetirizine could be obtained within a total incubation time of 6 hours\(^{64}\). In the presence of desloratadine, \(^{3}H\)levocetirizine binding to the H\(_1\)R increased gradually over time until a steady state in binding was obtained, whereas in the presence of rupatadine a clear overshoot in \(^{3}H\)levocetirizine binding was observed (figure 2). It is therefore qualitatively clear that rupatadine has a longer residence time on the H\(_1\)R than desloratadine. Fitting the data to the Motulsky-Mahan model\(^{113}\) did not lead to accurate determination of the \(k_{\text{off}}\) values, but indicated the \(k_{\text{off}}\) value for the binding of desloratadine to the H\(_1\)R to be > 0.03 min\(^{-1}\) (\(P = 95%\) in all 3 experiments) corresponding to a residence time of < 33 min. In the case of rupatadine, the \(k_{\text{off}}\) value for the binding to the H\(_1\)R was < 0.0033 min\(^{-1}\) (\(P = 95%\) in all 3 experiments), which corresponds to a residence time of > 300 min. Thus, rupatadine has a very long residence time at the H\(_1\)R, which is at least 10-fold longer than observed for desloratadine.

![Figure 2 - Radioligand association binding in co-incubation with rupatadine and desloratadine.](image)

**Figure 2** - Radioligand association binding in co-incubation with rupatadine and desloratadine. A homogenate of HEK293T cells expressing the H\(_1\)R was co-incubated with (A) 3.8 nM \(^{3}H\)mepyramine in the absence and presence of either 130 nM rupatadine or 4 nM desloratadine. In panel B, cell homogenates are co-incubated with 6.6 nM \(^{3}H\)levocetirizine in the absence and presence of either 6 nM rupatadine or 0.7 nM desloratadine. A representative graph is shown of 3 experiments with mean ± SD of duplicate values.

### 2.2 Design and synthesis of rupatadine analogs at the H\(_1\)R


To identify the structural features that drive the longer residence time of rupatadine as compared to desloratadine at the H₁R, various analogs were synthesized and pharmacologically characterized.

Rupatadine contains a 5-methylpyridin-3-yl group connected through a one-carbon spacer to the basic amine of desloratadine (figure 1). To study the SKR, we synthesized analogs with the methyl group on different positions of the pyridine ring (3-5) or the pyridine analog without the methyl group (6). Additionally, two positional isomers of 6 (7, 8) and two pyrimidines (9-10) were prepared. Moreover, the pyridine ring of rupatadine was changed into a phenyl ring with (11) or without (12) a 3-methyl group. Finally, to gradually bridge the transition to 2, a set of analogs was synthesized in which the basic amine of desloratadine was substituted with a range of increasingly smaller alkyl groups (13-24), varying in size, level of constrainment and point of attachment (with or without the one-carbon spacer).

The synthesis routes of rupatadine analogues are depicted in scheme 1 and benefit from a highly modular character all starting from commercially available starting material desloratadine. Compounds 4-8, 11-12 and 16 were obtained starting from nucleophilic substitution by 2 on different alkyl bromides in moderate to good yields (36-86%). Reductive amination of different aromatic aldehydes with 2 afforded 3, 9 and 10 (64-88% yield). Compounds 13-15, 17-20, 22 and 23 were synthesized from aliphatic carbonyl compounds in acceptable to good yields using a reductive amination (52-71%). Methyl-derivative 24 was obtained as the fumarate salt from aqueous H₂CO and NaBH(OAc)₃ in 60% yield. For the synthesis of cyclopropyl-substituted compound 21, a standard alkylation by cyclopropylbromide failed and we resorted to reductive amination conditions with (1-ethoxycyclopropoxy)triethylsilane to obtain the expected product albeit in low isolated yield (17%) 216.
2.3 Pharmacological characterization

2.3.1 H1R binding affinity

All rupatadine analogs with an aromatic group (3-12) and most analogs with aliphatic groups containing ≥ 5 carbon atoms (13-15, 18, 19 but not 16) show a diminished binding affinity for the H1R (pKi 7.7 – 8.9) compared to desloratadine. In contrast, most desloratadine analogs with aliphatic groups containing ≤ 4 carbons (17 and 22-24, but not 20 and 21) displayed similar or slightly higher binding affinities at the H1R compared to desloratadine (pKi = 9.0 – 9.4). Interestingly, analogs of desloratadine with cycloaliphatic groups have a higher binding affinity at the H1R when this functional group is separated from the basic amine with a one-carbon spacer, as exemplified by the 0.4 – 0.9 higher pKi values for 13, 14, 16 and 17 as compared to 18-21, respectively.

2.3.2 Analysis of binding kinetics

To explore the relative residence time of all analogs, a dual-point competition association was performed to determine the kinetic rate index (KRI) \(^{118}\). This methodology is based on the observed overshoot in kinetic competition radioligand binding when co-incubated with an unlabeled ligand with a longer residence time at the target than the radioligand (figure 2). The overshoot is quantified by measuring the radioligand binding at two time points. The ratio in \(^{3}H\)levocetirizine binding at both time points (1 and 6 hours) is > 1 for unlabeled ligands that cause an overshoot in \(^{3}H\)levocetirizine binding and hence have a relatively long residence time compared to \(^{3}H\)levocetirizine. Using this assay setup, a KRI value of 0.9 ± 0.1 was obtained for unlabeled levocetirizine, which therefore corresponds to a residence time that is similar to the radioligand. Desloratadine does not cause an overshoot in \(^{3}H\)levocetirizine binding (figure 2B) and has a KRI value of 0.8 ± 0.0. Conversely, rupatadine binds the H1R with a much longer residence time (figure 2B), which is indeed reflected by its KRI value of 2.3 ± 0.2 (table 1). The KRI values for all analogs are depicted in table 1, while figure 3 shows the data for the most relevant subset, i.e. that showing subtle variations in KRI values. All analogs with an aromatic substituent show KRI values > 1, indicative of a long residence time at the H1R (table 1). More notably, among the analogs with aliphatic substitutions on the piperidine ring, large differences in the KRI values were observed (figure 3).

Analogs with cycloaliphatic groups and a one-carbon spacer (13, 14, 16 and 17) show high KRI values, also indicating a long residence time on the H1R. However, structural analogs with the same cycloaliphatic group without the one-carbon spacer (18-21) show similar KRI values as desloratadine, indicative of a shorter residence time at the H1R. Additionally, analogs with small acyclic aliphatic substituents (22-24) had an average KRI value slightly larger than 1, implying an increased residence time at the H1R compared to desloratadine.
Table 1 - Characterizing the H1R binding of rupatadine and desloratadine analogs. Binding affinity (pKi) values were determined by competition binding experiments using [3H]mepyramine and KRI-values were determined by dual-point competition association experiments using [3H]levocetirizine. Depicted values represent the mean ± SEM of ≥ 3 experiments. * fumarate salt

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<th>Name</th>
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2.3.3 Kinetics of functional H1R-antagonism

To explore the observed trends in KRI values in more detail, the kinetics of functional H1R-antagonism was measured following a pre-incubation with a subset of test compounds. The functional recovery time of the H1R was previously shown to be correlated with the residence time of antagonists 217. As such, HeLa cells, with endogenous expression of the H1R, were pre-incubated with 10 times the K_i-concentration of the respective compound. Unbound ligands were then depleted by washing the cells, which were subsequently stimulated after different incubation times with 10 µM histamine. The intracellular calcium mobilization following an injection with histamine was determined with the calcium sensitive fluorescent-dye (Fluo4 NW).

Pre-incubating HeLa cells with desloratadine, which has a low (<1) KRI value, resulted in functional recovery of the H1R over time (figure 4A; supplementary table 1). However, cells pre-treated with rupatadine were completely unresponsive to histamine, at least for 2 hours after removing unbound rupatadine, suggesting very persistent target-engagement by rupatadine. In figure 4B, the functional recovery of the H1R is compared after pre-treating the cells with analogs containing cycloaliphatic N-substituents on the piperidine with or without a one-carbon spacer. Analogs with a one-carbon spacer (14, 16 and 17) completely abolished the histamine-induced calcium response for at least 2 hours, similarly to rupatadine. In line with the measured KRI values, removing the one-carbon spacer (19-21), allowed a relatively fast functional recovery of the histamine response.

Figure 3 - Aliphatic substituents on the basic amine of desloratadine cause differential binding kinetics at the H1R. A homogenate of HEK293T cells expressing the H1R was co-incubated with [3H]levocetirizine together with the respective ligands. Binding of [3H]levocetirizine was determined after 1 h and 6 h and the KRI value was determined as the ratio in [3H]levocetirizine binding at both time points (6 h/1h). The bars depict the mean and SEM of ≥ 3 experiments.
The differences in the kinetic binding profiles observed between the compounds were further explored on a structural level by docking studies. Therefore, reference compounds desloratadine, rupatadine, as well as all analogs (3-24) were docked using PLANTS into the doxepin-bound H1R crystal structure. In figure 5 the postulated binding modes of desloratadine, rupatadine and the representative couple 14/19 are depicted in comparison to the binding mode of the co-crystallized ligand doxepin. Desloratadine likely adopts a similar binding pose as was observed for doxepin in the H1R crystal structure (figure 5A). Rupatadine is also found to adopt a similar binding mode as doxepin, but targets an additional area of the H1R binding pocket towards the extracellular vestibule with its (5-methylpyridin-3-yl)methyl moiety (figure 5B). As the available space in the H1R pocket next to the amine-binding region is limited by I454 and Y458, the cyclopentyl substituent of 19 encounters a steric constraint (figure 5C). This results in a tilted binding mode compared to desloratadine, which is not observed for optimal binding of analogs with a methylene spacer between the desloratadine scaffold and the cyclopentyl group (14, figure 5D). The spacer allows the aliphatic group to turn towards the extracellular vestibule in the direction of H450 where more room is available, possibly preventing a steric clash with I454 and Y458 (figure 5D).

3 Discussion

A long drug-target residence time has been postulated to benefit the in vivo efficacy of several drugs for a broad number of drug targets, among which is the H1R. Optimization of the drug-target residence time is not routinely incorporated in drug development and the routinely used affinity-based optimization of the drug pharmacodynamics does not necessarily reflect an increase in the target residence time, which was also the case for ligands binding the H1R. Design strategies for optimizing the drug-target residence time of lead compounds are therefore not widely available. Since rupatadine is shown here to have a much longer residence time at the H1R than its close
structural analog desloratadine, despite a reduced binding affinity, it provides an opportunity for a detailed investigation of the SKR for this archetypical GPCR.

Analogs of rupatadine were designed to replace the (5-methylpyridin-3-yl)methyl group with other aromatic moieties (3-12). Interestingly, the apparent binding affinities of 3-12 are almost identical to the binding affinity of rupatadine. Additionally, all analogs have a long apparent residence time, as is reflected by the KRI > 1. Removing the aromatic character of the functional group by replacing it with a cyclohexyl group (13) does not affect the observed H1R pharmacodynamics either. Hence, the strong effect on the residence time by the (5-methylpyridin-3-yl)methyl group of rupatadine (compared to desloratadine) cannot be explained by the aromatic character, nor by the pyridine nitrogen atom and the methyl substituent.

