Universiteit
Leiden
The Netherlands

Mechanistic modelling of drug target binding kinetics as determinant of the time course of drug action in vivo

Witte, W.E.A. de

Citation
Witte, W. E. A. de. (2017, December 19). *Mechanistic modelling of drug target binding kinetics as determinant of the time course of drug action in vivo*. Retrieved from https://hdl.handle.net/1887/58472

Version:	Not Applicable (or Unknown)
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/58472

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/58472 holds various files of this Leiden University dissertation.

Author: Witte, W.E.A. de
Title: Mechanistic modelling of drug target binding kinetics as determinant of the time course of drug action in vivo
Issue Date: 2017-12-19

1. Introduction

The rates of drug-target association and dissociation are essential determinants of the time course of target binding and drug effect. This is most clearly illustrated by the irreversible binders aspirin and omeprazole, which have shown a long-lasting effect in clinical practice [1–3]. Numerous other examples confirm that drug-target binding kinetics are important drug characteristics, as reviewed by others [4–6].

The relevance of drug-target binding arises from their connecting role between pharmacokinetics and pharmacodynamics. More precisely, for a given drug concentration profile, the kinetics of drug-target binding determine the time course of target occupancy and thus the time course of drug effect. The basic concepts of target equilibration kinetics are well established. The simplest mechanism to describe drug-target binding is depicted in equation 1,

$$T + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} TL \qquad (1)$$

in which T = target concentration, L = ligand concentration, k_{on} = the second order association rate constant, and k_{off} = the first order dissociation rate constant. However, more complex mechanisms have been described in which target activation and G-protein binding (for GPCR's) are incorporated [5,7]. Affinity, the ratio of the dissociation and association rate constants ($K_D = k_{off}/k_{on}$), is related to binding kinetics, but informs only on the extent of binding at equilibrium and gives no information on the required time to reach a new equilibrium.

The important role of drug-target binding kinetics as a determinant of target occupancy profiles has been known for long, and both *in vitro* and *in vivo* measurements of association and dissociation kinetics have been reported from the eighties of last century [8–10]. However, with the development of high-throughput *in vitro* methods for binding kinetics, such as SPR, the interest in the use of binding kinetics in drug discovery has been rising in the past ten years. This has also led to the development of structure-kinetics relationships (SKR's) for some drug classes [11,12]. The recent attention for binding kinetics in drug discovery focuses mostly on the drug-target dissociation rate, since a slow dissociation rate is expected to give a prolonged duration of drug action and improved efficacy [5,6,13–16].

While most recent publications express an expected benefit of incorporating drug-target binding kinetics in drug discovery, more critical studies have also been published. On basis of basic pharmacokinetic/pharmacodynamic simulations, Dahl and Akerud indicated that the relevance of binding kinetics in drug treatment depends on a drug's pharmacokinetics [15]. Several other studies have indicated that multiple other physiological processes can influence the impact of drug-target binding kinetics on drug effect, including endogenous competition, diffusion-limited binding and signal transduction [17–19]. While these simulations might contain oversimplifications and cannot be applied to all cases of drug treatment, it is important to realize that the impact of drug-target binding kinetics on drug action depends on multiple kinetic processes in the human body.

To incorporate the role of drug-target binding kinetics in this complexity of kinetic processes, mathematical models have been developed to describe and predict the time profile of drug effects for several drugs and targets [20–26]. These models have been used to estimate drug-target binding kinetics on basis of pharmacokinetic and pharmacodynamic data, which supports the relevance of drug-target binding kinetics for drug action.

In summary, the available literature indicates a growing interest in the application of screening techniques for binding kinetics in drug discovery and a context dependency for the impact of drug-target binding kinetics on drug effect. This poses the question under which conditions the *in vitro* screening of binding kinetics would further drug discovery and development. To answer this question, this review aims to investigate the value of *in vitro* binding kinetics measurements for the prediction of *in vivo* target occupancy and drug effect, using available literature with emphasis on two questions:

- What is the relation between *in vitro* measured binding kinetics and *in vivo* measured binding kinetics?
- To what extent do binding kinetics contribute to target occupancy and drug effect profiles *in vivo*?

To that end, first, the available methods to measure drug-target binding kinetics both *in vitro* and *in vivo* will be addressed and discussed. Second, we will discuss to what extent the estimates of these *in vitro* and *in vivo* methods provide comparable results, and what experimental conditions are required to enable translation of *in vitro* to *in vivo* binding kinetics. Third, we will discuss binding kinetics in a broader perspective, i.e. in the context of the other determinants of target occupancy and drug effect. Finally, the integration of all kinetic processes will be discussed, as well as their implementation in the various phases of drug discovery and development.

2. In vitro methodological approaches to measure binding kinetics

2.1 Labeled-ligand assays

Various methods are available to determine *in vitro* kinetic binding parameters of compounds of interest at their respective target. In this review, we will use "ligand" to refer to compounds of interest (either labeled or unlabeled) and we will use "tracer" to refer to labeled or unlabeled compounds with known binding characteristics intended to inform about the binding of compounds of interest. The methods as discussed below are summarized in table 1.

2.1.1 Radiolabel-based assays

The most commonly used and straightforward method to characterize target binding is the use of radioligand binding assays. These assays use a radiolabeled ligand and can directly measure the association and dissociation rates of the radiolabeled ligand. In addition to traditional association and dissociation experiments, other kinetic radiolabel-based binding assays such as a competition association assay are emerging. This type of assay is an indirect assay based on a theoretical model developed by Motulsky & Mahan in 1984 by which one can quantitatively determine the binding kinetics of unlabeled ligands in a competitive assay using only one radiotracer [16,27–29]. The competition association assay can also be used in a higher-throughput fashion with the recently developed dual-point competition association assay. Only two timepoints are selected here to measure radiotracer binding; the ratio of binding at both time points gives a qualitative measure of the ligands' dissociation kinetics. This makes this simplified assay a suitable method for screening potential drug candidates with favorable dissociation kinetics [30].

2.1.2 Fluorescent label-based assays

Similarly, instead of using a radiolabeled tracer in a competition assay the tracer can also be fluorescently labeled and used in homogeneous time-resolved fluorescence (HTRF) assays. Similar to the radioligand competition assay, only one fluorescently labeled tracer is required and the binding kinetics of competitive ligands can be determined in an indirect fashion. This method is homogeneous since it requires no physical separation of bound and free ligand which enables continuous measurements and increases the throughput. HTRF assays are successfully applied in the determination of binding kinetics of dopamine D_2 receptor antagonist spiperone [31] and more recently for histamine H_1 receptor ligands [32] and GnRH receptor agonists [Nederpelt *et al.,* 2015, submitted for publication]. Of note, in addition to a fluorescently labeled tracer a fluorescently labeled receptor is needed for this method, as opposed to wild-type receptors for radioligand and radiotracer binding.

Table 1. Overview of *in vitro* methods to measure drug-target binding kinetics.

	***In vitro* methods**						
Technique	radioligand	radiotracer	HTRF	SPR	SAW	Organ bath	Washout
Throughput rate	Low	Low-medium	medium	medium	medium	Low	Low
Required labeling	Radiolabeled ligand	Radiolabeled tracer	Fluorescently labeled tracer	None	None	None	None
Receptor environment	Membrane fractions	Membrane fractions	Whole cells	Isolated	Isolated	Native tissue/ whole cells/ membrane fractions	Native tissue/ whole cells/ membrane fractions
Relation to ligand binding kinetics	Direct	Inferred from tracer binding	Inferred from tracer binding	Direct	Direct	Inferred from effect	Inferred from effect
Major confounding factors	Lack of intracellular environment	Lack of intracellular environment	Fluorescent labeling of target	Non-native target environment	Non-native target environment	Microkinetics, rebinding, signal transduction	Microkinetics, rebinding, signal transduction

SAW: Surface Acoustic Wave, HTRF: Homogenous Time-Resolved Fluorescence, SPR: Surface Plasmon Resonance

2.2 Label-free assays

Several label-fee methods can be applied for kinetic target binding measurements without the need of a labeled ligand or labeled tracer.

2.2.1 Surface plasmon resonance

The most instilled label-free measurement is surface plasmon resonance (SPR) spectroscopy [33]. This method has the potential to be medium-throughput and the capability to measure real-time quantitative binding kinetics of ligands for membrane proteins using relatively small quantities of protein. The traditional SPR method needs one immobilized binding component on a coated gold sensor chip during which the ligand in solution is flowed over the sensor chip. This induces a real time change in the refractive index on the sensor surface which is linear to the number of molecules bound [33–35].

2.2.2 Acoustic wave biosensor

Another label-free technology is the surface acoustic wave biosensor [36]. This methodology captures real-time mass changes on the surface, which result in a shifted phase and/or changed amplitude of a sound wave signal [37]. A disadvantage of these biophysical approaches for G protein-coupled receptors is that these receptors are integral membrane proteins that rapidly disintegrate when taken out of their natural environment, which is a prerequisite for these approaches. However, recent advances are made to overcome this problem [33].

2.3 Functional assays

Another way to determine drug-target binding kinetics is by use of functional assays. These assays provide an indirect measurement of binding kinetics by characterizing the time profile of drug effect. Although the use of functional assays is generally limited due to the indirect nature of these measurements, functional assays are valuable for the measurement of enzyme binding kinetics because of the direct relation between enzymatic product generation rates and enzyme inhibitor binding. Functional assays can be carried out in two different settings, either by resembling the classical ‘’organ bath’’ experiment or by washout experiments.

2.3.1 Organ bath

An organ bath experiment is only suitable to qualitatively examine binding kinetics of antagonists and requires pre-incubation of cells/tissues with antagonists prior their challenge with an agonist. With this method the distinction between so-called surmountable and insurmountable antagonists can be made, where the level of insurmountability by an antagonist is related to its receptor dissociation kinetics [18,38,39].

