Drug-target residence time : a case for the adenosine A1 and A2A receptors

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Chapter 2

Drug-target residence time
A case for G protein-coupled receptors

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About this chapter

A vast number of marketed drugs act on G protein-coupled receptors (GPCRs), the most successful category of drug targets to date. These drugs usually possess high target affinity and selectivity, and such combined features have been the driving force in the early phases of drug discovery. However, attrition has also been high. Many investigational new drugs eventually fail in clinical trials due to a demonstrated lack of efficacy. A retrospective assessment of successfully launched drugs revealed that their beneficial effects in patients may be attributed to their long drug-target residence times. Likewise, for some other GPCR drugs short residence time could be beneficial to reduce the potential for on-target side effects. Hence, the compounds' kinetics behavior might in fact be the guiding principle to obtain a desired and durable effect *in vivo*. We therefore propose that drug-target residence time should be taken into account as an additional parameter in the lead selection and optimization process. This should ultimately lead to an increased number of candidate drugs moving to the pre-clinical development phase and on to the market. This chapter contains examples of the kinetics behavior of GPCR ligands with improved *in vivo* efficacy and summarizes methods for assessing drug-target residence time.

2.1 Introduction

Drug-target residence time (RT) is an emerging concept in the drug research community. Its definition, coined by Copeland in 2006, stands for an experimental measure of the lifetime of a drug-target complex, reflected by its dissociation rate (k_{off}).[1] This is an important pharmacological feature as a drug is only effective when bound to, and influencing the activity of its physiological target.[1,2] Recently, several notable reviews including Swinney *et al.*,[2-5] Copeland *et al.*,[1,6,7], Vauquelin *et al.*,[8-11] and Zhang and Monsma,[12,13] to name a few, have emphasized the pivotal role of RT in the early phases of drug discovery. These discussions led to increasing recognition that drug-target residence time may even be of greater importance for a drug's effect in the patient than its affinity, since the body, unlike an *in vitro* setting, is an open system where the concentration of free drug fluctuates over time.[13,14] The dynamic flow *in vivo*—absorption, distribution, metabolism, and excretion (ADME)—often prevents the free drug from reaching equilibrium conditions that are otherwise readily obtained in a test tube.[13,14] In this sense, optimizing drug candidates based on equilibrium-derived parameters alone, such as affinity (K_i) / potency (IC_{50}), may not be ideal.[15] Drug-target residence time may be a useful additional parameter as it is thought to represent a surrogate marker of drug clinical efficacy: the longer the drug occupies the receptor, the more profound the drug may exert its effect.[1,12] Although this reasoning most certainly is too simplistic, there is substantial evidence, as witnessed in a survey of 50 drugs on 12 different drug targets, that about 70% of long residence time therapeutics displayed higher efficacy than comparable faster dissociating drugs.[5]

It needs to be pointed out that the criteria for 'long' or 'short' residence time may vary for different targets and for divergent clinical indications.[16] For therapies requiring prolonged target occupancy, a long RT drug offers advantages as it remains bound to the target and continuously exerts its pharmacological effect even when most of the free drug has already been eliminated from circulation.[17] Importantly, this simultaneously implies that a slowly dissociating drug will only offer a noticeably increased effect over a faster dissociating drug, when its dissociation half-life ($t_{1/2}$) outlasts its pharmacokinetic half-life, or when there are large fluctuations in the endogenous competitor (e.g., hormone or neurotransmitter) concentrations.[11] Further proof for the importance of RT comes from numerous examples

of drugs that are best-in-class due to slow dissociation from their target receptor. One example is the well-known muscarinic M_3 receptor antagonist, tiotropium, which is a long-acting, 24 hour, anticholinergic bronchodilator used in the management of chronic obstructive pulmonary disease.[29] On the other hand, there are cases where the mechanism-based toxicity outweighs the therapeutic advantages by long receptor occupancy. In this situation, a fast dissociating compound displaying short-lived intervention is preferred. This may be the case for the antipsychotic D_2 dopamine receptor antagonists, where long RT is not desired due to on-target toxicity associated with strong extrapyramidal motor effects. We will discuss this in a later part of this chapter.

2.2 Mathematical definitions of drug-target residence time

Drug-target residence time is equal to the reciprocal of the dissociation rate constant ($RT = 1 / k_{off}$).[1] Its value is interchangeable with the dissociation half-life ($t_{1/2} = \ln 2 / k_{off}$) with a factor of ln2, i.e., $RT = t_{1/2} / \ln 2$. Both can be used to represent the duration of the drug-target binary complex. For the convenience of discussion, we chose to use the dissociation half-life to describe the drug-target residence time in the following part of this chapter, since most of the kinetic values in literature were reported with $t_{1/2}$ values.

As mentioned above, it is necessary to determine the dissociation rate constant to calculate the RT or $t_{1/2}$. However, the definition of k_{off} depends on the specific mechanism of ligand-receptor interaction.[1,14] In general, three situations are well known. Equation 1 represents a simple one-step association and dissociation process, where the receptor (R) and ligand (L) encounter each other to form a binary complex (RL) with association rate k_1 (k_{on}) and dissociation rate k_2 (k_{off}), respectively.

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \tag{1}$$

In contrast, Equation 2 represents a more complicated process. Upon the binding of the ligand to the receptor, the initial complex (RL) undergoes a conformation isomerization leading to a much higher-affinity final complex

(RL*). This is also known as the 'induced-fit' mechanism. In this case, k_{off} entails both forward (k_3) and reverse steps (k_4) in receptor isomerization as well as the on- and off-rate constants for the free ligand bound to and unbound from the free receptor, such that $k_{off} = k_2 \cdot k_4 / (k_2 + k_3 + k_4)$. This isomerization step enhances the interaction between the ligand and the target, hence resulting in an extended overall $t_{1/2}$.

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RL^* \tag{2}$$

A third mechanism is represented in Equation 3, where the receptor is in equilibrium between two conformational states (R and R*) in the absence of ligand. Of these conformational states, the ligand specifically binds to one, R*, rather than the other (R), to form the R*L complex. This is also known as the 'conformation-selection' mechanism. The interconversion between conformational states R and R* is slow relative to the binding of the ligand to the R* state, thus it represents the rate-limiting step for the formation of the binary complex. For the dissociation rate constant, its value is equal to k_4.

$$R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R^* \underset{k_4}{\overset{k_3 \cdot [L]}{\rightleftharpoons}} R^*L \tag{3}$$

Among these three mechanisms, the first situation is most commonly encountered in ligand-GPCR binding kinetics, while the second mechanism can be the case for high-affinity ligand-receptor interactions, especially for enzyme inhibitors and receptor antagonists;[1,6,14] the third situation is rarely observed in ligand-receptor interactions.[14]

2.3 Experimental strategies to assess drug-target residence time

Measurement of drug-target complex lifetime, reflected by k_{off} or $t_{1/2}$, is not a new phenomenon *per se*. To date, there are several strategies available to examine the binding kinetics of ligand-GPCR interactions. The advantages and disadvantages / limitations of these experimental strategies to access drug-target residence time are briefly outlined in Table 2.1. Of these

Table 2.1 | The advantages and disadvantages / limitations of experimental strategies to access drug-target residence time.

Assay	*Advantages*	*Disadvantages / limitations*	*Ref.*
Direct kinetic radioligand binding assay	Straightforward; Robust; Quantitative measurement	Ligand of interest needs to be radiolabeled; Low throughput assay format	19-21
Indirect kinetic radioligand binding assay	Only one labeled ligand necessary; Quantitative measurement; Adjustable for high throughput screening	Need for suitable radiolabeled ligand; Intermediate filtration and / or 'washout' steps are needed	23-26
Competition association assay	Only one labeled ligand necessary; Quantitative measurement	Need for suitable radiolabeled ligand; Low throughput assay format	27-34
Dual-point competition association assay	Only one labeled ligand necessary; High throughput assay format; Easy readout for longer or shorter residence time compounds	Need for suitable radiolabeled ligand; Qualitative measurement	18,33
Dual-point IC_{50} / K_i value determination	Only one labeled ligand necessary; Adjustable for high throughput screening	Need for suitable radiolabeled ligand; Only selects long residence time compounds	30,34
Functional reversibility assay	No need for labeled ligands; Adjustable for high throughput screening	Qualitative measurement; Not suitable for binding kinetics at low temperatures.	35-46
Label-free kinetic measurement	No need for labeled ligands; Quantitative measurement	Need for protein purification and immobilization; Low throughput assay format	47-55

approaches, the most common and straightforward method is to radiolabel a compound of interest with high affinity and directly measure its on- and off-rate in a kinetic set-up of a radioligand binding experiment. Two examples, in the context of drug-target residence time, are radiolabeled aprepitant at the Neurokinin 1 (NK_1) receptor and tiotropium at the muscarinic M_3 receptor (M_3R). Both compounds have been well characterized by using this method.[19,20] Alternatively, a ligand can be fluorescently labeled to calculate its drug-target residence time. For instance, Keppler and coworkers have developed a so-called Tag-lite® platform, a combination of a Homogeneous Time-Resolved Fluorescence (HTRF®) detection method with a covalent labeling technology called SNAP-tag®.[21] One application note from Cisbio describes fluorescently labeled spiperone binding at the Tag-lite dopamine D_2 receptor.[22] Nevertheless, the process of radio- or fluorescent labeling is costly and labor intensive, which, as a consequence, confines kinetics determinations to a limited scope. Therefore, alternative approaches are highly desired (See Figure 2.1-2.4 for experimental schemes and representative results).

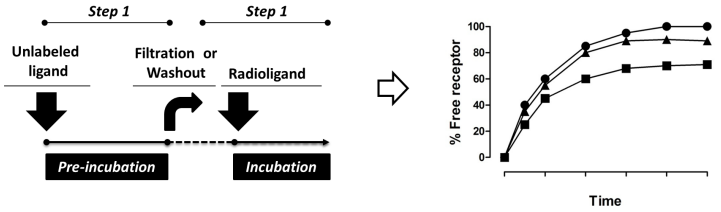

Figure 2.1 | Indirect kinetic radioligand binding assays: the receptor-bearing material is pre-incubated with a saturating concentration of unlabeled ligand, followed by an intermediate filtration and / or 'washout' step to initiate ligand dissociation. Subsequently, a fixed concentration of radioligand is added and incubated thereafter for a period before assay termination. This scheme is adapted from Vauquelin *et al*.[35] In this assay a slowly dissociating compound (squares) induces an apparent decrease in the amount of free receptor, compared to a fast dissociating compound (triangles), which allows the binding of a subsequently added radioligand; Control: no pre-incubation (spheres).