To further probe the SKR between rupatadine and desloratadine, a series of analogs was characterized that had different aliphatic substituents on the piperidine group (13-24). Strikingly, most aliphatic moieties afford an increase in the KRI compared to desloratadine, while the binding affinity remains similar or even decreases. For example, 13-15 contain relatively large aliphatic substituents (≥ 6-carbons) and have a slightly reduced binding affinity (pK, 8.6 – 8.9) and a high KRI (> 1.4) as compared to desloratadine. Moreover, analogs with small (≤ 3-carbons) acyclic aliphatic substituents (22-24) have a similar binding affinity but still a slightly higher KRI compared to desloratadine. This suggests that growing
an aliphatic group from the piperidine increases the residence time at the H1R. This trend is disrupted, however, for analogs that contain cycloaliphatic groups directly substituted on the amine (18-21) instead of being separated from the amine by a one-carbon spacer (13, 14, 16 and 17). Analogs without the methylene spacer (18-21) are marked by a diminished KRI and binding affinity compared to analogs with a methylene spacer, whereas the KRI values are of the same magnitude as desloratadine.

This cliff in the SKR trend was validated for a subset of analogs by studying the kinetics of functional H1R antagonism, which is known to reflect differential residence times at the H1R. Representative analogs for which the cycloaliphatic group is substituted with a one-carbon spacer (14, 16 and 17) completely inhibit the functional response of the H1R for at least 2 hours after removal of unbound ligands, as was observed for rupatadine. In contrast, analogs with the same cycloaliphatic groups without a one-carbon spacer (19-21) allowed a clear recovery of the H1R functional response, as was also observed for desloratadine. Hence, the relevance of the methylene-spacer for the binding kinetics of analogs with relatively large N-substituents was confirmed by the duration of functional H1R inhibition.

The observed residence time/affinity cliff corroborated with the binding poses of the representative couple 14 and 19 in the H1R binding pocket. The reduced flexibility of the cycloaliphatic group without a spacer (19) enforces a suboptimal fit due to steric hindrance (figure 5C, D). Growing an aliphatic group from the basic amine of desloratadine therefore only increases the residence time at the H1R when the shape of the H1R binding pocket, i.e. the steric constraints imposed by residues I4547.39 and Y4587.43, is not interfering with the binding position of the desloratadine scaffold.

Recently, it was shown that N-methylation of H1R ligands with a primary or secondary amine increased the binding affinity at the H1R by displacing a water molecule near I4547.39. However, this effect on the binding affinity was cancelled out for analogs with a chlorine moiety on the aromatic rings. Consistent with this finding, N-methylation of desloratadine (which contains a chlorine group), affording 24, had only modest effects on the H1R binding affinity. Interestingly, 24 did have a higher KRI compared to desloratadine but not to the same extent as was observed for larger aliphatic substituents (e.g. 13-17). Substitution with aliphatic or aromatic groups on the piperidine possibly reduces the resolvation of both the ligand and binding site during a dissociation event. For ligand dissociation from the CRF1R, for example, a low degree of ligand solvation during egress from the pocket was related to a long residence time at the receptor. For ligand dissociation from the CRF1R, for example, a low degree of ligand solvation during egress from the pocket was related to a long residence time at the receptor. Moreover, hydrophobic shielding of H-bonds can increase the lifetime of such interactions and consequently result in an increased residence time. Considering that N-substitution of H1R ligands was shown to interfere with the water network in the binding site and that the salt bridge between the basic amine of ligands and D1073.32 is crucial for a high binding affinity at the receptor, shielding this interaction pair might prevent a rapid egress of the ligands from the binding site.

4 Conclusion

Rupatadine has an extremely long residence time at the H1R, resulting in a longer duration of functional H1R antagonism compared to desloratadine. The presented SKR shows that aliphatic N-substitution of the piperidine ring from desloratadine is enough to obtain antagonists with a long residence time at the H1R. Analogs with large flexible cycloaliphatic or aromatic substituents, like the (5-methylpyridin-3-yl)methyl
substituent of rupatadine, have a long residence time at the H1R, provided that the substituent does not clash with I4547.30 and Y4587.43 in the binding pocket. Aliphatic N-substitution of H1R antagonists is therefore a potential strategy to optimize the residence time at the receptor. The presented SKR highlights that subtle structural changes of small-molecule ligands can have a profound effect on the binding kinetics at GPCRs.

5 Methods

5.1 Pharmacological assays

5.1.1 Radioligand binding experiments

Radioligand binding experiments were performed as described before with minor alterations.173 Cell pellets were produced from HEK293T cells expressing the N-terminally HA-tagged H1R and pellets were stored at -20 °C. Upon experimentation, cells were thawed, resuspended in radioligand binding buffer (50 mM Na2HPO4 and 50 mM KH2PO4, pH 7.4) and homogenized with a Branson sonifier 250 (Branson Ultrasonics, Danbury, CT, USA). Cell homogenates (0.5 – 3 µg/well) were then incubated with the respective ligands under gentle agitation, as specified for the various assay formats below. After the incubation time, binding reactions were terminated with the cell harvester (Perkin Elmer) using rapid filtration and wash steps over PEI-coated GF/C filter plates. Filter bound radioligand was then quantified by scintillation counting using Microscint-O and the Wallac Microbeta counter (Perkin Elmer).

In competition binding experiments, cell homogenates were incubated for 4 h at 25 °C with a single concentration 1.5 – 4nM [3H]mepyramine and increasing concentrations unlabeled ligands (10⁻⁵ M – 10⁻¹³ M). IC50 values were obtained by analyzing the displacement curves with GraphPad Prism 7.03 (GraphPad Software, San Diego, USA) and were converted to Ki values using the Cheng-Prusoff equation.115 The binding rate constants of [3H]levocetirizine (kindly provided by AstraZeneca) were determined using the previously described methodology, by using 4 different concentrations [3H]levocetirizine (1 – 35 nM) for a total incubation time of 360 min, with an incubation temperature of 37 °C (data not shown).173 This resulted in a $k_{on}$ of 3.7 ± 0.4 10⁶·min⁻¹·M⁻¹ and a $k_{off}$ of 0.022 ± 0.003 min⁻¹. In competitive association experiments with [3H]levocetirizine as radioligand, cell homogenates were incubated at 37°C for various incubation times with a single concentration 5 – 8 nM [3H]levocetirizine in the absence of unlabeled ligand as well as with three different concentrations of either desloradine (2 – 60 nM) or rupatadine (0.1 – 7 nM). The $k_{on}$ and $k_{off}$ values for the binding of [3H]levocetirizine are constrained during the analysis of the H1R binding kinetics of desloratadine and rupatadine. Kinetic binding rate constants as well as their asymmetrical 95% confidence intervals (95% CI) were determined using GraphPad Prism 7.03. Since the 95% CI values were very broad, values are depicted to be higher or lower than the 95% CI boundary value observed over all individually performed experiments. Graphs depict a representative graph with mean and SD of duplicate values showing, for clarity, only a single concentration unlabeled ligand.

Competitive association experiments with [3H]mepyramine as radioligand were performed as described before with minor alterations.173 Briefly, cell homogenates were incubated at 25°C for various incubation
times with a single concentration 2.5 nM – 5.5 nM [³H]mepyramine in the absence or presence of a single concentration unlabeled ligand (desloratadine [4 – 8 nM] or rupatadine [80 – 250 nM]).

In dual-point competition association experiments the kinetic rate index (KRI) at the H₁R are determined. Cell homogenates were incubated on a 96-well plate for 1 h and 6 h at 37°C, with a single concentration 4 – 11 nM [³H]levocetirizine together with a single concentration unlabeled ligand corresponding to one time the respective Kᵢ-value of that ligand at the H₁R. All conditions were measured in triplicate per experiment (n=3). Additionally, per top/bottom half of the 96-well plate, the same concentration [³H]levocetirizine was co-incubated with 10⁻⁵ M mianserin (n = 3), i.e. non-specific binding, and with radioligand in the absence of unlabeled competitors (n = 3). Specific binding levels were obtained by subtracting non-specific [³H]levocetirizine binding from total [³H]levocetirizine binding. The specific binding of [³H]levocetirizine in the presence of the respective unlabeled ligands were then used to determine the KRI-values. [³H]levocetirizine binding after 1 h in the presence of an unlabeled ligand was divided by the [³H]levocetirizine binding after 6 h in the presence of the respective ligand. KRI-values are a quantitative measure for the overshoot in radioligand binding, which results from co-incubating the radioligand with an unlabeled ligand that has a relatively low kᵢ. It is therefore crucial that the concentrations unlabeled ligands are comparable and lead to a sub-maximal inhibition of the radioligand. Therefore KRI-values were only accepted when the %-inhibition of [³H]levocetirizine binding (compared to the specific [³H]levocetirizine binding in the absence of competitor) was (1) less than 80% after either 1 or 6 h and (2) more than 20% after 6 h. In the case that data points had to be excluded, the concentration unlabeled ligands were attenuated (ranging from 1 x Kᵢ to 3 x Kᵢ concentrations).

5.1.2 Intracellular calcium mobilization assay

The functional recovery of the H₁R following antagonism was measured as described before. In short, HeLa cells, endogenously expressing the H₁R, were seeded 2∙10⁴ cells/well in a clear bottom 96-well plate which were pre-incubated overnight with a concentration antagonist corresponding to 10 times the respective Kᵢ at the H₁R (24 wells per antagonist). After 18-20 h, cells were labeled with the Fluo-4NW dye in the presence of the respective concentration antagonist for an hour. Both the excess dye-solution as well as the unbound antagonists were removed by washing the cells two times and cells were then reconstituted in HBSS buffer supplemented with 2.5 mM probenecid (t₀). Following the wash step, cells were stimulated every 5 min by histamine injection, into a single well, using the NOVOstar plate reader (BMG Labtech, Ortenberg, Germany), while simultaneously detecting the calcium mediated Fluo4NW fluorescence (λexcitation 494 nm and λemission 516 nm). For each well stimulated with histamine, a consecutive triton-x100 injection after 65 sec was used to lyse the cells leading to saturation of the Fluo4 NW with calcium. The histamine-induced peak-response was then normalized to basal levels of fluorescence (prior to histamine injection; 0) and saturated Fluo4 NW fluorescence (following Triton X-100 injection; 1). This led to a reproducible histamine induced response over time for HeLa cells pretreated with vehicle condition, which was set to a 100%. Histamine-induced peak-responses were plotted against the difference in time between t₀ and the subsequent histamine injection. The recovery time (RecT) was determined for antagonists by non-linear regression using the one-phase association model in GraphPad Prism 7.03.
5.2 Chemistry