2.3.2 Washout

Functional washout experiments are suitable for predicting binding kinetics of both agonists and antagonists. In this type of experiments, the rate of decrease in effect after removal of the free ligand by repeated washing (washout) is measured. Agonists with fast dissociation kinetics will readily wash out and will show a right-ward shift in their potency, whereas agonists with slow dissociation kinetics will show insignificant shifts in their potency, and *vice versa* for antagonists. It should be stated that control experiments are necessary to confirm that the long-lasting effect of the ligand is due to long target binding versus other effect-prolonging factors (such as exo-site binding, membrane partitioning, rebinding or signal transduction [40–42].

3.1 In vivo methodological approaches to measure binding kinetics

3.1.1 General principle of target occupancy measurements

To obtain drug-target binding kinetics *in vivo,* target occupancy and target site concentrations are required. For most *in vivo* and *ex vivo* approaches, the target occupancy of a drug is measured indirectly by using a tracer. The administered drug competes at the same target site with the tracer and the reduction in specific binding of the tracer is used to calculate the target occupancy of the drug. The tracer can be an antagonist (more common) or agonist to the target, and can be radiolabeled (more common) or non-radiolabeled. The advantages and disadvantages of each approach will be briefly discussed, and the characteristics of each approach are summarized in table 2. We focus here mainly on methods which are in use for measurement of binding kinetics in the brain, since most methods have been used primarily for the brain targets.

3.1.2 Tissue homogenate method with radiolabeled tracer

The traditional way of measuring CNS target occupancy in preclinical animals is the brain homogenate method. At a pre-determined time point after radiotracer administration, the animal is sacrificed and the brain regions of interest (e.g. striatum for D_2 receptors) and the reference region (e.g. cerebellum which has relatively low D_2 receptor density, for the correction of non-specific binding of radiotracer to and uptake in brain tissue) are collected. These brain regions are then dissolved in a scintillation cocktail and the drug-induced change in radioactivity of the tracer is measured by a liquid scintillation counter. Literature reports suggest that the target occupancy values obtained by this method are comparable to that obtained by positron emission tomography (PET) imaging [43]. Compared with PET/SPECT (single-photon emission computed tomography) imaging, this method is associated with much lower costs and allows higher throughput in screening different compounds or different doses of a single compound. Nevertheless, since this method involves the terminal use of animals, a continuous target occupancy time profile within the same animal cannot be obtained, and multiple animals are needed for a single target occupancy time profile. Moreover, in addition to the receptors expressed on the membrane surface, intracellular or internalized receptors would also become accessible to the tracer when the tissue is homogenized, which might hamper the accuracy of target occupancy assessment for membrane-bound receptors [44].

Table 2. Overview of *in/ex vivo* methods to measure drug-target binding kinetics.

	In vivo methods					***Ex vivo* methods**	
Technique	PET scan	SPECT scan	Beta-microprobe	Tissue homogenate method with radiolabelled tracer	Tissue homogenate method with non-radiolabelled tracer	Single time-point, tissue homogenate method with radiolabelled tracer	Single time-point, tissue slice method with autoradiography imaging
Subjects	Living humans or animals	Living humans or animals	Living animals	Animals sacrificed at a specific post-drug dosing time point	Animals sacrificed at a specific post-drug dosing time point	Animals sacrificed at a specific post-drug dosing time point	Animals sacrificed at a specific post-drug dosing time point
Equipments	Cyclotrons and PET scanner	SPECT scanner	Positron-sensitive probe	Scintillation counter	Liquid chromatograph /mass spectrometer	Scintillation counter	Autoradiographic film, storage phosphor imager or beta-imager
Radiolabelled tracer needed?	Yes	Yes	Yes	Yes	No	Yes	Yes
Simultaneous TO determination for multiple receptors?	Difficult	Yes	No	No	Yes	No	Yes
Relation to drug binding kinetics	Inferred from tracer binding	Inferred from tracer binding	Inferred from tracer binding	Inferred from tracer binding from multiple tissue samples	Inferred from tracer binding from multiple tissue samples	Inferred from tracer binding from multiple tissue samples	Inferred from tracer binding from multiple tissue samples
Major confounding factors	Anesthesia, tracer metabolite interference	Anesthesia, tracer metabolite interference	Tissue damage, tracer metabolite interference	Tracer dose, dosing time of tracer, tracer metabolite	Tracer dose, dosing time of tracer	Tracer incubation period and temperature	Tracer incubation period and temperature

PET: Positron Emission Tomography, SPR: Surface Plasmon Resonance, SPECT: Single Photon Emission Computed Tomography, TO: target occupancy

3.1.3 Tissue homogenate method with non-radiolabeled tracer using LC/MS assays

The procedures of this method are the same as that with radiolabeled tracer as described above, except that a non-radiolabeled tracer (cold tracer) is administered to the animal and the absolute amount of the tracer in the brain tissues is quantified by LC/MS. The first report was presented by Phebus and colleagues, in which the drug-induced target occupancy of D_2, serotonin 2A and NK-1 receptors in rat was quantified using non-radiolabeled tracers [45]. They also demonstrated in rats that for the eight D_2-antagonists they

had investigated, the doses required to achieve 50% target occupancy using this LC/MS method (cold raclopride as tracer) are comparable to those using the traditional brain homogenate method ([^{3}H]raclopride as tracer) [46]. This method offers several advantages; first, the parent, intact tracer in the brain tissue can be differentiated from the tracer metabolites, thus increasing the accuracy of tracer quantification. Second, the costs and hazards associated with radioactivity are avoided. Third, it allows separation and quantification of different tracers in one sample, and thus enables the simultaneous assessment of the target occupancy of different receptors [47].

The greatest concern of this method is the relatively high dose of the tracer that needs to be administered. Since the sensitivity of an LC/MS assay is lower than that of radioactivity counting, a much higher dose of the tracer is administered in order to achieve a quantifiable tissue concentration. This high tracer dose might distort the drug-induced target occupancy and might exert pharmacodynamics effects [48,49].

3.1.4 PET/SPECT imaging

PET and SPECT imaging are the most common approaches to measure drug target occupancy in living humans and other primates. After the administration of a very small dose of radiotracer for the desired target, scans are carried out by the PET or SPECT scanner before and after administration of the competing drug. The radioactivity at the region of interest is measured, from which the density of receptors (B_{max}) and the radiotracer binding affinity (K_D) are derived. The ratio of B_{max} and K_D is termed the binding potential. The target occupancy of the drug is calculated as the percentage reduction in binding potential after drug administration. Binding kinetic parameters (k_{on}, k_{off}) can be derived if the target occupancy and free drug PK at the binding site are available by fitting a mathematical model which describes binding kinetics according to scheme 1. However, the PET signal arises from the sum of free, specifically and non-specifically bound radiotracer, and free concentrations cannot be measured at the binding site. Instead of the free drug pharmacokinetics at the binding site, a reference tissue which is similar to the binding site but has no specific binding is commonly used [50,51]. PET/SPECT can be regarded as an *in vivo* version of autoradiography (discussed in the *ex vivo* section), with inferior spatial resolution but with the advantage that the pharmacokinetics of the tracer can be measured in a single experiment, or even in repeated studies on the same subject [52]. This also provides the possibility to obtain target occupancy values at different time points within the same subject. Over the past decade, there are considerable developments of both PET and SPECT systems with improved spatial resolution designed specifically for small-animal imaging (i.e. microPET and microSPECT).

A limiting factor in longitudinal PET/SPECT measurements is the half-life of the radioactive decay of the tracer (depending on the applied radiolabel), which can limit the duration of the experiment after tracer administration. This limited duration of the imaging decreases the suitability of PET/SPECT for measuring drugs with slow binding kinetics

One of the main concerns in PET/SPECT is that the anesthesia, applied to immobilize the animals before and during imaging, could hamper the accuracy of target occupancy assessment by, for example, altering the level of neurotransmitters [53]. Moreover, the use of anesthesia might also impose additional experimental variability (e.g. due to variable susceptibility to the anesthetic effect [54]).

Since both the tracer and the drug of interest interact with the same receptor, the observed effect cannot be completely attributed to the drug. Therefore, drug effect measurements are considered less useful, except for studies which are focused on the binding and effect of only the tracer. Depending on the target of interest, the required anesthesia can also interact with drug effects and make their measurement impossible or less useful.

3.1.5 Beta-microprobe

Another method of measuring a radiotracer in a living animal's brain is the use of a beta-microprobe. The microprobe captures beta/positron emission (similar to the PET detector) and is surgically implanted in the brain structures of interest, allowing *in vivo* measurement of local radioactivity concentrations within 1-2

millimeters from the probe. Reports on the application of beta-microprobe on target occupancy assessment are limited. Good correlations have been reported between *in vivo* beta-microprobe measurements and *ex vivo* brain homogenate and *in vivo* microPET measurements of respectively D_2 and $5HT_{1A}$ target occupancy in rat brain [55,56].

The potential advantages of beta-microprobe are that the target occupancy could be measured in awake, non-anesthetized animals and simultaneous assessment of drug-induced changes in behavior is allowed, which are critical for drugs that act on CNS receptors. Nevertheless, the surgical implantation procedures might interfere with the neurochemistry and the pharmacokinetics and pharmacodynamics of the drug and tracer. Implantation of the electrode into the brain would cause mechanical trauma and trigger both acute and chronic tissue responses, and the final outcome depends on factors such as the size, geometry and material of the probe, the insertion method, and the period after insertion [57]. Device implantation could also alter the release of neurotransmitters and neural activity [58]. While the previously developed beta-microprobes were based on a single pixel scheme that did not provide any spatial information on the radiotracer distribution [55], a new wireless probe was recently published, which contains 10 submillimeter pixels which allows the analysis of the spatial distribution of the radiotracer within the region of interest in freely moving rats [59].