A. Indirect kinetic radioligand binding assays

Contrary to the abovementioned, one-step, direct measurement of radiolabeled ligands' dissociation rates, indirect approaches usually contain two consecutive parts (Figure 2.1). Firstly, the receptor-bearing material is pre-incubated with a saturating concentration of unlabeled ligand (to occupy the majority of receptors), followed by an intermediate filtration and/or 'washout' step—methods vary in different labs[23-26]—to initiate ligand dissociation. Secondly, a fixed concentration of radioligand is added and incubated thereafter for a short period before assay termination. Thus, the unlabeled competitor's dissociation rate could be inferred from the recovery of radioligand binding. Furthermore, this method can be adopted and modified for high-throughput screening; an example is at the dopamine D_2 receptor (D_2R).[26]

B. (Dual-point) competition association assay

The competition association assay has been shown to be highly accurate in determining the binding kinetics of unlabeled ligands at the beta-

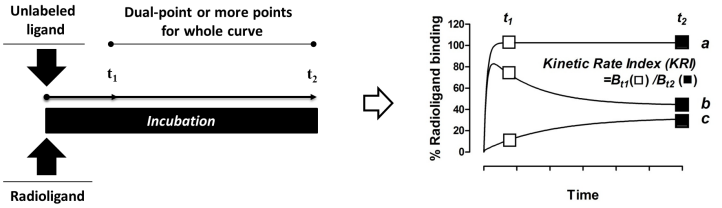

Figure 2.2 | (Dual-point) Competition association assay: an unlabeled competitor is co-incubated with a radioligand in a kinetic association experiment, and followed for two or more time points. ***a***: a radioligand association curve without co-incubation of an unlabeled competitor. ***b***: a co-incubated unlabeled competitor dissociates slower than the radioligand used. ***c***: a co-incubated unlabeled competitor dissociates faster than the radioligand used. B_{t1}: specific radioligand binding at the first time point (t_1); B_{t2}: specific radioligand binding at the second time point (t_2). Kinetic rate index (KRI) is defined as B_{t1} / B_{t2}. This scheme is adapted from Guo *et al.*[33]

adrenergic receptor,[27,28] and more recently, at the muscarinic M_3 receptor,[29] gonadotropin-releasing hormone (GnRH) receptor,[30] histamine H_1 receptor,[31] and adenosine receptors,[32,33] to name but a few. This method is based on a framework developed by Motulsky and Mahan,[34] where an unlabeled competitor is co-incubated with a radioligand during a kinetic association experiment (Figure 2.2). If the competitor dissociates faster from its target than the radioligand ($k_2 < k_4$), the specific binding of the radioligand will slowly and monotonically approach its equilibrium in time (Figure 2.2, ***c***). However, when the competitor dissociates slower ($k_2 > k_4$), the association curve of the radioligand will consist of two phases starting with a typical 'overshoot' followed by a decline until a new equilibrium is reached (Figure 2.2, ***b***).[34] In contrast, the association rates of either the competitor (k_3) or the radioligand (k_1) are usually less influential on the pattern of the curves.[34] Recently, the competition association assay has also been adopted and modified by Guo *et al.* for high-throughput kinetic screening studies at the adenosine A_1 receptor.[33] Briefly, in a so-called dual-point competition association assay, two time points are selected to measure radioligand binding: 1) at which the radiolabeled ligand just reaches equilibrium under control conditions in the absence of an unlabeled ligand (Figure 2.2, ***a*** and t_1), and 2) at which the incubation time is long enough for the labeled and unlabeled compound to equilibrate with the target (Figure 2.2, t_2). Next, the ratio of the binding at the first time point (B_{t1}) and that at the second time point (B_{t2}) is calculated, which Guo *et al.* defined as the 'kinetic rate index' (KRI).

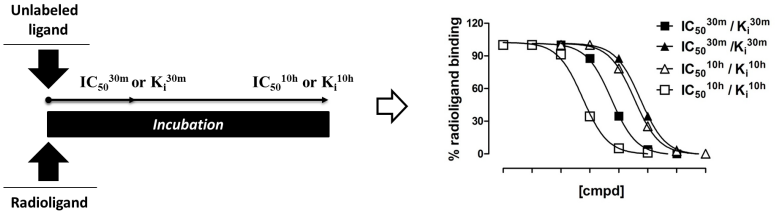

Figure 2.3 | Dual-point IC_{50} / K_i value determination: an unlabeled competitor is co-incubated with a radioligand for either a short time (in this case, e.g., 30 min) or a long time (in this case, e.g., 10 h) and their corresponding IC_{50} or K_i values are calculated. In the representative result, a slowly dissociation compound (squares) will have a more pronounced affinity shift than a fast dissociation compound (triangles).

In this manner, the compounds that quickly dissociate from their target will have a KRI below or equal to one (Figure 2.2, ***c***). Conversely, compounds that dissociate slowly from their target compared to the radioligand will have a KRI value larger than one, resulting from the typical 'overshoot' in the association curve (Figure 2.2, ***b***). Notably, the dual-point assay and KRI value can be applied to screen for candidate compounds with either long or short RT. This suggests the general applicability of this assay for other drug targets as well.

C. Dual-point IC_{50} / K_i value determination

Another method enabling kinetic screening was reported by Heise *et al.* They used a scintillation proximity assay (SPA) to measure an antagonist's affinity after 30 min or 10 h of incubation and examined their ratio ($K_i^{30\ min}$ / $K_i^{10\ h}$; Figure 2.3).[30] The theory behind this is based on the observation that displacement curves will shift leftward over time until a state of equilibrium is reached; the time to reach that state is determined by the dissociation rate of the slower unbinding process.[34] In other words, a slowly dissociating compound requires longer incubation times to reach equilibrium than a fast dissociating one, which is often neglected. This translates into markedly decreased experimental IC_{50} or K_i values over time. In this sense, the ratio of the dual-point IC_{50} or K_i values represents a surrogate of overall RT. However,

the principle of this approach also simultaneously implies its limited application, i.e. in screening long RT candidate compounds only, since for short RT compounds the state of equilibrium is quickly established and the K_i ratio derived thereof is hardly changed.

D. Fucntional reversibility assay

A ligand's binding kinetics profile could also be inferred from circumstantial evidence, which is commonly based on functional assays resembling the classical 'organ-bath' experiment. More specifically, it requires pre-incubating tissues / cells with antagonists (to allow them sufficient time to reach binding equilibrium conditions) before their challenge with an agonist (Figure 2.4).[35] As a result, fast dissociating antagonists can only cause a rightward shift of the agonist's concentration-dependent curve, since the receptor can quickly be liberated from the antagonist's blockade and thus readily respond to the subsequently administered agonist. These types of antagonists are categorized as 'surmountable' (Figure 2.4, pre-incubation experiment).[9,36,37] In contrast, slowly dissociating antagonists or, at the extreme, irreversible binders can induce not only a rightward shift but also a concomitant decline in maximal response of the agonist, due to sustained receptor blockade which, on a macroscopic scale, reduces the total receptor population available for an agonist's response.[38,39] Such antagonists are denoted as insurmountable (Figure 2.4, pre-incubation experiment).[9,36,37,40] Notably, such an insurmountable pattern can also be caused by a number of different mechanisms, such as allosteric interactions, receptor internalization or postreceptor functional response blockade.[41-44] None of these result from competitive antagonism. Nevertheless, these mechanisms can be distinguished in a co-incubation experiment, where a depressed maximal response can only be observed by a noncompetitive mechanism, but not for competitive antagonists, no matter how fast or slowly they dissociate from the target (Figure 2.4, co-incubation experiment). Thus, it is always necessary to perform a co-incubation experiment along with the pre-incubation experiment to clarify the mechanism of the insurmountable antagonism.[37,45,46] It is important to mention that the extent of the insurmountable effect, e.g. a decreased maximal response, is time dependent. During a short period of agonist exposure (following pre-incubation with a slowly dissociating antagonist), not all the

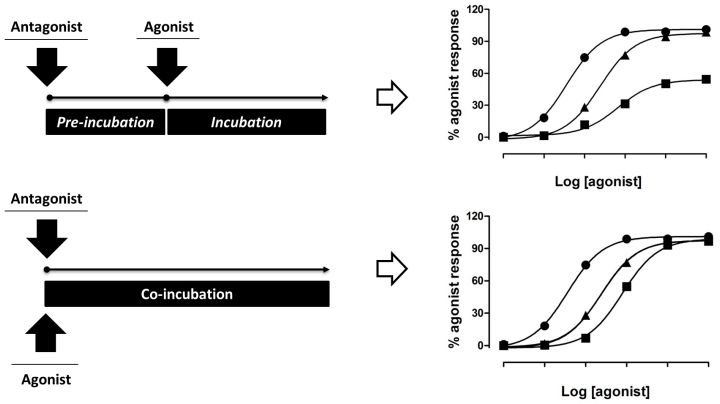

Figure 2.4 | Functional reversibility experiment: in the upper panel, tissues/cells are pre-incubated with an antagonist before challenge with an agonist. A slowly dissociating antagonist (squares) will cause a rightward shift of the concentration-response curve and a concomitant decline in maximal response of the agonist; a fast dissociating antagonist (triangles) will cause a rightward shift of the concentration-response curve without affecting the maximal response of the agonist. If as in the lower layer an antagonist is co-incubated with an agonist; both slowly and fast dissociating competitive antagonists will cause a rightward shift of the concentration-response curve without affecting the maximal response of the agonist. Otherwise they are non-competitive to the agonist; Control: agonist only (spheres). This scheme is adapted from Vauquelin *et al.*[35]

receptor sites are liberated before the response is measured, a state of so-called 'hemi-equilibrium'.[38,39,44] However, if the agonist exposure lasts longer, the occupancy of the receptors will readjust with time until, ultimately, both the agonist-receptor and the antagonist-receptor interactions reach equilibrium.[44] Evidently, the insurmountability of such slowly dissociating compounds will change if the response is measured at different time points.

E. Label-free kinetic measurements

One of the most well-established label-free measurements for kinetics is surface plasmon resonance measurement (SPR). In SPR, the protein is immobilized

on a coated gold chip, which is then exposed to the compound of interest under continuous flow. Ligand binding to the receptor immobilized on the chip's surface induces a real time change in the refractive index on the sensor surface. As such a change is linear to the number of molecules bound, it allows quantitative characterization of binding kinetics (for review, see[16,47,48]).

Another emerging label-free technology enabling kinetics measurement is by surface acoustic wave biosensor (SAW).[49] This methodology captures real-time mass changes on the surface, which result in a shifted phase and / or changed amplitude of the sound wave signal.[50] Besides these two biophysical assays, other equally valid technologies have also been reported elsewhere.[51]

However, these biophysical techniques have not been thoroughly tested on GPCRs. The difficulty is that these receptors are integral membrane proteins, and rapidly deteriorate when taken out of their natural environment, which is often a prerequisite for these newer biophysical approaches. Several strategies were developed to overcome this inherent difficulty. One of the successful examples is the application of a protein engineering method to generate a stabilized receptor (StaR), e.g. the adenosine A_{2A} receptor, minimally modified for thermostability.[52-54] The stabilized receptors can be purified in large quantities, whilst retaining correct folding, thus generating materials suitable for a broad range of structural and biophysical studies.[53] Another technique is to solubilize a given GPCR from whole cell pellets and capture it on antibody surfaces for analysis, exemplified by an SPR study on the CCR5 and CXCR4 chemokine receptors.[55] These solubilized receptor preparations could be useful materials for binding kinetics studies.

2.4 Revisit kinetics data on GPCRs

To date, nearly 800 genes encoding GPCRs have been identified, representing a substantial share of the human genome.[56] In general, all these receptors possess structurally conserved heptahelical transmembrane motifs (7TM) connected by alternating intracellular and extracellular loops, with the amino terminus located at the extracellular side and the carboxyl terminus at the intracellular side.[57] Despite these common features in their molecular structure, GPCRs are characterized by a relatively low overall sequence identity (less than 20%). According to phylogenetic classification, these receptors can be divided into five main families—*Rhodopsin* (Class A), *Secretin* (Class B), *Glutamate* (Class

Table 2.2 | Overview of GPCR ligand kinetics.