5.2.1 Synthesis of rupatadine analogs (3-24)

Anhydrous THF, DCM, DMF, and Et₂O were obtained by elution through an activated alumina column prior to use. All other solvents and chemicals were acquired from commercial suppliers and were used as received. ChemBioDraw Ultra 16.0.1.4 was used to generate systematic names for all molecules. All reactions were performed under an inert atmosphere (N₂). TLC analyses were carried out with alumina silica plates (Merck F₂₅₄) using staining and/or UV visualization. Column purifications were performed manually using Silicycle Ultra Pure silica gel or automatically using Biotage equipment. NMR spectra (¹H, ¹³C, and 2D) were recorded on a Bruker 300 (300 MHz), Bruker 500 (500 MHz) or a Bruker 600 (600 MHz) spectrometer. Chemical shifts are reported in ppm (δ) and the residual solvent was used as internal standard (δ ¹H NMR: CDCl₃ 7.26; DMSO-d₆ 2.50; CD₂OD 3.31; δ ¹³C NMR: CDCl₃ 77.16; DMSO-d₆ 39.52; CD₂OD 49.00). Data are reported as follows: chemical shift (integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad signal, m = multiplet, app = apparent), and coupling constants (Hz)). A Bruker microTOF mass spectrometer using ESI in positive ion mode was used to record HRMS spectra. A Shimadzu LC-20AD liquid chromatograph pump system linked to a Shimadzu SPD-M20A diode array detector with MS detection using a Shimadzu LC-MS-2010EV mass spectrometer was used to perform LC-MS analyses. An Xbridge (C18) 5 µm column (50 mm, 4.6 mm) was used. The solvents that were used were the following: solvent B (acetonitrile with 0.1% formic acid) and solvent A (water with 0.1% formic acid), flow rate of 1.0 mL/min, start 5% B, linear gradient to 90% B in 4.5 min, then 1.5 min at 90% B, then linear gradient to 5% B in 0.5 min, then 1.5 min at 5% B; total run time of 8 min. All compounds have a purity of ≥95% (unless specified otherwise), calculated as the percentage peak area of the analyzed compound by UV detection at 254 nm (values are rounded). Reverse-phase column chromatography purifications were performed using Buchi PrepChem C-700 equipment with a discharge deuterium lamp ranging from 200-600 nm to detect compounds using solvent B (acetonitrile with 0.1% formic acid), solvent A (water with 0.1% formic acid), flow rate of 15.0 mL/min and a gradient (start 95% A for 3.36 min, then linear gradient to 5% A in 30 min, then at 5% A for 3.36 min, then linear gradient to 95% A in 0.5 min, then 1.5 min at 95% A).

5.2.2 Detailed synthetic procedures and chemical validation

The Supplementary Information lists all detailed experimental procedures and chemical analyses including ¹H-NMR and ¹³C-NMR spectroscopy as well as high-resolution mass spectroscopy and LC-MS chromatography.

5.3 Molecular modeling

SMILES for compounds 1-24 were obtained from ChemBioDraw Ultra (version 16.0.1.4), and were subsequently used as input for ChemAxon’s calculator for protonation (pH = 7.4). A 3D conformation was then generated using Molecular Networks’ CORINA (version 3.49) and stored in Tripos MOL2 format (gold extension). The doxepin-bound H₁R structure was obtained from the protein data bank (PDB-code 3RZE) after which the fused T4-lysozyme was removed from the structure. The complex was further prepared for docking using MOE (Chemical Computing Group, version 2016.0802). Using PLANTS (version 1.2, 210)
each compound was docked into the H1R binding pocket 3-times with the following settings: search speed 1, cluster rmsd 1.0, cluster structures 10, and scored using the ChemPLP scoring function. The binding site was defined by the center of the co-crystallized ligand doxepin with a radius of 11Å. The resulting docking poses were visually inspected and the poses with the best overlap with each other as well as the doxepin reference compounds, which were also the highest-ranking poses for each compound. The binding mode figures were created with PyMol (version 1.8.0).

6 Acknowledgements

Hans Custers is gratefully acknowledged for his technical assistance.

7 Abbreviations

DCE, dichloroethane; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethylsulfoxide; GPCR, G protein-coupled receptors; H1R, histamine H1 receptor; KRI, kinetic rate index; RecT, recovery time; SKR, structure-kinetics relationship; THF, tetrahydrofuran; TEA, trimethylamine.
Supplementary information

**Supplementary Table 1 – Characterization of the kinetics of binding and functional inhibition for H1R antagonists.** Binding affinity (pKᵢ) values were determined by competition binding experiments and KRI-values were determined by dual-point competition association experiments. Recovery time of H1R function (RecT) was determined by the calcium mobilization assay, with steady state recovery expressed as the percentage of vehicle treated cells. Depicted values represent the mean ± SEM of N≥3.

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<th>KRI</th>
<th>RecT (min)</th>
<th>Steady state recovery (%)</th>
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**Synthesis**

Detailed synthetic procedures and chemical validation are furnished upon request.
Chapter 9 - Discussion
1 Measuring drug-target binding kinetics

An important focus within this thesis was the development of methods that allow quantification of the differences in ligand binding kinetics at the H1R (chapter 2 – 5). With new methods, we aimed to address the low throughput and robustness of conventional radioligand binding experiments (chapter 3 and 4). Moreover, we sought to develop methods that reflect the ligand binding kinetics at the H1R in a more relevant biological context (chapter 3 and 5). We are confident that this knowledge will not only benefit the detection of ligand binding kinetics at the H1R, but can be extended, at least in part, to other GPCR targets as well. Below, considerations and limitations are discussed that can be expected when measuring the binding kinetics. Understanding the limitations is crucial for selecting the appropriate method to measure drug-receptor binding kinetics.

1.1 Selecting appropriate incubation times when characterizing the kinetics of pharmacodynamics

As exemplified in figure 1, the binding kinetics of ligand to a receptor (e.g. chapter 4), but also the subsequent antagonism of the receptor (chapter 3 and 5), can often be described by a monophasic exponential function. To characterize the binding kinetics of e.g. a ligand to its receptor, it is important to measure ligand-receptor binding at time points on which the time-dependent differences in receptor occupancy are apparent. The relevant time points are therefore determined entirely by the equilibration rate ($k_{obs}$) of the respective exponential function. The slower the equilibration rate, the longer ligand binding should be measured to properly characterize its binding kinetics. Moreover, at equilibrium the

![Figure 1 – Time points to capture the exponential increase in binding and receptor modulation over time.](#)

Three one-phase exponential curves are depicted, with curve $a$ reflecting a $k_{obs}$ that is 10 fold greater than the $k_{obs}$ of curve $b$ and a 100 fold greater than the $k_{obs}$ of curve $c$. When quantifying $Y$ (receptor binding/activation) on time points $t_0$ and $t_2$ then curve $b$ can be distinguished from curves that increase either slower or faster, since in both cases, $Y$ will change at $t_2$. Conversely, when measuring $Y$ again at $t_0$ and $t_2$, curve $a$ can only be distinguished from curves that increase slower, whereas curve $c$ can only be distinguished from curves that increase faster. By increasing the kinetic resolution (add another time point $t_1$) or by increasing the total duration of detection (add time point $t_3$) more curves could be discriminated based on their observed exponential rate ($k_{obs}$).
receptor occupancy does not change and therefore all information on the kinetics of ligand-receptor binding is lost. Hence, the faster the equilibration rate of ligand-receptor binding, the higher the kinetic resolution needs to be to accurately measure the time dependent changes in receptor occupancy. Both the length of the binding reaction as well as the kinetic resolution is limited by the respective assay that is used. For filtration based radioligand binding reactions, a limited number of incubation times are employed (e.g. chapter 4) and both the incubation time as well as the kinetic resolution are therefore limited. Alternative methods have been recently developed that measure ligand binding continuously \(^{86,126,164,173,174}\). For example, when employing a fluorescent ligand and an energy donor fused to the N-terminus of the receptor, the binding of the ligand can then be measured continuously by the proximity based TR-FRET/BRET signal (chapter 2) \(^{86,126}\). Besides an improved throughput, a continuous detection will also increase the kinetic resolution providing a more robust fitting of the observed kinetics.

1.1.1 The kinetics of functional target modulation

Ligand binding kinetics as measured by radioligand binding experiments is often done with isolated cellular membranes as source of the receptor. However, it is unsure how well this reflects drug binding \textit{in vivo}. For example, a disconnect has been shown between the absolute residence times of antihistamines and the duration of functional H\textsubscript{1}R antagonism, as was measured in radioligand binding experiments and organ bath experiments, respectively \(^{10,88,220}\). Therefore, chapter 3 and 5 were dedicated to measure the kinetics of functional H\textsubscript{1}R modulation by antihistamines on living cells. Ideally, the used functional readout of the target can be measured continuously. Fluorescent or enzymatic reporters of the H\textsubscript{1}R response were considered as well as label free assays, that have been used to quantify H\textsubscript{1}R activity \(^{173,186}\). Besides a continuous detection, a stable receptor-induced functional response is also important. Responses detected using enzymatic reporters, for example, are more difficult to interpret kinetically since the magnitude of the readout is not only dependent on activity of the receptor but also on the change in substrate availability in time. In chapter 3, the histamine-induced β-arrestin2 recruitment to the H\textsubscript{1}R was measured by BRET, which is a ratiometric readout between the emission of the enzymatic energy donor (fused to the receptor) and the fluorescent energy acceptor (fused to β-arrestin2). The ratiometric signal has a normalized magnitude (relative to the output of the enzymatic energy donor), which facilitates data interpretation in kinetic experiments. However, since the signal of the H\textsubscript{1}R and other GPCRs is known to be desensitized upon activation by an agonist \(^{221}\), a decrease in signal can occur that would also convolute the ligand binding kinetics when measuring the response continuously. This is true, for example, for the H\textsubscript{1}R activated intracellular calcium mobilization and also for the DMR response over time (chapter 5). For β-arrestin2 recruitment to the H\textsubscript{1}R, as measured by BRET, a stable histamine-induced response was observed for at least 100 min. Moreover, considering that the β-arrestin2 binds directly to the H\textsubscript{1}R, an amplification of the signal is unlikely and the level of β-arrestin2 recruitment to the H\textsubscript{1}R was indeed found to be proportional to the level of agonist binding (chapter 3). Since the β-arrestin2-receptor complex was only stable upon continued agonist binding to the receptor (chapter 3), β-arrestin2 recruitment is a reversible measure for the level of receptor bound agonist. BRET based detection of β-arrestin2 recruitment to GPCR targets, therefore, seems a promising approach to measure the (competitive) binding kinetics of GPCR ligands in a medium throughput. However, it should be noted that for other GPCR receptors, the β-arrestin2-GPCR complex might be less stable over time as is observed for example for the β-arrestin2-H\textsubscript{1}R complex (data not shown).