3.2 Ex vivo approaches of target occupancy measurements

3.2.1 Tissue homogenate method with radiolabeled tracer

While for *in vivo* methods both the drug and the tracer are administered to the living animals, for *ex vivo* methods the tracer is added to the collected tissue from the drug-treated animal, and the amount of radiotracer bound to the target in the homogenate is measured by liquid scintillation counting. In this way tracers with unfavorable *in vivo* characteristics (e.g. slow equilibrium at target tissue, pharmacokinetic variability etc.) can be used and the costs of developing suitable tracers are reduced and the amount of tracer can be precisely controlled. However, the values of target occupancy obtained by this method are highly dependent on the binding conditions (particularly the time and temperature of tracer incubation) and tend to give an underestimation of drug-induced target occupancy [60]. This is mainly due to the dissociation of the drug from the receptor during the *ex vivo* tracer incubation and the tissue homogenization step, particularly for those drugs with a fast dissociation rate from the receptor. Therefore, a short incubation time and a radiotracer with a fast association rate is recommended [60].

3.2.2 Tissue slice autoradiography imaging

The procedures of this method are the same as that with tissue homogenate method described above, except that the animal tissue is sectioned into slices and the amount of radiotracer bound to the target is quantified by autoradiography. Unlike tissue homogenate, the tissue slice preparation maintains structural integrity. It offers higher spatial resolution than PET/SPECT imaging and thus allows the investigation of anatomical regions that are small in size. Traditionally, the radioactivity on the slice is captured by autoradiographic film, which requires a long exposure period (weeks) and thus is not considered as an efficient screening method for determining the target occupancy of compounds [61]. The introduction of storage phosphor imaging is a major improvement in *ex vivo* receptor autoradiography, which shortens the exposure time from weeks to days or even one day [62]. An alternative method is to use a beta-imager which uses a highly sensitive gaseous detector of beta particles. This allows the exposure time to be shortened to a few hours [63].

4. Comparison of *in vitro* and *in/ex vivo* measurements of binding kinetics

To investigate whether the current *in vitro* and *in vivo* measurements of binding kinetics deliver similar or translatable values, we performed a literature survey to identify compounds for which both *in vitro* and *in vivo* estimates of target association or dissociation rates were available. Since *in vivo* estimates are the least available, we started our search with *in vivo* estimates and continued to search for *in vitro* estimates of the

same compounds. Since the number of compounds for which we could find *in vitro* and *in vivo* estimates of their target binding kinetics was very low, we decided to list all estimates we could find and discuss the reliability and comparability of the estimates below. The results of this search are listed in table 3.

Based on table 3, we can start to answer our first question:

- What is the relation between *in vitro* measured binding kinetics and *in vivo* measured binding kinetics?

From the results in table 3, it can be directly seen that the difference between *in vitro* and *in vivo* estimates of target dissociation rates can be quite substantial (up to 30 fold) and inconsistent (the ratio varies from 0.2 to 31). This clearly indicates that the use of *in vitro* measured target binding kinetics to predict *in vivo* binding profiles is not straightforward. Apart from the studies in table 3, another study was published in which no *in vivo* values for k_{on} and k_{off} were included, but *in vitro* values were used to predict target occupancy profiles of the CRF1 receptor in rats for several antagonists [23]. Although the *in vivo* results were not highly informative for the identification of the binding kinetics for some compounds in this study, the target occupancy profiles could be predicted reasonably well.

To investigate the origin of the observed difference between *in vitro* and *in vivo* binding studies, the experimental details need to be taken into account to identify which results are less reliable or comparable.

4.1 Temperature

Firstly, all *in vitro* estimates of association and dissociation rates which are not obtained at 37 °C cannot be compared directly to *in vivo* estimates, since these rates are temperature dependent in a compound specific manner [85–87]. Therefore entry 3, 9, 12, 13, 17, 18 and 19 from table 3 cannot be used to compare *in vitro* and *in vivo* dissociation rates.

4.2 Influence of *in vivo* displacer/competitor dose

Another important factor in the comparison between *in vitro* and *in vivo* estimates of target dissociation rates is the method by which the dissociation is induced. Drug-target dissociation can be induced in *in vitro* studies either by continuous washing, the so-called “infinite dilution” method, or by displacement of the drug by adding an excess of a competing ligand. These methods can give quite different results since washing cannot displace all free ligand molecules and diffusion-limited binding (or “rebinding”) can occur. Thus, comparisons between *in vitro* and *in vivo* estimates should use the same method of dissociation measurement [19]. However, in the *in vivo* setting, continuous washing cannot be applied and the amount of competing compound which can be added is limited by its toxicological effects. In the analysis of *in vivo* drug-target binding studies, computational models can be used to correct for remaining drug concentrations or partial displacement. However, this is often not done and assumptions have to be made about the effect of a displacer dose or of a remaining drug concentration. For entry 1, 3, 4, 8 and 10 from table 3, the rationale for the displacer dose was not clear, and model-based analysis was not used. These entries should therefore not be used to compare *in vitro* and *in vivo* dissociation rates. For entry 1, the *in vitro* experiment did not use either a displacer or continuous washing, which makes it even less appropriate for comparison with the *in vivo* experiment.

For entries 14-19 in table 3, the *in vivo* drug-target binding kinetic parameters are estimated from PK and PD data without target occupancy measurements. This makes these estimates indirect and subject to influences of signal transduction kinetics and other factors between PK and PD. Therefore, entry 14-19 cannot be used for a direct comparison of *in vitro* and *in vivo* binding kinetic parameters.

Table 3. Literature data on estimated binding kinetics from *in vitro* and *in vivo* studies.

#	Drug (target)	$t_{1/2}$[a] assoc. *in vitro*	$t_{1/2}$[a] dissoc. *in vitro*	*In vitro* system [b]	$t_{1/2}$[a] assoc. *in vivo.*	$t_{1/2}$[a] dissoc. *in vivo*	Observed binding parameter (method)	Ref.	Ratio *in vivo*/ *in vitro*	
									assoc.	dissoc.
1	^{3}H-CGP 12177 (β-AR)	7	99	I	NA	50	dog heart (PET)	[64],[65]	NA	0.5
2	^{125}I-epidepride (D_2R)	267	13	II	NA	53	rhesus monkey striatum (SPECT)	[66]	NA	4
3	^{18}F-desmethoxy fallypride (D_2R)	3[c]	9[c]	III	NA	12	rhesus monkey striatum (PET)	[67]	NA	1
4	^{18}F-fallypride (D_2R)	1[c]	13	III	NA	169	rhesus monkey striatum (PET)	[68]	NA	13
5	^{18}F-fallypride (D_2R)	1[c]	13	III	NA	18[d]	rhesus monkey brain (PET)	[68],[69]	NA	1
6	^{18}F-fallypride (D_2R)	1[c]	13	III	NA	30[d]	rhesus monkey brain (PET)	[68],[70]	NA	2
7	^{18}F-spiperone (D_2R)	NA	56	IV/V	NA	50[e]	baboon striatum (PET)	[71],[72]	NA	0.9
8	^{3}H-spiperone (D_2R)	5	20	III	1690	231	rat striatum (homogenate)	[73]	338	12
9	Olanzapine (D_2R)	9[j,k]	18[j]	IV	234	16	rat brain (homogenate)	[74],[75]	26	0.9
10	^{123}I-iomazenil ($GABA_A$)	2	2	VI	46	4	baboon brain (SPECT)	[76]	23	2
11	^{123}I-iomazenil ($GABA_A$)	2	2	VI	NA	4[f]	human brain (SPECT)	[76],[77]	NA	2
12	^{3}H-flumazenil ($GABA_A$)	NA	15[g]	IV	NA	4[f]	mouse brain (homogenate)	[60],[78]	NA	0.3
13	^{11}C-flumazenil ($GABA_A$)	0.4	1[h]	VII	NA	2[i]	human brain (PET)	[79],[80]	NA	2
14	Nitrendipine (Ca^{2+} channels)	1[c]	2	VIII	320	47	human blood pressure	[81],[20]	320	24
15	Benidipine (Ca^{2+} channels)	1	112	VIII	16	3465	human blood pressure	[81],[20]	16	31
16	Benidipine (Ca^{2+} channels)	1	112	VIII	28[l]	60[l]	human blood pressure	[81],[21]	28	0.5
17	Buprenorphine (opioid)	26[j]	43[j]	I	6	68	human respiration	[82],[83]	0.2	2
18	Buprenorphine (opioid)	26[j]	43[j]	I	3	8	rat respiration	[82],[84]	0.1	0.2
19	Buprenorphine (opioid)	26[j]	43[j]	I	135	18	cat nociception	[82],[22]	5	0.4

[a] $t_{1/2}$ assoc.: concentration-dependent association half-life in min•nM (at a constant concentration of free ligand or free target and with absence of dissociation), $t_{1/2}$ dissoc.: dissociation half-life in min (with absence of association). Values are obtained by calculating $0.693/k_{on}$ and $0.693/k_{off}$, respectively.

[b] I = transfected CHO cells, II = rat striatal membranes, III = rat striatal homogenate, IV = rat brain homogenate, V = guinea pig brain homogenate, VI = baboon occipital homogenate, VII = rat brain P2 fraction, VIII = rat cardiac membranes.
[c] this value was obtained at 25 °C.
[d] displayed value is the average from all brain regions as reported in the reference.
[e] displayed value is the average from all experiments as reported in the reference.
[f] displayed value is the average from all brain regions as reported in the reference, except for the pons, which had an insufficient significance.
[g] this value was obtained at 4 °C.
[h] this value was obtained at 22 °C.
[i] displayed value is the average from the three-compartment estimation from all brain regions as reported in the reference.
[j] this value was obtained at room temperature.
[k] The published k_{on} values in this reference seem to be erroneously calculated. The value in this table is obtained by dividing the measured k_{off} over K_i.
[l] This value was based on a model fit on drug effect data of heart rate. The same model was also fitted on blood pressure which resulted in a similar but dose dependent estimate, which was ignored.
PET: Postiron Emission Tomography, SPECT: Single Photon Emission Computed Tomography, NA: Not Available.