Cmpd	*Target*[a]	$t_{1/2}$[b]	*Clinical indications*	*Status*[c]	*Ref.*
Ritanserin	5-HT_2 receptor	160 min (37 °C)	Cocaine-related disorders	Phase II	160
Risperidone	5-HT_7 receptor	Irreversible (37 °C)	Schizophrenia; Alcohol abuse	Marketed	161
Clonidine	α_{2A}-autoreceptor	27.6 s (37 °C)	ADHD	Marketed	99
Bopindolol	β_1-adrenoceptor	> 4 h (rat)	Hypertension	Marketed	162
Olodaterol	β_2-adrenoceptor	17.8 h	COPD	Marketed	163
Lofentanil	μ receptor	260 min (37 °C)	Analgesia	Marketed	164
Candesartan	AT_1 receptor	120 min (37 °C)	Hypertension	Marketed	43
UK432,097	A_{2A} receptor	173 min (5 °C)	COPD	Terminated[d]	32
Taranabant	CB_1 receptor	122 min (37 °C)	Obesity	Terminated[e]	25
Maraviroc	CCR5	> 136 h (4 °C)	HIV	Marketed	165
SCH527123	CXCR2	22 h	Asthma; Psoriasis	Phase II	166
PF-4850890	CRF_1 receptor	6.79 h (rat)	Depression; Anxiety	Preclinical	153
Clozapine	D_2 receptor	14.5 s (37 °C)	Schizophrenia	Marketed	135
AM432	DP_2 receptor	90 min (37 °C)	COPD	Preclinical	167
Uracil 3	GnRH receptor	> 43 h	Reproductive-endocrine axis disorders	Preclinical	168
Desloratidine	H_1 receptor	> 6 h	Allergy	Marketed	169
JNJ7777120	H_4 receptor	62 min	Inflammation	Preclinical	170
Tiotropium	M_3 receptor	27 h	COPD	Marketed	80
Aprepitant	NK_1 receptor	154 min	Chemotherapy-induced emesis	Marketed	19
Ibodutant	NK_2 receptor	28 min	Irritable Bowel Syndrome	Phase II	171
Osanetant	NK_3 receptor	10 min	Schizophrenia	Phase II	172
Almorexant	OX_2 receptor	139 min	Chronic primary insomnia	Phase III	173
Clopidogrel	P_2Y_{12} receptor	Irreversible (37 °C)	Stroke; Heart attack; Severe chest pain	Marketed	62
Apafant	PAF receptor	50 min (guinea pig)	Arrhythmias; Breast cancer; Inflammation	Preclinical	174
GSK1562590	UT receptor	> 1 h (rat)	Hypertension	Preclinical	175
SCH500946	Y_5 receptor	71 min	Obesity	Preclinical	176

[a] Official IUPHAR receptor name. [b] Dissociation half-life at room temperature and on human receptors unless mentioned otherwise. [c] Clinical status obtained from ClinicalTrials.gov.[179] [d] Discontinued due to lack of efficacy. [e] Discontinued due to high level of central nervous system side effects.[177,178]

C), *Adhesion* and *Frizzled/Taste2*.[56,58] Receptors from Class A to C have been most addressed in drug discovery efforts. The natural ligands of GPCRs are highly variable ranging from small ions and photons to large glycoproteins, which indicates the important and versatile roles these proteins play in a vast number of biological processes. These are also the reasons why GPCRs have developed into a very 'druggable' class of targets for more than 30% of currently available drugs.[59] However, in the introduction we also mentioned the tremendous attrition along R&D pipelines, and suggested that detailed mechanism-based studies of drug-target interaction should be introduced in the early phases of drug discovery to prevent 'fail late, fail expensive' scenarios. For this we examined the gigantic 'pool' of GPCR ligands and retrospectively analyzed the 'kinotype' (kinetics features) of some cases. Without claiming to be exhaustive we have summarized a series of prototypic GPCR ligands that have known kinetics information (Table 2.2). We listed one representative (candidate) drug per target (26 targets in total) including their clinical indications. Several conclusions can be drawn from this table. Firstly, the existing kinetics data for GPCR ligands is nowhere near the number of affinity-based evaluations in the early phases of drug discovery. The sum of targets presented here is far behind the total population of 'druggable' GPCRs, estimated to be 300.[60,61] Clearly, ligand-receptor binding kinetics has been overlooked and less investigated in the past. Secondly, the range of drug-target residence times, reflected by $t_{1/2}$ in the table, varies from seconds to hours. Most of these are in line with the clinical applications of the related drug. For instance, patients with allergy can benefit from the long-lasting effect by desloratidine ($t_{1/2}$ > 6 h) in relieving nasal congestion, or patients with COPD from taking tiotropium ($t_{1/2}$ = 27 h) for a durable bronchodilatory effect. In both cases, their clinical indications favor sustained target occupancy. On the contrary, for some marketed drugs, serious adverse effects could go along with the desired effects due to unwanted kinetic rates. This is exemplified by clopidogrel, which is an antiplatelet agent used to inhibit the formation of blood clots. However, its irreversible inhibitory effect may prolong or exacerbate bleeding.[62] Another case is represented by lofentanil for analgesia. Although its longer duration of action has an advantage for certain types of analgesia,[63] it is still unsuitable for most practical applications.[64] Instead, short-acting derivatives such as sufentanil or remifentanil are preferred for medical use in human surgical procedures.[65,66] One can speculate that such clinical limitations could be avoided or at least foreseen if kinetics were to be incorporated in the early phase of candidate selection.

2.5 Structure-kinetics and structure-affinity relationships

Thus far the abovementioned analyses in retrospect are still largely based on 'hindsight' information; hardly any structure-kinetics relationship studies (SKR), in prospect, have been reported previously. Therefore, we should like to propose a strategy for compound optimization that combines an extensive structure-kinetics relationship study (SKR) with a classical structure-affinity relationship study (SAR). It is envisioned that this added metric, i.e., binding kinetics or the 'kinotype' of a given compound, offers several advantages over the traditional and simple SAR paradigm:

1) A better understanding of the molecular mechanism of action. This includes detailed characterization of not only the bound states under equilibrium conditions (SAR) but also the entire drug-target interaction that comprises metastable intermediate states and transition states (SKR).[14,67] Such gained knowledge offers rationales for efficient drug design.

2) A more steered drug design and development. Different targets may have distinct kinetics preferences. Aiming for long or short RT compounds is a crucial consideration in early phase discovery to triage and advance the best leads towards clinical applications.[16]

3) An efficient translation from *in vitro* to *in vivo* settings. This may decrease the prevalence of the 'fail late, fail expensive' scenario, especially considering that lack of efficacy is one of the major reasons for drug attrition.[68,69]

In addition, a detailed SKR study also describes the association rate of the ligand-receptor interaction, which has been less investigated and is often thought to be diffusion-limited.[14] As a consequence, this could lead to the assumption that optimizing for a long residence time is tantamount to optimizing the binding affinity, since $K_D = k_{off} / k_{on}$ and k_{on} reaches an (identical) limit. However, Vilums *et al.* have recently reported a thorough SKR study on the CCR2 receptor, in which it was shown that this general assumption is not necessarily valid.[18] They documented that the compounds' on-rates varied significantly from 0.01 $nM^{-1} \cdot min^{-1}$ to 0.0003 $nM^{-1} \cdot min^{-1}$ —more than three orders-of-magnitude from the diffusion-limited value (approximately 10^{10} $M^{-1} \cdot min^{-1}$)[1]. It is also notable that the affinity difference of two diastereomers in that paper with K_i values of 3.6 nM and 289 nM, was predominantly caused by their 18-fold difference in the on-rate, rather than their very similar RTs (135 min and 77 min). Such valuable information can

only be obtained by a detailed SKR study, which has not been reported for many GPCRs so far.

Evidently, a detailed SKR offers added value to the traditional metric of affinity. This new strategy of combining SAR and SKR can be expected to further enhance the success of drug discovery and development. To further illustrate the importance of incorporating SKR into the pipeline of early phases of drug research, in the following part of this chapter we will primarily address small molecules targeting class A and B GPCRs, which have been reported to have optimized binding kinetics, more specifically dissociation kinetics. Specific evidence will be given for three 'long RT' targets and one 'short RT' target; muscarinic M_3, tachykinin NK_1 and CRF_1 receptor, and dopamine D_2 receptors, respectively, since these targets have been (most) extensively studied in this area.

2.6 Drug-target residence time for class A GPCRs

Muscarinic receptors

Muscarinic receptors, or muscarinic acetylcholine receptors (mAchRs), are a group of class A GPCRs, which are endogenously activated by acetylcholine.[70] To date, five subtypes of muscarinic receptors are identified, namely M_1-M_5.[71,72] These receptors can be further categorized into two broad groups based on their primary coupling efficiency to G proteins; M_2 and M_4 muscarinic receptors couple to the pertussis toxin-sensitive $G_{i/o}$ proteins, while M_1, M_3 and M_5 muscarinic receptors to $G_{q/11}$ proteins.[70,73] Of all five muscarinic subtypes, M_3 receptor is located in the smooth muscles of blood vessels throughout the body as well as in the lung. Hyperactivation of the lung M_3 receptor can cause over constriction of smooth muscle, such as that observed during bronchoconstriction. Thus, antagonizing M_3 receptor can lead to relaxation of airway smooth muscles and result in symptom relief of chronic obstructive pulmonary disease (COPD).[74,75] Additionally, for the treatment of chronic illness, long duration of action (preferably 24 h) is an important feature of drugs that enables both prolonged efficacy and patient compliance.[76] Therefore, the treatment of COPD can benefit from a long-

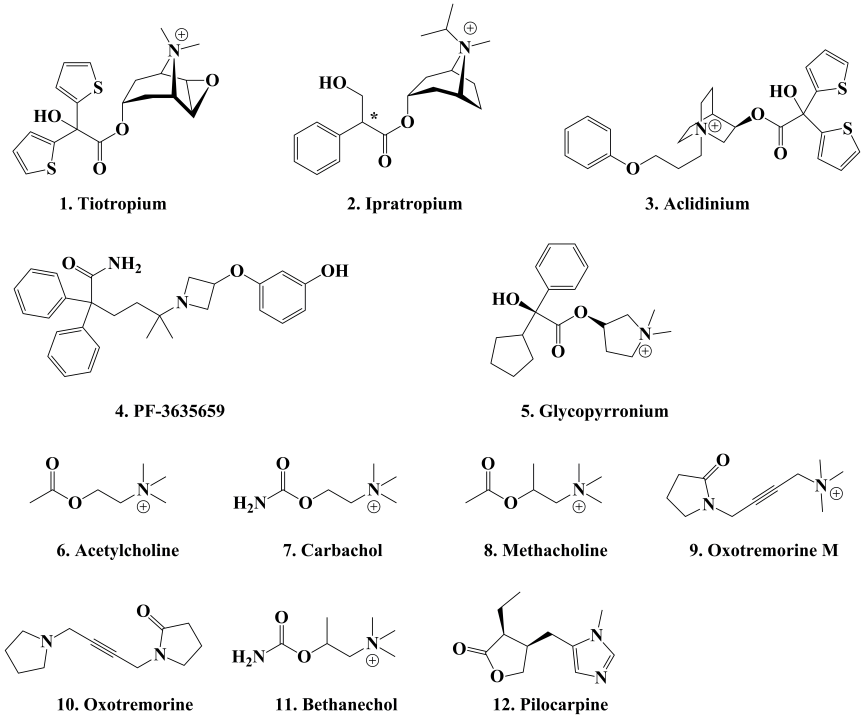

Figure 2.5 | Structures of small molecule M_3 muscarinic receptor ligands.

acting muscarinic antagonist (LAMA), as evidenced by tiotropium and other M_3 antagonists summarized below.