A downside of the BRET-based detection of GPCR signaling is that it requires heterologous expression of genetically manipulated proteins. Ideally, the ligand binding kinetics (or its downstream effect) is evaluated in a cell line with endogenous expression of the receptor, since this could for example affect
the level of insurmountable antagonism imposed by antagonists with a long residence time at the receptor. In chapter 5, we therefore set out to measure in HeLa cells the kinetics of receptor inhibition of the endogenously expressed H2R. This was done by a fluorescent-dye-based detection of the histamine-induced intracellular calcium levels or by label free detection of the histamine-induced DMR response. Since both histamine induced responses are rapidly desensitized, a continued detection of receptor antagonism was not possible. Therefore, to compare the kinetics of antagonism after a washout of unbound inhibitor, the histamine induced response was measured at different incubation times (chapter 5). An exponential decrease in histamine-antagonism was observed over time following washout of the inhibitor and, moreover, the observed recovery rate (k_{rec}) of receptor responsiveness correlated with the k_{off} of the respective antagonists. It was additionally clear that insurmountable antagonism of the H2R was achieved already at partial occupancy of the HeLa expressed H2R (i.e. observed for 1xKd concentrations antagonists, which are expected to occupy 50% of the receptor). The absence of a large receptor reserve might explain why absolute k_{rec} values for a lot of antagonists are fairly close to the k_{off} values (e.g. levocetirizine k_{rec} = 0.014 min^{-1}, k_{off} = 0.008 min^{-1}). For the DMR readout of H2R activity, a strong drift in the DMR signal was observed after washout of the inhibitor (up to 1 hour) and therefore it is difficult to quantify the kinetics of antagonism when the k_{rec} is very fast. Nonetheless, it was shown that using two time-points (1 and 2 hours) is for most antagonists enough for a good estimate of the k_{rec} which again correlated with the k_{off} of the respective antagonists. Hence, the kinetic resolution can be decreased (by eliminating the majority of evaluated time points) which will then benefit the assay-throughput. In conclusion, measuring the kinetics of GPCR antagonism on cell-lines with a physiologically relevant expression level can be achieved but at a relatively low throughput, and/or kinetic resolution, compared to assays that employ a continuous readout.

1.2 Limitations of ligand binding kinetics measurements

1.2.1 Incubation times and kinetic resolution

Continuous detection methods, but also methods using a finite incubation time for a few time points (vide supra), could be employed to obtain kinetic information in medium throughput. However, having a low kinetic resolution will make it more difficult to obtain robust fitting of the binding curves potentially causing a lower reproducibility. Additionally, choosing a limited number of time points will increase the chance that the equilibration of ligand-receptor binding is too fast/slow to be measured within the chosen time-frame (figure 1).

A good example is presented in chapter 5 in which the increased intracellular calcium levels in HeLa cells was used as a functional readout of H2R activation. It was observed that several antagonists that were pre-incubated with HeLa cells expressing the H2R, imposed insurmountable antagonism of the histamine response. Insurmountable antagonism indirectly reflects the remaining occupancy of antagonist on the receptor which could not be displaced by saturating concentrations agonist between two time-points: agonist stimulation and the functional readout (see also chapter 2). The decrease in antagonist occupancy can be converted into a k_{off}. If the antagonist is fully surmountable or insurmountable, than only a minimal or maximal k_{off} can be estimated, respectively. This was the case for H2R antagonism by both doxepin and levocetirizine (chapter 5, figure 4), which both imposes a fully insurmountable effect on the histamine induced calcium mobilization in HeLa cells. The inability to discriminate between the relative k_{off} of doxepin and levocetirizine at the H2R is in contrast to the tenfold difference in residence time (based on radioligand binding experiments) and is most likely related to the short window between agonist stimulation and the peak calcium levels in the cell (functional readout), which was observed after approximately 10 sec. Consequently, even relatively short residence time antihistamines remain largely
bound to the H1R within this time-frame and impose therefore a fully insurmountable inhibition of the histamine-response. By employing the same functional readout, the decrease in antagonism over time after washout was characterized every five minutes over a total incubation time of two hours (chapter 5: figure 5). In this case, the H1R recovery rates (k_{rec}) imposed by doxepin and levocetirizine could clearly be distinguished, reflecting the relative differences in k_{off}, whereas this difference was not reflected by their insurmountable antagonism of the histamine induced calcium mobilization (i.e. based on only two time points). Hence, the kinetic resolution and incubation time will directly determine which ligands can be discriminated based on their receptor-binding kinetics.

1.2.2 Determining the k_{on} and the k_{off} for drug-receptor binding

In many cases the observed binding kinetics is deconvoluted into the underlying rate constants of the ligand at the receptor (k_{on} and k_{off}), which are independent of the used ligand concentration. In such cases it is not only important to characterize the kinetics of ligand binding with the appropriate time-points, it

![Figure 2 - Probe dependency for the accuracy of unlabeled ligand k_{on} and k_{off} at the H1R. Binding rate constants of unlabeled ligands were determined in radioligand binding studies, using either [^3H]mepyramine or [^3H]levocetirizine as competitive probe. A correlation plot is depicted for the k_{on} (A) and k_{off} (B) as determined from competitive association experiments using [^3H]mepyramine (x-axis) or [^3H]levocetirizine (y-axis). Dashes lines represent a perfect correlation respective to the X-axis values and solid lines represent the linear regression lines. In C and D, the accuracy is depicted in which the Motulsky-Mahan model fitted the k_{on} (C) and k_{off} (D) by non-linear regression. To compare the accuracy of the fitted mean k_{on} and k_{off} values over a broad range, the relative magnitude of the error (SD), as derived from non-linear regression, was calculated for each individual replicate experiment and pooled for all ligands. The relative error was calculated by normalizing the SD by the mean (relative error = SD / mean). Subsequently, the relative error for the k_{on} and k_{off} were plotted against the corresponding mean k_{off} determined from the same competitive association curve. Data points derived from competitive association experiments that employed [^3H]levocetirizine are depicted in red. Additionally, data points from competitive association experiments with [^3H]mepyramine are shown, in which the total incubation time was either 80 min (blue) or 300 min (black). The arrows depict the k_{off} of the used probes with [^3H]levocetirizine in red and [^3H]mepyramine in blue as reported in table 1. Dashed lines represent a relative error of 1 (mean = SD).]
is also important that the observed kinetic signature provides sufficient information to determine both the \( k_{\text{on}} \) and the \( k_{\text{off}} \). Determining the binding rate constants is not always straightforward as became apparent, e.g., from the probe dependent \( k_{\text{on}} \) and \( k_{\text{off}} \) values that were measured for unlabeled ligands by competitive association experiments (chapter 4). In figure 2A and 2B, it is shown that similar trends in the \( k_{\text{on}} \) and \( k_{\text{off}} \) values are obtained when employing either \(^{3}H\)mepyramine or \(^{3}H\)levocetirizine as radioligand probe, but that the absolute values of the binding rate constants differed a lot in some cases. Upon closer inspection (figure 2C and 2D) it becomes clear that the accuracy of the fitted \( k_{\text{on}} \) and \( k_{\text{off}} \) values also depended on the mean \( k_{\text{off}} \) of the respective unlabeled ligand.

It seems therefore possible that the \( k_{\text{off}} \) values (as well as \( k_{\text{on}} \) values) differ when measured with different probes (figure 2B) because of the (incidentally) poor accuracy obtained when fitting this constant (figure 2D). When accuracy is low it seems logical to assume that this results in a poor reproducibility of the obtained \( k_{\text{off}} \) values, but this is not apparent for deviating values of the binding rate constants observed in figure 2B (compare error bars). Moreover, the relative differences in the \( k_{\text{off}} \) between ligands sometimes differed extensively depending on the used radioligand. For example, a 10-fold difference in the residence time was observed between S-cetirizine and VUF14544 when using \(^{3}H\)mepyramine as probe, whereas there was no difference when \(^{3}H\)levocetirizine was used as probe. Hence, reproducible \( k_{\text{off}} \) values for unlabeled ligands binding the H1R can be obtained in competitive association experiments that do not necessarily do justice to the relative differences between ligands.

An interesting case study is the competitive association between histamine and the probe \(^{3}H\)mepyramine. For histamine a \( k_{\text{off}} \) of \( 0.38 \pm 0.07 \text{ min}^{-1} \) was measured and a low micromolar affinity (\( pK_{d} = 4.3 \pm 0.0 \); chapter 3)\(^{62,173} \). Considering that \(^{3}H\)mepyramine has a > 10,000 times higher binding affinity than histamine (\( pK_{d} = 8.7 \pm 0.1 \)), the < 2-fold difference in \( k_{\text{off}} \) is surprisingly small (\( k_{\text{off}} = 0.22 \pm 0.01 \text{ min}^{-1} \)). Interestingly, consistent \( k_{\text{off}} \) values were obtained for histamine at the H1R in replicate competitive association experiments, with reasonable errors and 95% confidence intervals derived from non-linear regression analysis (table 1). When stimulating cells with histamine, stable β-arrestin2 recruitment at the H1R was observed over time (\( \geq 100 \text{ min} \)). By challenging the cells subsequently with high concentrations mepyramine, all H1R recruited β-arrestin2 was depleted within 2 min (chapter 3, figure 2). This suggests that histamine must at least dissociate within this time-frame. This was investigated closer by pre-incubating cells with histamine followed by an in-well injection of increasing concentrations mepyramine with a parallel detection of β-arrestin2 bound H1R, as depicted in figure 3. A mono-exponential decrease in β-arrestin2 bound H1R was observed with \( k_{\text{obs}} = 14 \pm 2 \text{ min}^{-1} \) for the highest concentration mepyramine. This implies that the histamine \( k_{\text{off}} \) is \( \geq 14 \pm 2 \text{ min}^{-1} \) (could be higher in the case that β-arrestin2 dissociation is rate limiting), suggesting that the \( k_{\text{off}} \) obtained in competitive association experiments is indeed underestimated. It should be noted however, that β-arrestin2 recruitment to the H1R is measured at 37°C, which is 12°C higher than the temperature in competitive association experiments, which has been shown, e.g. at the H1R\(^{64} \), to increase the \( k_{\text{off}} \). However, it is unlikely that this underlies a 36-fold difference in the \( k_{\text{off}} \), since only 7 – 10 fold differences in the \( k_{\text{off}} \) of other H1R ligands were observed over the same

| Table 1 – Best fitted values and reproducibility of the \( k_{\text{off}} \) of histamine at the H1R. Values are obtained from competitive association binding experiments for \(^{3}H\)mepyramine, histamine and a homogenate of HEK293T cells transiently expressing the H1R. Experiment is described in detail in chapter 3. |
|-----------------|-----------------|-----------------|
| \( k_{\text{off}} \) (min\(^{-1}\)) | N = 1 | N = 2 | N = 3 |
| SD              | 0.37 | 0.27 | 0.50 |
| 95% confidence interval | 0.006 to 0.73 | 0.18 to 0.37 | 0.25 to 0.75 |
temperature range. In conclusion, the \( k_{\text{off}} \) of histamine seems underestimated by competitive association experiments despite good reproducibility.