4.3 Most valid comparisons

To evaluate the difference between *in vitro* and *in vivo* estimates of association and dissociation rates, we should only use the most valid comparisons, restricting table 3 to entries 2,5,6,7 and 11. Now the ratio between *in vitro* and *in vivo* estimates varies between 0.9 and 4 which is considerably better, but based only on four compounds and two targets. Moreover, it should be noted that these entries include only one entry for which the comparison is made with human binding data. Also, all observations from table 3 originate from GPCRs and therefore, none of the studies used isolated receptors. One could speculate that the correlation between *in vitro* and *in vivo* estimates is better for membrane-bound targets than for soluble targets since the membrane-bound receptors are mostly measured in membrane fractions and therefore retain some of their natural environment, whereas soluble targets can be completely purified. However, the natural exposure of membrane-bound receptors to the differential composition of extracellular and intracellular fluids cannot be reproduced in homogenized *in vitro* experiments, while the homogeneous environment of soluble targets can be replicated *in vitro*.

4.4 Summary

The amount of available literature data to compare *in vitro* and *in vivo* estimates for drug-target dissociation rates in a valid manner is too low to draw general conclusions about the predictive value of the *in vitro* drug-target dissociation estimates. This is even more so for drug-target association rates. Moreover, differences in experimental approach and conditions, and differences in data analysis hamper the comparison of *in vitro* and *in vivo* binding kinetics data. These differences include most frequently a difference in temperature (i.e. *in vitro* experiment not at 37 °C), difference in dissociation method (washout vs. displacement) and analysis method (model-based parameter estimation vs. graphical methods). Therefore, the current *in vitro* estimates of drug-target binding kinetics cannot be translated reliably into *in vivo* binding kinetics due to a lack of available information on comparability and due to methodological differences between *in vitro* and *in vivo* experiments.

5. Missing links in the translation between in vitro and in vivo binding kinetics

The differential results that have been observed from *in vitro* and *in vivo* studies can be explained by a multitude of differences between the extremely complex *in vivo* situation and the much more simplified *in vitro* environment. Possible explanations include factors that are poorly understood, such as the *in vivo*

occurrence of complicated ligand interactions with multiple targets, allosteric binding sites, exosites and subcellular compartments or organelles, but also complex target interactions with other proteins (homo- and heterodimerisation), and other cell membrane and intra- and extracellular fluid constituents, such as ions. Moreover, the *in vivo* three-dimensional structure of multiple cell types is rarely replicated *in vitro* and unknown contributors to the observed *in vivo* target binding kinetics cannot be excluded.

However, the following section is focused on the better understood contributors to *in vivo* target binding kinetics and how these can be accounted for in the design and analysis of both *in vitro* and *in vivo* experiments

5.1 Experimental conditions in in vitro and in vivo studies of binding kinetics

As described in the previous section, the comparison of *in vitro* and *in vivo* binding kinetic parameters is often hampered by differential experimental conditions between in *in vitro* and *in vivo* studies. We will discuss here the most relevant experimental conditions which can hamper the translation between *in vitro* and *in vivo* measured binding kinetics. These are: *in vitro* temperature, *in vivo* displacement method and the presence of endogenous ligand.

5.1.1 Temperature

One very important *in vitro* and *ex vivo* experimental condition is the temperature. Since both drug-target association and dissociation rate are temperature dependent in a compound-specific manner [85–87], translation of binding kinetics from one temperature to another temperature cannot be done unless the temperature dependency has been determined for that specific compound. Moreover, since the target conformation might be temperature dependent as well, the Arrhenius plots of k_{on} and k_{off} are not necessarily linear. A few literature examples are available of linear Arrhenius plots for k_{on} and k_{off} [87–89]. Therefore, it is highly relevant to obtain *in vitro* binding parameters at 37 °C, or to obtain a linear Arrhenius plot at lower temperatures.

5.1.2 Displacer/competitor

Another condition that may affect translational success is the presence or absence of a displacer/competitor. To account for this, it is necessary to obtain both *in vitro* and *in vivo* estimates for k_{off} in the presence of a displacer. If both experiments are done in the absence of a displacer, translation can still be hampered because of differential diffusion rates and target clustering in the two experiments, leading to different diffusion-limited binding ("rebinding").

5.1.3 Endogenous ligand

The presence of an endogenous ligand is also influencing the rate of drug-target association and dissociation. An endogenous ligand can be present both *in vitro* and *in vivo.* To enable an accurate *in vivo* and *in vitro* estimation of drug-target k_{on} and k_{off} in the presence of an endogenous ligand, the concentration profile over time during the experiment and the binding kinetics of the endogenous ligand need to be known.

5.2 Integrated analysis of multiple determinants of in vivo target occupancy and drug effect

In order to use *in vitro* binding kinetic data to predict *in vivo* target occupancy and effect kinetics, all kinetic processes which influence the *in vivo* kinetics of drug effect need to be taken into account (see also section 7). These include pharmacokinetics, endogenous competition, diffusion-limited binding, non-specific binding, target turnover and signal transduction. Each of these processes will be discussed in the following section.

5.2.1 Pharmacokinetics

One of the clearest examples for the need to integrate all kinetic processes for the prediction of *in vivo* target occupancies is the role of pharmacokinetics: If the drug concentration in the human body has a constant profile, an equilibrium situation will be reached and a slow dissociation rate will not prolong the target occupancy anymore. On basis of a very simple relation between pharmacokinetics and binding

kinetics, one can expect a slow dissociation rate to be prolonging target occupancy only when its dissociation rate is slower than its elimination rate (figure 2, upper panels) [15,19]. However, this might be an oversimplification, and other processes need to be integrated as well [19].

5.2.2 Endogenous competition

Another process which is important for the role of binding kinetics is endogenous competition. The presence of a varying concentration of endogenous ligand can make a drug's binding kinetics more important, also when its dissociation half-life does not exceed its plasma elimination half-life (figure 1) [14,18,90–92]. Since endogenous ligands usually have a varying concentration, endogenous competition might be relevant for the binding kinetics of most agonists and antagonists. A hypothesis in this direction was already published by Kapur and Seeman before the recent interest in binding kinetics [92]. In their publication, fast dissociating dopamine antagonists were suggested to be less resistant to dopamine signaling, thereby preventing side effects from over-suppression of dopamine signaling.

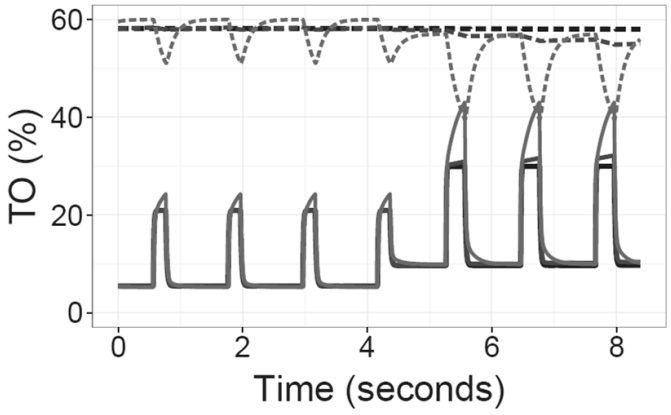

Figure 1. The influence of drug-target binding kinetics on drug (dashed lines) and dopamine (solid lines) target occupancy (TO) is influenced by endogenous competition, as simulated by Vauquelin et al [18]. *A constant drug concentration and pulsatile dopamine concentration are used, and the system is allowed to reach equilibrium before t = 0. The dopamine concentrations rise after 4 seconds to represent a high activity period. The drug target dissociation rate (k_{off}) changes from 181 min^{-1} (light grey) to 6.03 min^{-1} (dark grey), and 0.181 min^{-1} (black).*

5.2.3 Diffusion-limited binding

A kinetic process which has got only limited attention for its effect on target occupancy profiles is diffusion-limited binding. If the effective diffusion of a drug around its target is limited, the chance that it will re-associate to its target before diffusing into the tissue (often called 'rebinding') will increase and thus the target occupancy will decrease slower than expected from its binding kinetics and tissue concentration. Although the possible significance of diffusion and diffusion-limited binding (or 'rebinding') has already been indicated in studies with rats, humans and *in vitro* over three decades ago [9,10,93], there is no general practice of taking this into account in either *in vitro* or *in vivo* studies. As reported several times by Vauquelin and his colleagues, based on literature, experimental and theoretical findings, "rebinding" can have a significant impact on the estimated k_{off} value in *in vitro* and *in vivo* studies, and therefore needs to be taken into account in the design and analysis of these studies (figure 2) [19,40,94–96].