1. Tiotropium

Tiotropium is a long-acting anticholinergic bronchodilator, which is currently marketed under the trade name Spiriva™ (Figure 2.5, Compound 1).[77-79] Given its long *in vivo* duration of action, several groups, in retrospect, performed *in vitro* time-dependent IC_{50} value analysis to obtain the accurate affinity and binding kinetics of tiotropium.[29,80] It was found that equilibrium is reached only after 18-20 h in a radioligand binding assay. This phenomenon is consistent with the theory by Motulsky and Mahan that a long incubation time is needed for a radioligand to reach equilibrium in the presence of a slowly dissociating competitor.[34] Thus, it also explains that different affinities were published by different groups; after a period of 20 h incubation, the K_i value of tiotropium was 8 pM,[29,80] while after only 4 h incubation, its K_D value was 0.33 nM.[78] The $t_{1/2}$ of [^{3}H]-tiotropium was 34.6 h at room temperature, which was much longer than ipratropium (Figure 2.5, Compound 2; $t_{1/2}$ = 0.26 h, Table 2.3), a relatively short-acting agent requiring up to four doses per day.[74,78] Similarly, a kinetics study performed by Dowling and Charlton using a competition association assay has also demonstrated that tiotropium has a very long $t_{1/2}$ (7.7 h) at room temperature,[29] which, however, is shorter than the value (34.6 h) reported by Disse *et al.*[78] This may be due to the difference in their buffer for membrane preparation and / or the binding buffer for kinetic measurements, for instance, the presence of sodium ions. Likewise, tiotropium has an even shorter dissociation half-life (46.2 min) under mock physiological conditions at 37 °C, where the ionic strength (including Na^+, Ca^{2+}, K^+, Mg^{2+} and Cl^- ions) of the assay buffer was increased.[81] This example furthermore highlights the importance of careful choices in assay development for kinetic measurements. Another important pharmacological feature of tiotropium is its 'kinetic receptor subtype selectivity', namely a longer dissociation half-life at the M_3 receptor, 34.7 h, than at the M_1 and M_2 receptors (14.6 h and 3.6 h, respectively).[78] In contrast, tiotropium's affinities at these receptors were similar and in the subnanomolar concentration

Table 2.3 | Binding kinetics of M_3 muscarinic receptor ligands.

Nr	*Cmpd*[a]	*Affinity (K_i or pK_i)*[b]	*Dissociation half-life ($t_{1/2}$)*[c]	*Ref*
1.	Tiotropium	8 pM	34.6 h	29,78
2.	Ipratropium	0.69 nM	0.26 h	78
3.	Aclidinium	18 pM	10.7 h	80
4.	PF-335659	132 pM	> 24 h	96
5.	Glycopyrronium	10.04	6.3 h	80
6.	Acetylcholine	4.87	7.8 s	98
7.	Carbachol	4.09	6.3 s	98
8.	Methacholine	4.52	6.0 s	98
9.	Oxotremorine-M	4.61	7.2 s	98
10.	Oxotremorine	5.61	2.6 s	98
11.	Bethanechol	3.71	3.7 s	98
12.	Pilocarpine	4.78	3.0 s	98

[a] Compound names and synonyms used in original references. [b] Affinity, expressed as K_i values or pK_i as reported in original references; result on human receptor unless mentioned otherwise. [c] Dissociation half-life at room temperature.

range.[78] Taken together, these features make tiotropium the most widely used LAMA worldwide so far.[81,82]

To understand the molecular basis of the exceedingly long RT of tiotropium, Tautermann *et al.* performed a 'kinetic mapping' of its binding sites and the 'exit' channel from the M_3 receptor by testing the kinetics of tiotropium at mutant M_3 receptors.[83] They found that the slowly dissociating profile of tiotropium was attributed to interactions in the binding site particularly with $N508^{6.52}$ ($N507^{6.52}$ on the rat M_3 receptor, see Figure 2.6). Importantly, this residue appeared to 'anchor' tiotropium at the receptor via directly interacting with its hydroxyl-group and ester-group so as to restrict the movement of the ligand towards the exit channel. Consequently, tiotropium's RT was 45-fold longer on the wild type M_3 receptor than that of its analogue lacking the hydroxyl-group, while little difference was observed at the $N508^{6.52}$A mutant.

Furthermore, a recent structural biology study of the M_3 receptor (from rat) co-crystallized with tiotropium (PDB code: 4DAJ) provided a clear image for the molecular mechanism behind its long RT profile.[84] As presented in

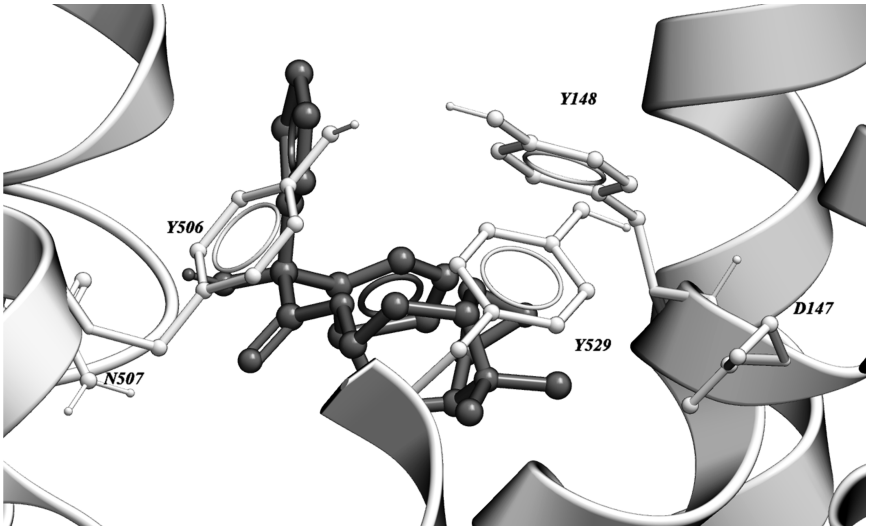

Figure 2.6 | The co-crystal structure of tiotropium in the rat M_3 receptor.[84] This figure was generated with ICM Browser v3.7 (Molsoft) from PDB code: 4DAJ. Tiotropium (black) binds within the pocket. Three tyrosine residues, Y148$^{3.33}$, Y506$^{6.51}$ and Y529$^{7.39}$, together prevent the ligand from moving out of the receptor. N507$^{6.52}$ interacts with the carbonyl and hydroxyl groups through H-bonds, while the ligand's typical quaternary ammonium group interacts with D147$^{3.32}$.

Figure 2.6, tiotropium binds deeply in the receptor core and is covered by an aromatic 'lid' comprised of three conserved tyrosines—Y148$^{3.33}$, Y506$^{6.51}$ and Y529$^{7.39}$. This aromatic 'lid' nearly occludes the ligand from the solvent thus preventing it from being 'wetted' by water molecules and then 'washed' off the binding pocket. In addition, the abovementioned N507$^{6.52}$ interacts with the carbonyl and hydroxyl groups of tiotropium through H-bonds, while the ligand's typical quaternary amine interacts with D147$^{3.32}$.

The elucidation of the co-crystal structure of tiotropium in the rat M_3 receptor also facilitated molecular dynamic simulations to characterize the pathway by which tiotropium binds to and dissociates from the rat M_3 receptor.[84] In contrast to a simple one-step dissociation process, the ligand appeared to firstly leave the orthosteric ligand binding pocket and then 'pause' at an extracellular 'vestibule', forming a loosely connected intermediate state, before tiotropium finally dissociated from the receptor. During this process,

the first step formed a large energy barrier that involved the aromatic 'lid' (Y148$^{3.33}$, Y506$^{6.51}$ and Y529$^{7.39}$) around the charged ligand head group opening up, while the second step posed a smaller energetic barrier. In this sense, the RT of tiotropium is greatly determined by the first step, which is rate-limiting for the ligand dissociation process.

As expected from the kinetic binding profile of tiotropium, an insurmountable antagonist behavior was observed in a functional reversibility experiment. This was detailed by Casarosa *et al.* during the preclinical evaluation of several long- and short-acting muscarinic antagonists, where tiotropium was pre-incubated for 15 min before the addition of the agonist muscarine in the inositol phosphate accumulation assay.[80] Fitting the dose ratios over a large range of tiotropium concentrations (10^{-10}-10^{-5} M) generated a Schild plot with a slope of 1.29, higher than unity. Such a value indicated the insurmountable M_3R antagonism of tiotropium, which was a result of a hemi-equilibrium due to its long M_3R residence time profile.[37]

Taken together, the *in vitro* results mentioned above combined with further evidence unveil the molecular basis of the long RT profile of tiotropium. These results correlate with this compound's long *in vivo* duration of bronchoprotection (> 24 h) as determined in a model of acetylcholine-induced bronchoconstriction in anesthetized dogs.[80] Moreover, despite the possible influence of drug metabolism and pharmacokinetic factors,[85] the clinical profile of tiotropium in COPD highlights an inextricable link between long receptor RT and long duration of action that enables a simple, once-daily dosage regime.[77,86,87]

2. Aclidinium

Aclidinium (Figure 2.5, Compound 3) is a long-acting, inhaled muscarinic antagonist recently approved by the FDA as a maintenance treatment for COPD.[88] The synthesis and biological evaluation of aclidinium and its analogues were elaborately reported by Prat *et al.* and Gavalda *et al.*[20,89] This antagonist was specifically designed for a maintained bronchodilatory efficacy with fewer unwanted systematic anticholinergic effects, such as dry mouth (reported in 11.6% of patients treated with tiotropium bromide versus 3.5%

of patients treated with placebo), glaucoma, constipation, increased heart rate, and urinary retention.[90] According to Prat *et al.*, aclidinium had a rapid hydrolysis in human plasma, hence minimizing the class-related systematic side effect. However, such a fast pharmacokinetics profile of aclidinium did not influence its long duration of action (over 24 h), as evaluated in an animal model.[20] This phenomenon was attributed to aclidinium's slowly dissociating profile from the M_3 receptor.

Compared to tiotropium, aclidinium had a similar affinity for the M_3 receptor (K_i = 18 pM), while its $t_{1/2}$ (10.7 h) was approximately two-fold shorter (Table 2.3).[80] In another study, Gavalda *et al.* used radiolabeled aclidinium and found a K_D value of 0.32 nM; combined with $t_{1/2}$ of 29.24 h, which was shorter than [^{3}H]-tiotropium (62.19 h).[20]

In vitro functional activity and *in vivo* duration of bronchoprotection of aclidinium was also examined by Gavalda *et al.*[20] More specifically, they used the isolated guinea pig trachea and treated it with carbachol to generate dose-dependent contraction. A 60 min pretreatment with aclidinium or tiotropium shifted the concentration-response curves of carbachol to the right. Along with the potency shift, tiotropium also suppressed the maximal agonist effect while aclidinium did not. Such a result is indicative of the relatively shorter RT of aclidinium than that of tiotropium at the M_3 receptor.

In addition to the characterization of aclidinium's functional activity, the onset of action of this compound was also studied in the carbachol-induced contraction assay. Notably, aclidinium displayed a faster onset of action ($t_{1/2}$ = 6.8 min, t_{max} = 35.9 min) than tiotropium ($t_{1/2}$ = 13.6 min, t_{max} = 61.2 min). For the *in vivo* duration of bronchoprotection, the effect half-life of aclidinium (defined as the time taken to reduce the maximal bronchoconstriction by 50%) was 29 h. This duration of action was considerably longer than that of ipratropium (8 h) yet shorter than tiotropium (64 h). Likewise, Casarosa *et al.* reported that aclidinium and tiotropium displayed different levels of bronchoprotection at 24 h after equi-effective dose administration (tiotropium = 35%, aclidinium = 21%).[80] Clearly, these *in vivo* results are in line with the *in vitro* binding kinetics.