### 1.2.3 Kinetic signatures of competitive association experiments

The described inaccuracy for the fitted binding rate constants (section 1.2.2) could either derive from the chosen time points, which perhaps insufficiently constrain the complete kinetic signature. However, it is also possible that the observed kinetic signature is well constrained by the chosen time points, whereas the observed kinetic signature does not provide enough information to stringently fit the \( k_{\text{on}} \) and the \( k_{\text{off}} \) of the unlabeled ligand. For example, in the more straightforward case of a single ligand binding the receptor, a monophasic increase in binding is observed for which the \( k_{\text{obs}} \) is described by equation 1. By changing the ligand concentration \([L]\), several \( k_{\text{obs}} \) can be measured which provide information to fit both the \( k_{\text{on}} \) and the \( k_{\text{off}} \) (as was done e.g. in chapter 3 for \[^{3}H\]mepyramine).

\[
k_{\text{obs}} = k_{\text{on}} \times [L] + k_{\text{off}} \quad (1)
\]

However, when four very high concentrations are used, than the \( k_{\text{off}} \) of the ligand has a negligible contribution to the \( k_{\text{obs}} \) values, which will then give an inaccurate estimation of the \( k_{\text{off}} \) (despite the fact that the \( k_{\text{obs}} \) might be fitted very stringently by the model).

The competitive binding between two ligands at the receptor is described by a more complex model derived by Motulsky and Mahan. Consequently, it is not always directly apparent whether a kinetic signature provides enough information to constrain the \( k_{\text{on}} \) and the \( k_{\text{off}} \) of the unlabeled ligand. The inaccuracies for fitting the \( k_{\text{on}} \) and \( k_{\text{off}} \) depicted in figure 2C and 2D are therefore discussed in more detail below.

![Log[mepyramine] vs. kobs (min⁻¹)](image)

**Figure 3 - Depleting the β-arrestin2 bound H₃R by histamine dissociation from the receptor.** Using the methodology described in chapter 3, the recruited β-arrestin2 at the H₃R is measured by BRET. Cells were pre-incubated for 1 hour with 10⁻⁵ M histamine, followed by a 10 min co-incubation with luciferase substrate and histamine. Consecutively, cells were challenged by an in-well injection of mepyramine (time = 0 sec) and BRET was detected for another 50 sec. A representative graph is shown from 3 experiments and the data reflects the mean ± SEM of triplicate values.
1.2.3.1 Short residence time unlabeled ligands (high $k_{off}$): use a probe with an even shorter residence time

From figure 2C and 2D it becomes apparent that fitting the binding rate constants of unlabeled ligands with a relatively short residence time (i.e. high $k_{off}$ on x-axis) is inaccurate (this is true for both the $k_{on}$ and the $k_{off}$). This inaccuracy is probe-dependent and seems to be relative to the residence time of the probe itself ($k_{off}$ of the respective probes are depicted by arrows in figure 2C and 2D), because when the unlabeled ligand has a shorter residence time than the probe ($k_{off}$ probe < $k_{off}$ unlabeled ligand), the accuracy decreases. Interestingly, for the competitive binding of [$^{3}$H]levocetirizine with either mepyramine (a short residence ligand) or levocetirizine (a long residence time ligand), the observed radioligand binding over time was captured well by the selected time-points (figure 4A and 4B, respectively). This seems to suggest that the kinetic resolution is not a limiting factor and that the kinetic signature therefore does not provide enough information to reliably fit the $k_{on}$ and the $k_{off}$. To probe this, the fitted values for the $k_{on}$ and the $k_{off}$ of unlabeled mepyramine (figure 4A) were constrained to 100-fold higher values than originally fitted by non-linear regression (C, dashed lines).

1.2.3.2 Long residence time unlabeled ligands (low $k_{off}$): increase the incubation time

From figure 2C and 2D it becomes apparent that fitting the $k_{off}$ (but not the $k_{on}$) of unlabeled ligands with a relatively long residence time (i.e. low $k_{off}$ on the x-axis) is inaccurate as well, which seems to be dependent on the used radioligand. In general, the total equilibration time for a competition association experiment increases with the longest residence time at the receptor from either the probe or the
competitive ligands. Competitive association experiments that employ unlabeled ligands with a long H1R-residence time therefore require a longer incubation time to reach a steady state in binding. Since the total incubation time is different between experiments using either [3H]mepyramine or [3H]levocetirizine (figure 4A and 4B), the used incubation times might be the limiting factor for an accurate fitting of the \( k_{\text{off}} \)-values (specifically in the case for unlabeled ligands with a long H1R-residence time).

To explore this, competitive association binding data was simulated (using Graphpad Prism 6) for a probe with the binding rate constants of [3H]mepyramine, using 12 different time points (reflecting a typical radioligand binding kinetics experiment). Different \( k_{\text{off}} \) values for the ‘unlabeled ligand’ were simulated (with a random SD of 10%) while keeping the binding affinity (= \( k_{\text{off}}/k_{\text{on}} \)) constant. Representative curves are shown (\( k_{\text{off}} \) ‘unlabeled’ = 0.018 min\(^{-1}\)) for simulated incubation times of 80 min (figure 5A) or 300 min (figure 5B). Graphs were simulated 300 times for each set of conditions and were analyzed with the Motulsky-Mahan model (as, e.g., in chapter 4). The distribution of the fitted \( k_{\text{off}} \)-values are presented in figure 5C and 5D. It is indeed shown that for the ‘unlabeled ligands’ with a long residence time (low \( k_{\text{off}} \) on the x-axis) that the variability was much greater when an incubation time of 80 min was simulated than
for a 300 min incubation time. Hence, the incubation time plays an important role for the accuracy of the fitted $k_{\text{off}}$-values.

1.3 The optimal assay depends on the desired binding kinetics of the lead compound.

It was discussed in the last paragraphs that measuring the kinetics of ligand binding roughly depends on two things: (1) do the time-points of detection capture the differential receptor binding/activation over time and (2) does the kinetic signature provide enough information regarding the pharmacodynamic parameter of interest. The former depends largely on the used assay whereas the latter depends mostly on the experimental design (section 1.2.3). When selecting an assay it is mostly preferred to have a continuous detection of ligand binding. However, also for assays with a continuous readout, the maximal incubation time can be hampered. For example, BRET-based detection of fluorescent-ligand binding depends on the presence of a substrate and the total incubation time is therefore limited by the consumption of the substrate. But also TR-FRET based detection of fluorescent-ligand binding was found previously to be convoluted over time due to bleaching of the fluorophore. Moreover, most methodologies do not allow a continuous detection of the respective pharmacodynamic process, whereas much more assays could be tailored in a way to detect two different time-points (section 1.1.1). Dual-time-point assays are therefore a versatile approach for establishing the relative kinetics of ligand binding. Moreover, in controlled settings, using a dual time-point approach (e.g. DMR and insurmountable antagonism (chapter 5) or KRI (chapter 8) to measure the relative binding kinetics of ligands can result in very meaningful data. In chapter 8, for example, the dual-point competition association method was very effective to determine (with medium throughput) the relative differences in the $H_1R$-residence time of analogs of desloratadine and rupatadine. The exact quantification of the residence time was not immediately necessary since both desloratadine and rupatadine functioned as a good benchmark for the relative residence time of their analogs.

Additionally, the success of dual-time-point assays in terms of throughput was already highlighted by a study that measured the relative residence time of more than 1800 antagonists at the $D_2R$. To do so, membranes expressing the receptor were pre-incubated with antagonist. Consecutively, membranes were separated from unbound ligands using filtration and membranes were then incubated with radioligand. The rate of dissociation from the $D_2R$ of the unlabeled ligands determines the available receptors for binding the radioligand, which is evaluated after a 5 min incubation time. The amount of radioligand is therefore an indirect measure, distinguishing between unlabeled ligands with various degrees of dissociation within this 5 min incubation time. However, theoretically, the incubation time could be easily tailored to reflect the differences in residence time for any relevant timespan (figure 1).

Major drawback of such an approach is the required knowledge of the optimal drug-target residence time. If it is known what drug-target residence time would be required to elicit an $in\ vivo$ response than this would be the perfect incubation time in the above example for ranking the relative drug-target residence times. However, research describing the effects of the respective drug-target residence time $in\ vivo$ is lacking, making it often unclear what the desired residence time would be.

A popular rational for increasing the residence time in the literature is to retain a prolonged receptor occupancy after the clearance of unbound drug. In cases in which drug-target residence time could increase the therapeutic window, effectiveness would also depend on the rate in which new unbound drug target is synthesized, subsequently lowering the occupancy of the total receptor population. For example, it was shown $in\ vitro$ and $in\ vivo$ that the inhibitor of BTK1, despite its 167 h residence time had a >50% reduction in occupancy within a day due to re-synthesis of the kinase. Increasing the drug-
target residence time far beyond the time needed for re-synthesis of the target will therefore not have an increased effect on the therapeutic window.

Another example where drug-target residence time could make a difference is for drug-targets that can be antagonized in an insurmountable fashion. This can occur when the presence of the agonist is transient (e.g. neuronal signaling), enabling the long-residence time antagonist to outlast the presence of the agonist while bound to the target\textsuperscript{19,175}. In this way signaling will be blocked by the antagonist even when there is a very high concentration agonist. However, once an antagonist would already have a full and insurmountable inhibition of the agonist \textit{in vivo} by outlasting the pulse of agonist exposure, a further increase in residence time would be trivial for the imposed insurmountable antagonism. However, pinpointing the required residence time is not just dependent on the timing and frequency of the agonist pulse but also on the number of receptors and transduction efficiency, which will be cell type dependent\textsuperscript{132}. Therefore determining the minimum residence time for complete insurmountable antagonism might not be as straightforward.

In conclusion, long residence time drugs might have a kinetic advantage \textit{in vivo} but if its advantage is at some point limited by the biological system, a further increase in the residence time would be unimportant. Hence, the relation between the kinetics of drug target-binding and its imposed effect \textit{in vivo} requires much more attention. Breakthroughs here will not only help in establishing selection criteria in early drug discovery but as discussed it could also enhance the throughput in which information on the drug-target binding kinetics can be obtained by dual point assays. This would also make it easier to use functional assays for drug optimization in which it is difficult to quantitatively determine the kinetic binding rate constants of drugs, but easier to measure the relative effects on agonist signaling\textsuperscript{223,224}. Moreover, when using functional assays to measure the duration of action of a test-set of ligands, this could capture already some of the biological limits, which arguably could be more valuable information for drug optimization than just the drug-target residence time.