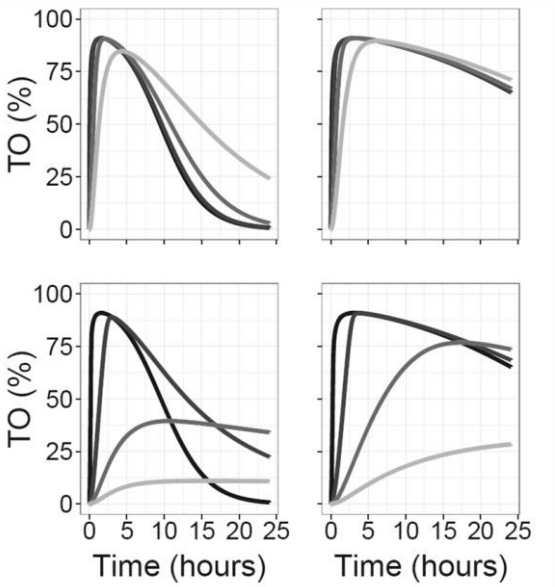

Figure 2. The influence of drug-target binding kinetics on target occupancy (TO) depends on both pharmacokinetics and diffusion-limited binding, as simulated by Vauquelin et al [19]*. The drug target dissociation rate (k_{off}) changes from 83 hr^{-1} (black) to 2.1 hr^{-1} (dark grey), 0.35 hr^{-1} (grey), and 0.087 hr^{-1} (light grey). The drug elimination rate constant is 0.35 hr^{-1} for the left panel and 0.087 hr^{-1} for the right panel.*

5.2.4 Non-specific binding

Another kinetic process which can influence the profile of target occupancy is non-specific binding. Non-specifically bound drug can act as a reservoir which releases drug upon decreasing free drug concentrations, thereby decreasing the effective elimination rate. Moreover, if the release of non-specifically bound drug is slow, this can become the rate determining factor for the rate of drug elimination from either the plasma or the target tissue (figure 3) [97,98].

5.2.5 Target turnover

The rate of target synthesis and degradation can also influence the profile of target occupancy, since the breakdown of occupied target and synthesis of new (unoccupied) target decreases the occupied fraction. Thus, target turnover provides a suitable explanation for the limited duration of the antiplatelet effect of the irreversible binder aspirin [1]. Moreover, target synthesis and degradation can be regulated and function as feedback mechanisms [99–103]. A high rate of target turnover can limit the impact of a decreasing dissociation rate constant and can increase the impact of the association rate constant (figure 4).

5.2.6 Signal transduction

Apart from these multiple factors which influence the target occupancy profiles, another step is required to predict effect kinetics from target occupancy profiles. To do this, the kinetics of all signal transduction steps need to be taken into account. The significance of signal transduction kinetics with respect to binding kinetics has been indicated by a simulation study of binding kinetics, enzyme inhibition and several signal transduction pathways [17]. However, since signal transduction can have various mechanisms and includes feedback mechanisms, the influence of signal transduction on the role of drug-target binding kinetics can differ greatly between targets.

Although the kinetics of signal transduction can be important, direct relationships between target occupancy and drug effect have been characterized for a few targets. However, *in vivo* target occupancy and drug effect are rarely measured simultaneously, and mathematical models are often required to estimate the relationship between target occupancy and effect from pharmacokinetic and pharmacodynamic data.

One example where *in vivo* target occupancy and drug effect were measured simultaneously for the dopamine D_2 receptor demonstrated the typical hyperbolical relationship [104]. However, linear relationships between target occupancy and effect have been used by mathematical models as well, for example to describe drug effect for calcium channel blockers and DPP-4 inhibitors [20, 26].

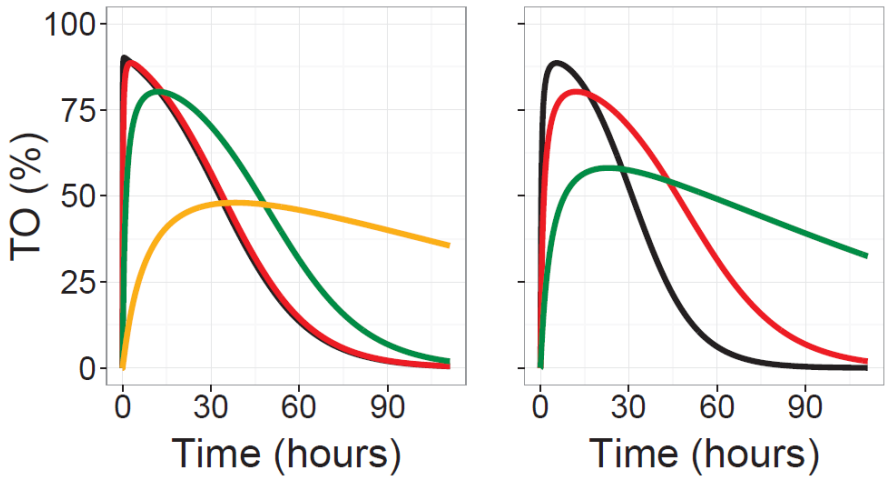

Figure 3. The target occupancy (TO) profile can be influenced by non-specific binding of the drug, as simulated for lipid and protein binding in the brain by Peletier et al [97]. *The drug target dissociation rate constant (k_{off}) is 36 $hr^{-1}$for all lines. For the left panel, the drug-protein dissociation rate constant changes from 1000 sec^{-1} (black) to 100 sec^{-1} (red), 10 sec^{-1} (green), and 1 sec^{-1} (orange). For the right panel, the drug-lipid dissociation rate constant changes from 500 sec^{-1} (black) to 100 sec^{-1} (red) and 20 sec^{-1} (green). The drug-protein and the drug-lipid affinity change in the same way as the dissociation rate constants, since both drug-protein and drug-lipid association rate constants remain unchanged.*

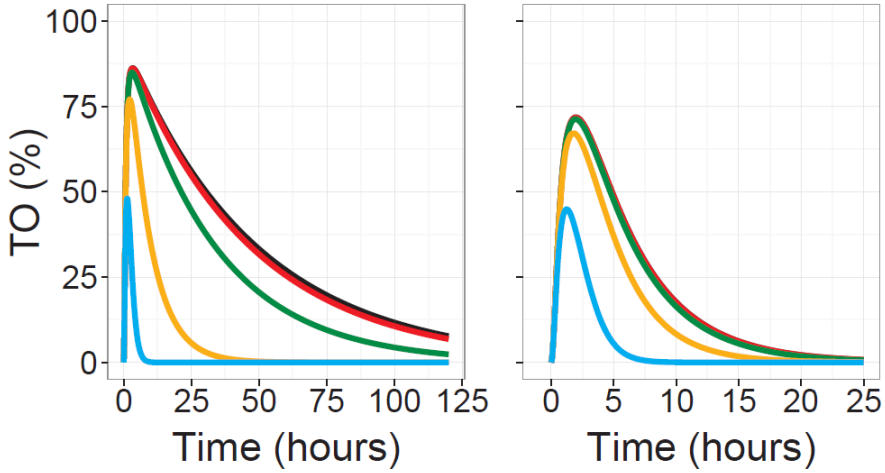

Figure 4. The influence of drug-target binding kinetics on target occupancy (TO) can be influenced by target turnover, as described by the model of Hong et al. for the antiplatelet effect of aspirin and ibuprofen [1]. *The drug target dissociation rate (k_{off}) changes from 0 (black line representing aspirin) to 0.001 hr^{-1} (red), 0.01 hr^{-1} (green), 0.1 hr^{-1} (orange) and 1 hr^{-1}(blue). The target degradation rate constant (k_{deg}) is 0.02 hr^{-1}(as identified for aspirin and ibuprofen) for the left panel and 0.2 hr^{-1} for the right panel (note the different time scale). The target synthesis rate constant (k_{syn}) was adjusted accordingly to obtain a steady state target concentration of 25 nM for both panels: $k_{syn} = 25 * k_{deg}$.*

5.2.7 Integrated mathematical modeling

To incorporate all the processes as described above for the prediction of target occupancy and drug effect and to use these predictions for the selection of the best drug candidates, quantitative mathematical description and integration of all these processes is essential.

Mathematical models have made use of drug-target binding kinetics in the previous decades to describe and predict the time course of drug effect [20–26]. These mathematical models most often use differential equations to describe the rate with which concentrations change, rather than describing the absolute value of a concentration for any time point. The use of differential equations requires solving of the differential equations for each time profile and each initial value, but it also allows the integration of numerous processes in a relatively simple way. As an example, the decrease in drug concentration due to elimination is often described by an equation like equation 2, where dC/dt is the change in drug concentration per time unit, C is the drug concentration and k_{el} is the elimination rate constant.

$$\frac{dC}{dt} = -k_{el} * C \quad (2)$$

Equation 2 means that if k_{el} = 0.1/min, for example, the drug concentration decreases with 10% every minute (if you solve the equation by taking time steps of 1 minute). For compartmental models, differential equations are used to describe the concentration in each compartment, and each compartment is considered to be homogeneous. For example, if the distribution of a drug over the body is fast or limited, the concentration profile of a drug in plasma can often be described by a one-compartment model with absorption and elimination. Such a compartmental approach can be used to describe drug-target binding by adding one or more compartments which represent the drug-target complex and assuming homogeneous distribution of the target in one of the pharmacokinetic compartments. This approach has been used for the simulations of figure 1-4, where binding was simulated from a single compartment for figure 2 and 4, from a brain compartment for figure 3 while a constant concentration was used for figure 1. Although these simulations are based on simplifying assumptions such as homogeneity, they provide a conceptual insight in the impact of the described processes on the relation between drug-target binding kinetics and target occupancy.

A special field where drug-target binding kinetics are taken into account as standard practice is the field of target mediated drug disposition (TMDD). TMDD describes the pharmacokinetics of drugs (mostly biologicals) which are distributed and eliminated predominantly when bound to their target. In this situation, binding kinetics are required to describe the drugs pharmacokinetics, since the pharmacokinetics depend on the binding and the dissociation is often relatively slow [105–108]. Mathematical analysis of a TMDD model revealed that k_{on} had a more pronounced impact on the maximal target occupancy than k_{off} [109]. Another field where drug-target binding kinetics are commonly incorporated in mathematical models is in the analysis of positron emission tomography (PET) data (see table 3). In all these examples and in the simulations shown in figure 1-4, mathematical models have demonstrated their potential to further our understanding of the role of drug-target binding kinetics in their complex physiological context.