Furthermore, compared to tiotropium's once-daily dose regime, aclidinium required a twice-daily dose regime for a 24 h bronchodilation effect in clinical trials.[91-95] Such a difference appears to be derived from their divergent RTs at the M_3 receptor. In addition, aclidinium's faster onset of action, mostly due to its fast association rate, can provide quicker symptom relief as compared to tiotropium.

3. PF-3635659

The synthesis and biological evaluation of PF-3635659 (Figure 2.5, Compound 4) and its analogues was reported by Glossop *et al.*[96] This study nicely exemplifies a systematic combination of both a structure-affinity and structure-kinetics relationship study for early-phase drug design and development. More specifically in this study, Glossop *et al.* demonstrated that a *gem*-dimethyl substitution adjacent to the basic amine moiety of PF-3635659 exerted a profound effect on the dissociation rate. As evidence, the $t_{1/2}$ of the initial *gem*-dimethyl-free compound (38 min) was extended to over 1440 min, indicating the importance of the *gem*-dimethyl substitution for attaining ligand receptor RT. Further chemical modifications on the heterocyclic ring and the right side of the phenyl ring induced corresponding changes in ligand affinity and kinetics, which eventually led to the discovery of the candidate compound, i.e., PF-3635659 (Compound 47 in the original paper). Following-up binding kinetics characterization of this compound in a competition association assay revealed that it had an on-rate of 1.05×10^7 $M^{-1}\cdot min^{-1}$, slower than tiotropium and ipratropium ($k_{on} = 2.12 \times 10^8$ $M^{-1}\cdot min^{-1}$ and 1.00×10^9 $M^{-1}\cdot min^{-1}$) and an off-rate of 1.39×10^{-4} min^{-1}, translating into a $t_{1/2}$ of more than 24 h (Table 2.3).

Importantly, the *in vivo* onset and duration of action data for PF-3635659 were in line with its *in vitro* binding kinetics. The profiling of PF-3635659 in a conscious dog model of bronchoconstriction illustrated a slower onset of action compared to ipratropium and tiotropium, with yet a longer duration of action (> 24 h) than ipratropium.[96] This compound has now progressed into a phase II clinical trial. Data from healthy volunteers demonstrated that PF-3635659 provided efficacious bronchodilation over 24 h from a single inhaled dose, thus confirming the suitability of PF-3635659 as a novel once-daily inhaled muscarinic M_3 receptor antagonist for the treatment of COPD.

4. Glycopyrronium

Glycopyrronium (Figure 2.5, compound 5) is the cation and active moiety of glycopyrronium bromide, of which the dry-powder formulation is also known as NVA237.[81] *In vitro* binding kinetics profiling of this compound

in a radioligand competition association assay revealed a dissociation rate of 0.11 ± 0.02 h^{-1} from the M_3 receptor, equal to a dissociation $t_{1/2}$ of 6.3 h (Table 2.3). This value was much longer than the short-acting M_3 antagonist ipratropium (0.22 h) but shorter than tiotropium (27 h).[80] However, glycopyrronium had a more rapid rate of onset of action (3- to 4.8-fold) than tiotropium, as determined in an *in vitro* calcium assay and rat tracheal strip assay.[81] This profile was similar to that obtained with ipratropium bromide in preparations of guinea-pig and human airways.[97]

Notably, glycopyrronium also displayed higher equilibrium binding and kinetic selectivity for M_3 versus M_2 receptors as compared to both tiotropium and ipratropium.[80,81,97] Its kinetic selectivity resulted from a 16.5-fold longer receptor RT at the M_3 receptor than at the M_2 receptor, which was higher than tiotropium (10.4-fold) and ipratropium (7.3-fold). Such a property may provide a better therapeutic window.

5. M_3 receptor agonists

The RTs of seven M_3 agonists (Figure 2.5, Compound 6-12) were recently reported by Sykes *et al.*[98] These agonists are acetylcholine, carbachol, methacholine, oxotremorine-M, oxotremorine, bethanechol and pilocarpine, and are not very selective. The data, however, were only collected on the M_3 receptor subtype. Among the seven agonists, oxotremorine displayed the shortest $t_{1/2}$ at the M_3 receptor (2.6 s), while acetylcholine had the longest receptor RT ($t_{1/2}$ = 7.8 s). In general, these agonists' off-rates were much faster than that of the abovementioned M_3 antagonists (Table 2.3). Further *in vitro* functional characterization of the M_3 agonists demonstrated that they had different relative agonist efficacies. Notably, Sykes *et al.* found that there was no relationship between agonist efficacy and the equilibrium binding affinity of each agonist. Interestingly, when efficacy was compared with the dissociation rate constant, the two were highly correlated. This result suggests a relationship between the duration of agonist binding at the receptor and the intrinsic efficacy, a finding also reported by Guo *et al.* and Hoeren *et al.* at the adenosine A_{2A} receptor and at the α_{2A}-adrenoceptor.[32,99] Importantly, such a finding again emphasizes that equilibrium models and assays alone are not sufficient to describe a dynamic signaling system; instead, a combination of kinetic models alongside the traditional affinity determination might be

preferred to better capture the nature of efficacy and to develop efficacious drugs for GPCRs.[98]

In summary, several case studies of long acting M_3 receptor antagonists suggest their sustained pharmacological effect lies in these compounds' long receptor occupancy time. The design and development of long residence time antagonists may provide further candidate drugs with once-daily application for COPD. We also included the kinetics data of several M_3 agonists, since they provide important information that agonist efficacy may be correlated to its drug-target residence time rather than the commonly tested equilibrium affinity. This further indicates the importance of combining both binding and kinetics information in the early phase of drug design and development.

Tachykinin receptors

Tachykinin receptors comprise three different subtypes, namely the NK_1R, NK_2R and NK_3R, which can be activated by tachykinin peptides, including Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB).[100-102] The three members of the neurokinin receptor class are differentiated by their varying affinities for these three tachykinins, which are SP > NKA > NKB for the NK_1R, NKA > NKB > SP for NK_2R and NKB > NKA > SP for NK_3R (for reviews, see[100-102]). All three subtypes are functionally coupled to $G_{q/11}$, which leads to the activation of phospholipase C-IP_3/DAG signaling and results in elevation of intracellular Ca^{2+} levels.[102] The localization of the tachykinin receptors varies between subtypes in the human body: the NK_1R and NK_3R are prominent both in the central nervous system (CNS) and in peripheral tissues, while the NK_2R is widely expressed in the smooth muscle of the gastrointestinal, respiratory, and urinary tracts and to a much lesser extent in the CNS.[102-104] A large body of preclinical evidence has suggested that pharmacological or gene manipulation of the NK_1R has potential clinical utility in the treatment of chronic pain, migraine, neurogenic inflammation and emesis.[105-107] These diseases could benefit from therapeutics with a long duration of NK_1R antagonism.

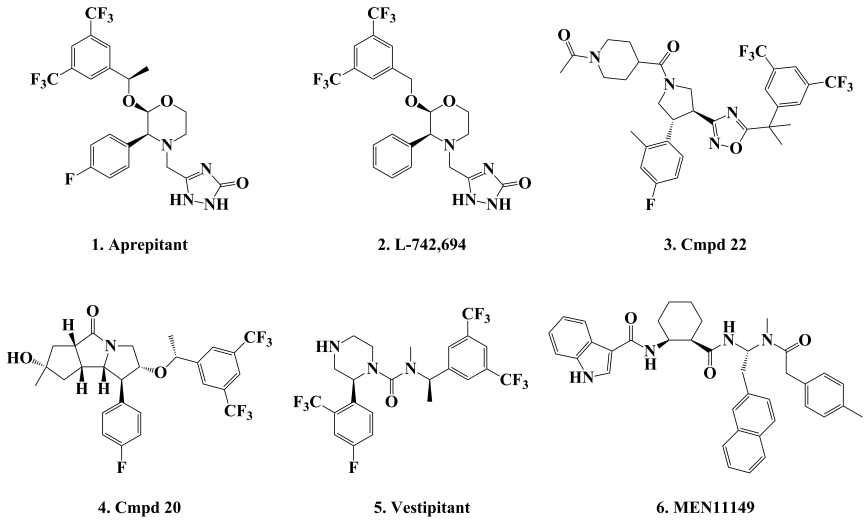

Figure 2.7 | Structures of small molecule NK_1 receptor antagonists.

1. Aprepitant

Aprepitant, also known as L-754,030, MK0869 or Emend (Figure 2.7, Compound 1),[19,108-110] is the only tachykinin receptor antagonist on the market to date. It is a selective, slowly dissociating NK_1 antagonist with a $t_{1/2}$ of 154 min at 22 °C (Table 2.4).[19] In comparison, it was found by Cascieri *et al*. that the dissociation rate of the radiolabeled NK_1's natural ligand SP (i.e., ^{125}I-[Tyr^8]-SP) was too rapid to be measured accurately at a comparable temperature.[111] At 15 °C, this peptide dissociates with $t_{1/2}$ = 46 min and 0.2 min for the G protein-coupled and -uncoupled receptor states, respectively.[112] Evidently, aprepitant had a much longer residence time on the NK_1 receptor than the natural ligand which has been speculated to be the reason for its high potency and *in vivo* efficacy.

As expected from a slowly dissociating ligand, aprepitant's activity was found to be insurmountable in functional assays compared to other, fast dissociating NK_1R antagonists. This functional effect was associated with its long-lasting *in vivo* efficacy, a finding shared by many authors.[19,108,110] One of the most elaborate studies reported on aprepitant was conducted by

Table 2.4 | Binding kinetics of NK_1 receptor antagonists.

Nr	*Cmpd*[a]	*Affinity (K_i, nM)*[b]	*Dissociation half-life ($t_{1/2}$)*[c]	*Ref.*
1	Aprepitant	0.019	154 min (22 °C)	19
2	L-742,694	0.037	27 min (22 °C)	111
5	Vestipitant	0.027	59.7 min (37 °C)	119

[a] Compound names and synonyms used in original references. [b] Affinity, expressed as K_i values that were reported in original references; results on human receptor unless mentioned otherwise. [c] Dissociation half-life at different temperatures indicated in brackets.

Lindström *et al.*[108] Several functional experiments (i.e., pre-incubation, co-incubation and washout experiments) were used to reaffirm that aprepitant was a competitive yet pseudo-irreversible antagonist with slow functional reversibility. Next to the measurement of its functional interactions *in vitro*, the authors also examined the pharmacokinetic/pharmacodynamics relationship of aprepitant *in vivo* by using the gerbil foot tap (GFT) assay, a model reflecting central NK_1R activation.[113] It was found that the duration of GFT inhibition by aprepitant outlasted the time course of its plasma or brain concentrations, indicating that its long duration of action was indeed more likely due to a sustained receptor blockade rather than a slow pharmacokinetic half-life. In contrast, two other structurally unrelated NK_1 antagonists, CP-99994 and ZD6021, demonstrated rapid functional reversibility that was in line with their pharmacokinetic profile.[108] Furthermore, these authors also examined *ex vivo* receptor occupancy by autoradiography, reaffirming that time-dependent inhibition of GFT by aprepitant correlated well with NK_1 occupancy in the gerbil brain, a finding that was shared by Duffy *et al.* in 2002.[109] Taken together, all *in vitro* and *in vivo* results provide strong evidence that aprepitant is a slowly dissociating NK_1R antagonist.