2 Molecular understanding of the differences in drug-receptor binding rate constants

2.1 Structure kinetics relationships for H\textsubscript{1}R ligand binding

In chapter 6, 7 and 8 close analogs were compared to identify structural elements of H\textsubscript{1}R ligands that corroborated with a long residence time binding at the H\textsubscript{1}R. Two structural motifs were found to enhance the ligand-H\textsubscript{1}R residence time (figure 6). One of these motifs is the carboxyl group which was introduced in VUF14454 on different positions, affording e.g. VUF14506 and VUF15288, to bind a cationic part of the H\textsubscript{1}R extracellular vestibule (figure 6, chapter 6). The other motif is a variation on the prototypical aromatic rings of antihistamines, which is a well conserved H\textsubscript{1}R-ligand pharmacophoric feature\textsuperscript{36}. When these aromatic rings were fused into a tricyclic ring structure, this greatly increased the residence time at the H\textsubscript{1}R as depicted in figure 6 for cyclizine, VUFH1896 and BS7617 (chapter 7). Interestingly, the clinically used antihistamine olopatadine possesses both structural motifs, which results in an H\textsubscript{1}R residence time that exceeds the ligand-receptor residence times of almost all tested ligands in this thesis (chapter 4). Moreover, for desloratadine, another clinically used tricyclic antihistamine, the residence time at the H\textsubscript{1}R could be further increased by substituting the piperidine with an aliphatic or aromatic group, as observed for, e.g., VUF16140 (figure 6, chapter 8). In the respective chapters, hypotheses for the increased residence time often relied on transition state theory, which can explain the observed disconnect between ligand binding affinities and ligand residence times\textsuperscript{15,62,72,194,206}. Transition state theory proposes that an
intermediate instable complex is formed, separating the ligand-bound from the unbound receptor state\textsuperscript{62,225}. When a solvated ligand has to enter a hydrophobic binding pocket, this molecule must at some point cross a transition in which the ligand is not fully solvated and not yet bound in the binding site. This is especially true for deeply buried hydrophobic binding sites like that of the H\textsubscript{3}R\textsuperscript{37,38,62}. Moreover, repelling forces and conformational constrains might destabilize the ligand even further during its association path to the stable binding position.

As discussed in the introduction, the Eyring equation\textsuperscript{73} can describe the transition state of a binding reaction, from which it follows that the transition state energy is related to the $k_{on}$, the binding energy is related to the $K_d$ and the $k_{off}$ is dependent on the sum of the two energies (figure 7). This implies that the $k_{on}$ and $K_d$ can be modulated separately to obtain the optimal residence time ($1/k_{off}$), suggesting moreover that the stabilizing interactions observed in crystal structures that are found to shape ligand binding affinity (chapter 1; section 4.1) do not provide enough information to predict the ligand residence time. A better molecular understanding of differential transition state energies between ligands binding the

\begin{itemize}
\item \textbf{VUF14454} RT = 2.0 ± 0.1 min
\item \textbf{VUF14506} RT = 23 ± 2 min
\item \textbf{VUF15288} RT = 18 ± 1 min
\item \textbf{VUF14454} RT = 2.5 ± 0.4 min
\item \textbf{VUFH1896} RT = 9 ± 1 min
\item \textbf{BS7617} RT = 30 ± 4 min
\item \textbf{olopatadine} RT = 190 ± 50 min
\item \textbf{desloratadine} RT = 190 ± 40 min RecT = 57 ± 7 min
\item \textbf{VUF16140} RecT >> 57 min
\end{itemize}

\textbf{Figure 6 - Chemical features of antihistamines that promote a long residence time on the H\textsubscript{3}R.} Residence time (RT) values are obtained from chapter 6 and 7 and functional recovery time (RecT) values are obtained from chapter 8. More than 90% of the H\textsubscript{3}R antagonism imposed by VUF16140 was retained for longer than 120 min after washout of the ligand and an exact recovery time could therefore not be obtained.
same receptor could therefore be valuable to guide the design of new drug molecules. However, studying the transition state depends highly on \textit{in silico} simulations, since the transition state is per definition unstable and structural information cannot be obtained with the currently available \textit{in vitro} methods.

Molecular dynamics (MD) simulations have been performed to characterize the association of ligands binding the β2-adrenergic receptor (β2AR)\textsuperscript{207}. It was found that desolvation of both the ligand and the receptor imposed the strongest barrier for ligand binding, which constrained ligand association 15 Å away from the stable binding position in the extracellular vestibule of the receptor. There was an additional transition barrier along the association pathway of the ligand from the extracellular vestibule to the stable binding position in which steric hindrance might play a bigger role. Under the assumption that the ligand leaves the receptor via the same pathway, the rate of ligand-dissociation from the binding site is expected to be equally dependent on these unfavorable transition states. However, since the residence time of a drug-receptor complex is often in the range of minutes to hours, it is difficult to simulate the unbinding of drugs with MD simulations in which time scales are in the milliseconds range\textsuperscript{62}.

Various \textit{in silico} methods were recently developed to observe ligand dissociation in MD simulations by introducing a bias that forces the ligand out of the binding site. These \textit{biased} MD experiments suggested, e.g., that the long residence time of corticotropin releasing factor receptor 1 (CRF\textsubscript{1}R) ligands, as measured \textit{in vitro}, correlated with a reduced degree of ligand solvation along the egress pathway\textsuperscript{218}. This indicates that solvation of ligand and receptor might indeed impose a strong transition barrier for dissociation as was suggested for the β2AR. Moreover, it was found that hydrogen bonds between ligand and receptor which were sterically shielded from water molecules were more stable and long-lived, which potentially could increase the drug-receptor residence time\textsuperscript{219}. In \textit{unbiased} MD simulations of the muscarinic M\textsubscript{3} receptor (M\textsubscript{3}R) it was shown that steric hindrance by aromatic residues blocked the exit pathway for the long-residence-time-ligand tiotropium, preventing its dissociation from the receptor. Tiotropium was shown to interact by its hydroxyl group with the N508\textsuperscript{6.52} and a close analogue of tiotropium lacking this alcohol moiety (i.e. the alcohol group was replaced by a hydrogen) was found to dissociate much faster from the M\textsubscript{3}R\textsuperscript{194}. MD simulations showed that opening of the egress pathway was accompanied by solvation of N508\textsuperscript{6.52}, which occurred less frequent for tiotropium than for its analogue without an alcohol group. Both the opening of the egress pathway and solvation of N508\textsuperscript{6.52} were considered to be essential for tiotropium unbinding\textsuperscript{226}. In general, solvation and steric hindrance seem to be plausible transition state barriers for the binding and unbinding of GPCR-ligands.

The transition state of ligand binding provides plausible explanations for the aforementioned SKRs among ligand analogs at the H\textsubscript{1}R (chapter 6 – 8). The desolvation energy is likely increased for ligand analogs with a carboxyl group (e.g. VUF14506), potentially explaining the low $k_{on}$ as a consequence of a high transition state barrier (chapter 6). Moreover, resolution of the binding pocket upon dissociation of desloratadine-analogs that contain aliphatic/aromatic N-substitutions (e.g. VUF16140) might be unfavorable due to hydrophobic shielding of the interaction between the basic amine and D107\textsuperscript{3.32}, which could explain the long residence time of these analogs at the H\textsubscript{1}R (chapter 8). Finally, steric constraints during ligand association and dissociation might play a role for ligands that contain a rigid tricyclic aromatic core as is exemplified for example by the relatively low $k_{on}$ of BS7617 (chapter 7). However, it is likely wrong to interpret the measured $k_{on}$ as a direct consequence of only the transition state energy. It has been
described, for example, that the relative collision rate between ligand and receptor as well as local ligand concentrations near the receptor might also affect the measured $k_{\text{on}}$. Finally, the mechanism of ligand binding to the receptor can also affect the observed binding rate constants. Convoluting factors that might shape the measured binding rate constants are therefore discussed in more detail below.

### 2.2 Ligand diffusion and rebinding

The transition state can only fully explain the differences in $k_{\text{on}}$ between ligands binding to the same receptor when the collision rate between the different ligands and the receptor is the same. Factors that might influence the collision rate between molecules are therefore discussed.

The diffusion rate limit of two molecules colliding is expected to be in the order of $10^{-9}$ M$^{-1}$s$^{-1}$ for two medium-sized soluble proteins$^{62,225,227}$. However, when only a part of the surface is participating in the binding interaction, the amount of effective collisions will decrease dramatically. This will therefore reduce the diffusion-rate limit to the order of $10^{-5}$-$10^{-6}$ M$^{-1}$s$^{-1}$ $^{225,227}$. In the case of ligand binding to the H$_1$R, the situation becomes even more complex because the ligand is free to diffuse but the receptor can only diffuse laterally over the membrane. Moreover, simulations show that a molecule colliding with the membrane are likely to collide a couple of times more before diffusing away, which could potentially increase the amount of effective binding events$^{10,228}$. In general, the rate of successful collision between ligand and receptor is determined by many factors and can, e.g., be influenced by receptor density, localization of the receptor and the geometrical shape of the membrane$^{10}$. The above variables are expected to alter the $k_{\text{on}}$ of all ligands similarly. However, there are also ligand-specific effects on the collision rate with the receptor as a consequence of the underlying differences in physicochemical properties. One well-characterized example is that long-range coulomb interactions are known to increase the diffusion-limited rate of collision. This charge-mediated accelerated binding was shown for various ligands binding to their respective targets and was found to be determined by charge of the ligand molecule and receptor$^{91,229}$. Another difference in the diffusion of ligands towards the target-receptor could be caused by ligand affinity for the membrane. This might result in lateral diffusion of the ligand over the membrane, which confines both ligand and receptor to a 2D surface, increasing therefore the chance of collision$^{10}$. If a ligand can only slowly diffuse away from the receptor after dissociation as a consequence of e.g. ligand retention on the membrane, there will be a higher chance for the ligand to bind back to a (nearby) receptor. This rebinding could be an alternative mechanism to prolong the apparent drug-target occupancy and albeit different than the drug-target residence time, these mechanisms could be easily mixed up when experiments are not designed to distinguish between the two (see also chapter 2). Related to rebinding it might also be possible that ligands can accumulate in direct vicinity to the drug target or in an exosite at the receptor interface$^{10,230}$. This will affect the local ligand concentrations and consequently the observed association rate and ligand binding affinity for the receptor$^{189}$. Moreover in the case that ligand concentrations in bulk solvent are depleted, the exosite (or any proximal site of ligand retention) can only be adopted by ligands that dissociate from the receptor, which could therefore function as a diffusion barrier and hence increase the level of rebinding$^{10}$.

Also for the histamine H$_1$ receptor enrichment of ligand concentrations near the receptor might affect the observed binding dynamics. For the H$_1$R antihistamines azelastine and levocetirizine (long residence time)
but also for mepyramine and dextrocetirizine (short residence time), it was shown in organ bath experiments that functional inhibition of the H\textsubscript{1}R was retained much longer than could be explained by the residence time determined in radioligand binding experiments on cell membranes\textsuperscript{64,84,87}. Nonetheless, the relative differences between ligand residence times seemed to coincide with the duration of functional receptor inhibition. This suggests that an independent mechanism prolongs the duration of target inhibition which works synergistically with the residence time of antihistamines. Tissue incorporation could be an explanation for this mechanism, especially since it was already observed before that the long acting antihistamine fexofenadine had prolonged bioavailability in tissue compared to plasma concentrations\textsuperscript{231}. Moreover, it was shown that the insurmountable mode of antagonism of azelastine on isolated guinea pig trachea was abrogated when the epithelial layer was removed, indicating that this long duration of action might result from epithelial layer-mediated rebinding of azelastine\textsuperscript{87}.