6. Conclusion

On basis of the sparse amount of available literature estimates for drug-target binding kinetics, no conclusions can be made on how well *in vivo* binding kinetics are reflected in *in vitro* experiments. Moreover, differences in conditions, methodology and analysis avoid the comparison of available *in vitro* and *in vivo* estimates in many cases.

Next to the relation between *in vitro* and *in vivo* estimates of binding kinetics, the relation between *in vivo* binding kinetics and *in vivo* target occupancy and effect kinetics is also uncertain. This relation can be influenced by pharmacokinetics, endogenous competition, target tissue diffusion, non-specific binding, signal transduction and other factors. A quantitative integration by means of mathematical models can greatly enhance our understanding of the role of drug-target binding kinetics in this context.

This implies that more scientific support is required for the rational selection and development of drug-candidates on basis of *in vitro* estimates of drug-target binding kinetics.

7. Expert opinion: Towards an integrated approach for translational binding kinetics analysis in drug discovery and development.

The aforementioned determinants of target occupancy are related to each other, and need to be taken into account in an integrated manner. The use of compartmental modeling, as applied commonly in PK/PD modeling, is an important tool to facilitate the integration of all kinetic processes which are involved in the generation of drug effect (figure 5). The value of such models for all stages of drug discovery and development is increasingly recognized [110–112].

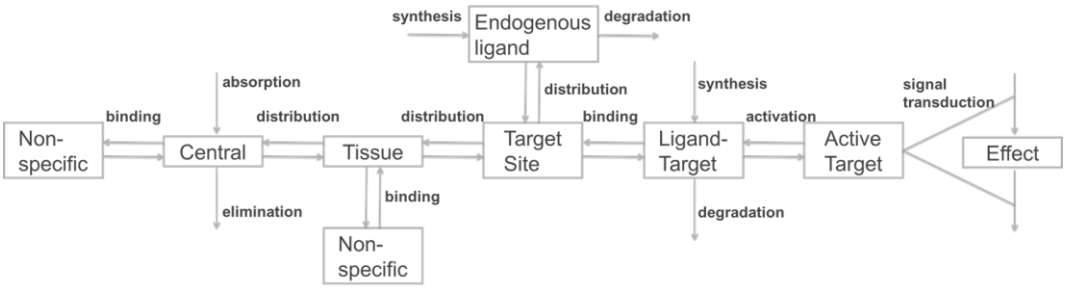

Figure 5. Schematic representation of the interconnected kinetic processes which determine target occupancy and effect kinetics. The central compartment represents the blood, the target site compartment represents the direct environment around the target, from where binding occurs.

The feasibility of such an integrative approach in (the early phases of) drug discovery and development may seem limited by its complex and time-consuming nature. However, it is important to note that some of these kinetic processes are system-specific processes (e.g. endogenous competition, target turnover and signal transduction). These system-specific processes are equal for all new compounds and will not decrease the screening throughput in drug discovery. The integrated analysis of these system-specific processes can thus be used to define which drug specific kinetic parameters (e.g. drug-target association and dissociation rate, non-specific binding rates and pharmacokinetic parameters) are most relevant per target/drug class to screen for. During the drug discovery process, the screening can start with only the most important parameter and be gradually extended to the other relevant parameters to refine the compound selection. The integrated analysis of all contributors to drug effect will not only improve compound selection, but it will also enable drug-candidate optimization on the most relevant parameters and optimization of drug dosing and sampling times in (pre)clinical investigations.

7.2 Context-dependency of binding kinetics values

To enable the integrated analysis of the kinetic processes which determine a drug's effect kinetics, specific information on all of these separate processes is required. This urges the performance of both *in vitro* and *in vivo* experiments which deliver drug- and system specific parameters for all kinetic processes. This is necessary to avoid experiments which inform only on the combined effect of multiple processes and thus deliver context-dependent information. For example, if an *in vivo* binding study is analyzed to determine only the rate with which the target occupancy inclines and declines after a certain dose, process-specific information is lacking because no specific information is collected about the pharmacokinetics or binding kinetics. This applies also to *in vitro* experiments. If a washout experiment is used to estimate the dissociation rate without a competing ligand, the obtained estimate can be a combined parameter for both dissociation and diffusion, because 'rebinding' can occur [96]. The occurrence of multiple kinetic processes during one experiment is not necessarily problematic, as long as the results can be analyzed in a process-specific manner to enable optimal translation to different experimental or clinical conditions. This type of

process-specific analysis can be obtained by using physiologically based PK/PD models with process-specific parameters.

7.3 Need for an integrated approach

Although experiments are available and in use to estimate the rate constants of the above mentioned kinetic processes, there is hardly any information available about what the relative contribution of each of those processes is in the determination of target occupancy profiles during the various scenarios of drug treatment. To enable the prediction of *in vivo* target occupancy and effect profiles, integrated analysis of experimental data and increased theoretical insight in the role of all contributors to target occupancy and effect are required. Mathematical models which describe the mechanisms of all relevant processes can be of great value to both analyze experimental data and simulate various cases of drug treatment in a comprehensive and integrated fashion. Increasing knowledge of the drivers of drug effect is of critical importance to select the best drug candidates in drug discovery, to optimize drug therapy in drug development and improve the health of those in need of medicines.

8. Acknowledgements

The K4DD project is supported by the Innovative Medicines Initiative Joint Undertaking (IMI JU) under grant agreement no 115366, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution.

References

1. Hong Y, Gengo FM, Rainka MM, et al. Population pharmacodynamic modelling of aspirin- and ibuprofen-induced inhibition of platelet aggregation in healthy subjects. Clin Pharmacokinet 2008;47(2):129–37
2. Katashima M, Yamamoto K, Tokuma Y, et al. Comparative pharmacokinetic/pharmacodynamic analysis of proton pump inhibitors omeprazole, lansoprazole and pantoprazole, in humans. Eur J Drug Metab Pharmacokinet 1998;23(1):19–26
3. Äbelö A, Holstein B, Eriksson UG, et al. Gastric acid secretion in the dog: A mechanism-based pharmacodynamic model for histamine stimulation and irreversible inhibition by omeprazole. J Pharmacokinet Pharmacodyn 2002;29(4):365–82
4. Copeland RA. The dynamics of drug-target interactions: drug-target residence time and its impact on efficacy and safety. Expert Opin Drug Discov 2010;5(4):305–10
5. Tummino PJ, Copeland RA. Residence Time of Receptor-Ligand Complexes and Its Effect on Biological Function. Biochemistry 2008;47(20):5481–92
6. Swinney DC. The role of binding kinetics in therapeutically useful drug action. Curr Opin Drug Discov Devel 2009;12(1):31–9
7. Kinzer-Ursem TL, Linderman JJ. Both ligand- and cell-specific parameters control ligand agonism in a kinetic model of g protein-coupled receptor signaling. PLoS Comput Biol 2007;3(1):84–94
8. Leysen JE, Gomeren W. Different kinetic properties of neuroleptic receptor binding in the rat striatum and frontal cortex. Life Sci 1978;23(5):447–52
9. Perry DC, Mullis KB, Oie S, et al. Opiate antagonist receptor binding in vivo: evidence for a new receptor binding model. Brain Res 1980;199(1):49–61
10. Syrota A, Paillotin G, Davy JM, et al. Kinetics of in vivo binding of antagonist to muscarinic cholinergic receptor in the human heart studied by positron emission tomography. Life Sci 1984;35(9):937–45
11. Guo D, Xia L, van Veldhoven JPD, et al. Binding Kinetics of ZM241385 Derivatives at the Human Adenosine A2A Receptor. ChemMedChem 2014;9(4):752–61
12. Schneider E V, Böttcher J, Huber R, et al. Structure-kinetic relationship study of CDK8/CycC specific compounds. Proc Natl Acad Sci U S A 2013;110(20):8081–6
13. Copeland RA, Pompliano DL, Meek TD. Drug-target residence time and its implications for lead optimization. Nat Rev Drug Discov 2006;5(9):730–9
14. Vauquelin G, Van Liefde I. Slow antagonist dissociation and long-lasting in vivo receptor protection. Trends Pharmacol Sci 2006;27(7):356–9
15. Dahl G, Akerud T. Pharmacokinetics and the drug-target residence time concept. Drug Discov Today 2013;18(15-16):697–707
16. Guo D, Hillger JM, IJzerman AP, et al. Drug-target residence time - a case for G protein-coupled receptors. Med Res Rev 2014;34(4):856–92
17. Yin N, Pei J, Lai L. A comprehensive analysis of the influence of drug binding kinetics on drug action at molecular and systems levels. Mol Biosyst 2013;9(6):1381–9
18. Vauquelin G, Bostoen S, Vanderheyden P, et al. Clozapine, atypical antipsychotics, and the benefits of fast-off D2 dopamine receptor antagonism. Naunyn Schmiedebergs Arch Pharmacol 2012;385(4):337–72
19. Vauquelin G. Rebinding : or why drugs may act longer in vivo than expected from their in vitro target residence time. Expert Opin Drug Discov 2010;5(10):927–41
20. Shimada S, Nakajima Y, Yamamoto K, et al. Comparative Pharmacodynamics of Eight Calcium Channel Blocking Agents in Japanese Essential Hypertensive Patients. Biol Pharm Bull 1996;19(3):430–7
21. Yun H-Y, Yun M-H, Kang W, et al. Pharmacokinetics and pharmacodynamics of benidipine using a slow receptor-binding model. J Clin Pharm Ther 2005;30(6):541–7
22. Steagall PVM, Pelligand L, Giordano T, et al. Pharmacokinetic and pharmacodynamic modelling of intravenous, intramuscular and subcutaneous buprenorphine in conscious cats. Vet Anaesth Analg 2013;40(1):83–95
23. Ramsey SJ, Attkins NJ, Fish R, et al. Quantitative pharmacological analysis of antagonist binding kinetics at CRF1 receptors in vitro and in vivo. Br J Pharmacol 2011;164(3):992–1007
24. Yassen A, Olofsen E, Dahan A, et al. Pharmacokinetic-Pharmacodynamic Modeling of the Antinociceptive Effect of Buprenorphine and Fentanyl in Rats : Role of Receptor Equilibration Kinetics. J Pharmacol Exp Ther 2005;313(3):1136–49
25. Äbelö A, Andersson M, Holmberg AA, et al. Application of a combined effect compartment and binding model for gastric acid inhibition of AR-HO47108: A potassium competitive acid blocker, and its active metabolite AR-HO47116 in the dog. Eur J Pharm Sci 2006;29(2):91–101
26. Landersdorfer CB, He YL, Jusko WJ. Mechanism-based population pharmacokinetic modelling in diabetes: Vildagliptin as a tight binding inhibitor and substrate of dipeptidyl peptidase IV. Br J Clin Pharmacol 2012;73(3):391–401
27. Guo D, Mulder-Krieger T, IJzerman AP, et al. Functional efficacy of adenosine A_2A receptor agonists is positively correlated to their receptor residence time. Br J Pharmacol 2012;166(6):1846–59