2. L-742,694

Aprepitant was originally developed from L-742,694 (Figure 2.7, Compound 2),[109,111,112] which was shown to be a pseudo-irreversible yet competitive antagonist by Cascieri *et al.* in 1997.[111] The $t_{1/2}$ of [^{3}H]-L-742,694 (27 min, Table 2.4) at 22 °C is considerably shorter than the half-life of aprepitant but longer than the natural ligand SP.[19,111,112] Its functional interaction was

further characterized by Cascieri *et al*. by using both human and rat NK_1 receptor.[111] Interestingly, its nature of NK_1 receptor antagonism was species related as L-742,694 was insurmountable on the hNK_1 receptor but not on the rNK_1 receptor. A follow-up mutational analysis revealed that mutating $H197^{5.39}$ in TM5 into Serine reduced L-742,694 affinity and simultaneously abolished its functional insurmountability at the hNK_1 receptor, making it a key amino acid for L-742,694 interaction and function. When testing several structural analogues of L-742,694, the authors found that the addition of the triazoline moiety (as in L-742,694) converted a surmountable antagonist into an insurmountable one, possibly by slowing the dissociation rate.[111]

3. Compound 22

Aprepitant has formed the basis for the discovery of more potentially long acting NK_1R antagonists. Young *et al*. developed a series of pyrrolidine-carboxamides and oxadiazoles based on aprepitant, aiming for feasible clinical candidates for a once-daily dosing regimen.[114] Their screening strategy was to find compounds with enduring functional activity *in vitro* and long duration of action *in vivo* at lower drug levels as to obtain an improved safety profile. Overall, Compound 22 (Figure 2.7, Compound 3) was found to be the best compound with efficacious functional activity in an IP-1 functional assay and long duration of action (over 24 h) in the GFT model. During the course of compound optimization, the authors were also able to establish a kinetically informed structure-activity relationship. They concluded that both receptor binding affinity and functional insurmountability are highly sensitive to both absolute and relative stereochemistry,[114] which was reaffirmed for another compound series by Morriello *et al*.[115]

4. Compound 20

Morriello *et al*. employed virtually the same screening technique as described by Young *et al*.[114,115] to develop both a series of long acting 5,5-fused pyrrolidines[115-117] and later a series of 5,5,5-fused tricyclic antagonists with even slower dissociation rates, with cyclopentane compound 20 (Figure 2.7,

Compound 4) being the most active.[118] The authors found that increasing the overall compound rigidity as well as modifying some other specific molecular components, such as by the addition of a substituent on the pyrrolidine nitrogen, increased functional insurmountability.

5. Vestipitant and its analogues

Vestipitant (Figure 2.7, Compound 5) has a $t_{1/2}$ of 59.7 min (Table 2.4, 37 °C) from the human NK_1R.[119] It is an insurmountable yet competitive antagonist with long duration of action (over 8 h) in the *in vivo* GFT model.[119] Compared to other structural analogues of vestipitant, it appeared that certain features such as the benzylic substitution patterns and its stereochemistry were important for vestipitant's mode of antagonism,[119] which was reaffirmed in another publication by Di Fabio *et al.*[120] Surprisingly, even a small, hydrophobic substituent, for instance a methyl substituent, played an important role in determining the magnitude of an antagonist's insurmountability and duration of action.[120]

6. MEN1149

This peptidomimetic compound (Figure 2.7, Compound 6) set the example that long acting small molecule antagonists can be developed from short acting peptide antagonists, in this case from FK888, by simple structural modifications. The main modification introduced, originally to increase metabolic stability, was the β-aminocycloalkyl carboxylic acid residue replacing FK888´s prolyl group.[121] Interestingly, these modifications turned the purely surmountable FK888 into the insurmountable MEN1149 with much slower reversibility during organ bath washout assays and a longer duration of action *in vivo* of over 3 h.[121] The authors speculated that introduction of the β-amino cycloalkyl carboxylic acid caused a slower rate of dissociation which was likely to be responsible for the change in type antagonism, but only provided circumstantial evidence and no direct kinetics measurements.[121]

Overall, the examples summarized above provide a strong indication that NK_1R are amongst the targets for which slowly dissociating antagonists could offer therapeutic advantages.

Dopamine receptors

Dopamine receptors are a group of class A GPCRs, which are named after their endogenous ligand dopamine.[122] To date, five subtypes of dopamine receptors have been identified, namely D_1 to D_5.[123] These receptors can be further categorized into D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) subfamilies, based on their similarities in sequence, pharmacology and ability to stimulate or inhibit adenylyl cyclase activity: the D_1-like subfamily couples to G_s and mediates excitatory neurotransmission, while the D_2-like (D_2, D_3 and D_4) subfamily couples to $G_{i/o}$ and mediates inhibitory neurotransmission.[124] Dopamine receptors, particularly D_1 and D_2 receptors, are prominent in the vertebrate CNS,[123,125] indicating their functional importance in neuronal signaling (for reviews, see[126-131]). To date, numerous drugs targeting the D_2 receptor are available on the market. Antagonizing the dopamine D_2 receptor has been reported as the molecular foundation of schizophrenia treatment.[132] Currently, all known marketed anti-schizophrenia drugs bind to the D_2 receptor and are supposed to modulate dopamine hyperfunction.[26,133] In general, these drugs can be categorized into two groups. The first generation or the so-called typical antipsychotics (e.g., haloperidol) can effectively treat positive schizophrenia symptoms (i.e., hallucination and delusions), yet they invoke concomitant on-target adverse effects, such as extrapyrimidal symptoms (EPS) and hyperprolactinaemia, through excessive blockade of the D_2 receptor. The second generation, also known as 'atypical' antipsychotics, are defined according to their 'clozapine-like' properties, i.e., low incidence of the on-target/mechanism-based side effects, which are mostly attributed to their short residence times at the D_2 receptor.[134] Other theories have also been proposed to explain 'atypicality'. One of these is the multi-receptor hypothesis or polypharmacology.[180] Many atypical antipsychotics were found to have high affinity at other neurotransmitter receptors in addition to D_2 receptors, in particular at serotonin 5-HT_{2A} receptors, while typical antipsychotics, like haloperidol, bind more specifically to D_2 receptors.[135] However, fully occupying serotonin 5-HT_{2A} receptors with different antipsychotics at

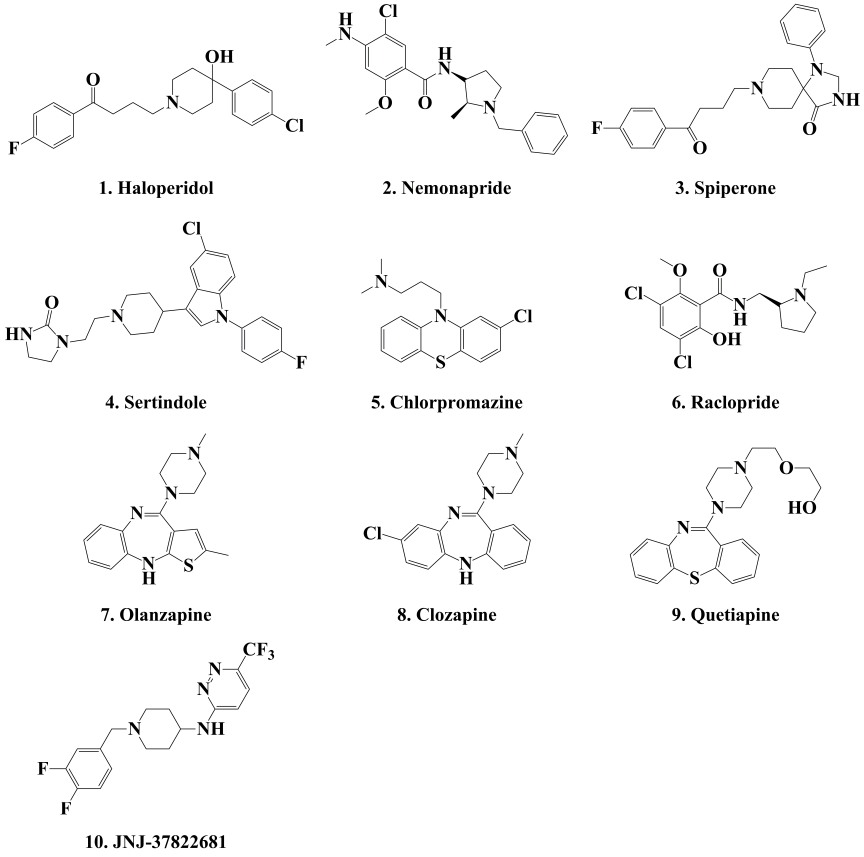

Figure 2.8 | Structures of small molecule D_2 receptor antagonists.

clinically relevant doses did not induce EPS to the same level.[134] Hence, the atypicality is more likely connected to the compounds' short residence times at the D_2 receptor than their antagonism at the 5-HT_{2A} receptors.

Additionally, it is worth mentioning that interaction of atypical antipsychotics with additional receptors may cause undesired off-target side-effects—distinct from its on-target side-effect (e.g., EPS and hyperprolactinaemia), such as $5HT_{2C}$ receptors (weight gain), α_1-adrenoceptors (orthostatic hypotension, reflex tachycardia, and hypnosedation), α_2-adrenoceptors (tachycardia), histamine H_1 receptors (sedation and weight gain), and muscarinic receptors (blurred vision, dry mouth, constipation, and cognitive impairment).[135] These side-effects can logically be minimized by decreasing the compound's affinity or residence time on the off-targets.

Contrary to aforementioned cases of other GPCRs, where long RT ligands are desired for optimal clinical outcomes, short residence time D_2 receptor antagonists are preferred, since they will supposedly induce less on-target 'mechanism-based toxicology'.[25,35] In the following part of this section we will focus on published binding kinetics data of dopamine D_2 antagonists and the related benefit from short RT in clinical applications.

1. Haloperidol and other slowly dissociating D_2 receptor antagonists

Haloperidol (Figure 2.8, Compound 1) was approved by the U.S. Food and Drug Administration (FDA) in 1967 and marketed as Haldol in U.S. and other countries. Haloperidol exhibited high-affinity antagonism for human dopamine D_2 receptor (1.8 nM) with a k_{off} of 0.01 s^{-1}, equal to a $t_{1/2}$ of 72.4 s (Table 2.5).[135] Notably, its $t_{1/2}$ was substantially changed when the kinetics were tested at rat striatal membranes (i.e., $t_{1/2}$ = 42 min), indicating significant species differences.[134] One might argue that the absolute value of haloperidol's $t_{1/2}$ is much smaller than other classical slowly dissociating GPCR antagonists, such as aforementioned NK_1R antagonist aprepitant ($t_{1/2}$ = 154 min). It should be realized that the time scale for endogenous dopamine concentration burst can be within a millisecond to second range, which is much shorter than the $t_{1/2}$ of haloperidol.[136] In this sense, the receptor blockade by haloperidol is already too lengthy to allow normal physiological responses to fast and small changes in the neurotransmitter concentration.

Table 2.5 | Binding kinetics of D_2 receptor antagonists.