2.3 Transition state energy versus local ligand concentrations

The transition state theory suggests that residence time is ultimately determined by both the energy of binding and height of the transition state barrier (figure 7) as can be determined by measuring the \(K_a\) and the \(k_{\text{on}}\), respectively. Based on this rational it has been proposed to use structure activity relations for both metrics to come to a rationalized optimization of the residence time\textsuperscript{206}. However, interpreting the differences in the \(k_{\text{on}}\) as measured in pharmacological experiments might not be as straightforward, since

![Figure 7 - A transition state for ligand-receptor binding reactions.](image)

The bound and unbound states are separated by a transition state (\(T\)) representing a high free energy coordinate along the reaction path. The difference in free energy between (\(T\)) and the unbound state (\(1\)), i.e. \(\Delta G_{\text{on}}\), determines the association rate constant (\(k_{\text{on}}\)) and the difference in free energy between (\(T\)) and the bound state (\(2\)), i.e. \(\Delta G_{\text{off}}\), determines the dissociation rate constant (\(k_{\text{off}}\)). Moreover the energy of binding is determined by the free energy difference between (\(1\)) and (\(2\)), i.e. \(\Delta G_a\), which determines the ligand binding affinity (\(K_a\))\textsuperscript{62}. When the energy of ligand solvation is increased, e.g. in the case of a more hydrophobic ligand, the free energy of the unbound state becomes higher as well (red line). Consequently, the |\(\Delta G_a\)| and hence the binding affinity, will increase. However, in the case that ligand is desolvated in the transition state (\(T\)), the stability of (\(T\)) is unaffected by the increased solvation energy and the |\(\Delta G_{\text{on}}\)| is therefore decreased and hence the \(k_{\text{on}}\) is increased.
it can be convoluted by the aforementioned changes in local ligand concentrations or ‘effective’ ligand diffusion towards the receptor.

An interesting example for when the transition state energy might not be directly related to the $k_{on}$ is when the ligand binding affinity (pK$_d$) seems to correlate with the log $k_{on}$\textsuperscript{133,189,232}. Such an observation is counterintuitive from a thermodynamic point of view, since the $k_{on}$ should be dependent only on the transition state energy whereas the binding affinity is expected to depend on the free energy of binding (figure 7). For $\beta_2$AR ligands, it was postulated that this correlation between the log $k_{on}$ and the pK$_d$ was driven by the ligand affinity for phospholipids (i.e. the cellular membrane), which will affect the local ligand concentrations near the receptor. Since ligand concentrations are assumed to be homogenous by the used pharmacological models, underestimating the effective ligand concentrations near the receptor are expected to cause overestimations of the $k_{on}$ as well as the pK$_d$. Local ligand concentrations near the receptor were therefore estimated based on the affinity for phospholipids and data was re-analyzed using this new information. The corrected $k_{on}$-values hardly differed between the various ligands and a correlation was no longer found between the log $k_{on}$ and the binding affinity. Moreover, the corrected pK$_d$-values now correlated with log $k_{off}$-values instead. This suggests that the $k_{off}$ is fully driven by the binding affinity and the observed differences in the $k_{on}$ are caused by local ligand concentrations near the $\beta_2$AR as a result of their physicochemical properties.

Although increased local ligand concentrations could explain the observed differences in the $k_{on}$ at the $\beta_2$AR, an alternative hypothesis can be formulated. Ligand binding is shaped by the interactions that are created upon binding, but is also negatively dependent on all the interactions that are made by the unbound ligand and receptor, which have to be broken in order to accommodate the ligand-receptor interactions (e.g. the interactions with the solvent). In the above example, the affinity of the $\beta_2$AR-ligands for phospholipids was additionally related to its hydrophobicity. This could be estimated from the predicted tendency of ligands to dissolve in n-octanol over that in water (clogP). This highly correlated with the measured membrane affinity, which means that increased membrane affinity observed for the $\beta_2$AR-ligands might be driven by the reduced solvation energy in buffer. However, if this is true, this would likely also increase the affinity for other hydrophobic adhesion sites, like the binding pocket of the $\beta_2$AR. Moreover, since desolvation of the ligand has been implicated to be an important bottleneck in ligand association \textsuperscript{207}, reducing the absolute desolvation energy could increase the $k_{on}$. In other words, by increasing the gibbs free energy of the unbound state (i.e. increasing the solvation energy as depicted by the red line in figure 7) the ln $k_{on}$ is expected to decrease whereas the lnK$_d$ is expected to increase. This would explain, complying with transition state theory, why $k_{on}$ and the binding affinity might be correlated. Moreover, for ligands binding the hERG channel \textsuperscript{222} it was found that the observed correlation between the log $k_{on}$ and the pK$_a$ was not related to the affinity of ligands for phospholipids, suggesting that local concentrations do not fully explain the observed differences in the $k_{on}$ between ligands.

### 2.4 Receptor isomerization as rate limiting step in ligand-receptor binding dynamics

For the binding of $[^{3}H]$mepyramine to the H$_2$R, a one-step binding reaction according to law of mass action fits well with the observed binding kinetics of this ligand (chapter 3). However, in many cases such a model does not allow adequate fitting of the observed binding data, as was discussed in the context of
antihistamines in chapter 2, section 2.2.3. A one-step binding mechanism is often an oversimplification of ligand binding, which is probably only a good model when the binding site is freely accessible and is not limited by the conformational dynamics of the receptor. Two often used models to describe the effect of conformational isomerization of the receptor on ligand binding are induced fit and conformational selection (figure 8). The conformational selection mechanism entails an isomerization step of the receptor prior to ligand binding. Conversely, for an induced fit binding mechanism the ligand first engages the receptor into a relatively instable receptor-ligand complex after which a subsequent isomerization step of the receptor increases the complex stability.

The induced fit mechanism and conformational selection are two extreme cases that assume an exclusive progression of ligand-receptor binding through one route. However, there is no reason to believe that a composed binding mechanism could not exist, in which conformational selection and induced fit are parallel binding mechanisms. GPCRs are highly dynamic proteins and upon ligand binding a subset of the conformational spectrum of a GPCR is stabilized, as was shown in the context of the β2AR. For an induced fit-based binding mechanism, receptor conformations are stabilized that prevent ligand dissociation. However, these receptor conformations with an enclosed binding site should then be a fraction of all receptor conformations and likewise pose a barrier for ligand association. For example, within the M3R a hydrophobic binding site was enclosed by several aromatic tyrosine residues which

Figure 8 – Conformational isomerization of the receptor affects the mechanism of ligand binding. In the depicted ligand binding reaction, conformational isomerization of the receptor (between R and R*) is required to obtain an optimal fit of the ligand (L) in the receptor (R*L). Conformational isomerization of the receptor can precede ligand binding (conformational selection) or follows a weak binding interaction between ligand and receptor (induced fit). The $k_r$ and $k_{-r}$ depict the forward and reverse rate, respectively, of conformational receptor isomerization and the binding rate constants of the ligands are depicted by the $k_{on}$ and $k_{off}$.
prevents ligand egress from the binding site (vide supra)\textsuperscript{226}. Assuming that receptor isomerization into an ‘open-lid’ conformation is a rate-limiting step, the bound tiotropium in the ‘closed-lid’ conformation is obtained by an induced fit like mechanism\textsuperscript{233}. However, it was also observed that the unbound M\textsubscript{3}R adopted the same ‘closed-lid’ receptor conformation\textsuperscript{226}, which would then also pose a barrier for ligand association and accordingly, full occupancy of the receptor depends on conformational selection as well. It seems, therefore, unlikely that ligand-receptor binding ever goes purely via an induced fit mechanism, which is considered to be the dominant mechanism of ligand binding\textsuperscript{233}. However when a subset of inaccessible receptor conformations is sufficiently enriched upon binding of the ligand, an induced fit mechanism might be approximated.

Understanding how receptor-isomerization might affect the ligand binding kinetics could be valuable information for rationalizing the design of drug molecules. An example would be to design drugs that interact with ‘lid’ residues of the target protein and in this way stabilize a closed conformation of the receptor, as has been implicated for inhibitors of HIV integrase\textsuperscript{233,236}. Recently, maraviroc was shown to have a strong induced fit component for binding the chemokine receptor CCR\textsubscript{5}\textsuperscript{117}. This was elegantly shown by [$^{3}$H]maraviroc dissociation experiments that were initiated after pre-incubating CCR\textsubscript{5} expressing membranes with the radioligand for various incubation times. When increasing the pre-incubation time, slower [$^{3}$H]maraviroc dissociation was observed, suggesting therefore a time dependent increase in stability of the bound ligand as is expected for an induced fit-like mechanism. Since maraviroc has a very long apparent residence time (> 15 hr after a 4 hr pre-incubation), designing drugs that bind their respective receptor via a similar mechanism could be a good strategy for increasing the duration of target engagement \textit{in vivo}.

In conclusion, the rate in which the receptor can adopt the conformations that are needed for a ligand to bind can be rate limiting, rather than the kinetics of drug-receptor binding itself. In some cases this will be reflected within pharmacological experiments. However, since the mechanism of binding can be infinitely complex, it can never be excluded that an apparent one-step ligand binding mechanism might in fact harbor a rate-limiting receptor isomerization step. This is even more difficult when analyzing the ligand binding rate constants with a Motulsky-Mahan approach, for which it is unsure how an aberrant binding mechanism could be reflected in the observed probe binding over time. Therefore it might be difficult to separate a transition state based increase in the drug-receptor residence time from a receptor isomerization based increase in the drug-receptor residence time.

\subsection*{2.5 Interpretation of structure kinetic relationships}

From the above discussions it becomes clear that it is difficult to rationalize the observed drug binding kinetics to a particular molecular mechanism. Although transition state theory can be a useful tool to rationalize structure kinetic relationships, it will not in all cases be sufficient to fully explain the observed differences in kinetic binding profiles. The discussions of SKR-studies in chapter 6 – 8 are therefore revisited here.

\subsubsection*{2.5.1 Carboxylic substitution of antihistamines}
In chapter 6 it was established that growing a carboxyl group from the prototypical H1R ligand VUF14454 (‘1’, see figure 6), reduced the $k_{on}$ at the H2R for all tested analogs (bearing a carboxyl group) with a concomitant increase in the residence times for most analogs. One example is VUF14506 (‘4c’, see figure 6) which has a 50-fold lower $k_{on}$ and a 10-fold higher residence time at the H2R compared to VUF14454. Since differences in the $k_{on}$ are thought to (sometimes) reflect the differences in the transition state energy of ligand binding, it was proposed that the carboxyl group of (e.g.) VUF14506 destabilizes the transition state resulting in a prolonged residence time at the receptor.