28. Motulsky HJ, Mahan LC. The Kinetics of Competitive Radioligand Binding Predicted Mass Action by the Law of Mass Action. Mol Pharmacol 1984;25(1):1–9
29. Zweemer AJM, Nederpelt I, Vrieling H, et al. Multiple binding sites for small-molecule antagonists at the CC chemokine receptor 2. Mol Pharmacol 2013;84(4):551–61
30. Guo D, van Dorp EJH, Mulder-Krieger T, et al. Dual-point competition association assay: a fast and high-throughput kinetic screening method for assessing ligand-receptor binding kinetics. J Biomol Screen 2013;18(3):309–20
31. Mathis G, Pierre N, Tardieu J. Determination of association(kon) and dissociation(koff) rate constants of Spiperone on the Dopamine D2 receptor using the Tag-lite® platform. 2011. p. SBS 17th Annual Conference. Orlando, USA
32. Schiele F, Ayaz P, Fernández-Montalván A. A universal, homogenous assay for high throughput determination of binding kinetics. Anal Biochem 2014;468:42–9
33. Patching SG. Surface plasmon resonance spectroscopy for characterisation of membrane protein-ligand interactions and its potential for drug discovery. Biochim Biophys Acta 2014;1838(1A):43–55
34. Rich RL, Myszka DG. Higher-throughput, label-free, real-time molecular interaction analysis. Anal Biochem 2007;361(1):1–6
35. Shiau AK, Massari ME, Ozbal CC. Back to basics: label-free technologies for small molecule screening. Comb Chem High Throughput Screen 2008;11(3):231–7
36. Gronewold TMA, Baumgartner A, Hierer J, et al. Kinetic Binding Analysis of Aptamers Targeting HIV-1 Proteins by a Combination of a Microbalance Array and Mass Spectrometry (MAMS). J Proteome Res 2009;8(7):3568–77
37. Gronewold TMA. Surface acoustic wave sensors in the bioanalytical field: recent trends and challenges. Anal Chim Acta 2007;603(2):119–28
38. Kenakin T, Jenkinson S, Watson C. Determining the potency and molecular mechanism of action of insurmountable antagonists. J Pharmacol Exp Ther 2006;319(2):710–23
39. Vauquelin G, Fierens F, Verheijen I, et al. Insurmountable AT 1 receptor antagonism : the need for different antagonist binding states of the receptor Glutamate signalling in the lung. Trends Pharmacol Sci 2001;22(7):343–4
40. Szczuka A, Wennerberg M, Packeu A, et al. Molecular mechanisms for the persistent bronchodilatory effect of the beta 2-adrenoceptor agonist salmeterol. Br J Pharmacol 2009;158(1):183–94
41. Lindström E, von Mentzer B, Påhlman I, et al. Neurokinin 1 receptor antagonists: correlation between in vitro receptor interaction and in vivo efficacy. J Pharmacol Exp Ther 2007;322(3):1286–93
42. Le MT, Pugsley MK, Vauquelin G, et al. Molecular characterisation of the interactions between olmesartan and telmisartan and the human angiotensin II AT1 receptor. Br J Pharmacol 2007;151(7):952–62
43. Atack JR, Wong DF, Fryer TD, et al. Benzodiazepine binding site occupancy by the novel GABAA receptor subtype-selective drug 7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine (TPA023) in rats, primates, and humans. J Pharmacol Exp Ther 2010;332(1):17–25
44. Laruelle M. Measuring Dopamine Synaptic Transmission with Molecular Imaging and Pharmacological Challenges : The State of the Art. Neuromethods 2012;71:163–203
45. Chernet E, Martin LJ, Li D, et al. Use of LC/MS to assess brain tracer distribution in preclinical, in vivo receptor occupancy studies: dopamine D2, serotonin 2A and NK-1 receptors as examples. Life Sci 2005;78(4):340–6
46. Barth VN. Typical and atypical antipsychotics: Relationships between rat in vivo dopamine D(2) receptor occupancy assessed using LC/MS and changes in neurochemistry and catalepsy. Section Title: Mammalian Hormones. 2006.
47. Need AB, McKinzie JH, Mitch CH, et al. In vivo rat brain opioid receptor binding of LY255582 assessed with a novel method using LC/MS/MS and the administration of three tracers simultaneously. Life Sci 2007;81(17-18):1389–96
48. Hume SP, Gunn RN, Jones T. Pharmacological constraints associated with positron emission tomographic scanning of small laboratory animals. Eur J Nucl Med 1998;25(2):173–6
49. Kung M-P, Kung HF. Mass effect of injected dose in small rodent imaging by SPECT and PET. Nucl Med Biol 2005;32(7):673–8
50. Hume SP, Myers R, Bloomfield PM, et al. Quantitation of carbon-11-labeled raclopride in rat striatum using positron emission tomography. Synapse 1992;12(1):47–54
51. Tantawy MN, Jones CK, Baldwin RM, et al. [(18)F]Fallypride dopamine D2 receptor studies using delayed microPET scans and a modified Logan plot. Nucl Med Biol 2009;36(8):931–40
52. Lancelot S, Zimmer L. Small-animal positron emission tomography as a tool for neuropharmacology. Trends Pharmacol Sci 2010;31(9):411–7
53. Müller CP, Pum ME, Amato D, et al. The in vivo neurochemistry of the brain during general anesthesia. J Neurochem 2011;119(3):419–46
54. Schiffer WK, Mirrione MM, Biegon A, et al. Serial microPET measures of the metabolic reaction to a microdialysis probe implant. J Neurosci Methods 2006;155(2):272–84

55. Ginovart N, Sun W, Wilson AA, et al. Quantitative Validation of an Intracerebral beta-Sensitive Microprobe System to Determine in Vivo Drug-Induced Receptor Occupancy Using [11C]Raclopride in Rats. Synapse 2004;52(2):89–99
56. Balasse L, Maerk J, Pain F, et al. PIXSIC, a Pixelated β(+)-Sensitive Probe for Radiopharmacological Investigations in Rat Brain: Binding Studies with [(18)F]MPPF. Mol imaging Biol 2014;
57. Polikov VS, Tresco PA, Reichert WM. Response of brain tissue to chronically implanted neural electrodes. J Neurosci Methods 2005;148(1):1–18
58. Wang Y, Michael AC. Microdialysis probes alter presynaptic regulation of dopamine terminals in rat striatum. J Neurosci Methods 2012;208(1):34–9
59. Märk J, Benoit D, Balasse L, et al. A wireless beta-microprobe based on pixelated silicon for in vivo brain studies in freely moving rats. Phys Med Biol 2013;58(13):4483–500
60. Li J, Fish RL, Cook SM, et al. Comparison of in vivo and ex vivo [3H]flumazenil binding assays to determine occupancy at the benzodiazepine binding site of rat brain GABAA receptors. Neuropharmacology 2006;51(1):168–72
61. Schotte A, Janssen PF, Gommeren W, et al. Risperidone compared with new and reference antipsychotic drugs: in vitro and in vivo receptor binding. Psychopharmacology (Berl) 1996;124(1-2):57–73
62. Lengyel K, Pieschl R, Strong T, et al. Ex vivo assessment of binding site occupancy of monoamine reuptake inhibitors: methodology and biological significance. Neuropharmacology 2008;55(1):63–70
63. Langlois X, te Riele P, Wintmolders C, et al. Use of the beta-imager for rapid ex vivo autoradiography exemplified with central nervous system penetrating neurokinin 3 antagonists. J Pharmacol Exp Ther 2001;299(2):712–7
64. Baker JG, Hall IP, Hill SJ. Pharmacological characterization of CGP 12177 at the human beta(2)-adrenoceptor. Br J Pharmacol 2002;137(3):400–8
65. Delforge J, Syrota A, Lançon JP, et al. Cardiac beta-adrenergic receptor density measured in vivo using PET, CGP 12177, and a new graphical method. J Nucl Med 1991;32(4):739–48
66. Kessler RM, Votaw JR, Schmidt DE, et al. High affinity dopamine D2 receptor radioligands. 3. [123I] and [125I]epidepride: in vivo studies in rhesus monkey brain and comparison with in vitro pharmacokinetics in rat brain. Life Sci 1993;53(3):241–50
67. Mukherjee J, Yang ZY, Brown T, et al. 18F-desmethoxyfallypride: a fluorine-18 labeled radiotracer with properties similar to carbon-11 raclopride for PET imaging studies of dopamine D2 receptors. Life Sci 1996;59(8):669–78
68. Mukherjee J, Yang Z-Y, Lew R, et al. Evaluation of d -Amphetamine Effects on the Binding of Dopamine D-2 Receptor Radioligand , 18F-Fallypride in Nonhuman Primates Using Positron Emission Tomography. Synapse 1997;27(1):1–13
69. Christian BT, Narayanan T, Shi B, et al. Measuring the in vivo binding parameters of [18F]-fallypride in monkeys using a PET multiple-injection protocol. J Cereb blood flow Metab 2004;24(3):309–22
70. Vandehey NT, Moirano JM, Converse AK, et al. High-affinity dopamine D2/D3 PET radioligands 18F-fallypride and 11C-FLB457: a comparison of kinetics in extrastriatal regions using a multiple-injection protocol. J Cereb blood flow Metab 2010;30(5):994–1007
71. Leysen JE, Gommeren W. Drug-Receptor Dissociation Time , New Tool for Drug Research : Receptor Binding Affinity and Drug-Receptor Dissociation Profiles of Serotonin-S2 , Dopamine-D2 , Histamine-HI Antagonists , and Opiates. Drug Dev Res 1986;131:119–31
72. Perlmutter JS, Kilbourn MR, Welch MJ, et al. Non-Steady-State Measurement of in vivo Receptor Binding with Positron Emission Tomography :“Dose Response” Analysis. J Neurosci 1989;9(7):2344–52
73. Leslie CA, Bennett JP. [3H]spiperone binds selectively to rat striatal D2 dopamine receptors in vivo: a kinetic and pharmacological analysis. Brain Res 1987;407(2):253–62
74. Kapur S, Seeman P. Antipsychotic agents differ in how fast they come off the dopamine D2 receptors. Implications for atypical antipsychotic action. J Psychiatry Neurosci 2000;25(2):161–6
75. Johnson M, Kozielska M, Pilla Reddy V, et al. Mechanism-based pharmacokinetic-pharmacodynamic modeling of the dopamine D2 receptor occupancy of olanzapine in rats. Pharm Res 2011;28(10):2490–504
76. Laruelle M, Abi-Dargham A, Ai-Tikriti MS, et al. SPECT Quantification of [123I] lomazenil Binding to Benzodiazepine Receptors in Nonhuman Primates : II . Equilibrium Analysis of Constant Infusion Experiments and Correlation with In Vitro Parameters. J Cereb blood flow Metab 1994;14(3):453–65
77. Millet P, Graf C, Moulin M, et al. SPECT quantification of benzodiazepine receptor concentration using a dual-ligand approach. J Nucl Med 2006;47(5):783–92
78. Sakiyama Y, Saito M, Inoue O. Acute treatment with pentobarbital alters the kinetics of in vivo receptor binding in the mouse brain. Nucl Med Biol 2006;33(4):535–41
79. Sihver W, Sihver S, Bergström M, et al. Aspects for In Vitro Characterization of Receptor Ligands : Receptor Binding Using 11C-Labeled A Detailed Study with the Benzodiazepine Receptor Antagonist [11C] Ro 15-1788. Nucl Med Biol 1997;24(8):723–31