Nr	*Cmpd*[a]	*Affinity (K_i, nM)*[b]	*Dissociation half-life ($t_{1/2}$)*[c]	*Ref*
1	Haloperidol	1.8	72.4 s	135
2	Nemonapride	0.025 (rat)	355 min (rat)	134
3	Spiperone	0.1 (rat)	200 min (rat)	134
4	Sertindole	1.2 (rat)	47 min (rat)	134
5	Chlorpromazine	1.3 (rat)	35 min (rat)	134
6	Raclopride	1.6 (rat)	28 min (rat)	134
7	Olanzapine	6.4 (rat)	18 min (rat)	134
8	Clozapine	137	14.5 s	135
9	Quetiapine	67.6 (rat)	23 s (rat)	139
10	JNJ-37822681	158	6.5 s	135

[a] Compound names and synonyms used in original references. [b] Affinity, expressed as K_i values that reported in original references; result on human receptor unless mentioned otherwise. [c] Dissociation half-life at room temperatures and on human receptors unless mentioned otherwise.

Next to the result from the direct kinetic binding assay at both human and rat D_2 receptor preparations, haloperidol was found to have long-lasting D_2 blockade by using an indirect kinetic measurement. Only 48% (lower than that of clozapine) of the total receptor population was recovered and able to bind to subsequently added [^{3}H]-spiperone after pre-incubating haloperidol for one hour with the receptor.[135]

The binding kinetics of other slowly dissociating D_2 receptor antagonists were elaborately studied by Kapur and Seeman.[134] The authors investigated the affinity and the kinetics of nine typical and atypical antipsychotics (main results are included in Table 2.5; compound structures in Figure 2.8). Among these compounds, nemonapride and spiperone had a $t_{1/2}$ of 355 min and 200 min, respectively, indicative of D_2 receptor blockade for more than 3 h; these two compounds were followed by sertindole (47 min), haloperidol (40 min), chlorpromazine (35 min), raclopride (28 min) and olanzapine (18 min) in terms of their $t_{1/2}$ values. In contrast, the two atypical antipsychotics clozapine and quetiapine displayed faster dissociation half-lives, i.e., less than 1 min. Furthermore, it is important to note that the k_{off} values varied a 1000-fold from 0.002 min^{-1} to 3.013 min^{-1}, yet less variation was found in k_{on}. Apparently, the rate at which antipsychotic agents come off the receptor (k_{off})

causes the variation in their affinity for the D_2 receptor, while differences in k_{on} do not account for that. Moreover, since atypical antipsychotics display relatively faster dissociation than typical ones, it can be inferred that the atypical antipsychotic effects are mostly attributed to their high k_{off} values for the D_2 receptor.

2. Clozapine

Clozapine was the first atypical antipsychotic drug on the market (Figure 2.8, Compound 8). Compared to haloperidol, clozapine had a relatively low affinity for the D_2 receptor (K_i = 137 nM) and a 5-fold shorter $t_{1/2}$ of approximately 14.5 s (Table 2.5).[135] This (partially) explained the observation by Kapus and Seeman, that clozapine reached equilibrium 100 times faster than haloperidol in the displacement assay.[134] Such a fast molecular interaction enabled clozapine to have a fast-on and then fast-off blockade of the D_2 receptor. Therefore, the receptor was readily responsive to the endogenous dopamine burst upon physical stimulation, hence lowering the risk of EPS or hyperprolactinaemia. This mechanism was further supported by the *in vivo* observation that clozapine caused a surmountable blockade of striatal dopamine receptors in contrast to cataleptogenic neuroleptics (the insurmountable control).[137] Moreover, clinical positron emission tomography (PET) analysis of a subject receiving clozapine (400 mg) revealed transiently high D_2 receptor occupancy (i.e., 80%) that rapidly declined to low levels (i.e., 15%) within 48 h. In comparison, D_2 receptor occupancy by haloperidol (5 mg) peaked at approximately 80%, but it declined more slowly over time, showing D_2 occupancy of over 10% even 3–5 days later.[138] This reaffirmed that fast kinetics may be favorable for a low incidence of mechanism-based, on-target, toxicology.

3. Quetiapine

Quetiapine (Figure 2.8, Compound 9), also known as Seroquel, Xeroquel or Ketipinor, is another marketed atypical antipsychotic approved for

schizophrenia treatment. Similar to the molecular properties of clozapine, Caboni *et al*. found that quetiapine displayed low D_2 receptor affinity (K_i = 67.6 nM) and a fast dissociation rate (1.8 min^{-1}), equal to a $t_{1/2}$ of 23 s, at a rat striatum homogenate (Table 2.5).[139] In the subsequent *in vivo* profiling, Caboni *et al*. performed time-dependent analysis of plasma prolactin levels, a surrogate of peripheral D_2 receptor blockade, after orally treating rats with vehicle or different dose of quetiapine or haloperidol. They found that compared to haloperidol treatment quetiapine displayed a short-lived increase of prolactin level, which was in line with its short RT profile at the D_2 receptor. Moreover, serial PET scans evaluating the D_2 receptor occupancy of quetiapine demonstrated that this compound dissociated very rapidly from the D_2 receptor.[140] Taken together, all *in vitro* and *in vivo* results provided strong evidence that quetiapine is a fast dissociating D_2 receptor antagonist.

4. JNJ-37822681

JNJ-37822681 (Figure 2.8, Compound 10) was developed by Janssen Pharmaceuticals. One of the most elaborate studies on JNJ-37822681 has been reported by Langois *et al.*.[135] The compound was initially selected from an indirect kinetic screening assay (for method, see[23,135]), where JNJ-37822681 was estimated to have a k_{off} similar to clozapine. Subsequently, a kinetic [^{3}H]-JNJ-37822681 binding experiment revealed that this compound had an extremely short $t_{1/2}$ (6.5 s, Figure 2.5) at the D_2 receptor, which was approximately 2-fold and 11-fold shorter than clozapine and haloperidol, respectively. In the follow-up *in vivo* profiling, JNJ-37822681 displayed a larger specific margin (compared with haloperidol) between the inhibition and blockade of apomorphine-induced behaviors, which represents a model for psychosis. This result was further reaffirmed in phase IIb clinical trials, where JNJ-37822681 displayed an efficacious antipsychotic effect but low propensity of EPS and prolactin liability.[141,142]

5. Overall structural determinants for binding kinetics at the dopamine D_2 receptor

Compared to the residence times of M_3R or NK_1R antagonists, D_2 receptor antagonists are preferred to have a short RT to circumvent on-target side effects. It is therefore highly desired to analyze the structural determinants that increase ligand dissociation from the receptor for future rational drug design. For this reason, Tresadern *et al.* performed a comprehensive statistical analysis of the binding kinetics of over 1800 D_2 receptor antagonists and examined their physicochemical properties that potentially discriminate fast dissociating compounds from slowly dissociating ones.[26] They discovered that faster dissociating antagonists were in general less lipophilic and had lower molecular weight. This seems to hold true for other fast dissociating GPCR ligands as well, a finding shared by Miller *et al.*[143] However, it is also important to note that other descriptors, for instance polar surface area, partial positive charge or the amount of hydrogen bond donor and acceptor groups, caused different effects on the ligand's dissociation, depending on the particular cluster of chemicals that was analyzed. This suggested that local effects of a specific chemical scaffold on the binding kinetics could be dominant over other physiochemical descriptors. Thus, instead of intuitively modifying a compound' lipophilicity, molecular weight or other molecular properties, a more specific structure-kinetics relationship study is needed to direct future drug design and discovery.

In summary, examples presented here demonstrate the benefit of fast dissociating D_2 receptor antagonists for lowering the risk of mechanism-based toxicology. Such an advantage over long RT antagonists is supported by evidence from preclinical and clinical aspects of the typical and atypical antipsychotics. This case study on the D_2 receptor further emphasizes the necessity of characterizing a ligand's binding kinetics profile in the early phases of candidate drug selection.

2.7 Drug-target residence time for class B GPCRs

Corticotrophin releasing factor type-1 receptor

Corticotrophin releasing factor (CRF) is a 41 amino-acid peptide that exerts its effects through activation of CRF receptors, which are members of the family B GPCRs.[144] The CRF receptor subfamily has two subtypes, namely CRF_1 and CRF_2. Both receptors are primarily coupled to G_s protein, resulting in the activation of adenylate cylase and increased cAMP levels.[145,146] Since CRF acts on the pituitary gland to regulate the hypothalamic-pituitary-adrenal axis and the CNS to modulate behavior responses to stress, it has caught considerable attention in drug discovery for psychiatric diseases.[144-147] In particular, antagonism of the CRF_1 receptor has been proposed for rebalancing a dysfunctional stress axis.[147-149] Although highly potent *in vitro* and efficacious in animal models, early reported compounds such as CP-154,526 and NBI-27914 are highly lipophilic, which leads to compound accumulation in tissues or long elimination half-life, and thus high risks of toxicity.[150] For this reason, efforts have been specifically directed towards discovering less lipophilic, fast clearance CRF_1 receptor antagonists to decrease the incidence of toxicity but with maintained drug efficacy. A long residence time CRF_1 receptor antagonist is therefore highly desired, which could provide the benefit of a long duration of action and at the same time less accumulation on the unwanted targets. Such compounds would exemplify new mechanisms for depression treatment beyond classical monoamine modulators.[148,151]

1. The binding kinetics of CRF_1R antagonists and their *in vivo* pharmacodynamics

One of the most elaborate binding kinetics studies at the CRF_1 receptor was performed by Fleck *et al.*[151] In this article, the authors investigated a series of CRF_1 receptor antagonists regarding their *in vitro* binding kinetics and *in vivo* pharmacodynamics. They found that the k_{off} and k_{on} values of 12 CRF_1

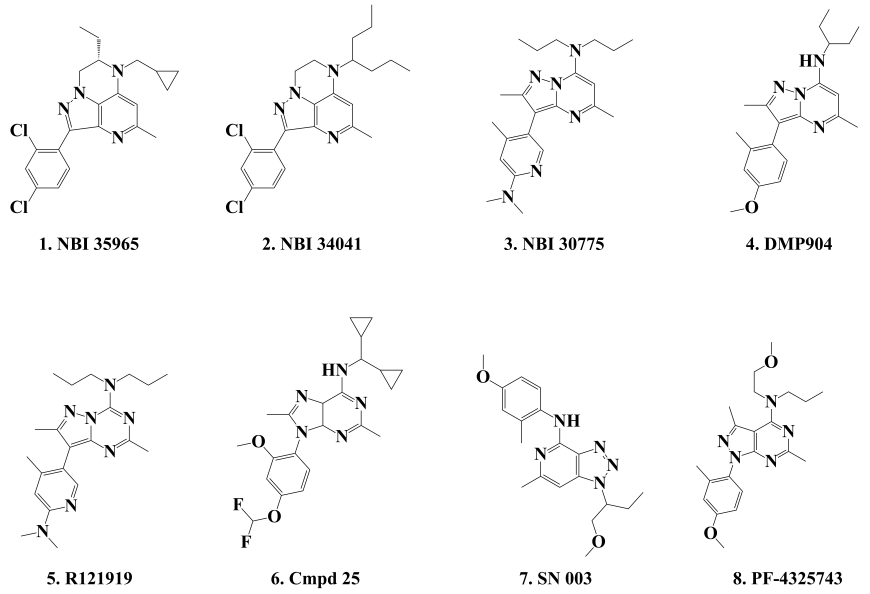

Figure 2.9 | Structures of small molecule CRF_1 receptor antagonists.

receptor compounds varied 170-fold and 13-fold, respectively, which resulted in a maximal change of around 510-fold in the kinetically derived affinity ($K_D = k_{off} / k_{on}$) at 37 °C. Notably, this finding was in stark contrast with the previous reported K_i values measured in radioligand displacement assays, where little difference was observed between compounds. This discrepancy was mostly attributed to insufficient incubation time to reach equilibrium for compounds with long residence time profile. For instance, less than 40% of NBI 30775 dissociated from the CRF_1 receptor after 2 h, far from equilibrium, hence the K_i values were underestimated. Furthermore, several lead compounds displayed divergent dissociation half-lives; NBI 35965 with 16 min, NBI 34041 with 53 min and NBI 30775 with 130 min (Figure 2.9, Compound 1-3; Table 2.6). This ranking of their binding kinetics, in fact, was consistent with the ranking of their *in vivo* pharmacodynamic profiles, at least in adrenalectomized rats, where NBI 34041 and NBI 30775 produced sustained suppression of adrenocorticotropin for 4 h and 6 h, respectively, while NBI 35965 induced a more transient effect (less than 2 h). In addition, all three compounds (NBI 35965, NBI 34041 and NBI 30775)

Table 2.6 | Binding kinetics of CRF_1 receptor antagonists.