Interestingly, the $k_{on}$ of VUF14454 is $9.6 \times 10^9$ M$^{-1}$s$^{-1}$ approximating therefore the diffusion rate limit, which is therefore higher than expected (not all collisions between ligand and receptor are expected to result in binding) $^{225,227}$. This suggests that the $k_{on}$ could be convoluted by high local ligand concentrations, which would increase the observed $k_{on}$ and the observed $K_i$ by assuming a constant ligand concentration during analysis $^{189}$. Moreover, the high lipophilicity and basicity of this compound are suggested to enhance membrane binding, which support this hypothesis $^{237}$. Mepyramine is another (lipophilic/basic) antihistamine with a high $k_{on}$ in the diffusion limited range ($9.6 \times 10^9$ M$^{-1}$s$^{-1}$). Likewise, it is possible that [$^{3}H$]mepyramine accumulates near the receptor, however, integration into the membrane should at least be rapidly reversible considering the low non-specific binding of the radioligand to cellular membranes after rapid wash steps (e.g. chapter 3). Additionally, upon removing unbound mepyramine by a washout, a full and rapid recovery of the H1R induced intracellular calcium mobilization was apparent (chapter 5).

Since VUF14506 has an additional carboxyl group compared to VUF14454, which will likely influence its ability to partition into the membrane, enrichment of ligand-concentrations near the receptor might be more likely to occur for VUF14454 than for VUF14506. The observed difference in $k_{on}$ between VUF14454 and VUF14506 ($k_{on}$ VUF14454 > 50x $k_{on}$ VUF14506) could therefore result from an underestimated local concentration VUF14454 near the receptor, which would be additionally linked to an overestimation of the $K_i$ of VUF14454 binding the H2R. Such a line of thought suggests that VUF14506 has an increased binding affinity at the H2R compared to VUF14454 when corrected for the local ligand concentrations of the latter. Moreover, the difference in $k_{on}$ between VUF14506 and VUF14454 might not depend on a difference in the transition state energy. Thus, the long residence time at the H2R of VUF14506 compared to VUF14454 could result from an increased energy of binding and not by an unfavorable transition state barrier (contrary to what was hypothesize, vide supra). Hence, it cannot be concluded with certainty that the increased H2R residence time upon substitution with a carboxyl group is either driven by a more favorable energy of binding or rather by a more unfavorable transition state energy. Future studies could e.g. examine the binding kinetics of antihistamines with and without a carboxyl group, on both solubilized and membrane incorporated H2R, to study the effect of membrane enriched ligand concentrations in more detail.

### 2.5.2 Aliphatic substitution of desloratadine

Substituting desloratadine with a cyclopentyl group on the piperidine (VUF16140), decreased the apparent $pK_a$, but increased the residence time at the H2R, as reflected by the prolonged H2R antagonism (RecT, figure 6). This therefore implies a lower $k_{on}$, which suggests a transition state energy driven increase in the drug-receptor residence time. Moreover, considering the high lipophilicity of the substituent for
VUF16140, the decreased $k_{on}$ is probably not resulting from decreased ligand concentration at the membrane.

In general, substituting desloratadine with aromatic or aliphatic rings, like rupatadine, increased the apparent residence time and decreased the apparent pK$_i$ (e.g. chapter 8: table 1). To determine the pK$_i$ accurately in competition binding experiments, the total incubation time should be approximately five times the dissociation half‐live or longer. Lower incubation times will not be sufficient to reach a binding equilibrium and the pK$_i$ will then be underestimated. Since the dissociation half‐live of rupatadine (figure 6) was found to be $>300$ min at $37^\circ$C, the pK$_i$ of rupatadine is underestimated by the 4 h competition binding experiment at $25^\circ$C. This suggests that the reported pK$_i$-values of rupatadine based analogs, with aliphatic or aromatic substitution on the piperidine group (e.g. VUF16140), were underestimated in chapter 8. Hence, although a transition state-based increase in residence time is apparent for VUF16140 compared to desloratadine, this observation is probably convoluted in part by the underestimated binding affinity of ligands with a long H$_1$R residence time.

### 2.5.3 Tricyclic antihistamines

In chapter 7 it was shown that fusing the two aromatic rings of antihistamines, like cyclizine, into a tricyclic ring system increased the residence time at the receptor. This increase in the residence time seemed to result from an increase in the binding affinity (i.e. increased energy of binding) together with a decrease in the $k_{on}$, (i.e. increased energy of the transition state). Therefore, the transition state energy seems to at least partially influence the residence time of tricyclic antihistamines on the H$_1$R (although differences in e.g. membrane partitioning cannot be excluded). Due to the rigidity of the tricyclic core it is tempting to speculate that steric interference between ligand and receptor could decrease the binding rate constants. This could be based on a steric barrier, like a narrow access pathway towards the binding pocket, which would fit well with the idea of an unfavorable transition state. However, if a steric constraint is mostly resolved by slow conformational changes of the receptor, then the ligand-receptor binding rate constants could be convoluted by the kinetics of receptor isomerization. Interestingly, radioligands of the tricyclic antihistamines doxepin (figure 9) and desloratadine have been described. The kinetic binding profile for both ligands at the H$_1$R did not seem to follow a one-step binding mechanism according to law of mass action. In dissociation experiments the level of $[^3]$Hdoxepin bound H$_1$R decreased in a bi-exponential manner. Moreover, desloratadine showed only modest dissociation over a 6 hour timespan, whereas the $k_{off}$ that was extrapolated from association experiments would predict a full dissociation within that time frame. Interestingly, in chapter 5, functional recovery of the H$_1$R was incomplete only after pre-incubation with desloratadine and doxepin. It was hypothesized that the high affinity of these ligands allowed residual binding of the receptor by the trace amount of ligands available after washout (chapter 5). Conversely, the residual inhibition of the H$_1$R could also be explained by a biphasic exponential decrease in H$_1$R-antihistamine complexes. In such a scenario a biphasic exponential recovery of H$_1$R function could be expected, in which the fast exponential phase might be well resolved within the 2 hour experiment, but the slow exponential phase might not be. This is supported by the observation that inhibition with all the tricyclic antihistamines resulted in an incomplete recovery of the H$_1$R functional response after washout, independent of the observed binding affinity for these ligands (chapter 5 and 8). This included for example VUF16135 (figure 9) which has a lower H$_1$R binding affinity (pK$_i$ = 7.1 ± 0.0 and
8.4 ± 0.2, respectively) than mepyramine (pKᵢ = 8.8 ± 0.0; figure 9), while mepyramine does allow a full functional recovery of the H₁R after washout. It is therefore hypothesized that dissociation of tricyclic antihistamines from the H₁R involves rate-limiting isomerization steps of the receptor.

2.5.4 Future prospects for SKR studies on the H₁R

When comparing ligand analogs for possible structure kinetics relationships, it is hard to exclude potential effects of their physicochemical properties on the ligand-receptor collision rate and on the local concentrations of ligand near the receptor. Because the H₁R is a membrane protein and much larger in size than the small molecule ligands that are known to bind the receptor, the relative diffusion of the receptor is negligible compared to the diffusion of the ligand ²²⁵. Moreover, the receptor concentration should not affect the binding kinetics too much (which is normalized to the B_max in pharmacological models) as long as there is an abundant amount of ligand (i.e. there is no ligand depletion; chapter 2). When comparing the ligand binding kinetics at a receptor which is altered by point mutations, observed kinetic differences are therefore probably not related to differences in the ligand-receptor collision rate. Instead, the observed kinetic differences will likely reflect the changed interaction between ligand and receptors directly. Ligands like R-hydroxyzine and levocetirizine (structure of antihistamines are depicted

Figure 9 – Structures of H₁R antihistamines.
in figure 9) have up-to 5 fold differences in the \( k_{on} \) rates when comparing ligand binding to the wild type H\(_1\)R with binding to the mutant receptors K191\^{5.39}\text{A} and T194\^{5.42}\text{A}. Furthermore, unpublished data shows that triprolidine binds the K191\^{5.39}\text{A} mutant with a 10-fold increased \( k_{on} \) as compared to triprolidine binding to the wild type H\(_2\)R. The binding rate constants of ligands at the H\(_2\)R seem therefore to be influenced by interactions along the binding pathway, which is suggestive of a transition state barrier. Hence, it is likely that the differential \( k_{on}\)-values for ligands binding to the H\(_2\)R, is mediated (at least in part) by differences in the transition state energies. Future studies should therefore focus on probing the structure of the receptor as well as the ligand, to further explore the role of a transition state in the ligand-receptor binding kinetics.

2.6 Final notes on the interpretation of ligand-receptor binding rate constants

The work described in this thesis clearly shows that the differences in receptor binding affinity and residence time for ligands at H\(_1\)R are not necessarily related. Since the \textit{in vivo} efficacy of clinically used antihistamines might depend (to some extent) on the residence time (chapter 1), the ligand binding kinetics can have added value for the development of new antihistamines. However, for many other drug targets it is unclear what the preferred kinetic binding profile would be (section 1.3). For a general implementation of the drug-target binding kinetics as a tool to optimize lead compounds in drug development, future research should address first the relationship between the preferred kinetic target binding profile of a drug and the \textit{in vivo} efficacy.

The design of new molecules with a specific kinetic binding profile at the H\(_1\)R requires a detailed molecular understanding of the dynamic interaction between ligand and receptor. The discussion in section 2 exemplifies that interpretation of the measured binding rate constants using pharmacological experiments is challenging. Pharmacology often reduces the complexity of the binding reaction to a one-step binding reaction according to law of mass action, yielding the two kinetic binding rate constants \( k_{on} \) and \( k_{off} \). However, the measured binding rate constants can also be shaped by, e.g., the kinetics of conformational changes in the receptor or changes in the effective diffusion of drugs towards the receptor. Therefore, the notion that the \( k_{on} \) and \( K_d \) values could be used as separate metrics for the optimization of the residence time (1/\( k_{off} \)) of drugs at the H\(_2\)R is probably too simplistic (section 2.3). For example, increased local ligand concentrations near the receptor could affect the \( k_{on} \) and the \( K_d \) simultaneously (section 2.3). However, it would still be good practice to monitor the \( k_{on} \) as well as the \( k_{off} \) in SKR studies that aim to optimize the residence time. For example, substituting VUF14454 (figure 6) with a carboxylic acid group, had in some cases hardly any effect on the residence time (chapter 6). Yet, even when there was no increase in the residence time, a large decrease in the \( k_{on} \) was apparent, which suggests that ligand substitution with a carboxyl group could increase the residence time at the H\(_1\)R if the drop in affinity could be mitigated, as was indeed the case for e.g. VUF14506 (figure 6).

In the end, we found several structural motifs that convey a long residence time to ligands binding the H\(_1\)R (figure 6). These motifs might destabilize the transition state of ligand binding by affecting the solvation energies and steric interactions between ligand and receptor (section 2.1). This hypothesis requires further exploration by probing the relevant interactions between drugs and the H\(_1\)R (e.g. mutagenesis studies; \textit{in silico} modelling). A better understanding of the underlying mechanism that
resulted in the observed ligand SKR at the H1R will potentially help to translate the observed SKR to other GPCR drug-targets.
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