80. Koeppe RA, Holthoff VA, Frey KA, et al. Compartmental analysis of [11C]flumazenil kinetics for the estimation of ligand transport rate and receptor distribution using positron emission tomography. J Cereb blood flow Metab 1991;11(5):735–44
81. Ishii A, Toyama J. Binding Properties of (+/-)[3H] Benidipine Hydrochloride to Rat Heart Membranes. J Cardiovasc Pharmacol 1993;21(2):191–6
82. Cassel JA, Daubert JD, DeHaven RN. [(3)H]Alvimopan binding to the mu opioid receptor: comparative binding kinetics of opioid antagonists. Eur J Pharmacol 2005;520(1-3):29–36
83. Yassen A, Olofsen E, Romberg R, et al. Mechanism-based PK/PD modeling of the respiratory depressant effect of buprenorphine and fentanyl in healthy volunteers. Clin Pharmacol Ther 2007;81(1):50–8
84. Yassen A, Kan J, Olofsen E, et al. Mechanism-Based Pharmacokinetic-Pharmacodynamic Modeling of the Respiratory-Depressant Effect of Buprenorphine and Fentanyl in Rats. 2006;319(2):682–92
85. Casadó V, Allende G, Mallol J, et al. Thermodynamic analysis of agonist and antagonist binding to membrane-bound and solubilized A1 adenosine receptors. J Pharmacol Exp Ther 1993;266(3):1463–74
86. Langlois X, Megens A, Lavreysen H, et al. Pharmacology of JNJ-37822681, a specific and fast-dissociating D2 antagonist for the treatment of schizophrenia. J Pharmacol Exp Ther 2012;342(1):91–105
87. Treherne JM, Young JM. Temperature-dependence of the kinetics of the binding the histamine H 1 -receptor : comparison with the kinetics of [3H] -mepyramine. Br J Pharmacol 1988;94(3):811–22
88. Wallace RM, Young JM. Temperature Dependence Related Compounds of the Binding of [3H]Mepyramine to the Histamine H1 Receptor. Mol Pharmacol 1983;23(1):60–6
89. Sakai S. Effect of hormones on dissociation of prolactin from the rabbit mammary gland prolactin receptor. Biochem J 1991;279:461–5
90. Endres CJ, Kolachana BS, Saunders RC, et al. Kinetic modeling of [11C]raclopride: combined PET-microdialysis studies. J Cereb blood flow Metab 1997;17(9):932–42
91. Morris ED, Yoder KK. Positron emission tomography displacement sensitivity: predicting binding potential change for positron emission tomography tracers based on their kinetic characteristics. J Cereb blood flow Metab 2007;27(3):606–17
92. Kapur S, Seeman P. Does Fast Dissociation From the Dopamine D 2 Receptor Explain the Action of Atypical Antipsychotics ?: A New Hypothesis. Am J Psychiatry 2001;158(3):360–9
93. Wiley HS. Anomalous Binding of Epidermal Growth Factor to A431 Cells Is Due to the Effect of High Receptor Densities and a Saturable Endocytic System. J Cell Biol 1988;107(2):801–10
94. Packeu A, Wennerberg M, Balendran A, et al. Estimation of the dissociation rate of unlabelled ligand-receptor complexes by a “two-step” competition binding approach. Br J Pharmacol 2010;161(6):1311–28
95. Vauquelin G, Van Liefde I. Radioligand dissociation measurements: potential interference of rebinding and allosteric mechanisms and physiological relevance of the biological model systems. Expert Opin Drug Discov 2012;7(7):583–95
96. Vauquelin G, Charlton SJ. Long-lasting target binding and rebinding as mechanisms to prolong in vivo drug action. Br J Pharmacol 2010;161(3):488–508
97. Peletier LA, Benson N, van der Graaf PH. Impact of protein binding on receptor occupancy: a two-compartment model. J Theor Biol 2010;265(4):657–71
98. Proost JH, Wierda JM, Meijer DK. An extended pharmacokinetic/pharmacodynamic model describing quantitatively the influence of plasma protein binding, tissue binding, and receptor binding on the potency and time course of action of drugs. J Pharmacokinet Biopharm 1996;24(1):45–77
99. Kim KM, Valenzano KJ, Robinson SR, et al. Differential regulation of the dopamine D2 and D3 receptors by G protein-coupled receptor kinases and beta-arrestins. J Biol Chem 2001;276(40):37409–14
100. Macey TA, Gurevich V V, Neve KA. Preferential Interaction between the Dopamine D2 Receptor and Arrestin2 in Neostriatal Neurons. Mol Pharmacol 2004;66(6):1635–42
101. Paspalas CD, Rakic P, Goldman-Rakic PS. Internalization of D2 dopamine receptors is clathrin-dependent and select to dendro-axonic appositions in primate prefrontal cortex. Eur J Neurosci 2006;24(5):1395–403
102. Dang VC, Christie MJ. Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. Br J Pharmacol 2012;165(6):1704–16
103. Skinbjerg M, Liow J-S, Seneca N, et al. D2 dopamine receptor internalization prolongs the decrease of radioligand binding after amphetamine: a PET study in a receptor internalization-deficient mouse model. Neuroimage 2010;50(4):1402–7
104. Haraguchi K, Ito K, Kotaki H, et al. Prediction of drug-induced catalepsy based on dopamine D1, D2, and muscarinic acetylcholine receptor occupancies. Drug Metab Dispos 1997;25(6):675–84
105. Mager DE, Jusko WJ. General pharmacokinetic model for drugs exhibiting target-mediated drug disposition. J Pharmacokinet Pharmacodyn 2001;28(6):507–32
106. Eppler SM, Combs DL, Henry TD, et al. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin Pharmacol Ther 2002;72(1):20–32

107. Jin F, Krzyzanski W. Pharmacokinetic model of target-mediated disposition of thrombopoietin. AAPS J 2004;6(1):86–93
108. Retlich S, Duval V, Graefe-Mody U, et al. Impact of target-mediated drug disposition on Linagliptin pharmacokinetics and DPP-4 inhibition in type 2 diabetic patients. J Clin Pharmacol 2010;50(8):873–85
109. Aston PJ, Derks G, Raji A, et al. Mathematical analysis of the pharmacokinetic-pharmacodynamic (PKPD) behaviour of monoclonal antibodies: Predicting in vivo potency. J Theor Biol 2011;281(1):113–21
110. Zhang L, Sinha V, Forgue ST, et al. Model-based drug development: the road to quantitative pharmacology. J Pharmacokinet Pharmacodyn 2006;33(3):369–93
111. Stone JA, Banfield C, Pfister M, et al. Model-Based Drug Development Survey Finds Pharmacometrics Impacting Decision Making in the Pharmaceutical Industry. J Clin Pharmacol 2010;50(S9):20S – 30S
112. Van der Graaf PH, Gabrielsson J. Pharmacokinetic – pharmacodynamic reasoning in drug discovery and early development. Future Med Chem 2009;1(8):1371–4