Nr	*Cmpd*[a]	*Affinity (K_i, nM)*[b]	*Dissociation half-life ($t_{1/2}$)*[c]	*Ref*
1.	NBI 35965	2.3	16 min (37°C)	151
2.	NBI 34041	1.7	53 min (37°C)	151
3.	NBI 30775	0.36	130 min (37°C)	151
4.	DMP-904	1.5	5.5 h	152
5.	R121919	12	12.8 h	152
6.	Cmpd 25 (Pfizer)	11	10.7 h	152
7.	SN003	3.64 (rat)	0.35 h (rat)	153
8.	PF-4325743	8.22 (rat)	0.37 h (rat)	153

[a] Compound names and synonyms used in original references. [b] Affinity, expressed as K_i values that were reported in original references; result on human receptor unless mentioned otherwise. [c] Dissociation half-life at room temperature and on human receptor unless mentioned otherwise.

had similar pharmacokinetics. This confirmed that the unique sustained adrenocorticotropin suppression evoked by NBI 30775 was derived from its prolonged CRF_1 receptor occupancy rather than its pharmacokinetics.

Functional reversibility assays were also performed to kinetically profile CRF_1 receptor antagonists.[152,153] Briefly, cells expressing recombinant CRF_1 receptor were incubated with CRF in either the presence or absence of an antagonist to measure the suppression of the maximal cell response to CRF by the tested antagonist. Although CRF binds to a different site on the receptor than the non-peptide small molecule antagonists, the insurmountability of the non-competitive antagonists still correlated to their off-rates. In particular, Miller *et al*. plotted the antagonists' dissociation half-lives against their suppression of the maximal CRF_1 receptor response to its endogenous ligand. They found a good correlation between the two parameters, although the exact molecular mechanism of this link is yet to be discovered.[152] By using this assay, at least in a qualitative manner, Miller *et al*. investigated several other long RT compounds reported in literature. They found that DMP-904 and R121919 almost completely suppressed the maximal CRF response (Figure 2.9, Compound 4 and 5). Both compounds were found to have slow dissociation half-lives, i.e., 5.5 h and 12.8 h, respectively, which were calculated from k_{off} values determined in a competition association assay.[152] In comparison, shorter residence time CRF_1 receptor antagonists (Figure 2.9,

Compound 7 and 8) such as SN003 ($t_{1/2}$ = 0.35 h, Table 2.6) or PF-4325743 ($t_{1/2}$ = 0.37 h, Table 2.6) did not significantly decrease the maximal CRF response.[153]

2. Overall structural determinants for binding kinetics at the CRF_1 receptor

A detailed structure-kinetics relationship (SKR) study was performed by Fleck *et al.*, in which the authors performed a retrospective binding kinetics investigation on a set of CRF_1 receptor ligands including the initial, low-affinity chemical starting points up to the lead compounds. Key features of the high affinity or long residence time CRF_1 receptor ligand are represented in Figure 2.10A (adapted from the models developed by Gilligan *et al.*, Kehne and De Lombaert, and Fleck *et al.*[151,154,155]) and the SKRs are summarized as follows: 1) *ortho*-position substitution (R_1) on the low ring was necessary to enforce a twisted bioactive conformation to enhance ligand-receptor interactions. This can be illustrated by the conformation of CP-376395 in the recently elucidated CRF_1R crystal structure (PDB code: 4K5Y, Figure 2.10B). Apparently, the pose of the compound's trimethylphenoxy moiety is rotated to fit in a hydrophobic pocket formed by $F284^{5.51}$, $L287^{5.54}$, $T316^{6.42}$, $L319^{6.45}$ and $L320^{6.46}$ with the pyridine nitrogen at the core to form a hydrogen bond with $N283^{5.50}$.[156] In contrast, the association rate of an *ortho*-substitutent free analogue decreased 33-fold compared to its chloro-substituted analogue, suggesting a substantial barrier for the free rotating ligand to fit into the binding pocket. 2) Adding a methyl moiety at R_2 resulted in an increased k_{on} and decreased k_{off}, indicating a raised probability of successful collision between this group and a rigid binding pocket on the receptor, combined with a stabilized ligand-receptor conformation. This finding was in agreement with other published results which suggested that the methyl-group interacted with a hydrophobic pocket in the receptor.[154,155,157,158] 3) Chemical modifications on the upper aliphatic groups at R_3 demonstrated varying effects on the binding kinetics depending on their size, branching pattern and the stereochemical configuration, which may affect the compound's interaction with $F203^{3.44}$ and $Y327^{6.53}$, two aromatic residues gating the small access channel from the extracellular part (Figure 2.10B).[156]

In another SKR study, Miller *et al.* started from a bicyclic purine core and

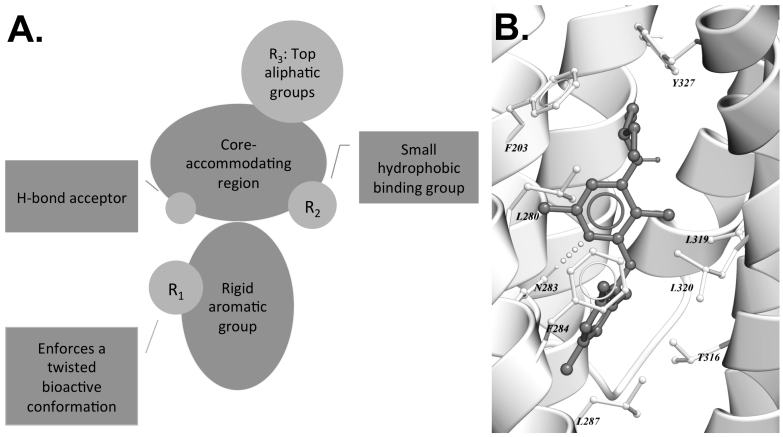

Figure 2.10 | (A) Diagram of the putative functional groups implicated in structure-kinetics relationships of non-peptide antagonists. This diagram was adapted from literature,[160,162,163] based on the comprehensive analysis of ligand SAR. (B) The co-crystal structure of CP-376395 in the human CRF_1 receptor. This figure was generated with ICM Browser v3.7 (Molsoft) from PDB code: 4K5Y. CP-376395 (black) binds deeply within the pocket. Two aromatic residues, F203$^{3.44}$ and Y327$^{6.53}$, together prevent the ligand from moving out of the receptor. The pose of the compound's trimethylphenoxy moiety is rotated to fit in a hydrophobic pocket formed by F284$^{5.51}$, L287$^{5.54}$, T316$^{6.42}$, L319$^{6.45}$ and L320$^{6.46}$ with its pyridine nitrogen at the core to form a hydrogen bond with N283$^{5.50}$.[156]

performed a series of chemical modifications on the substitution patterns at the low ring and the upper branched groups. Follow-up functional investigations *in vitro* led to the identification of compound 25 (Figure 2.9, Compound 6) which displayed 79% suppression of the maximal CRF response. Binding kinetics characterization of this compound revealed that it had a slow $t_{1/2}$ of 10.7 h, correlating well with its insurmountable antagonist behavior.[152]

2.8 Concluding remarks

We reviewed the existing kinetics data for a number of GPCRs. The examples presented make a strong case for the value of measuring drug-target residence time, or, more generally speaking, the kinetic profile of interaction for

compound optimization in the early phases of the drug discovery process. Thus, a strategy is warranted that comprises both a structure-kinetics relationship study (SKR) and a classical structure-affinity relationship study (SAR). This may offer added value to the traditional metric of affinity-based drug evaluation.

This chapter further summarized the factors influencing drug-target residence time, in particular, to find a universal molecular determinant or a certain 'hot spot' on GPCRs for designing kinetics-favorable ligands. Several kinetics-related statistical analyses indicated that manipulating physicochemical properties such as lipophilicity or molecular weight can lead to tuned drug-target residence times.[26,143] Alternatively, this could be achieved by modulating the rigidity / flexibility of a drug candidate.[117] However, these 'intuitive', 'one size-fits-all' approaches are very likely too simple and may increase the concern of binding to collateral targets, thereby resulting in unwanted side effects.[67,159] In this sense, fine-tuning drug-target residence time should probably be more specific. Despite this challenge, progress can still be made by gaining knowledge from both the ligand and target perspectives, such as by ligand modifications with subtle changes to probe relevant pharmacophores, mutation studies, co-crystallization and molecular dynamic studies to establish the corresponding 'anchoring' sites on the receptor.

We also summarized the kinetic methodologies in this chapter. Several screening schemes are available and new assays are emerging, which enable quick identification of compounds of interest. Quantitative characterization of a compound's binding kinetics can be obtained in a kinetic binding assay or competition association assay. Functional reversibility experiments prevail in drug discovery as well, which offer an alternative way to interpret a ligand's kinetics profile. In addition, *ex vivo* receptor occupancy experiments or *in vivo* duration of action experiments can translate the *in vitro* binding kinetics into the effect in an *in vivo* setting, which provides further evidence for a compound's drug-target residence time. Notably, it is also necessary to take into account the PK profile of the candidate compound, particular for one aiming at long drug-target residence time, since RT can only prolong the duration of action only when the RT outlasts the PK.[2,15]

The compounds summarized in the present chapter are predominantly GPCR antagonists. This reflects a lack of kinetic information on agonists in the literature. However, more kinetics studies focused on the latter are emerging. For instance the case studies at the M_3 and A_{2A} receptors

explored the molecular basis of the compounds' intrinsic efficacy from a kinetic perspective.[32,98] In addition to examining an agonist's mechanism of action, one can also specifically optimize an agonist's kinetic properties for therapeutic benefit. This can be exemplified by the administration of long-acting agonists at the GnRH receptor to intentionally desensitize the GnRH receptor and therefore antagonize gonadotropin secretion in hormone-dependent cancers.[181] Such a strategy could be a therapeutic substitute for targets where no antagonist is available. On the other hand, one needs to take into consideration that agonist-induced desensitization or internalization by prolonged or repeated administration can cause drug tolerance, for instance opiate drugs targeting μ-opioid receptors.[182] In such a case, it might be tempting to search for shorter residence time agonists to prevent their target's internalization. Thus, a study that compares agonist binding kinetics and GPCR desensitization / internalization kinetics would be of great interest.

Taken together, a rapid expansion of the field of GPCR binding kinetics may be anticipated in the near future, which hopefully will lead to the identification of novel and better-in-class drugs for clinical applications.

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